Calcium Diffusion in Uterine Smooth Muscle Sheets

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ABSTRACT The potassium contracture in the longitudinal muscle of estrogen-treated rat uterus was kinetically investigated. The rates of tension development after Ca addition and relaxation after Ca removal were measured under the high-potassium depolarization. Both rates decreased with an increase in preparation thickness. The relaxation rate had only a slight dependence on temperature. On the contrary, both relaxation and contraction rates in a contraction induced by an electrical stimulation strongly depended on temperature, but not on preparation size. These results suggest that the Ca diffusion process in the extracellular space is the rate-limiting step in relaxation of Ca-dependent contracture under potassium depolarization. The diffusion model, in which the effect of the unstirred layer was considered, could quantitatively explain the experimental results. The apparent diffusion coefficient in the muscle sheet was estimated to be \( \sim 3 \times 10^{-7} \text{cm}^2/\text{s} \). The difference from that in aqueous solution is discussed.

INTRODUCTION

Recent studies have shown that the understanding of excitation-contraction coupling in smooth muscles depends on knowledge of the calcium-controlling system composed of the sarcolemmal membrane and intracellular organelles (Bolton, 1979). Calcium movements have been mainly investigated by use of the radioactive tracer \(^{45}\text{Ca}\). The rate of Ca movements obtained by this method was, however, much slower than expected from the time course of tension change. If tension could be regarded as a measure for the change of intracellular or extracellular calcium concentration, the kinetic analysis of the time course of tension development may give useful information on the Ca movement in smooth muscles.

Longitudinal smooth muscles of rat uteri have been shown to produce external Ca-dependent contracture under potassium depolarization (Edman and Shild, 1962; Ogasawara et al., 1980). This contracture was defined as “Ca contracture” in the present paper. Removal of external Ca induced the relaxation of the contracted muscle and the rate of relaxation was dependent on the ionic composition in relaxant solution (Osa, 1975) and on the hormonal state of the uterus (Ogasawara et al., 1980). The present study was designed...
to investigate the Ca movements by means of the kinetic analysis of time course of the Ca contracture.

This kind of experiment required an assessment of the homogenate, because a muscle strip is composed of a number of smooth muscle cells and connective tissue. First, we verified the functional homogeneity of individual cells in the muscle preparation. Subsequently, the rate-limiting step in tension changes of Ca contracture was determined by investigating the effects of preparation thickness and temperature on the rate of tension changes. The experimental results suggested that the rate-limiting step was the Ca diffusion process in the extracellular space. A theoretical model was proposed that could explain the experimental results quantitatively.

MATERIALS AND METHODS

Wistar-strain virgin rats weighing 200–250 g were used 2 wk after ovariectomy. 10 μg of estradiol-17β benzoate (Sigma Chemical Co., St. Louis, MO) dissolved in 0.5 ml sesame oil was subcutaneously injected. After 4 d the uterus was removed from the stunned rat and was immediately immersed in a control Locke-Ringer solution whose composition is given later. Longitudinal muscle sheets were dissected from the uterus under a stereoscopic microscope. Their sizes were 2–3 mm in length, 0.1–1 mm in width, and 0.04–0.2 mm in thickness.

The preparation, both edges of which were tied with silk threads, was mounted in the acrylic chamber described in Fig. 1. The size of cylinder at the preparation site in the chamber was 3 mm long in the direction of flow and 2 mm in diameter. The test solution passed through a thermoregulated water jacket was circulated through the chamber at the constant flow rate of 1.2 cm/s. The exchange of solution at the preparation site depended on the flow rate and was finished within 0.6 s. The
temperature of the perfused solution was measured using a thermistor thermometer attached to the chamber. During changes of the perfused solutions, the distinction between solutions of different composition was carried out by insertion of a short air gap between them.

Isometric tensions were converted into electrical signals by a strain gauge transducer (TB612-T; Nihon Kohden, Tokyo, Japan), and amplified signals were recorded with a pen recorder and a recticorder (RJG-3004; Nihon Kohden). The latter records were used for kinetic treatments. The resting load was <10% of the maximum tension of evoked contraction under the experimental condition. The term “evoked contraction” is used in this paper for a contraction induced by an electrical stimulation. An electrical stimulation with 1-s duration pulses was applied through a pair of Ag-AgCl electrodes.

Electrical activity was recorded by conventional glass microelectrodes filled with 3 M KCl. The muscle preparation of 7 mm in length and 1 mm in width was fixed in the partition chamber described by Abe and Tomita (1968).

The control Locke-Ringer solution contained 154 mM NaCl, 8 mM NaHCO3, 5.6 mM KCl, 1 mM CaCl2, and 5.5 mM glucose. The isotonic potassium solution was prepared by replacement of NaCl and NaHCO3 in the control solution with equimolar KCl and KHCO3. The external CaCl2 concentration was varied without compensation for ionic strength. Solutions were equilibrated with a gas mixture of 95% O2–5% CO2 in the reservoirs and the pH was 7.2–7.4.

The thickness and transverse section areas of muscle sheets were measured by a microscopic method. After tension measurements, the Ca-free fixating solution containing 1% glutaraldehyde and 4% formaldehyde was passed through the chamber for 10 min. The fixed preparation was taken from the chamber and was immersed in the fixating solution overnight. After a wash with tap water, the preparation was stained with eosin B solution in order to make preparation of slices from the small and thin preparation easy, and embedded in polyethyleneglycol (1,500 mol wt, and subsequently 4,000 mol wt; Wako Pure Chemical, Osaka, Japan). Slices of 20 μm thickness were transversely cut at the interval of 100 μm along the whole preparation by a microtome. Polyethyleneglycol in the slice was washed out with control solution on the glass slide. The average values of thickness and transverse section area were obtained by the use of the photograph of several slices along the whole muscle strip.

**RESULTS**

**Functional Homogeneity of Cells in the Muscle Sheet**

Electron microscope studies have shown that a longitudinal rat uterus muscle consisted of bundles containing a number of smooth muscle cells, fibroblast cells, and connective tissues (Garfield and Daniel, 1974). The size of a single muscle fiber has been estimated to be several micrometers in diameter and several tens of micrometers in length, so that a muscle strip contains $10^6$–$10^7$ cells per unit area (square centimeters) in the transverse section (Garfield and Daniel, 1974; Kao, 1977). A measured tension of a muscle sheet can therefore be regarded as a summation of tensions developed by all cells of the muscle sheet. This indicates that a kinetic analysis of tension change by use of a muscle sheet is effective when physiological functions of all cells constituting the muscle sheet are uniform. In the present experiments, the functional homogeneity was checked by the following three methods.
First, the amplitudes of evoked or spontaneous contractions were measured for the muscle preparation of different thickness (Fig. 2A). The tension increased in proportion to the transverse section area up to $1 \times 10^{-3}\text{cm}^2$, and the slope of the line gave the tension per unit area as $4.0 \pm 0.6 \text{N/cm}^2$ (mean $\pm$ standard deviation, $n = 8$) in the control solution at $38^\circ\text{C}$. This value lends to the tension of a single muscle cell of $\sim 0.8 \mu\text{N}$, if the number of cells per unit transverse section area (square centimeter) is taken as $5 \times 10^6$. The linear relationship between tension and transverse section area was also obtained under the different experimental conditions, at $27^\circ\text{C}$ or in the test solution containing...
2 mM CaCl₂. These linearities suggest that each cell develops a uniform tension and is uniformly distributed in the muscle sheet.

Second, electrical activity was recorded simultaneously with mechanical activity (Fig. 2B). The duration of the plateau in the action potential (lower trace) was comparable to that of the contraction (upper trace). Electrical activity recorded by microelectrode corresponds to the activity of a single muscle cell at the surface of the muscle sheet. On the other hand, mechanical activity corresponds to a summation of activities of all cells constituting the muscle sheet. The agreement in durations of electrical and mechanical activities therefore suggests that each individual cell has the same pattern in tension development and that all cells in the preparation synchronize electrically.

Finally, the time courses of tension development and relaxation in evoked or spontaneous contractions were compared in preparations of different sizes (Fig. 3). Because the physical meaning of tension change is unknown at this moment, the half-time was used as a measure of the rate of tension change (Fig. 3D). Both half-times were found to be independent of preparation thickness. This result confirms that the individual cell contracts with the same time course and suggests that the diffusion process in the extracellular space is not included in tension development processes in the evoked and spontaneous contractions.

**Ca Contracture under the Potassium Depolarization**

When the control solution was replaced with the Na-free and Ca-free isotonic K-Locke-Ringer solution, the longitudinal muscle exhibited a transient contraction and gradually relaxed to the resting level (Fig. 4A). Then the sustained contracture was induced by adding CaCl₂ to the perfused solution, and its amplitude depended on the external Ca concentration (Fig. 4B). At concentrations <0.1 mM the relative tension increased linearly with an increase in external Ca concentration. The relative tension was slightly larger at 37 than at 23°C. This relationship between relative tension and external Ca concentration was independent of preparation thickness at the transverse section area of <1 x 10⁻³ cm². When the thicker preparation was used, however, reproducible data could not be obtained, probably because it took a long time to obtain the steady contracture. For this reason and the requirement of homogeneity of the preparation, muscle sheets of <1 x 10⁻³ cm² and thinner than 200 µm were used hereafter.

**Effect of Preparation Thickness on the Rate of Tension Development and Relaxation**

Fig. 5 shows the time course of tension development when 1 mM CaCl₂ was added to perfused isotonic K solution. It is noted that there is a time lag of a few seconds before the tension development. This time lag may be an indication of the unstirred layer at the surface of the preparation, as discussed later. Because the time course of tension development could not be described as a single exponential curve, the half-time of tension development, $t_{0.5}$, was plotted against the thickness, as shown in Fig. 5C. The half-time was longer
than that of the evoked contraction and increased slightly with an increase in thickness. On the other hand, that of the evoked contraction was independent of thickness.

**Figure 3.** Time course of tension development and relaxation in spontaneous and evoked contractions. A. The original record by a recticorder. Lower trace represents the electrical stimulus. $F_0$ means the peak tension. B. Semilogarithmic plot of tension development. Cross: spontaneous contraction of the muscle in which the transverse section area is $3.3 \times 10^{-4} \text{cm}^2$; dot: evoked contraction, $1.2 \times 10^{-4} \text{cm}^2$. C. Semilogarithmic plot of relaxation. $F = F_0$ at $t = 0$. Dot: $1.2 \times 10^{-4} \text{cm}^2$; cross: $6.2 \times 10^{-4} \text{cm}^2$. D. Relationship between the half-time of tension development (filled symbols) and relaxation (open symbols). Different symbols (○, □, △, ▽) correspond to different animals.

Fig. 6 shows the time course of relaxation after removal of external Ca ions. In every preparation of different thickness, the semilogarithmic plot of the relative tension curved near the half-tension and hereafter became linear. The half-time of relaxation, $t_{0.5}^R$, was markedly longer than those of evoked
contractions and tension development in Ca contracture, and increased with an increase in preparation thickness (Fig. 6C).

Effect of Temperature on the Rate of Tension Development and Relaxation

Rates of both tension development and relaxation in evoked contractions strongly depended on temperature, and $Q_{10}'s$ (38 and 28°C) were 2.0 and 3.2, respectively (Figs. 7A and C). The high sensitivity of the rates to temperature suggests that the rate-limiting process in the tension change in the evoked contraction implicates chemical reactions (Laidler, 1965). On the other hand, the relaxation rate of Ca contracture was almost independent of temperature and $Q_{10}$ was <1.2 (Fig. 7D). This result confirms that the rate-limiting process in relaxation is a physical process like diffusion. The rate of tension development in Ca contracture has a temperature dependency similar to that of
evoked contractions (Fig. 7B). This may be due to the same order of the rates of tension development in the evoked contraction and Ca contracture, especially in thin preparations (cf. Fig. 5C; note that \( t_{0.5} \) contains a time lag of a few seconds).

**Figure 5.** Time course of tension development in Ca contracture after introduction of CaCl₂. A. The original record. \( F_s \) means the steady tension. B. Semilogarithmic plot of the relative tension in the preparation dissected from the same tissue. Temperature is 37°C. C. The half-time of tension development as a function of thickness of the muscle sheet. Bars represent the SD of thickness. Crosses represent the half-time of tension development in evoked contractions.

*Diffusion Model*

In the above results, the dependency of thickness and temperature on the rate of tension changes suggests that the rate-limiting step in the tension change of
Ca contracture is the diffusion process of the Ca ion in the extracellular space of the muscle sheet. Thus, the problem is equivalent to the nonsteady diffusion problem of the Ca ion into or from a plane sheet. The schematic model is represented in Fig. 8. This model contains the unstirred layer at the surface of the muscle sheet.
First, we discuss the case of no unstirred layer, i.e., $\delta = 0$. The time course of the Ca concentration profile in the muscle sheet after addition of CaCl$_2$ can be readily calculated as represented in the Appendix and as shown in Fig. 9A. If we assume that the individual muscle cell in the sheet develops the tension in accordance with the tension-Ca concentration relationship described in Fig. 4B, the Ca concentration profile can be converted to the tension profile, as shown in Fig. 9B. Integration of the curves with respect to thickness at each time gives the time course of the total amount of Ca ion having penetrated into the muscle sheet and that of tension development (Fig. 10A, a and b). In a similar way, the time course of the Ca content and relaxation after removal of CaCl$_2$ was calculated and is shown in Fig. 10A (b and c). These results clearly explain the experimental results (Figs. 5B, 6B, 10B, circle), in which the rate of relaxation is remarkably slower than that of tension development.

**Figure 7.** Temperature dependency of the half-time of tension development (A and B) and relaxation (C and D). A and C: evoked contraction. B and D: Ca contracture.
It is noted that the Ca content takes the same time course in either case after addition and removal of Ca ions.

The differences between the time courses in the Ca content and tension are caused by the nonlinear relationship between the tension and Ca concentration. If the relationship is linear, both time courses of tension development and relaxation will fit to that of the Ca content. To examine this hypothesis,

![Diagram of calcium diffusion](image)

**Figure 8.** Schematic model for the diffusion. A. Transverse section of the muscle sheet. B. Longitudinal section. The muscle sheet is 2 unit thick and contacts the unstirred layers of thickness δ at both surfaces. $C_1$ represents the Ca concentration in the bulk solution. $D$ and $D_0$ mean the diffusion coefficients of the Ca ion in the muscle sheet and in the unstirred layer, respectively.

the rate of tension change in Ca contracture induced by 0.1 mM CaCl₂ was measured because the tension-Ca concentration relationship is approximately linear at concentration of <0.1 mM. The results in Fig. 10B (triangle) agree with the expected curve and also confirm the diffusion-limiting hypothesis.

**Evaluation of the Diffusion Coefficient of Ca Ion**

The comparison between the observed and calculated results enables us to evaluate the diffusion coefficient of Ca ion in the muscle sheet. The half-time...
of relaxation can be represented as a function of the apparent diffusion coefficient of the Ca ion, $D$, and the half-thickness of the muscle sheet, $l$. When $F/F_0 = 0.5$ in curve c in Fig. 10A, the half-time is represented by

$$d_{0.5}^R = 0.745 \frac{l}{D}$$

(1)

In Fig. 6C, $d_{0.5}^R$ was plotted as a function of $2l$ for different values of $D$. The experimental data fit to the theoretical data when the value of $D$ is taken as $2-3 \times 10^{-7}\text{cm}^2/\text{s}$. Using $3 \times 10^{-7}\text{cm}^2/\text{s}$ for $D$, the theoretical time courses of relaxation were plotted in Fig. 6B for the different thicknesses of preparations.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure9}
\caption{Calculated concentration profile of the Ca ion (A) and tension profile (B) in the half-muscle sheet at various time $Dt/l^2$. Initial concentration is $C_0$ in the muscle sheet and $C_1$ at the surface. Ordinate is the tension relative to that at $x = l$. Abscissa is the reduced distance. The Ca concentration profile was converted to the tension profile by use of the Ca concentration-tension relationship described in Fig. 4B.}
\end{figure}

Considering experimental errors, especially that of thickness, the agreement between the experimental and theoretical results is satisfactory.

**Effect of the Unstirred Layer**

It has been reported that the effect of an unstirred layer cannot be neglected in investigating a material transport across a thin membrane (lipid bilayer membrane: Everitt and Haydon, 1969; Andreoli and Troutman, 1971; ion-exchange membrane: Mackay and Meares, 1959; small intestine: Winne, 1973; Thomson and Dietschy, 1980). The effect of the unstirred layer on the Ca diffusion in the muscle sheet was theoretically derived. The schematic model has been already represented in Fig. 8. We assume the unstirred layer of thickness $\delta$ in which the diffusion coefficient of the Ca ion is $D_0$. The detail of the theory is described in the Appendix. Fig. 11A is the calculated curve for
Figure 10. Comparison between the theoretical and experimental results. A. Calculated dependence of the total Ca content in the muscle sheet and that of the tension on $Dt/l^2$. a, tension development; b, total Ca content entered or remained in the muscle sheet; c, relaxation. B. Comparison with the experimental results. Ca contracture was induced by the solution containing 1 mM CaCl$_2$ (circles) and 0.1 mM (triangles). The observed data were drawn for tension development (filled symbols) and for relaxation (open symbols). Solid curves are the theoretical curves taking $D$ as $2.1 \times 10^{-7}$ cm$^2$/s; a, tension development; b, Ca content; c, relaxation.

The time course of the Ca content. Here a dimensionless parameter $L$ is defined by

$$L = \frac{l}{\delta} \frac{D_0}{D}$$

This graph shows that the rate of the change in the Ca content, that is, the rate of the tension change, decreases with a decrease in $L$ even though $D$ and $l$ are constant. Under the experimental conditions, the decrease of $L$ corresponds to the decrease of $l$ or the increase of $\delta$. It is expected from the
theoretical result that the unstirred layer affects the rate of the tension change when the preparation is thin or the unstirred layer is thick. In fact, the observed time course of relaxation was slower than the calculated one in the thin preparation (Fig. 6B, a).

![Diagram](image)

**Figure 11.** The effect of the unstirred layer on the time course of tension change. A. The calculated curves for the time course of Ca content in the smooth muscle sheet. A dimensionless parameter $L$ is defined by Eq. 2 in the text. The effect of the unstirred layer increased with a decrease in the value of $L$. The curve at $L = \infty$ corresponds to the curve b in Fig. 10A. B. Comparison with the experimental results. The time course of relaxation was plotted for the different flow rate of perfused solution; ○, 0.5 cm/s, ●, 1.6 cm/s. Each plot is the average of three observations. The thin preparation of 50 μm thickness was used. Solid curves are the calculated results taking $D$ as $3 \times 10^{-7}$ cm$^2$/s.

To examine the effect of the unstirred layer thickness, the flow rate of the perfused solution was changed, because the thickness of the unstirred layer depends on the flow rate or the stirring rate of the external solution. The relaxation rate became faster with an increase in the flow rate (Fig. 11B). The
value of $L$ is evaluated to be between 2 and 5 at the flow rate of 1.6 cm/s. Assuming $D_0$ to be $6 \times 10^{-6}$ cm$^2$/s, and taking $D$ as $3 \times 10^{-7}$ cm$^2$/s and $l$ as $2.5 \times 10^{-3}$ cm, Eq. 2 gave the thickness of the unstirred layer as 100–250 μm. This value is similar as reported for the other membranes; e.g., several tens of micrometers for the lipid bilayer membrane (Andreoli and Troutman, 1971) and the ion-exchange membrane (Mackay and Meares, 1959), 100–1000 μm for the intestinal mucosal membrane (Thomson and Dietschy, 1977), and 119 μm for the rabbit gallbladder (Diamond, 1966).

If 150 μm is used as the value of $\delta$, $L$ is estimated to be 6.7, 10, and 13.3 when $l$ is 50, 75, and 100 μm, respectively. The relaxation rate of the thick preparation was slightly affected by the flow rate as expected.

The time lag in tension development after addition of CaCl$_2$ can be also explained by the unstirred layer effect. This time lag corresponds to the time in which the Ca ion diffuses across the unstirred layer and the Ca concentration at the surface of muscle sheet reaches at the critical concentration developing tension, ~0.01 mM. This time is approximately given by

$$t = 0.06 \frac{\delta^2}{D_0}$$

(Crank, 1975). If $\delta = 150$ μm and $D_0 = 6 \times 10^{-6}$ cm$^2$/s are used, the time lag is estimated to be 2.25 s, which agrees with the observed value of ~2–3 s shown in Fig. 5B.

**DISCUSSION**

The Ca movement in uterine smooth muscles was analyzed by use of the tension change in Ca contracture under the high-potassium depolarization. Similar studies have been reported by Milligan (1965) for a skeletal muscle and by Sekiguchi and Chujyo (1978) for a guinea pig taenia coli. The present study is characterized by the following three points: (a) the functional homogeneity of the individual cell in the preparation was examined and the checked preparations were used for experiments; (b) the rate-limiting step was established by investigation of the effects of thickness and temperature on the rate of tension change; and (c) the effect of the unstirred layer was considered.

A study on smooth muscles is accompanied by difficulties caused by the small size of a single cell. If the uniformity of the individual cell in the tissue is confirmed, the measurement by use of tissues will be available to estimate functions of the individual smooth muscle cell. Morphological studies have shown that the shapes and sizes of isolated cells are diverse (Bagby et al., 1971; Cooke and Fay, 1972). However, Fay’s result (1977), that the peak tension developed by the isolated cell from the toad stomach is comparable to that measured from the intact tissue when the tensions are normalized for the cross section area, suggests the functional homogeneity of the individual cell for tension development. Our results support his theory about the longitudinal muscle of the estrogen-treated rat uterus.

When the Ca movement is estimated from the tension change in Ca contracture in which the external Ca concentration is changed, we cannot
neglect the Ca diffusion process in the extracellular space. Our results suggest that the rate-limiting step in relaxation is the Ca diffusion process because the rate of relaxation is strongly dependent on preparation thickness and little dependent on temperature. This is also confirmed by another finding that the rate of relaxation in Ca contracture is remarkably slower than that in spontaneous and evoked contractions that do not include the Ca diffusion process in the extracellular space. Our results, however, did not eliminate the possibility that the rates of tension change in Ca contracture in single cells might be slower than those of evoked contractions because of the different mechanisms for Ca movements during Ca contracture and during evoked contractions.

The Ca movement in smooth muscles has been investigated by use of the radioactive tracer $\text{Ca}^{45}$, and its time scale was on the order of several tens of minutes (guinea pig taenia coli: Schatzman, 1961; Chujyo and Holland, 1963; Goodford, 1965; Bauer et al., 1965; rat uterus: van Breemen et al., 1966). In these measurements a thick tissue preparation was used so that one part of the washout curve of $\text{Ca}^{45}$ consisted of the Ca diffusion process in the extracellular space. However, most of investigators presumed that the effect of the diffusion process finished within only several minutes, assuming the same diffusion coefficient of Ca ion in the extracellular space as that in aqueous solution. On the contrary, our results suggest that the diffusion coefficient of Ca ion in the tissue is smaller than that in aqueous solution by a factor of 20. This means that the effect of the Ca diffusion on the washout curve will dominate during a 20-fold-longer period than expected. The large effect of the Ca diffusion on the $\text{Ca}^{45}$ washout curve is supported by several data: the size dependency, e.g., the comparison of thin strip (Goodford, 1965) and thick strip (Chujyo and Holland, 1963), the low sensitivity to temperature (Goodford, 1965; van Breemen et al., 1966), and the resemblance of the washout curve to that from the connective tissue (Bozler, 1963).

The last point obtained by our experiments is the effect of the unstirred layer, which is especially important when a thin strip is used. Recently, Hirata et al. (1981) have reported the $\text{Ca}^{45}$ washout from the isolated smooth muscle cells, in which the time course of the washout is slow, on the order of 10 min. One reason for this slow Ca movement may be the unstirred layer.

The comparison between the experimental and theoretical results gave the apparent diffusion coefficient of the Ca ion in the extracellular space. The apparent diffusion coefficient of Ca ion in the uterine muscle sheet was estimated to be $3 \times 10^{-7} \text{cm}^2/\text{s}$. The self-diffusion coefficient of Ca ion in the dilute aqueous solution with only CaCl$_2$ present has been found to be $7.78 \times 10^{-6} \text{cm}^2/\text{s}$ (Wang, 1953) and $1.2 \times 10^{-5} \text{cm}^2/\text{s}$ (Harned and Owen, 1958). The actual value in the test solution may be reduced by the effect of other monovalent ions. Kushmerick and Podolsky (1969) have reported that the diffusibility of ions and nonelectrolytes except Ca ion are reduced by a factor of 2 in the skeletal muscle sarcoplasm relative to the aqueous solution. Therefore, the actual value of the diffusion coefficient in the test solution is taken to be $6 \times 10^{-8} \text{cm}^2/\text{s}$, as estimated by Hodgkin and Keynes (1957). The
observed value is remarkably smaller than that in aqueous solution by a factor of 20. There are some reasons for the reduced diffusibility in the muscle sheet. The experimental error may be caused mainly by an error of measurement of thickness owing to the shrinkage of a preparation during fixation and embedding process. If the maximum error by the shrinkage is taken as 50%, the diffusion coefficient will be reduced by a factor of only 2.25.

The muscle sheet is assumed to be homogeneous in theory, but the mean free path of diffusion of the Ca ion increases because of the zigzag pathways in the muscle. Harris and Burn (1949) introduced a correction factor of $\lambda$ and represented the apparent diffusion coefficient as $D_a/\lambda^2$ where $D_a$ is the true diffusion coefficient in the extracellular space. They evaluated $\lambda$ as $\pi/2$, i.e., $\lambda^2 = 2.5$, considering a packed cylinder model, and Sekiguchi and Chujyo (1978) experimentally obtained $\lambda^2$ as 3.1 for the packed cylinder composed of resin columns.

Another reason for the reduced diffusibility in the muscle sheet is the effect of Ca binding on the specific Ca binding site in the extracellular space. Van Breemen and McNaughton (1970) have shown by means of the La method that 80% of the total amount of bound Ca in rabbit aorta is present in the extracellular binding site. The effect of Ca binding on Ca efflux from squid axons has been reported by Baker and McNaughton (1978). The general diffusion equation considering the effect of Ca binding is described in the Appendix. Under the experimental conditions it is expected that the apparent diffusion coefficient will be reduced by a factor of $(1 + X_0/K_d)$ where $X_0$ is the concentration of the binding site and $K_d$ is the dissociation constant. The values of $X_0$ and $K_d$ of estrogen-treated rat uterus are unknown. Weiss (1978) has reported that rabbit aortic muscle has two affinity sites of Ca binding; a high-affinity site of $X_0 = 1.27$ mmol/g tissue and $K_d = 0.0234$ mM, and a low-affinity site of $X_0 = 9.0$ mmol/g tissue and $K_d = 3.214$ mM. Because the low-affinity site mainly affects Ca diffusion under the experimental condition as described in the Appendix, if we assume $X_0 = 10$ mM and $K_d = 3$ mM, a reducing factor $(1 + X_0/K_d)$ gives a result of 4.3.

Our result for the value of apparent diffusion coefficient in the extracellular space is smaller than the value reported by Milligan (1965), $2 \times 10^{-6}$ cm$^2$/s for the skeletal muscle, and by Keatinge (1972), $1.7 \times 10^{-6}$ cm$^2$/s for the vascular smooth muscle. The difference may be caused by the difference of method and preparation. The difference for preparations may be caused by the difference of $X_0$ and $K_d$ in the Ca binding site. With respect to this point, it should be noted that we used a Mg-free test solution. Mg ions may competitively bind to the Ca binding site and thus affect the diffusibility of the Ca ion.

**APPENDIX**

**Assumptions**

(a) The muscle sheet is regarded as an infinite plane sheet of thickness $2l$. According to Crank (1975), the edge effect can safely be neglected with thin membranes ($l/a \ll \lambda^2$).
0.2, where \( \sigma \) is the width of the muscle sheet. The preparation used in this experiment satisfied this condition.

(b) The muscle sheet is filled with a homogeneous medium. This assumption is wrong because the sheet is composed of complex structure. The apparent diffusion coefficient should be corrected by some factor as described in the Discussion.

(c) The term of potential gradient, \( \partial \psi / \partial t \), is neglected. This effect may be compensated by the large amount of mobile ions contained in the test solution.

(d) The thickness of the muscle sheet does not change during contracture.

(e) Uptake of Ca ion by cells is negligible.

**Basic Diffusion Equation**

We consider one-dimensional diffusion in the infinite muscle sheet described in Fig. 8. A diffusion equation is represented by

\[
\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2}
\]

where \( C \) is the concentration of the Ca ion, \( t \) is the time, \( x \) is the distance with respect to the direction of thickness, and \( D \) is the apparent diffusion coefficient.

If the unstirred layer is absent and the initial and boundary conditions are represented by

\[
C = C_0, \quad x = \pm l, \quad t > 0
\]

\[
C = C_0, \quad -l < x < l, \quad t = 0
\]

the solution of Eq. A1 becomes

\[
\frac{C - C_0}{C_1 - C_0} = 1 - \frac{4}{\pi} \sum_{n=0}^{\infty} \frac{(-1)^n}{2n + 1} \exp \left( \frac{-D(2n + 1)^2 \pi^2 t}{4l^2} \right) \cos \left( \frac{(2n + 1)\pi x}{2l} \right)
\]

(Crank, 1975).

If the unstirred layer is present on the surface of the muscle sheet, the initial and boundary conditions are

\[
C = C_1, \quad x = \pm l \pm \delta, \quad t > 0
\]

\[
C = C_0, \quad -l - \delta < x < l + \delta, \quad t = 0
\]

\[
-\frac{D}{\delta} \frac{\partial C}{\partial x} = D_0 \frac{C_1 - C_0}{\delta}, \quad x = \pm l, \quad t > 0
\]

where \( D_0 \) is the diffusion coefficient of the Ca ion in the unstirred layer and \( \delta \) is the thickness of the unstirred layer. It is noted that the boundary condition (A7) can be used only when \( D \ll D_0 \). In this case the solution of Eq. A1 is

\[
\frac{C - C_0}{C_1 - C_0} = 1 - \sum_{n=1}^{\infty} \frac{2L \cos(\beta_n x / l) \exp(-\beta_n^2 D_0 l^2)}{(\beta_n^2 + L^2 + L) \cos \beta_n}
\]

where the \( \beta_n \)'s are the positive roots of

\[
\beta \tan \beta = L
\]

and

\[
L = \frac{l}{\delta} \cdot \frac{D_0}{D}
\]

(Crank, 1975).
The concentration profile represented by Eqs. A4 and A8 can be calculated with a computer. The Ca content entered into the muscle sheet after introduction of CaCl₂, \( M \), is obtained by integration of the concentration profile with respect to the distance:

\[
\frac{M}{M_0} = \int_0^l C(x, t) \, dx. 
\]  
(A11)

The concentration profile \( C(x, t) \) is converted to the tension profile \( F(x, t) \) using the Ca concentration-tension relationship. By integration of the tension profile, the time course of tension development is obtained:

\[
\frac{F_s - F}{F_s} = \frac{\int_0^l F(x, t) \, dx}{\int \{F(t, 0) - F(0, 0)\}}. 
\]  
(A12)

In a similar way, we can get the time course of the Ca content remaining in the muscle sheet after removal of CaCl₂, \( M/M_0 \), and relaxation, \( F/F_0 \).

**Effect of Ca Binding**

If the binding site for the Ca ion is present in the muscle sheet and its dissociation equilibrium is rapidly established rather than the diffusion, the diffusion equation becomes,

\[
\frac{\partial(C + C_x)}{\partial t} = D \frac{\partial^2 C}{\partial x^2}, 
\]  
(A13)

where \( C \) is the concentration of free Ca ion, and \( C_x \) is the concentration of bound Ca ion. \( C_x \) is represented by

\[
C_x = \frac{X_0 C}{C + K_d}, 
\]  
(A14)

where \( X_0 \) is the concentration of total binding sites, and \( K_d \) is the dissociation constant.

Substituting Eq. A14 in Eq. A13, we get the following nonlinear partial differential equation:

\[
\frac{\partial C}{\partial t} = D_a(C) \frac{\partial^2 C}{\partial x^2}, 
\]  
(A15)

where

\[
D_a(C) = \frac{D}{f(C)}, 
\]  
(A16)

and

\[
f(C) = 1 + \frac{X_0 K_d}{(C + K_d)^2}. 
\]  
(A17)

The same type of equation is given for the gas diffusion in a glassy polymer that can absorb the gas, and is solved by a numerical method (Vieth and Sladek, 1965).

Eq. A16 means that the apparent diffusion coefficient \( D_a \) is a function of the Ca concentration and is always smaller than the diffusion coefficient \( D \) with the binding site absent because of \( f(C) \gg 1 \). If \( C \ll K_d \), Eq. 17 reduces to

\[
f(C) = 1 + \frac{X_0}{K_d}. 
\]  
(A18)
Thus the apparent diffusion coefficient is independent of the Ca concentration and is reduced by a factor of Eq. A18 relative to $D$. If $C \gg K_d$ and $X_0 \ll C$, Eq. A17 becomes $f(c) \approx 1$ so that the effect of Ca binding on the diffusibility of Ca ion can be neglected.

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