CAFFEINE-INDUCED CALCIUM RELEASE FROM SARCOPLASMIC RETICULUM OF A SKELETAL MUSCLE

TATSUHIKO SEKIGUCHI AND HIROSHI SHIMIZU

Faculty of Pharmaceutical Science, University of Tokyo, Bunkyo-ku, Tokyo, 113, Japan

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Ca efflux from sarcoplasmic reticulum (SR) of a skinned skeletal muscle fiber was measured quantitatively using \(^{45}\text{Ca}\). The time course of Ca efflux was fitted by superimposing two exponential curves.

Caffeine caused Ca efflux from SR at very low concentration of free Ca ion. In order to study the relation of the Ca efflux mechanism to Ca-induced Ca release, caffeine was introduced to SR under controlled free Ca concentration. The results showed that caffeine induced Ca release from SR.

**Keywords** — caffeine; calcium release; skeletal muscle; skinned fiber; sarcoplasmic reticulum; caffeine-induced; calcium-induced

INTRODUCTION

It is well known that caffeine works on the sarcoplasmic reticulum (SR) of muscle cell and induces Ca release.\(^1\) This is the cause of so-called “caffeine contracture”. Endo\(^2\) studied the relationship between this Ca release mechanism and Ca-induced Ca release which was discovered by Endo \textit{et al.} \(^3\) and Ford and Podolsky.\(^4\) He concluded that caffeine increased the affinity of Ca channels for Ca and enhanced Ca-induced Ca release mechanism. Using isolated SR vesicles, there have been several studies supporting this point of view.\(^5\)–\(^7\) On the other hand, there has been a report suggesting that 5 mM caffeine induced Ca-insensitive Ca release from SR.\(^8\)

Generally speaking, skinned fibers provide a more physiological model system of SR than biochemically isolated SR because the former preserves the structurally physiological SR, including the junction between transversal tubules and SR. Thus, it is still important to study the Ca release mechanism using a skinned fiber SR.

In the present paper, using a skinned fiber SR, we quantitatively measured caffeine-induced Ca release in the presence of controlled concentration of free Ca and studied the relationship between caffeine-induced Ca release and Ca-induced Ca release.

MATERIALS AND METHODS

Fiber Preparation and Mounting — Semitendinosus muscles dissected from a female African clawed frog, \textit{Xenopus laevis}, were suspended in a cold Amphibian Ringer's solution (115.5 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl\(_2\), 3.1 mM NaH\(_2\)PO\(_4\) + Na\(_2\)HPO\(_4\) buffer, pH 7.0). Single fibers about 100 µm thick were dissected and split longitudinally into two equal parts in the relaxing solution. A skinned fiber with no visible damage and about 10 mm long was fixed with collodion to stainless steel rods at both ends and then was immersed in the relaxing solution. About 5 min later, the fiber was treated with 25 mM caffeine for over 30 s in order to deplete “physiological” Ca from SR. The fiber was put back into the relaxing solution for about 5 min and was ready for experiments.

Experimental Chambers and Temperature Control — The experimental chamber had 15 wells of 3 different dimensions. The \(^{45}\text{Ca}\) loading well was 4 mm long, 4 mm wide and 4 mm deep. The washing wells (7 mm × 4 mm × 2 mm) were for washing \(^{45}\text{Ca}\) or Mg salt of adenosine triphosphate (MgATP) around SR. The test wells (5 mm × 4 mm × 2 mm) were for the measurement of \(^{45}\text{Ca}\) efflux. With these wells of various sizes, \(^{45}\text{Ca}\) diffused to the portion of the fibers that was not immersed in the loading solution were trapped through a washing procedure and will influence only slightly the measurement of Ca efflux. The experimental check of the point is described below. The amounts of solution applied to 3 kinds of wells were 80, 100 and 60 µL, respectively. From a diameter of 0.1 mm, the volume of a skinned fiber was estimated as about 0.04 µL. Thus, the amounts of solution in the wells were sufficient to be regarded as a reservoir. The chamber was made of aluminium.
and its surface was coated with Teflon in order to make it hydrophobic. It was attached to a micromanipulator, which could be moved up and down.

The chamber was placed in a box made of polymethylmethacrylate board and the box was covered with styrofoam board for insulation. The temperature in the box was controlled by a radiator through which thermo-regulated ethylene glycol flowed. All the experiments were carried out at 9 ± 1°C.

_Solutions_ — The relaxing solution contained 20 mM K₂SO₄, 2 mM ethylene glycol bis(β-aminoethyl)ether)-N,N',N'-tetraacetic acid (EGTA), 4 mM MgSO₄, 4 mM ATP, 100 mM sucrose and 20 mM 3-[(N-morpholino)propane sulfonic acid (MOPS), pH 7.0. The releasing solution contained 25 mM caffeine in addition to the constituents in the relaxing solution. The washing solution contained 20 mM K₂SO₄, 2 mM EGTA, 10 mM adenosine monophosphate (AMP), 100 mM sucrose and 20 mM MOPS, pH 7.0. Test solutions also contained various concentrations of CaSO₄, MgSO₄, caffeine or procaine. The buffered ⁴⁵Ca solution (the loading solution) was used to load the skinned fibers with a radioactive tracer containing 20 mM K₂SO₄, 4 mM MgSO₄, 4 mM ATP, 100 mM sucrose, 2 mM EGTA, 1 mM ⁴⁵CaCl₂ and 20 mM MOPS, pH 7.0 (pCa 6.46); stock ⁴⁵CaCl₂ solution was prepared from a high specific activity ⁴⁵CaCl₂ (New England Nuclear, Boston, MA, U.S.A.) diluted 14-folds with CaCl₂ and had a final activity of about 20–50 μCi/ml. Concentrations of free ions were calculated with a high speed computer in the Computer Center of the University of Tokyo using the method of Perrin and Sayce, taking into account the multiple equilibrium of all types of ions. The scintillation mixture contained 667 ml of toluene, 333 ml of Triton X-100, 6 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene in 1 l solution.

Adenine nucleotides and procaine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.), caffeine from Wako Pure Chemical Industries, Ltd. (Osaka), EGTA and MOPS from Nakarai Chemical Ltd. (Kyoto). Other chemicals were of analytical grade.

_Measurement of Time Course of ⁴⁵Ca Efflux_ — The methods used for the measurement of the time course of ⁴⁵Ca efflux from SR were essentially the same as those of Stephenson, except in regards to the fiber-immersion technique and the method of the measurement of the remaining ⁴⁵Ca in SR.

A skinned fiber was exposed to the loading solution for 5 min and then was rinsed for 15 s in the relaxing solution to trap ⁴⁵Ca in the myoplasm. Then, the fiber was transferred to the washing solution and was rinsed for 30 s three times. By this washing procedure, about 7% of ⁴⁵Ca initially loaded in SR was lost and the remaining 93% of ⁴⁵Ca was considered as 100% for the normalization of the data described later. The fiber was then transferred at a defined time to a series of the test solution containing CaSO₄, MgSO₄, caffeine or procaine. These solutions contained only negligible amounts of MgATP so that the measurement of unidirectional Ca efflux across the SR membrane could be made. Finally, at the end of a measured time course, the fiber was exposed to the releasing solution containing 25 mM caffeine to deplete almost all the Ca in SR. Each fiber was passed through these loading-releasing procedure 3–6 times. At the end of the use of the fiber, it was treated with 0.4% triton X-100 to completely extract ⁴⁵Ca from SR. We defined this state as [Ca in SR] = 0. The amount of ⁴⁵Ca released into each solution was measured with a liquid scintillation counter (Packard Tri-Carb 3225) and was expressed as a fraction of the total amount of ⁴⁵Ca, which was the sum of the amount of ⁴⁵Ca released in the test and in the releasing solutions. The fraction remaining in the SR at the end of each treatment was obtained by sequentially back-adding to the fraction remaining at the end. In order to estimate the influence of ⁴⁵Ca-binding in the fiber, the fiber was passed through the same procedure from ⁴⁵Ca loading to the caffeine treatment. The ⁴⁵Ca that appeared in each solution was also measured and subtracted from each count of experiments. This procedure was applied to all the fibers used.

The surface of our experimental chamber was hydrophobic and the meniscus of the solution protruded above the surface of the wells. The length of the fiber immersed in the solutions was limited to 4, 7, and 5 mm for the loading, washing and the test solutions, respectively. Since the whole length of fiber was 10 mm, end regions of several millimeters were not exposed to the solutions. The contribution of SR in these regions
was checked as follows. After a lateral half of a fiber was loaded with \(^{45}\text{Ca}\), the other half was exposed to the releasing solution. At this time, \(^{45}\text{Ca}\) released from the unloaded half was only 7.3% of that of the loaded half. Then, we were certain that the contribution from the unexposed portion was minor. With our immersion technique, the amount of tracer contamination was reduced to less than 1% of the total amount of \(^{45}\text{Ca}\) released from SR, while it was about 10% when the whole fiber was immersed with rods.

Stephenson\(^8\)) used a solution containing 0.05% Triton X-100 + 5 mM EGTA for the measurement of Ca remaining in SR. But with Triton X-100, SR was so heavily damaged that the fibers could not be used for further experiments. We used 25 mM caffeine in place of Triton X-100, because we found that with 25 mM caffeine, 93.2 ± 1.2% of Ca initially loaded in the SR was released with high reproducibility (see Results). Our method enabled us to measure the time courses of Ca efflux from SR repeatedly using the same skinned fiber. As described above, a single skinned fiber could be used for 3 – 6 time course experiments. The fraction which was released by 25 mM caffeine was independent of the number of the fiber recycling at least up to 6 times.

Results are expressed as the mean ± S.D.

RESULTS

Determination of Rate Constants of Ca Efflux with High Concentration of Caffeine

We first studied how much Ca in SR was released when treated with 25 mM caffeine. After a fiber was loaded with \(^{45}\text{Ca}\) for 5 min at pCa 6.46, it was treated for 3 min with the releasing solution containing 25 mM caffeine. The fiber was then transferred to a solution containing 0.4% Triton X-100 in order to extract residual \(^{45}\text{Ca}\) from the SR. We measured the amount of \(^{45}\text{Ca}\), in the releasing solution (\(F_{\text{CR}}\)) and in the extraction solution (\(F_{\text{TX}}\). The value, \(F_{\text{CR}} + F_{\text{TX}}\) was measured as 1.8 ± 0.2 μmol/ml fiber (\(n = 20\): This value was obtained that the volume of SR was 10% of that of the fiber.\(^1\)) This Ca content was in good agreement with that reported by Ford and Podolsky,\(^1\)) 2 – 3 μmol/ml fiber. The fraction of Ca released by 25 mM caffeine was expressed as \(F_{\text{CR}}/(F_{\text{CR}} + F_{\text{TX}})\). From 20 observations, this value was independent of the number of loading-washing procedures performed. In addition, the amount of Ca not released by 25 mM caffeine within 3 min had no relationship to the initial amount of Ca loaded in SR, as reported by Makino et al.\(^1\)). On the basis of this result, we used the solution containing 25 mM caffeine to measure the Ca remaining in SR after treatment with a series of test solutions.

Figure 1 shows a typical time-dependent Ca efflux curve plotted in semilogarithmic scale (open circles). The measurement of Ca efflux from SR was performed in a solution containing no MgATP to obtain unidirectional Ca movement, Ca efflux, in the absence of Ca-pump activity. The amount of Ca remaining in SR at a given time was expressed in terms of relative values to that at time zero (\(= 100\). Figure 1 indicates the Ca efflux curve for the external Ca of 3.16×10\(^{-5}\) M. As indicated in the figure the Ca efflux curve was fitted well by superimposing two exponential curves. Consequently, the relative amount of Ca in SR at time \(t\) (s), \(C(t)\), may

![FIG. 1. Semilogarithmic Plot of Ca Efflux from a Skinned Fiber SR](image-url)
be denoted in percent as
\[ C(t) = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) \]
with \( A_1 + A_2 = 100 \).

For the curves shown in Fig. 1, the parameters were determined by the least square method as, \( A_1 = 13.2, A_2 = 86.6, k_1 = 1.46 \times 10^{-8} \text{ s}^{-1} \) and \( k_2 = 4.24 \times 10^{-8} \text{ s}^{-1} \). The same type of analysis was available for every Ca efflux curve. As a result, \( A_1 (14.8 \pm 2.08, n = 30) \) and \( k_1 (2.54 \pm 0.73 \times 10^{-3} \text{ s}^{-1}, n = 30) \) were independent of the composition of medium (the concentration of free Ca, caffeine, Mg or procaine), while \( k_2 \) depended on it. Therefore the faster process (\( k_2 \), closed circles) is attributed to the efflux of Ca from SR and the slower process to passive Ca efflux (Ca leakage), since the rate constant of the slower process was similar to that of Ca leakage. When the concentration of Ca or caffeine was low, the value of \( k_2 \) approached that of \( k_1 \) and consequently it was difficult to separate these two processes in the Ca efflux curve. In such cases, the mean values of \( k_1, 2.54 \times 10^{-3} \text{ s}^{-1} \) and \( A_1, 14.8 \) were used for the slower process.

Hereafter, the term “rate constant” is meant for the faster process, \( k_2 \).

**Ca-Dependent Ca Efflux and Caffeine-Dependent Ca Efflux**

In order to determine the natures of Ca efflux from SR of skinned fiber preparations, the dependence of Ca efflux on free Ca and caffeine concentrations was studied. Figure 2 shows the dependence of the rate constant of Ca efflux on the external free Ca concentration. Ca buffered solution consisted of 2 mM EGTA and various concentrations of CaSO₄ (0.5—4 mM). Concentrations of free Ca were calculated for each case taking into account of the multiple equilibrium. Up to about pCa 5.5, the rate constant had a positive dependence on the external free Ca concentration (A-phase). But beyond that optimal concentration, a negative dependence was observed (I-phase). These features agreed with previous workers’ results. Hill’s coefficient was 1.75 and 0.85 for A-phase and I-phase, respectively. The result can be interpreted as follows. The Ca channel apparently has two types of Ca-binding sites, A-site and I-site. The channel opens when two Ca ions bind to A-site and closes when one Ca ion binds to I-site with dissociation constants of \( 8.9 \times 10^{-7} \) and \( 1.7 \times 10^{-8} \), respectively.

Figure 3 shows the dependence of the rate constant on caffeine concentration in the presence of 2 mM EGTA (pCa > 8) in the external solution. With higher concentrations of caffeine, Ca was released with larger rate constants. This result was in good agreement with that of Stephenson. The dissociation constant was calculated as \( 6.14 \times 10^{-8} \) M.

**The Effect of Caffeine on Ca-Dependent Ca Release**

As shown in Fig. 3, caffeine appears to induce Ca release from SR. In order to clarify the mecha-
FIG. 4. Effect of Caffeine on $\text{Ca}^{2+}$-Dependent Ca Efflux

Data are taken in the presence of 2 mM (□), 5 mM (▲), 10 mM (△) and 25 mM (■) caffeine, under the same conditions as in Fig. 2. Control curve is taken from Fig. 2.

nism of caffeine-induced Ca release, the effect of caffeine on Ca-dependent Ca efflux was examined.

The results are summarized in Fig. 4. As the concentration of caffeine was raised from 2 mM to 5, 10 and 25 mM, the rate constant in the A-phase increased, while there was little change in the I-phase. In addition, the slope of the A-phase decreased as the concentration of caffeine was increased and finally, at 25 mM caffeine, became virtually independent of the external free Ca concentration. This suggested that caffeine induced Ca efflux independently of external free Ca.

In another condition where Ca-induced Ca release process was enhanced (in the presence of 4 mM ATP), Ca was released with 3 - 4 fold larger rate constants than the largest rate constant obtained in the present study (about 0.1 s$^{-1}$). Thus, the diffusion of Ca was not a rate-limiting step in the case. Even if the concentration of external EGTA was changed, the Ca efflux induced by 5 and 25 mM caffeine was independent of the concentration of EGTA between 2 and 10 mM (Fig. 5). This indicates that EGTA concentration of 2 mM was sufficient to control the Ca concentrations in myoplasm and the rate constants measured were almost free from the contribution of the Ca efflux which was induced by the released Ca.

In addition, the contribution of ATP which is known as an enhancer of Ca-induced Ca release$^{15}$ was negligible. This was ascertained as follows. A fiber was treated with hexokinase-glucose system to deplete ATP. Hexokinase transfers phosphate from ATP to glucose, producing adenosine diphosphate (ADP) and glucose-6-phosphate. The ability of ADP to potentiate Ca-induced Ca release is weaker than that of

FIG. 5. Effect of External EGTA Concentration on Ca Efflux Induced by Caffeine

The concentration of caffeine is 5 mM (▲) and 25 mM (■). Experimental conditions are the same as in Fig. 3.

FIG. 6. Effect of Caffeine on $\text{Ca}^{2+}$-Dependent Ca Efflux after Treatment with 1 mg/ml Hexokinase and 5 mM Glucose for 3 min

The caffeine concentration is 5 mM (▲) and 25 mM (■). The curve marked with open circles is taken from Fig. 2.
ATP. The fiber actively loaded with Ca was exposed to the washing solution containing 1 mg/ml hexokinase and 5 mM glucose for 3 min. The concentration of ATP at that time was negligible, judging from the hexokinase activity. The fiber then underwent the Ca efflux processes described in Materials and Methods in the presence of 10 mM EGTA. The result is shown in Fig. 6. The dependence of the rate constant for Ca efflux on the external free Ca concentration was less in the presence of 5 and 25 mM caffeine than in their absence and the values were only slightly affected. The closed circles in Fig. 3 indicate the rate constant of caffeine-induced Ca efflux after treatment with hexokinase-glucose. Also in the case, there was no appreciable difference in the presence or the absence of the hexokinase-glucose system.

From these facts, it was concluded that the rate constants shown in Fig. 4 were free from artifacts such as an apparent decrease of rate constants due to diffusional barrier or unexpected enhancement of Ca release.

If caffeine-induced Ca release is the result of enhancement of Ca-induced Ca release, the feature of rate constants in the presence of free Ca and caffeine will be quite different from Fig. 4. Thus, it is concluded that caffeine-induced Ca release by itself. Detailed analysis is presented in the Discussion section.

**Effect of Mg and Procaine on Caffeine-Induced Ca Release**

It is well known that Mg ion and procaine inhibit Ca-induced Ca release. The effect of these drugs for caffeine-induced Ca efflux was also studied (Fig. 7). With 1 mM MgSO₄ (0.24 mM free Mg), the caffeine-dependence curve was shifted to higher concentrations. This is a typical change showing competitive inhibition. On the other hand, 3 mM procaine reduced the maximum rate constant about 50% and did not change the half maximal saturation caffeine concentration, indicating that procaine inhibits caffeine-induced Ca release noncompetitively.

![Diagram](attachment:image_url)

**FIG. 7.** Effect of Mg⁴⁺ or Procaine on Caffeine-Dependent Ca Efflux

The ordinate represents the concentrations of caffeine in a logarithmic scale. Data are obtained in the presence of 1 mM MgSO₄ (0.24 mM Mg²⁺) (●) and 3 mM procaine (△). Experimental conditions are the same as in Fig. 3. Control curve (○) is taken from Fig. 3.

![Graph](attachment:image_url)

**FIG. 8.** Schematic Models of the Effects of Ca²⁺ and Caffeine on Ca Efflux

(a) The model for mechanism [A]. The affinity of Ca channel for Ca²⁺ is increased to N-fold by the binding of caffeine. (b) The model for mechanism [B]. The channel opens by caffeine binding. Ca channel is represented as “Ch”. Two types of Ca binding sites are expressed on the right side (A-site) and the left side (I-site) of “Ch”. The binding site for caffeine is expressed on the lower part of “Ch”. K₁, K₂, and K₉ are dissociation constants of the channel for Ca²⁺ (A-site), Ca²⁺ (I-site) and caffeine, respectively. The states surrounded by the broken line correspond to open states, and the others are closed states.
DISCUSSION

In the present study, the mechanism of caffeine-induced Ca efflux from the sarcoplasmic reticulum of a skinned skeletal muscle was studied. As described previously, two schemes have been suggested for the mechanism of caffeine-induced Ca release; [A] caffeine increases affinity of the Ca channel for Ca ion and enhances Ca-induced Ca release mechanism; [B] caffeine directly affects the Ca channel and induces Ca release. In the case of mechanism [A], it is expected that, at very low concentrations of free Ca, Ca would not be released by the application of caffeine, while it would be in case of mechanism [B].

For better understanding of the difference between these two mechanisms, the kinetic schemes for mechanism [A] and [B] are shown in Fig. 8a and 8b, respectively. As described previously, the channel opens when two Ca ions bind to the A-site and closes when one Ca ion binds to the I-site. Then, in scheme A, the open states are $Ca_2$-Ch and $Ca_2$-Ch-Caf while in B, Ch-Caf, $Ca_3$-Ch and $Ca_2$-Ch-Caf are the open states. It is assumed here that the rate constant of Ca release is proportional to the fraction of the Ca channel in the open state and that Ca is released only through the Ca channel. In scheme A, assuming that the binding constant of the channel for Ca ion increases $N$-fold by the binding of caffeine, the fraction of open channel, $R_a$, is expressed as

$$R_a = \frac{(X_1 + NX_1 Y)}{S_a}$$

with

$$S_a = 1 + X_1 + X_1 X_2 + Y + NX_1 Y + NX_1 X_2 Y$$

In scheme B,

$$R_b = \frac{(X_1 + Y + X_1 Y)}{S_b}$$

with

$$S_b = (1 + X_1 + X_1 X_2)(1 + Y)$$

where

$$X_1 = \frac{[Ca^{2+}]}{K_1^2}$$

$$X_2 = \frac{[Ca^{2+}]}{K_2}$$

$$Y = \frac{[caffeine]}{K_{caf}}$$

$N$ : constant

The dissociation constants $K_1$, $K_2$ and $K_{caf}$ are obtained from the experimental results (Fig. 2 and 3) as $8.9 \times 10^{-7}$, $1.7 \times 10^{-5}$ and $6.1 \times 10^{-3}$ M, respectively. With these parameters, the fraction of open channel was calculated for both schemes and the results are shown as Fig. 9a and 9b. In scheme A, as expected, the Ca channel is almost in the closed state at very low concentrations of free Ca and the slope of the A phase is the same at every caffeine concentration. On the other hand, in scheme B, the channel is almost in an open state at low Ca and high caffeine concentrations, and a decreased slope is observed at the higher concentration of caffeine. The experimental result shown in Fig. 4 is explained by mechanism B with the parameters obtained.

**FIG. 9. Calculations on the Basis of the Schematic Models Described in Fig. 8**

(a) and (b) are results of the calculation based on the models shown in Fig. 8 (a) and (b), respectively. Theoretical curves are drawn on the assumption that the rate constant is proportional to the fraction of the open state of channels. Points refer to the same experimental data as in Fig. 4. The dissociation constants, $K_1 = 8.9 \times 10^{-7}$ M, $K_2 = 1.7 \times 10^{-5}$ M and $K_{caf} = 6.14 \times 10^{-3}$ M, are obtained from Figs. 2 and 3. In (a), the caffeine concentration was fixed at 5 mM and the value of $N$ was changed. The points are the data of 5 mM caffeine. In (b), calculation was done for all caffeine concentrations examined. The points are those in Fig. 4.
from independent experiments (Fig. 2 and 3).

There have been reports supporting mechanism [A]. But no one except Endo observed that Ca release was negligible at the low free Ca and high caffeine concentrations. In the caffeine-method which Endo used, the Ca content in SR was measured by the contraction of skinned muscle fiber by application of caffeine. Thus, it is difficult to measure quantitatively very high and very low Ca content in SR and there is a possibility that caffeine might release Ca with a rate constant at very low concentrations of free Ca. The results shown in Fig. 4 are free from contributions such as slow Ca diffusion or contamination of ATP and it is concluded that caffeine directly affects the channel and induces Ca release. Stephenson reported caffeine-induced Ca release in the presence of MgATP. In that report, Ca release was measured under a condition where the Ca uptake system was functioning. It was difficult to clarify the definite nature of caffeine action. In the present study, Ca uptake was completely suppressed, and it was possible to determine kinetic parameters such as dissociation constants for Ca ion or caffeine. Using a scheme similar to Fig. 8, the dissociation constants of channels for Mg ion and procaine were calculated as $5 \times 10^{-5}$ and $3 \times 10^{-5}$ M, respectively. The concentration of EGTA sufficient to buffer Ca during the Ca efflux process was 10 mM according to Stephenson. On the other hand, it was about 2 mM in our case (Fig. 5). This may be due to the thickness of a skinned fiber preparation. Since we used only half of a single fiber, ions might diffuse more easily in our preparations than in those of Stephenson.

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