

# Enhanced Permeability to Sugar Associated with Muscle Contraction

## *Studies of the role of $Ca^{++}$*

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**ABSTRACT** When contractures were induced in isolated frog sartorius muscles with 4 mM caffeine, there was an increase in permeability of the muscle cells to 3-methylglucose. This observation suggests that the changes in permeability to sugar that are known to occur in electrically stimulated muscles may not be intimately related to the depolarization phase of the tissue response. Contractures that were elicited by exposing the muscles to a high concentration of  $K^+$  were also associated with an increased permeability to sugar. As the concentration of  $^{45}Ca$  in the medium was raised, more  $^{45}Ca$  entered the muscles during potassium contractures, and the contractures lasted longer, in agreement with the observations of other investigators. There was also a greater change in permeability to sugar when potassium contractures were elicited in the presence of higher concentrations of  $Ca^{++}$ . The possibility that the enhanced permeability to sugar may be related to changes in the intracellular concentration of  $Ca^{++}$  is discussed.

### INTRODUCTION

Contraction of skeletal muscle is associated with an increase in permeability to sugar *in vivo* (1, 2). In order to investigate this phenomenon in greater detail, the rate of penetration of 3-O-methyl-D-glucose, a nonmetabolizable compound that resembles glucose in the kinetics of its entry into muscle cells (3-5), was measured in isolated frog sartorius muscles after electrical stimulation (6). It was found (6) that permeability increased as a function of the frequency and duration of stimulation, but that changes in the amount of work performed during a given number of isotonic twitches did not modify the effect on sugar transport. In the present investigation changes in permeability to sugar have been measured after eliciting caffeine or potassium contractures in frog sartorius muscles, and an attempt has been made to examine the alterations in permeability under conditions that were designed to affect the  $Ca^{++}$  content of the cells.

## EXPERIMENTAL PROCEDURE

*Chemicals*

$^{45}\text{Ca}$  was obtained from the New England Nuclear Corporation and had a specific activity of approximately  $350 \mu\text{c}$  per  $\mu\text{mole}$ . The source and purity of 3-O-methyl-D-glucose- $^3\text{H}$  and mannitol- $^{14}\text{C}$  and the general procedure used for measuring radioactivity have been described elsewhere (5, 6). Nonradioactive 3-O-methyl-D-glucose was generously donated by Dr. William L. Glen of Ayerst, McKenna and Harrison, Ltd., Montreal, Canada.

*Incubation Media*

The normal frog Ringer's solution used in the present investigation was a modification of the Krebs-Henseleit solution (7), and had the following composition: 87 mM NaCl, 25 mM  $\text{NaHCO}_3$ , 1.52 mM KCl, 1.19 mM  $\text{KH}_2\text{PO}_4$ , 1.19 mM  $\text{MgSO}_4$ , and 1.3 mM  $\text{CaCl}_2$ . Potassium Ringer's solution was made by substituting potassium ion for all of the sodium ion of normal Ringer's solution.

The normal frog Ringer's solution had a tonicity of approximately 240 milliosmols per liter. Mannitol was used for measuring the extracellular space and for adjusting osmolarity to a constant value, usually 260 milliosmols per liter, throughout a given experiment. Muscle cells tend to swell during exposure to high concentrations of potassium ion (8); in order to diminish this uptake of water the tonicity of experimental and control media was raised with mannitol to 287 milliosmols per liter in experiments involving potassium contracture. When the concentration of calcium ions was altered, or when 3-methylglucose was included in the medium, the osmolarity was kept constant by appropriate alterations in the concentration of mannitol. For experiments in which the concentration of calcium ions was varied, phosphate ions were omitted from the incubation medium.

*Handling of Tissues*

Female *Rana pipiens* were obtained during the months of November through April from J. R. Schettle Biologicals of Stillwater, Minn., and were kept in water at  $4^\circ\text{C}$ . After low spinal transection of frogs, sartorius muscles weighing approximately 70 to 130 mg were dissected out and were kept overnight in Ringer's solution at  $4^\circ\text{C}$  prior to use (4).

Muscles were generally incubated in stoppered 25 ml Erlenmeyer flasks containing 5 ml of medium and a gas phase of 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . The flasks were shaken 110 times a minute in a Dubnoff incubator. For electrical stimulation, or when tension was to be measured during contractures, muscles were attached to a Lucite rod (6) under a resting tension of 2 g. In experiments involving potassium contracture, muscles were incubated in sodium Ringer's solution at the desired concentration of  $\text{Ca}^{++}$  for 20 min before and after exposure to potassium Ringer's solution having the same concentration of  $\text{Ca}^{++}$ . Finally, the initial rate of penetration of 3-methylglucose- $^3\text{H}$  was measured as described previously (5, 6), using an 8 mM concentration of substrate. It was possible to measure permeability to sugar following the end of potassium contractures because the effects persisted for several hours.

For studies of potassium contracture, it was desirable to have at least a minimum amount of  $\text{Ca}^{++}$  in the medium in order to ensure normal excitability of the muscles (9). On the other hand, for measuring the accumulation of  $^{45}\text{Ca}$  in muscle, it was preferable to keep the amount of nonradioactive calcium in the extracellular space low so that the specific activity of the  $^{45}\text{Ca}$  would not be diminished at the time that muscles were immersed in the radioactive medium. Accordingly, when the rate of accumulation of  $^{45}\text{Ca}$  was to be studied at different intervals after the onset of a potassium contracture, muscles were incubated initially for 10 min at  $19^\circ\text{C}$  in sodium Ringer's solution containing 1 mM  $\text{CaCl}_2$ , then in potassium Ringer's solution containing 1 mM  $\text{CaCl}_2$  for either 1 or 3 min, and then for 3 min in potassium Ringer's solution containing 10 mM  $^{45}\text{CaCl}_2$ . The concentration of  $^{45}\text{Ca}$  refers to the sum of radioactive and nonradioactive forms of calcium in the medium; 1  $\mu\text{c}$  per ml was present in each case. The basal rate of accumulation of  $^{45}\text{Ca}$  was determined by

TABLE I  
INCREASE IN PERMEABILITY TO 3-METHYLGLUCOSE  
IN RESPONSE TO CAFFEINE CONTRACTURE

Muscles were incubated for various periods of time in Ringer's solution containing 4 mM caffeine. The initial rate of penetration of 3-methylglucose,  $v$ , was then measured, and is expressed as micromoles of sugar per milliliter of intracellular water per hour. Each value is the mean for three muscles, and the standard error of the mean is given.

Duration of exposure to caffeine	$v$
<i>min</i>	$\mu\text{moles/ml/hr}$
0	$1.4 \pm 0.2$
15	$2.6 \pm 0.3$
30	$3.0 \pm 0.5$
60	$4.4 \pm 0.9$
120	$6.0 \pm 0.5$

incubating paired control muscles for 20 min in sodium Ringer's solution containing  $^{45}\text{Ca}$ ; the rate was constant for approximately 30 min.

After incubation with  $^{45}\text{Ca}$ , all muscles were washed for 90 min with four changes of nonradioactive Ringer's solution that contained the same concentration of  $\text{Ca}^{++}$  as the radioactive incubation media, according to the procedure of Bianchi and Shanes (10). Each washed muscle was then ground with 1 ml of 5% trichloroacetic acid, which extracted essentially all of the radioactivity from the tissue. Duplicate 0.1 ml aliquots of each extract were added to 2 ml of ethanol and 10 ml of scintillator solution (5) and counted in a liquid scintillation counter.

## RESULTS

### *Effect of Caffeine Contracture on Permeability to Sugar*

Incubation of sartorius muscles in the presence of 4 mM caffeine caused a significant increase in permeability to 3-methylglucose (Table I). Isometric tensions up to 25 g were observed in these muscles. Although there is some

evidence that caffeine can cause irreversible microscopic lesions in muscle cells (11), the increase in permeability to 3-methylglucose exhibited some specificity, as shown by the finding that permeability to mannitol, the extracellular marker used in these studies, was not altered. Studies by Bianchi (12), Frank (13), and Herz and Weber (14) suggest that caffeine contractures may be brought about by the release of  $\text{Ca}^{++}$  within the myoplasm from storage sites in the muscle. Omission of  $\text{Ca}^{++}$  from the incubation medium did not prevent caffeine from producing its effect on permeability to 3-methylglucose. However, it was of interest to explore further the relationship of the  $\text{Ca}^{++}$  content of the medium to the rate of penetration of sugar under other conditions.

*Permeability to Sugar in Resting and Electrically Stimulated Muscles*

Incubation of frog sartorius muscles overnight at 4°C and then for 2 hr at 19°C in a  $\text{Ca}^{++}$ -free Ringer's solution containing 4 mM EDTA did not affect the permeability of resting cells to 3-methylglucose. Moreover, varying the concentration of  $\text{Ca}^{++}$  in the medium from 0.5 to 15 mM affected neither the permeability to sugar of resting muscles that were incubated at 19°C for 2 hr, nor the increase in permeability that resulted from stimulation for 30 min at a frequency of 30 shocks per minute, an amount of stimulation that caused a submaximal increase in permeability.

*Entry of  $^{45}\text{Ca}$  during Potassium Contractures*

In order to examine the relationship between the intracellular concentration of  $\text{Ca}^{++}$  and permeability to sugar, it was desirable to find a more effective means of altering the concentration of free  $\text{Ca}^{++}$  in skeletal muscle cells in a graded manner. It seemed possible that potassium contractures might be suitable for this purpose, because raising the extracellular concentration of  $\text{Ca}^{++}$  is known to increase the duration of contracture (9, 15). In the present studies there was a progressive increase in the duration of potassium contractures as the concentration of  $\text{Ca}^{++}$  was raised from 0.2 to 15 mM. The changes in isometric tension that occurred when muscles were exposed for 3 min to potassium Ringer's solution containing either 0.5 or 10 mM  $\text{Ca}^{++}$  are shown in Fig. 1. It is likely that the tension curves developed by these muscles are, at least in part, a reflection of changes in the concentration of  $\text{Ca}^{++}$  in the myoplasm (16); the initial portion of the mechanical response is probably related to the rapid release of  $\text{Ca}^{++}$  from the sarcoplasmic reticulum, whereas the isometric tension exerted during later stages of the contracture appears to be influenced by the influx of  $\text{Ca}^{++}$  from the surrounding medium (16, 17). The predominant effect of raising the concentration of  $\text{Ca}^{++}$  was to sustain tension at a higher level for a longer period of time (Fig. 1). This result supports the concept that physiologically effective amounts of  $\text{Ca}^{++}$  enter skeletal muscle cells during a potassium contracture.

This concept was tested by measuring the accumulation of  $^{45}\text{Ca}$  in muscles. The rate of accumulation of  $^{45}\text{Ca}$  under different conditions is assumed to reflect the relative ease with which  $\text{Ca}^{++}$  can pass from the medium into the cells, but it is not a measure of the net  $\text{Ca}^{++}$  balance of the cells because efflux of nonradioactive  $\text{Ca}^{++}$  occurs during the period of observation. Muscles were not attached to a supporting rod, and no tension was developed during these contractures. Contractures were initiated by placing muscles in potassium Ringer's solution containing 1 mM nonradioactive calcium. After 1 or 3 min the muscles were transferred to potassium Ringer's solution containing 10 mM  $^{45}\text{Ca}$ , and the accumulation of  $^{45}\text{Ca}$  that occurred in a 3 min period was meas-

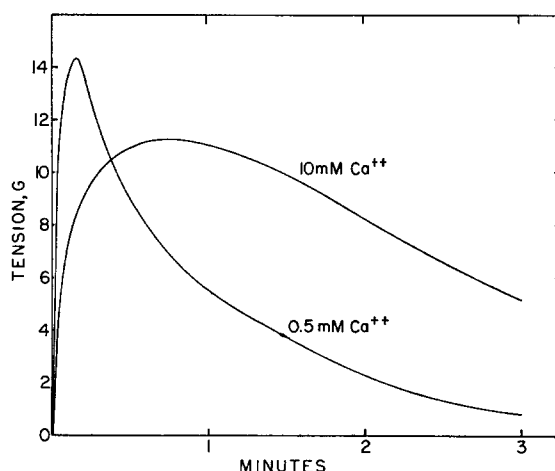


FIGURE 1. Effect of  $\text{Ca}^{++}$  concentration on isometric tension during potassium contractures at  $19^{\circ}\text{C}$ . Curves represent average values of continuous recordings obtained on six paired muscles.

ured as described under Experimental Procedure. In six muscles an average value of  $149 \pm 10 \mu\text{moles}$  of  $^{45}\text{Ca}$  accumulated per g of muscle when the accumulation took place during the 2nd through the 4th min of contracture. When accumulation was studied in paired muscles during the 4th through the 6th min of contracture, an average value of  $26 \pm 5 \mu\text{moles}$  of  $^{45}\text{Ca}$  per g was observed. The basal rate of accumulation for four muscles exposed to sodium Ringer's solution containing 10 mM  $^{45}\text{Ca}$  was  $10.2 \pm 1.5 \mu\text{moles}$  per g per 3 min. Thus, the rate of accumulation of  $^{45}\text{Ca}$  was markedly increased during the early portion of a potassium contracture, and declined rapidly during the contracture, in accord with the findings of Bianchi and Shanes (10). However, in contrast to their observations, the rate of accumulation remained significantly higher than the basal value for more than 3 min after onset of the contracture. The use of a high concentration of  $^{45}\text{Ca}$ , 10 mM, may have facilitated

the demonstration of an increased rate of accumulation over longer periods of time in the present experiments.

Lorković (15) has shown that the amount of  $^{45}\text{Ca}$  accumulating in muscles during a potassium contracture is augmented when the external concentration of  $^{45}\text{Ca}$  is raised from 2 to 5 mM. The data of Table II show that raising the concentration of  $^{45}\text{Ca}$  in the medium from 1 to 10 mM caused an approximately fivefold increase in the accumulation that occurred during 3 min of exposure to potassium Ringer's solution. The amount of accumulation that occurred during a 3 min-contraction in the presence of 10 mM  $^{45}\text{Ca}$  in Table II is greater than the accumulation that occurred in the experiment described in the preceding section. Two factors that may help to account for this difference are:

TABLE II  
ACCUMULATION OF  $^{45}\text{Ca}$  IN MUSCLE DURING  
POTASSIUM CONTRACTURES IN THE PRESENCE OF  
DIFFERENT CONCENTRATIONS OF  $^{45}\text{Ca}$

Muscles were rinsed in Ringer's solution without added  $\text{Ca}^{++}$  for 10 min at  $19^\circ\text{C}$ , then equilibrated with sodium Ringer's solution containing 1 or 10 mM  $^{45}\text{Ca}$  for 7 min, and then incubated for 3 min in potassium Ringer's solution containing the same concentration of  $^{45}\text{Ca}$ . Finally, the muscles were washed with Ringer's solution four times, and a trichloroacetic acid extract was counted as described under Experimental Procedure. Basal accumulation of  $^{45}\text{Ca}$  was measured in sodium Ringer's solution. There were eight muscles in each group.

$^{45}\text{Ca}$ in medium	Condition of muscle	Accumulation of $^{45}\text{Ca}$
<i>mM</i>		<i>mμmoles/g/3 min</i>
1	Basal	$0.8 \pm 0.1$
1	Contracture	$59 \pm 4.0$
10	Basal	$7.4 \pm 0.9$
10	Contracture	$289 \pm 17$

a) the extracellular space of the muscles used for the experiments of Table II was filled with  $^{45}\text{Ca}$ , in sodium Ringer's solution, prior to inducing the contracture; (b) an earlier portion of the contracture was studied in Table II.

Although the basal rate of accumulation of  $^{45}\text{Ca}$  in muscles incubated in sodium Ringer's solution was quite low, even this small amount of accumulation may have been accounted for, in part, by binding of  $^{45}\text{Ca}$  to connective tissue (18). Therefore, it is possible that the true rate of basal penetration into the cells was lower than the observed rate of accumulation. On the other hand, it is reasonable to assume that most of the increment of accumulation that occurred upon exposure to potassium Ringer's solution represents an actual entry of  $^{45}\text{Ca}$  into the cells.

*Effect of Potassium Contracture on Permeability to 3-Methylglucose*

The permeability of muscle to 3-methylglucose was found to be increased significantly as a result of a potassium contracture. In addition, permeability continued to increase even after the muscle was transferred to sodium Ringer's solution at the end of the contracture; this phenomenon is similar to the further increase of permeability that was previously observed following a course of brief, intense electrical stimulation (6). When permeability to 3-methylglucose was measured in Ringer's solution immediately following 3 min of exposure to potassium Ringer's solution, the average rate of penetration of sugar for eight muscles was  $2.0 \pm 0.3$   $\mu$ moles per ml of cell water per hr. In contrast, when paired muscles were allowed to rest in sodium Ringer's solution for 20 min at 19°C after contracture and prior to measuring permeability, the rate of penetration of sugar was  $3.7 \pm 0.4$ ; this value is significantly higher than the preceding one ( $p < 0.01$ ). The basal rate of penetration of sugar was  $0.9 \pm 0.2$ .  $\text{CaCl}_2$  was present at a concentration of 5 mM in the media used for these experiments, as well as in the media for the following experiments of this section.

When muscles were left in sodium Ringer's solution until they had developed their full change in permeability to sugar following a potassium contracture, no further increase or decrease of permeability was found during several hours of additional incubation at 19°C. Thus, 20 min following the second of two 4 min potassium contractures spaced 20 min apart, the rate of penetration of 3-methylglucose was  $7.6 \pm 0.5$   $\mu$ moles per ml of cell water per hr, and 3 hr later the rate in paired muscles was  $7.3 \pm 0.5$ . This prolonged maintenance of a constant, elevated level of permeability after potassium contractures is similar to the situation that was found previously after cessation of electrical stimulation (6).

After two 3 min potassium contractures, exposure to potassium Ringer's solution for an additional 20 min, either continuously or intermittently, caused a decline rather than an increase in permeability. The reason for this gradual inhibition of permeability by a higher concentration of  $\text{K}^+$  is not known; Kipnis and Parrish (19) have reported that high concentrations of  $\text{K}^+$  impair the uptake of 2-deoxyglucose by fully insulinized rat diaphragms. In order to minimize this inhibitory effect, short periods of exposure to potassium Ringer's solution were employed for most of the present studies on sugar transport.

*Effect of  $\text{Ca}^{++}$  Concentration in the Medium on the Change in Permeability to Sugar Associated with a Potassium Contracture*

When muscles were exposed to potassium Ringer's solution for 3 min, the increase in permeability to 3-methylglucose was five times greater at 5 mM  $\text{Ca}^{++}$  than at 0.2 mM (Fig. 2). Further increases in the concentration of  $\text{Ca}^{++}$  had

little additional effect. It is important to bear in mind that, although the muscles were exposed to potassium Ringer's solution for 3 min in each instance, contractures lasted for only about 1 min at a  $\text{Ca}^{++}$  concentration of 0.2 mM, whereas they lasted for the whole 3 min at 2 mM and higher concentrations.

Contractures of more comparable duration are obtained at 0.5 and 10 mM  $\text{Ca}^{++}$  when the period of exposure to potassium Ringer's solution is limited to 2 min (Fig. 1). When sodium Ringer's solution is substituted for potassium Ringer's solution at the end of 2 min, tension returns to base line values in

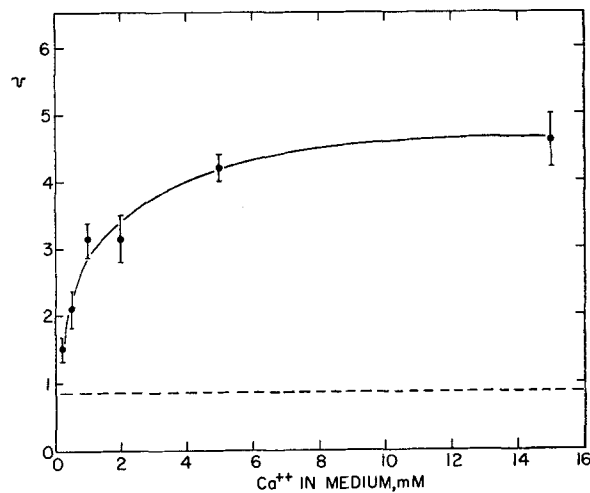


FIGURE 2. Changes in permeability to 3-methylglucose resulting from potassium contractures in the presence of varying concentrations of  $\text{Ca}^{++}$ . Muscles were incubated for 20 min at 19°C in Ringer's solutions containing different concentrations of  $\text{Ca}^{++}$  ranging from 0.2 to 15 mM. Each muscle was then transferred for 3 min to potassium Ringer's solution containing the same concentration of  $\text{Ca}^{++}$ . Next, the muscles were again placed for 20 min in sodium Ringer's solution of the same composition as that used originally. Finally, the initial rate of penetration of 3-methylglucose was measured. Each point represents the mean of four to eight muscles, and vertical bars indicate two times the standard error. The basal rate of penetration of 3-methylglucose in unstimulated muscles incubated in normal Ringer's solution is shown by the dotted line.

approximately 5 sec at 0.5 mM  $\text{Ca}^{++}$  and in 20 sec at 10 mM  $\text{Ca}^{++}$ . When muscles were exposed to potassium Ringer's solution containing 0.5 mM  $\text{Ca}^{++}$  for 2 min, the average rate of penetration of 3-methylglucose, measured 20 min after the contracture, was  $1.82 \pm 0.22$   $\mu\text{moles per ml of cell water per hr}$  in ten muscles; the rate in paired muscles that underwent contracture in the presence of 10 mM  $\text{Ca}^{++}$  was  $2.86 \pm 0.26$   $\mu\text{moles per ml per hr}$ . The difference between the means is significant ( $p < 0.01$ ). In these studies of sugar permeability the muscles were free of attachments, and shortened in potassium Ringer's solution without developing tension.



## DISCUSSION

Isolated frog sartorius muscles become more permeable to sugar when contractions are elicited with electrical stimulation (6). Contractures caused by caffeine are also accompanied by significant increases in permeability to sugar (Table I). Since caffeine at low concentrations produces contractures without depolarizing the plasma membrane (20), the results suggest that the changes in permeability to sugar that occur after electrical stimulation may not be intimately related to the depolarization process. Furthermore, changes in permeability to sugar develop relatively slowly after electrical stimulation, and are not limited to the period of depolarization (6).

Interaction of actin and myosin at multiple sites along the filaments, which is responsible for the shortening of muscle fibers (21), appears to involve the breakdown of ATP (22). More ATP and phosphocreatine are broken down per twitch when the work load is increased (23, 22, 6). In contrast, varying the work performed per twitch does not modify the change in permeability to sugar that accompanies contraction (6). This last observation suggests that alteration of permeability to sugar, unlike the breakdown of high-energy phosphate compounds, is not a function of the number of interactions that take place between actin and myosin filaments. Chemical analyses have also shown that the magnitude of the change in permeability to sugar that occurs after a given series of isotonic twitches is not related to the amount of lactate formed or to the extent of breakdown of creatine phosphate (6). Since the changes in permeability to sugar that are associated with muscle contraction are not directly dependent upon membrane depolarization or the intrinsic contractile process, it is possible that the third fundamental event in contracting cells, a change in  $\text{Ca}^{++}$  concentration, mediates the changes in permeability. The observations that have been described suggest the hypothesis that an increase in concentration of  $\text{Ca}^{++}$  in the myoplasm is a step that is common to two separate pathways, one leading to muscle contraction, and the other to an alteration of permeability to sugar.

The concept that calcium ions are involved in the alterations of permeability to sugar receives some support from studies of potassium contractures. Contractures in potassium Ringer's solution result in an enhanced permeability to sugar and this effect is greater in the presence of higher concentrations of  $\text{Ca}^{++}$  (Fig. 2). The amount of  $^{45}\text{Ca}$  that enters frog sartorius muscles during a potassium contracture is also augmented at higher concentrations of  $^{45}\text{Ca}$  in the medium (Table II; reference 15). Moreover, in agreement with the reports of Frank (9) and Lorković (15), it has been found (Fig. 1) that the duration of potassium contractures is prolonged when the concentration of  $\text{Ca}^{++}$  in the medium is raised. These results suggest that calcium ions that enter muscle cells during a potassium contracture contribute significantly towards maintaining a

high level of free  $\text{Ca}^{++}$  in the myoplasm, and that the amount of  $\text{Ca}^{++}$  that enters can be varied by altering the external concentration.

In the initial experiments the duration of potassium contracture was not the same at all concentrations of  $\text{Ca}^{++}$ . However, in other studies, when groups of muscles that contracted for approximately 2 min were compared, it was still found that permeability to sugar was significantly greater in muscles that contracted in the presence of 10 mM  $\text{Ca}^{++}$  than in muscles that contracted in 0.5 mM  $\text{Ca}^{++}$ . These muscles were free of any attachments, so that neither group developed any tension. The exact duration of contracture in individual fibers of an intact muscle is difficult to control or measure, but the findings suggest that the changes in permeability to sugar that accompany potassium contractures may reflect the extent of elevation of concentration of  $\text{Ca}^{++}$  in the myoplasm as well as the duration of this rise.

The influx of  $\text{Ca}^{++}$  from the medium is believed to make only a minor contribution to any rise in concentration of  $\text{Ca}^{++}$  that occurs in the myoplasm of electrically stimulated skeletal muscle cells (24–26). In skeletal muscle cells that have been stimulated electrically, contraction appears to be initiated by calcium ions that are released from a cellular storage site, possibly the endoplasmic reticulum (21, 26–29).

In electrically stimulated muscles, the brief duration of twitches suggests that calcium ions remain elevated for only a fraction of a second after each excitation (16). However, prolonged stimulation at frequencies ranging from 3 to 20 shocks per minute leads to different plateau values of permeability to sugar that are proportional to the frequency of stimulation (6). This relationship suggests that changes in the concentration of  $\text{Ca}^{++}$  in the myoplasm do not alter permeability by a direct interaction with the cell membrane, but initiate another reaction of slower time course, and that this second reaction then affects permeability in a graded fashion. The occurrence of a further increase in permeability to sugar following the end of a potassium contracture or following a brief period of intense electrical stimulation (6) supports this concept. The nature of this hypothetical reaction of slower time course remains to be elucidated.

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