

# The Influence of Hydrogen Ion Concentration on Calcium Binding and Release by Skeletal Muscle Sarcoplasmic Reticulum

YOSHIAKI NAKAMARU and ARNOLD SCHWARTZ

From the Division of Myocardial Biology, Baylor College of Medicine and the  
Fondren-Brown Cardiovascular Research and Training Center, Methodist Hospital,  
Houston, Texas 77025

**ABSTRACT** Calcium release and binding produced by alterations in pH were investigated in isolated sarcoplasmic reticulum (SR) from skeletal muscle. When the pH was abruptly increased from 6.46 to 7.82, after calcium loading for 30 sec, 80–90 nanomoles (nmole) of calcium/mg protein were released. When the pH was abruptly decreased from 7.56 to 6.46, after calcium loading for 30 sec, 25–30 nmole of calcium/mg protein were rebound. The calcium release process was shown to be a function of pH change: 57 nmole of calcium were released per 1 pH unit change per mg protein. The amount of adenosine triphosphate (ATP) bound to the SR was not altered by the pH changes. The release phenomenon was not due to alteration of ATP concentration by the increased pH. Native actomyosin was combined with SR in order to study the effectiveness of calcium release from the SR by pH change in inducing superprecipitation of actomyosin. It was found that SR, in an amount high enough to inhibit superprecipitation at pH 6.5, did not prevent the process when the pH was suddenly increased to 7.3, indicating that the affinity of SR for calcium depends specifically on pH. These data suggest the possible participation of hydrogen ion concentration in excitation-contraction coupling.

## INTRODUCTION

Since the initial experiments of Huxley and his coworkers, which clearly indicated that local stimulation could evoke muscle contraction (1–3), it has been generally assumed that contraction of striated muscle is caused by a release of calcium from sites on the terminal cisternae of the sarcoplasmic reticulum (SR) by passage of local current through this membrane area (4–7).

In a preliminary paper (8) we reported that isolated SR from both skeletal muscle and heart muscle rapidly released previously bound calcium when the

pH of the medium was abruptly increased. Different experiments leading to somewhat similar conclusions have been reported recently (9, 10). Using a triple-barreled electrode technique, Carter et al. (11) suggested that intracellular pH (pH<sub>i</sub>) of rat skeletal muscle may be a function of membrane potential, a prediction made some time ago by Conway (12). While controversial (13), if this is true, propagation of depolarization through excitable intracellular membranes might be accompanied by specific pH changes that may release calcium from the sarcoplasmic reticulum.

The purpose of this paper is to ascertain conditions for the reversible release and binding of calcium ions by isolated SR as influenced by pH in the presence of adenosine triphosphate (ATP). Since it is important that the magnitude and velocity of calcium release by alterations in pH are great and high enough, respectively, to cause rapid contraction, superprecipitation of native actomyosin, as influenced by pH and affinity of sarcoplasmic reticulum for calcium, was studied.

#### METHODS

##### *A. Preparation of Skeletal Muscle "Microsomes" (SR; Relaxing System)*

Sarcoplasmic reticulum fragments were prepared from dog skeletal muscle by a minor modification of procedures employed in this laboratory (14, 15). 5 g of muscle were cut into small pieces and, after washing three times, were homogenized in 20 ml of 10 mM NaHCO<sub>3</sub> with a Polytron PT-20 (Brinkmann Instruments Inc., Westbury, N. Y.), twice each for 10 sec at a rheostat setting of 1.5. The remainder of the procedure was essentially the same as previously described (14, 15).

##### *B. Preparation of Native Actomyosin*

300 g of dog skeletal muscle were washed with ice-cold distilled water, finely minced, and suspended in 3 vol of Weber-Edsall solution consisting of 0.6 M KCl, 0.1 M Na<sub>2</sub>CO<sub>3</sub>, and 0.04 M NaHCO<sub>3</sub> at a pH of 8.4. The suspension was kept at 2°C for 24 hr and was then diluted to 2.5 vol by the addition of 0.6 M KCl and was passed through several layers of cheesecloth. The resulting filtrate was slowly added to cold distilled water to make a final concentration of 0.2 M KCl, and the pH was adjusted to 6.5 with 1 M acetate. After 1 hr the resulting turbid solution was centrifuged at 2500 g for 15 min and the pellet was dissolved in 0.6 M KCl (final concentration) by adding 2 M KCl and adjusting the pH to 6.8. After 3 hr the solution was centrifuged at 20,000 g for 15 min to remove any denatured actomyosin and other proteins. The ionic strength of the supernate was lowered to 0.25 M KCl by adding cold distilled water slowly with careful stirring. After centrifugation at 7000 g for 10 min the resulting precipitate was dissolved by raising the ionic strength to 0.6 M KCl. The same precipitation and solubilization procedure was repeated and the final actomyosin solution in 0.6 M KCl was stored with an equal volume of glycerin at -18°C. Just before use, the suspension was washed first with 10 vol of distilled water, then twice with 50 mM KCl, and finally suspended in 0.2 M KCl.

### C. Measurement of Calcium Binding Activity of Sarcoplasmic Reticulum

After preincubation for 2 min at 37°C, the reaction was started by the addition of 0.2 ml of the SR suspension (0.5–0.8 mg protein) to 0.8 ml of a solution containing (in final concentration) 40 mM tris(hydroxymethyl)amino methane (Tris)-maleate (pH is indicated under each figure), 4 mM MgCl<sub>2</sub>, 0.1 mM <sup>45</sup>CaCl<sub>2</sub> (representing about 15,000 cpm), and 2 mM adenosine triphosphate (ATP) (2Na-2Tris form). 5 sec before termination of the reaction, 0.8 ml of the reaction mixture was pipetted and passed through a Millipore filter membrane (HA 0.45 μ; Millipore Corp., Bedford, Mass.). The filtration required about 2 sec. The filtrate (0.1 ml) and the filter membrane were added separately to vials containing 10% Bio-Solve Solubilizer (BBS-3) and Fluoralloy (Beckman Instruments, Inc., Fullerton, Calif.) in a toluene base and counted in a Beckman liquid scintillation spectrometer (Beckman Instruments, Inc.). Extravesicular contaminating calcium, contributed by both the SR preparation (20 nmole Ca<sup>++</sup>/mg protein) and other added ingredients (3 nmole Ca<sup>++</sup>/ml), was measured by the murexide method (14, 15) as follows: EGTA [ethylene glycol bis(β-iaminoethyl ether) *N,N,N',N'*-tetraacetate] was added to the reaction medium until no change of murexide was observed. In other experiments, contaminating calcium was bound to the SR by the addition of ATP (without added calcium). From the decrease of absorption of murexide at 507 and 542 nm, the total amount of contaminated calcium was calculated. Contaminated calcium by other ingredients was measured without SR by EGTA titration. Contaminated calcium from SR was calculated as a difference. These amounts indicate free calcium concentration in the solution and must be taken into account in the calculation of calcium binding by the Millipore filter method because of dilution of specific radioactivity of added <sup>45</sup>Ca. An amount of total calcium (43 nmole/mg), which includes the contaminated free calcium contributed by the SR and calcium *bound* to SR, was also titrated by EGTA in the presence of murexide after complete decomposition in 10 N H<sub>2</sub>SO<sub>4</sub> at 170°C for 2 hr, with dropwise addition of 3% H<sub>2</sub>O<sub>2</sub>, and subsequent neutralization by 1.6 M KOH. Controls were carried out in the same manner without SR. An amount of calcium bound by SR (23 nmole/mg protein) was estimated as a difference between the total calcium and the contaminated free calcium. This amount was not, however, included in the correction of the contamination, since determination of exact exchangeability of this amount is unclear.

### D. Measurement of Superprecipitation of Native Actomyosin

The principle is based upon the turbidimetric procedure developed originally by Ebashi (16). Actomyosin, suspended in 0.2 M KCl, was homogenized with a glass homogenizer and Teflon pestle each time before pipetting. 3 ml of reaction mixture, containing 40 mM Tris-maleate at the desired pH, 100 mM KCl, 4 mM MgCl<sub>2</sub>, and 3 mg protein of actomyosin, were thoroughly homogenized in a small glass homogenizer and immediately introduced into the chamber of an American Instrument Co., Inc., Silver Spring, Md. dual-beam spectrophotometer. A stationary vibrator with a diaphragm on top was placed over the chamber for rapid mixing and to avoid gradual aggregation of the actomyosin. Calcium and ATP were added by injection through the rubber diaphragm into the reaction chamber.

## RESULTS

*Effect of Abrupt Increase of pH in the Presence and in the Absence of ATP*

In preliminary experiments we found that the apparent optimum pH for calcium binding by sarcoplasmic reticulum was between 6.1 and 6.3 (8). This agrees with the data of Sreter (17) and suggests that diminished calcium

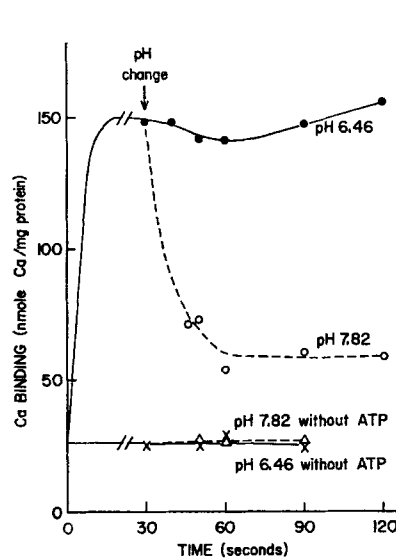


FIGURE 1

FIGURE 1. Calcium release from skeletal muscle sarcoplasmic reticulum by pH increase. Conditions: 40 mM Tris-maleate (pH 6.46), 4 mM MgCl<sub>2</sub>, 10<sup>-4</sup> M <sup>45</sup>CaCl<sub>2</sub>, 2 mM ATP, 90 mM KCl, and 0.85 mg protein, at 37°C. Solid lines represent experiments in which pH was maintained at 6.46. Dashed lines represent experiments in which pH was increased by the addition of 20 μl of 1 M Tris to 7.82 at 30 sec after calcium binding at pH 6.46.

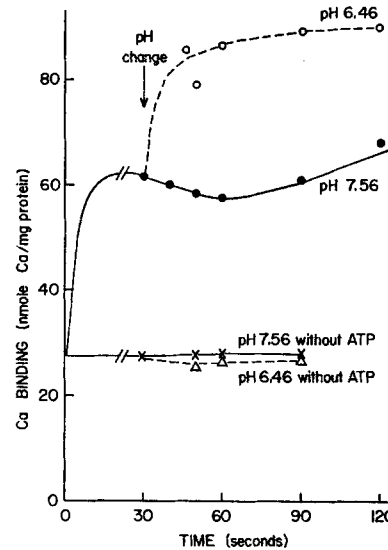


FIGURE 2

FIGURE 2. Calcium binding to skeletal muscle sarcoplasmic reticulum by pH decrease. Conditions: 40 mM Tris-HCl (pH 7.56), 4 mM MgCl<sub>2</sub>, 10<sup>-4</sup> M <sup>45</sup>CaCl<sub>2</sub>, 2 mM ATP, 120 mM KCl, and 0.72 mg protein, at 37°C. Solid lines represent experiments in which pH was maintained at 7.56. Dashed lines represent experiments in which pH was decreased by the addition of 20 μl of 0.2 M maleic acid to 6.46 at 30 sec after calcium binding at pH 7.56.

binding at higher pHs might be explained in either of two ways: (a) a simple decrease of net calcium binding, or (b) an increase in calcium release induced by a more alkaline pH. If the latter were true, it would be expected that calcium bound to the SR might be immediately released upon an abrupt elevation of pH, in the presence of ATP. The experiment shown in Fig. 1 was designed to demonstrate the latter possibility. Calcium was bound first at pH 6.46. When this pH was maintained, calcium release was found to be minimal, if any. However, when the pH was rapidly increased from 6.46 to 7.82 by the

addition of 20  $\mu$ l of 1 M Tris 30 sec after initiation of calcium binding by ATP, an abrupt release of calcium was observed. The amount of calcium released by this alteration of pH was from 80 to 90 nmole of calcium/mg protein. It is noteworthy that the release process appears to be rapid; furthermore, in the absence of ATP no change was observed by the pH alteration. The latter strongly suggests that it is only that fraction of calcium bound in an energy-dependent process that can be released by alterations of pH.

We tested to see if a change in pH might lower ATP concentration. The latter could be responsible for an *apparent* calcium release when the pH was increased from 6.5 to 7.8, since the concentration of ATP is critical for activation of calcium binding (18). We added increasing increments of ATP, enough to adjust the concentration of ATP at pH 7.8 to approximately the same amount present at pH 6.5. Calcium release was not prevented (and in fact was the same as at standard conditions) even in the presence of an ATP-regenerating system, suggesting that calcium release by pH change is not due to diminution of ATP or to alteration of ADP or inorganic phosphate. The data further suggest that elevation of pH probably causes a diminution of calcium affinity to an "energized" sarcoplasmic reticular membrane and imply that the diminished affinity might be "reversibly" restored by decreasing the pH. The experiment depicted in Fig. 2 indicates that this is a possibility.

#### *Effect of Abrupt Decrease of pH in the Presence and in the Absence of ATP*

At a pH of 7.56, 60 nmole of calcium/mg protein were bound at equilibrium conditions. This may be contrasted with the 150 nmole of calcium/mg of protein bound at equilibrium at a pH of 6.46 (Fig. 1). When the pH was abruptly decreased from 7.56 to 6.46, 30 sec after the initiation of calcium binding by ATP, 25–30 nmole of calcium/mg protein were bound by the SR, in addition to the 60 nmole of calcium/mg protein already bound (Fig. 2). Again, in the absence of ATP, alterations of pH in this range did not cause any significant change in calcium binding by the sarcoplasmic reticulum (Fig. 2).

#### *ATP-<sup>14</sup>C Binding to Sarcoplasmic Reticulum as a Function of pH*

A number of investigators (6, 19–21) have suggested that calcium binding by the SR requires ATP, possibly because the nucleotide causes a conformational change resulting in an exposure of calcium binding sites. It is possible, therefore that the amount of ATP binding to the SR membrane might be significantly changed by the alterations in pH and that the pH-induced changes in calcium binding may be a secondary event. The results in Table I, however, indicate that the amount of ATP-<sup>14</sup>C bound to the SR before binding of calcium was unchanged by alterations in pH. Consequently, the data shown in Figs. 1 and 2 cannot be directly due to alterations in ATP binding to the sarcoplasmic reticulum.

TABLE I  
ATP-<sup>14</sup>C BINDING BY SARCOPLASMIC RETICULUM AND pH

pH	ATP- <sup>14</sup> C binding
	<i>nmole ATP/mg protein</i>
6.5	0.32
7.8	0.33
After 30 min, pH is changed from 6.5 to 7.8	0.32

0.3 ml of SR suspension (8.2 mg/ml) was added to 0.7 ml of reaction mixture containing (as a final concentration) 40 mM Tris-maleate (pH 6.5), 4 mM MgCl<sub>2</sub>,  $2 \times 10^{-5}$  M ATP-<sup>14</sup>C (about 40,000 cpm), 2 mM phosphoenol pyruvate, and 0.1 mg pyruvate kinase (ATP-regenerating system), at 37°C. After 50 sec the reaction mixture was centrifuged at 150,000 *g* for 5 min and the supernatant was discarded. The inside of the centrifuge tube was then carefully wiped. The pellet was dissolved with 0.3 ml of Hyamine overnight and added to a scintillation vial containing 1.5 ml of ethanol, in addition to scintillation medium (see Methods). Each value for ATP-binding represents the average of three experiments.

*Possible Relationship Between pH and Calcium Release from Sarcoplasmic Reticulum*

The pH was increased from 6.45 to various levels 30 sec after the initiation of calcium binding by ATP and bound calcium was measured 20 sec after the abrupt pH changes. The amount of calcium released from the SR was proportional to pH in a range between 6.45 and 7.7; the calcium released was 57 nmole/mg of sarcoplasmic reticulum per 1 pH unit. These data suggest that approximately 85 nmole of calcium would be expected to be released from 1 mg of SR during a change of the membrane potential from 90 mv, provided intracellular pH is, in fact, a function of the membrane potential (11, 12). In another experiment (data not shown), using dual-beam spectrophotometry, calcium release from the SR induced by pH changes was measured. The release of calcium was 70 nmole/mg protein per 1 pH unit change. These values are well within the proposed alterations in calcium required for muscle contraction (22). The velocity of calcium release at the initial stage of pH change is still not clear, since the Millipore filter method only allows measurement at a minimum of 10 sec after the pH alteration. Nevertheless, the data in Figs. 1 and 2 indicate that both calcium binding and release by pH alterations are rapid processes. Comparing these data with those obtained by the murexide method (8), 46 nmole of calcium/mg protein were released 5 sec after the elevation of pH from 6.63 to 7.56 (30 sec after ATP addition), which is similar to the amount of calcium bound in the first 5 sec after ATP addition, namely 54 nmole of calcium/mg protein.

*Superprecipitation of Native Actomyosin as Influenced by Sarcoplasmic Reticulum and pH*

In order to determine whether the amount of calcium released by the pH changes might be enough to cause contraction, superprecipitation of native actomyosin was estimated in the presence and in the absence of sarcoplasmic reticulum. At a pH of 6.5, the addition of 80 nmole of calcium instantaneously caused superprecipitation; 0.68 mg protein of SR completely inhibited superprecipitation at this pH for 6 min after ATP addition (Fig. 3). Thereafter,

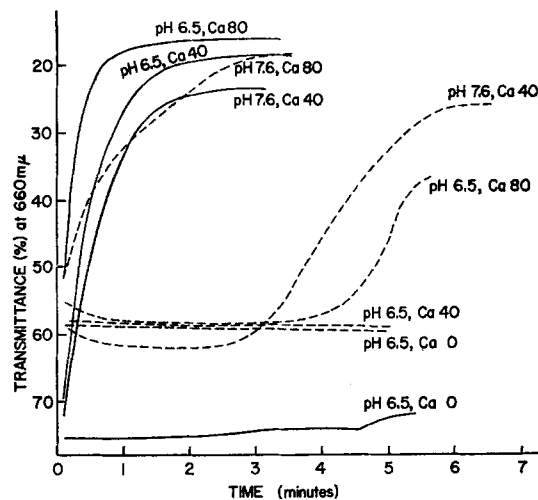


FIGURE 3. Effects of sarcoplasmic reticulum and pH on the superprecipitation of native actomyosin of skeletal muscle. Turbidity change was recorded at 660 nm after initial stirring (see Methods). Conditions: 40 mM Tris-maleate (pH 6.5 or 7.6), 4 mM MgCl<sub>2</sub>, 100 mM KCl, 1.3 mM ATP, 3 mg actomyosin protein/3 ml, and 0.68 mg sarcoplasmic reticulum protein/3 ml. Reaction was started by the addition of ATP. Dashed lines represent experiments with SR, solid lines without SR. The amount of calcium indicated in the figure is expressed as nanomoles.

gradual superprecipitation occurred, implying a slow release of calcium from the sarcoplasmic reticulum. The same experiment repeated at pH 7.6, however, shows that the same amount of SR could not inhibit superprecipitation in the presence of 80 nmole of calcium. When the addition of calcium was decreased to 40 nmole, this amount of SR (0.68 mg of protein) completely inhibited superprecipitation for 5 min. This suggests that the inhibition of superprecipitation by SR at 7.6, in the presence of 80 nmole of calcium, was not caused by the high pH itself. It is concluded that the affinity of the SR for calcium at pH 7.6 is low and, hence, at a sufficiently high concentration of calcium, superprecipitation of actomyosin can occur. When calcium concentration is significantly lowered, however, and the same concentration of SR is

maintained, insufficient calcium is available for interaction with actomyosin due to some binding of calcium by the sarcoplasmic reticulum. Employing the rubber diaphragm mixing device through which microinjections could be made, the rate of superprecipitation with respect to pH alterations was continuously measured. At a pH of 6.5, the turbidity changes were more rapid and greater (line 1, Fig. 4) than at pH 7.6 (line 3, Fig. 4) in the presence of 80 nmole of calcium. When SR was present, superprecipitation was completely inhibited at pH 6.5 (line 4), although some degree of turbidity increase was observed at the initial stage. The same amount of sarcoplasmic reticulum, however, did not prevent superprecipitation at pH 7.6 (line 2). These observations coincide with the data shown in Fig. 3. When the pH was abruptly

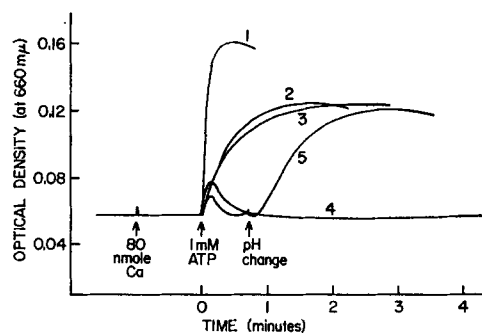


FIGURE 4. Superprecipitation of skeletal muscle native actomyosin by calcium released from sarcoplasmic reticulum by pH change. Turbidity change was recorded at 660 nm with continuous mixing by a stationary vibrator. Conditions: 40 mM Tris-maleate, 4 mM MgCl<sub>2</sub>, 100 mM KCl, 3 mg actomyosin protein/3 ml, and 0.68 mg SR protein/3 ml at 25°C. Line 1, pH 6.5 without SR; line 2, pH 7.6 with SR; line 3, pH 7.6 without SR; line 4, pH 6.5 with SR; line 5, with SR; pH was changed from 6.5 to 7.3. Reaction was started by the addition of 1 mM ATP.

changed from 6.5 to 7.3 by the addition of 20  $\mu$ l of 1 M Tris 45 sec after the addition of ATP, superprecipitation was evoked (line 5, Fig. 4) at the same rate as in the experiments shown in lines 2 and 3 with the exception of a few seconds lag time after the pH change. This indicates that a sufficient amount of calcium to cause superprecipitation was released by a pH increase of 0.8 units. The fact that the same results were obtained when the pH was altered by the addition of KOH, instead of Tris, excludes the possibility of specific effects of Tris. The reason for the slight lag after the pH change is not clear. It may be that calcium release by [H<sup>+</sup>] alteration requires a few seconds. If this is true, this delay would be greater than that required to explain muscle twitch. On the other hand, it should be pointed out that the mixing was not enough to raise the pH rapidly and uniformly, because at this stage after ATP addition, the characteristics of actomyosin might be different with respect to viscosity and elasticity. Still another possibility to consider is that the lag may



be due to a greater clearing response by the addition of ATP at pH 7.3 than a pH 6.5. The data shown in Fig. 5 indicate that turbidity development at pH 6.5 was, in fact, more rapid and greater than at pH 7.6. In the absence of calcium, some decrease of turbidity (clearing response) was observed immediately after the addition of ATP, at a pH of 7.6, whereas such increase was not observed at a pH of 6.5. Thus, this significant difference may contribute to the explanation of the lag in optical density increase when the pH was changed from 6.5 to 7.3 (Fig. 4).

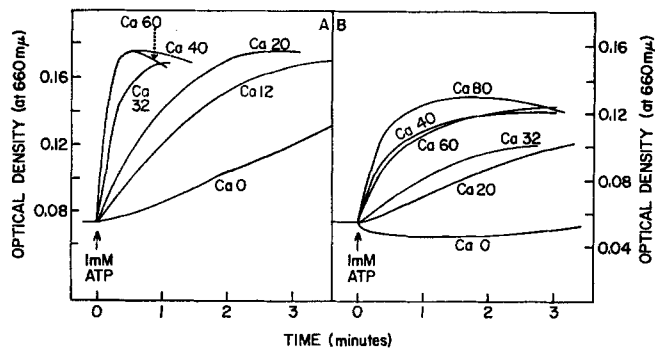


FIGURE 5. Superprecipitation (turbidity development) of skeletal muscle native actomyosin at different pH values. A = pH 6.5; B = pH 7.6. Conditions: 40 mM Tris-maleate, 4 mM  $MgCl_2$ , 100 mM KCl, 2.4 mg actomyosin protein/3 ml at 25°C. Calcium, expressed as nanomoles, was added 1 min before ATP addition.

#### DISCUSSION

The excitation event presumably releases enough calcium from the internal membrane system to initiate the contractile process. Quantitatively, more than 25 nmole of calcium/mg protein (22) must be liberated from the SR in a short period after excitation in order to effect excitation-contraction coupling (7). Conway (12) predicted that in resting skeletal muscle intracellular pH should be lower than extracellular pH. Considering the relationship between the extracellular and intracellular ionic compositions and the Donnan equilibrium, intracellular pH should be approximately 6.0. Contrary to this suggestion, most intracellular pH measurements (dimethyl oxide [DMO],  $CO_2$ , and glass microelectrode procedures) have yielded values from 6.8 to 7.0 (13). These are, interestingly, still lower than extracellular pH. Employing triple-barreled electrodes, it was recently reported that intracellular pH appears to be directly related to the membrane potential of skeletal muscle and to extracellular pH (11), supporting Conway's prediction. If hydrogen ions move across the membrane according to charge distribution, it would be expected that a gradient of hydrogen ions across the excitable membrane would rapidly change with each excitation event (23).

The results presented in this paper suggest that an equilibrium between intra- and extravesicular calcium ions is a function of extravesicular pH in the range 6.4-7.8. Moreover, the achievement of calcium equilibrium, when the pH was rapidly changed, was compared to the initial rate of calcium binding after the addition of ATP (8). The amount of calcium released from the SR by the abrupt changes in pH was enough to cause superprecipitation of native actomyosin. One advantage of postulating pH as an effector or "trigger" in the binding or release of calcium ions from intracellular membranes is that calcium movements can be controlled under conditions wherein concentrations of ATP and metal ligands would remain relatively constant. Although sodium-induced calcium release following membrane depolarization has been suggested (24-26), Katz and Repke (27) reported no *specific* alteration in calcium release from cardiac "microsomes" by either sodium or potassium. We too have not found any significant effect of sodium or potassium on calcium release from isolated SR (data not shown).

Calcium release by pH change described in this paper might be a reflection of a rapid physiological excitation, where protons or electron movement are limited near the excitable membrane (23) or SR membrane and this charge transfer might cause specific changes of intracellular membranes. It remains to be determined whether selective pH changes within the intracellular milieu actually occur. We apply caution, however, to these suggestions since (a) the system described is an *in vitro* artificial one and (b) the amount of calcium released sufficient to cause contraction *in situ* depends on the amount of fully "relaxed" actomyosin present, the amount of extravesicular calcium, the quantity of reticulum "filled" with calcium, the amounts of sarcoplasmic reticulum and actomyosin present in muscle, and undoubtedly many other factors.

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