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Surface Charge and Calcium Binding in Sarcoplasmic Reticulum Membranes

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Abstract

Vesicles of fragmented sarcoplasmic reticulum membranes have been adsorbed on to 2.68 μ latex spheres. Observation of these vesicle containing spheres in the presence of an electric field allows a calculation of the electrophoretic mobility of the vesicles. Following this determination, the net membrane surface charge has been estimated. The mobility of sarcoplasmic reticulum membranes exhibited a dependency on pH. At an ionic strength of 0.10 a mobility (pH = 7.0) of -0.67 ± 0.10 μ/sec/volt/cm was observed. At pH = 7.0 and $\Gamma/2 = 0.150$ the net excess negative charge density was 2.0×10^{-2} coul/m^2. This is equivalent to one charge per 10^3 A^2 (assuming a uniform charge distribution). With an average vesicle volume of 2.8×10^8 A^3 and a surface area of 2×10^6 A^2 the surface of one vesicle would contain a net of approximately 2×10^3 negative charges. While the mobility did not change during uptake of calcium by the vesicles, both glutaraldehyde fixation and lecithin extraction by phospholipase C greatly altered the mobility of the vesicle membrane. Calcium binding and uptake both exhibited a dependence on pH.

Introduction

The ability of fragmented sarcoplasmic reticulum membranes to bind and transport calcium has been extensively studied [1-9]. The fragmented membranes, which form into vesicles ranging in diameter from 0.025 to 0.24 μ, contain cation binding sites. Fragmented S-R from rabbit skeletal muscle has been shown to have a cation binding capacity of 350 μeg/g protein at pH = 7.0 [12]. In the presence of ATP some of these sites show a greatly increased affinity for Ca^{++}.

A number of investigators [5, 10-15] have postulated that the first step in the uptake of calcium by the sarcoplasmic reticulum is the binding of calcium to the FSR membrane. This is supported in

part by the observation that the ATP induced binding of calcium is too rapid to be explained by a transport mechanism [10]. Carvalho [16] has proposed that the "uptake is a binding process in which calcium interacts with fixed binding sites in the membranes and remains bound to maintain electrical neutrality of the membrane phase." The idea of calcium binding to the membrane as a result of site modification induced by ATP binding has also been advanced [15]. The above observations suggested that the determination of the electrophoretic mobility of FSR membranes might provide an indication of surface charge alterations occurring as a result of the binding process.

Information on the surface charge properties of living cell membranes has been obtained by the microscope method of electrophoresis developed by Northrup and Kunitz [17]. This method has been used to characterize the surface charge in normal and pathological states; to investigate the character of ionic groups at the outer surface of intact cells; and to quantitate the changes in surface charge resulting from specific chemical alteration of the cell surface as for example, through the action of enzymes.

Isolated FSR vesicles are not large enough to be examined directly by the microscope method of electrophoresis. In this investigation the vesicles are first adsorbed on to 2.68 μ latex spheres. The spheres containing the adsorbed FSR vesicles can be clearly observed by the method of Northrup and Kunitz. Furthermore, it is generally assumed, following the work of Abramson and Moyer [18], that the electrophoretic mobility of proteins adsorbed to inert particles is essentially the same as the mobility of the protein in solution. Bull [19, 20], in an extension of these studies, concluded that while the agreement between the mobilities of adsorbed proteins and proteins in solution is not exact, they differ by only a small amount. Accordingly, in the present investigation, we are assuming that the electrophoretic mobility of the adsorbed FSR membrane vesicles is approximately the same as their mobility in solution.

In addition to a determination of the mobility (and an estimation of the net surface charge) in the normal state, we have also carried out mobility measurements on FSR vesicles following treatment with glutaraldehyde, triton X-100, phospholipase C, and caffeine. All of these agents alter calcium uptake in isolated FSR vesicles. We have estimated their effect on the net surface charge of the vesicles.

Methods

Preparation of FSR and Measurement of Calcium Transport

Fragmented sarcoplasmic reticulum was obtained from the abdominal muscles of the lobster (*Homarus americanus*), by a

method which has been previously described [9]. Measurements of calcium uptake were performed in the manner described by Fanburg and Gergely [21] using Tricine as a buffer. Microsomal protein was measured by a Folin method with bovine serum albumin as a standard.

Electron Microscopy

Aliquots of the FSR suspension containing two drops of a suspension of polyvinyl Tolelene latex spheres (Dow Chemical Co., Midland, Michigan) (2.68 ± 0.015 μ diameter) (Fig. 1) per 0.30 mg of FSR protein, were fixed by the addition of 10% glutaraldehyde (pH 7.0) to the cuvette (final concentration 2%). After 20 min of fixation at 24°C, the suspension was centrifuged at 10,000 *g* and the resulting pellet was postfixed with 1% osmium tetroxide for 1 h. The pellet was dehydrated in acetone and embedded in a specially prepared resin which was a mixture of Araldite (Ciba Products Co., Summit, N.J.) and Epon 812 (Shell Chemical Co., N.Y.). Sections were prepared with a diamond knife. They were poststained in lead citrate (Fig. 2).

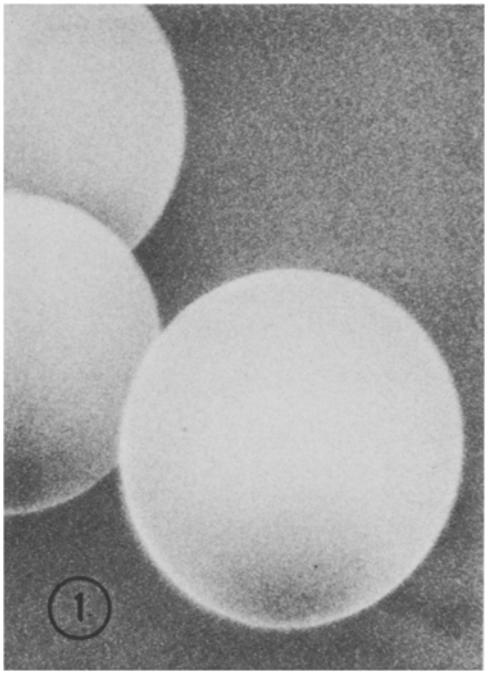

Figure 1. Scanning electron micrograph of 2.68 micron latex spheres. x10,000.

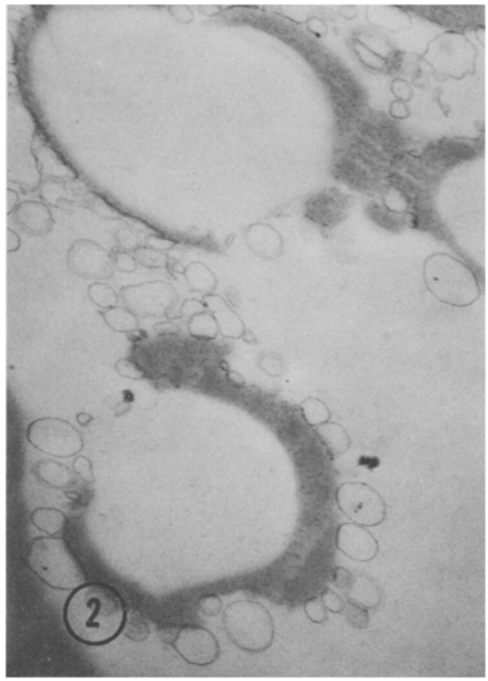

Figure 2. Electron micrograph of pellet section containing spheres with adsorbed FSR vesicles. ×7,500.

Microelectrophoresis

The microelectrophoresis apparatus and method of calibration has previously been described in detail [22]. The location of the stationary layer was calculated and then checked empirically using human red blood cells with a mobility of -1.09 ± 0.05 μ/sec/volt/cm (Ionic strength = 0.145). This calibration was repeated at frequent intervals.

Polyvinyl Tolulene latex spheres were added to the FSR suspension in a solution containing either 100 mM Acetate, 100 mM Tricine or 100 mM Tris buffer, 5 mM $MgCl_2$, and approximately 0.15 mg of FSR protein. Mobility was determined using increasing quantities of latex spheres until the minimum quantity which gave consistent results was determined. This was found to be one drop of the latex suspension per 0.15 mg of FSR protein. Electron microscopy (see above) of spheres containing adsorbed S-R vesicles showed few spheres ($<5\%$) without adsorbed vesicles (Fig. 3).

In a typical run latex spheres were added to a solution containing

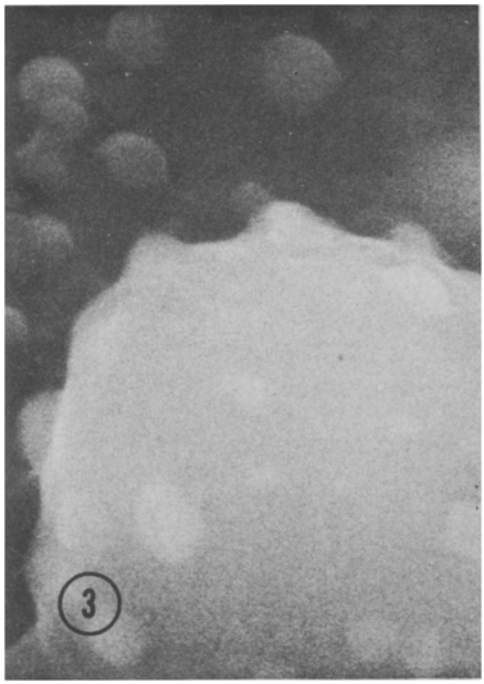

Figure 3. Scanning electron micrograph of spheres containing adsorbed FSR vesicles. x15,000.

100 mM Tris, 100 mM Acetate, or 100 mM Tricine at a preset pH. 0.15 mg of FSR protein was added to this suspension and it was vigorously stirred. The pH was again determined and the solution placed in the electrophoresis cell. Following measurement of the mobility of the spheres containing the adsorbed FSR vesicles, the pH was again determined. If the sample showed a change in pH greater than 0.2 pH units, the run was discarded.

Adsorption of FSR Vesicles on to Latex Spheres

The latex spheres had a diameter of 2.68 ± 0.015 μ (Fig. 1). Following mixing in the FSR suspension, the major portion of the sphere surface was covered with adsorbed FSR vesicles (Figs. 2 and 3). Latex spheres alone had a mobility of -4.80 μ/sec/volt/cm at pH = 7.0. The maximum negative mobility of spheres containing adsorbed FSR vesicles (pH = 7.0) was -3.25 μ/sec/volt/cm (Ionic strength less than 0.010). The spheres containing adsorbed FSR vesicles were stable for periods greater than several hours as evidenced by a constant value of mobility.

Mobility Measurements at Low Ionic Strength

The mobility experiments performed at low ionic strength must be examined with particular caution due to the possibility that the results were influenced by particle surface conductance. As a result of ionic conduction through the double layer, the electrical potential gradient across a particle undergoing electrophoretic motion is reduced. This phenomenon is known as "surface conductance" and is most evident at ionic strengths below 0.01 [23]. Above this value its effect on mobility was found to be negligible [24].

The Dependence of Surface Charge Properties on Ionic Strength

The electrokinetic behavior of charged sarcoplasmic reticulum vesicles will be a function of the ionic strength of the suspending medium. (Theoretically this follows from the proportionality of the Debye length to the reciprocal square root of the ionic strength.) As the ionic strength is lowered, the coulombic screening of charged groups is reduced. Also, ionic groups further from the surface of shear no longer possess counterions that move with the cell as a hydrodynamic unit. These ionic groups are thus "unmasked" and can contribute to the surface charge measured by electrophoretic methods. We have studied the mobility of FSR vesicles at various ionic strengths including experiments at $\Gamma/2$ less than 0.01.

Results

pH Dependence of Mobility at Various Ionic Strengths

The electrophoretic mobility of sarcoplasmic reticulum membranes exhibited a marked dependency on pH (Fig. 4). At an ionic strength of 0.10 (100 mM KCl) a mobility of -0.67 ± 0.10* was observed at pH = 7.0. The negative mobility increased at higher pH, reaching a value of -0.95 at pH = 9.0. At this ionic strength the mobility remained constant from pH 7.0 to 5.0. The mobility decreased abruptly below pH 5.0 and reached a value of -0.05 (nearly isoelectric) at pH = 4.0. The results in a lower ionic strength medium were quite different. A mobility of -2.23 at pH = 7.0 was observed at an ionic strength = 0.01. In this solution the mobility was relatively constant from pH = 9.0 to 6.0. Below pH = 6.0 the mobility dropped sharply reaching a value of -1.23 at pH = 4.0.

Experiments were also carried out at very low ionic strength (less than 0.01). For these experiments, latex spheres containing adsorbed FSR vesicles were added to double-distilled water and the pH adjusted using

* All values of mobility will be in μ/sec/volt/cm.

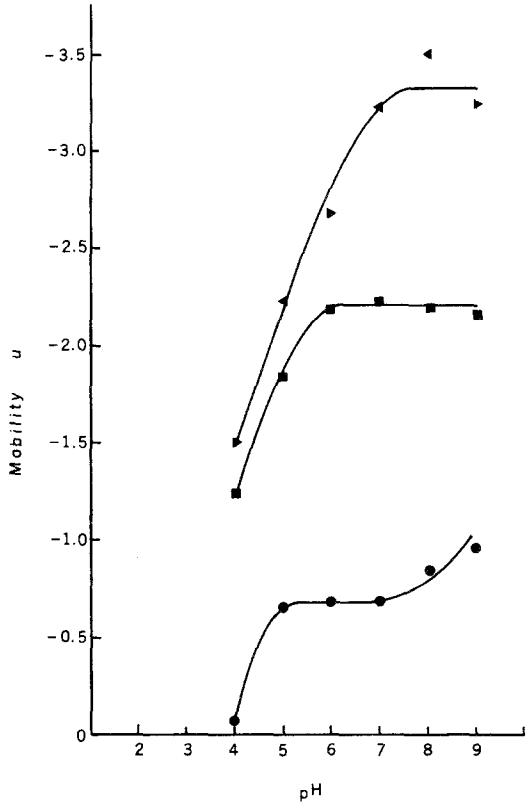

Figure 4. Electrophoretic mobility of absorbed FSR vesicles as a function of pH. Mobility, μ, is in microns/sec/volt/cm. Temp. 24° C. Circles, 100 mM KCl, 100 mM buffer.* Squares, 10 mM KCl, 100 mM buffer. Triangles, adsorbed FSR vesicles in distilled water.

* (Buffer was Tris pH = 4 and 5, Tricine pH = 6 and 7, Acetate pH = 8 and 9.)

0.01 M solutions of HCl or NaOH. The effect on the ionic strength of ions added with the FSR as well as the effect of dissolved CO_2 was not calculated. At this very low ionic strength the mobility showed a direct dependency on pH from a pH = 4.0 to 7.0. The mobility changed only slightly at higher pH's. The mobility had a value of −3.25 at pH = 7.0.

The mobility and therefore the surface charge properties of the sarcoplasmic reticulum membranes showed remarkable stability under the experimental conditions described above. Electrophoretic measurements were completed within 10 min of adjusting the pH. The values of mobility for a given pH could be reproduced following measurement at another pH (i.e. the charge properties of the membrane were not altered during the measurements of mobility).

The Effect of Divalent Cations on Electrophoretic Mobility

Sarcoplasmic reticulum membranes exhibit a high affinity for divalent cations [2]. The electrophoretic mobility measured in a solution containing 10 mM calcium or 10 mM magnesium (Ionic strength = 0.03) had a value of −0.50 at pH = 7.0 (Fig. 5). The mobility was approximately constant from pH = 9.0 through pH = 6.0. The mobility decreased below pH = 6.0 reaching the isoelectric point at a pH of approximately 4.7. At pH = 4.0 the net charge on the membrane became positive with a mobility of +0.75.

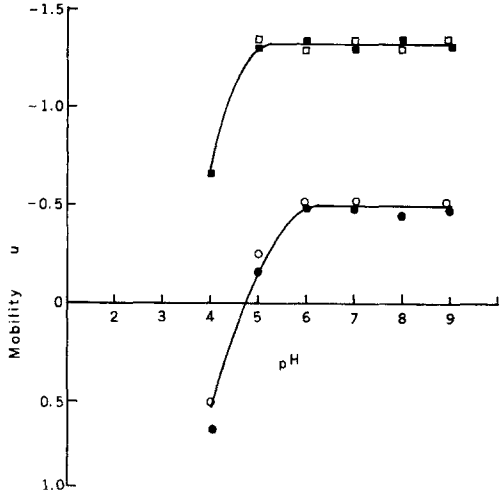

Figure 5. Electrophoretic mobility of adsorbed FSR vesicles in the presence of divalent cations. Solid circles, 100 mM buffer, 10 mM $CaCl_2$. Open circles, 10 mM $MgCl_2$, 100 mM buffer. Solid squares, 100 mM buffer, 1 mM $CaCl_2$. Open squares, 100 mM buffer, 1 mM $MgCl_2$.

At an ionic strength of about 0.003 and a divalent cation concentration of 1 mM, the membrane mobility showed little change over a wide range of pH. The mobility at pH = 7.0 was −1.26. Approximately this same value was maintained from pH = 9.0 through pH = 5.0. Below pH = 5.0 the mobility decreased to −0.63 at pH = 4.0. Both magnesium and calcium effected the mobility to the same extent. It is apparent, therefore, that shifts in bound protons are compensated for by divalent cation binding or release over a wide range of pH.

Dependence of Electrophoretic Mobility on Divalent Ion Concentration at Constant Ionic Strength

The mobility of the FSR vesicles at different divalent ion concentrations was examined at pH = 7.0 and an ionic strength of 0.150.

The mobility was a direct function of the divalent ion concentration, decreasing from a value of −0.66 at 0 mM to −0.02 at 50 mM bulk divalent ion concentration (Fig. 6). The relationship was approximately linear with a slope of 0.013 μ/sec/volt/cm/mM divalent cation.

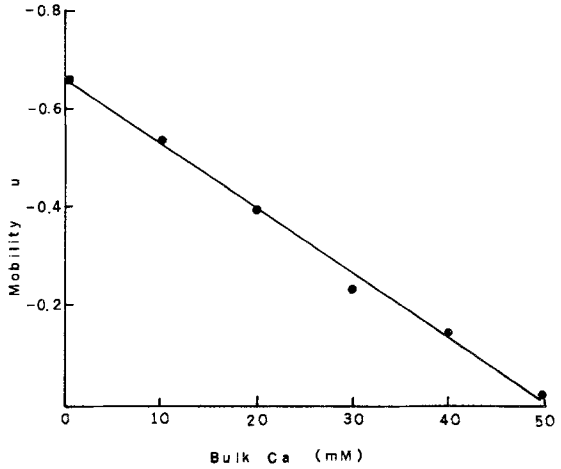

Figure 6. Mobility of adsorbed FSR vesicles as a function of divalent cation concentration at constant ionic strength. $\Gamma/2 = 0.150$. Temp. 24° C.

From the measurements of mobility at constant ionic strength, the surface charge density was calculated using the Gouy-Chapman equation:

$$\sigma^2 = NDkT/2000 \P \sum_i [C(\exp - 1200 \P enZ_i u/DkT - 1)].$$

Where N (Avogardro, s number) = 6.03×10^{23} moles^{-1}, D (dielectric constant–water 25 C) = 78.54, k (Boltzmann's constant) = 1.38×10^{-16} ergs/degree, T = 298 K, C_i and Z_i are the concentration and charge of the species i, e (charge on the electron) = 4.8×10^{-10} esu, n (viscosity of water at 25 C) = 0.89×10^{-2} poise, u (electrophoretic mobility) in μ/sec/volt/cm. The assumptions involved in the use of this equation have been discussed by Overbeek [25] and by Haydon [26].

The charge density at 0.150 ionic strength (pH = 7.0) was calculated to be 5.8×10^{-3} esu/cm^2 in the absence of diavalent cations (Fig. 7). The negative charge density decreased in a nearly linear fashion with increasing divalent cation concentration. The surface showed a decrease of approximately 1.0×10^{-3} esu/cm^2 for each 10 mM of increase in divalent ion concentration.

A surface charge density of 2.0×10^{-2} coul/m^2 is equivalent to one charge per 10^3 A^2 assuming a uniform charge distribution. Assuming an average vesicle volume of 2.8×10^8 A^3 [27] and a surface area of

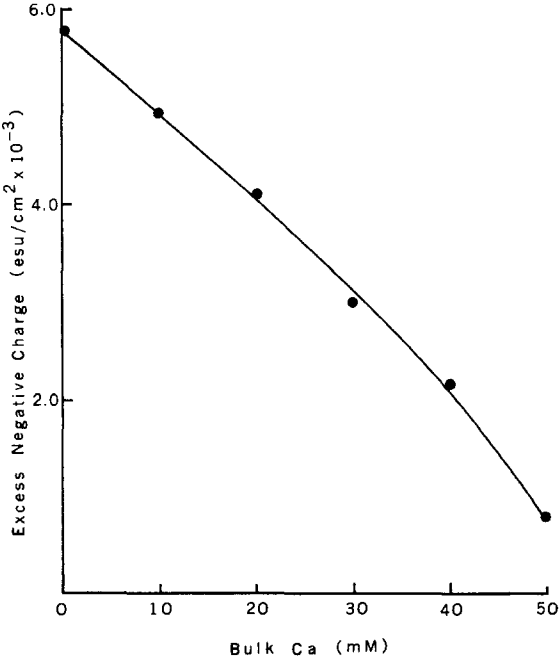

Figure 7. Net excess membrane negative charge as a function of calcium concentration. (Computed using Gouy-Chapman equation.) Temp. 24° C.

2×10^6 A^2, the surface of one vesicle would contain a net of approximately 2×10^3 negative charges.

Mobility Change During Calcium Uptake

The electrophoretic mobility of FSR membranes during calcium binding and uptake was determined (Table I). Following the determination of the mobility of FSR in the reaction mixture, the mobility was again determined after addition of 0.12 mM Ca.

TABLE I. Electrophoretic mobility of adsorbed FSR vesicles during calcium uptake. (Mobility μ, is in microns/sec/volt/cm, $\Gamma/2 = 0.10$, Temp. = 24°C

	0 mM Potassium oxalate	5 mM Potassium oxalate
Vesicles in 100 mM Tricine, 100 mM KCl, 5 mM ATP, and 5 mM $MgCl_2$	−0.87 ± 0.10	−0.68 ± 0.10
0.12 mM $CaCl_2$ added	−0.83	−0.63
0.12 mM $MgCl_2$ added instead of 0.12 mM $CaCl_2$	−0.80	−0.69

Measurements were carried out in reaction mixtures containing oxalate as well as non-oxalate containing mixtures. The presence of active calcium binding and uptake was determined isotopically. The results in experiments with and without oxalate were similar in that in neither case was evidence found for any change in mobility during calcium binding and uptake. The value of mobility measured was determined by the ionic strength and the cations present. The same final value of mobility was found with the addition of 0.12 mM Mg as with the Ca addition.

Mobility of FSR Membranes Following Glutaraldehyde Fixation

Glutaraldehyde has been used as a stabilizing agent in electrophoretic studies on erythrocytes [28]. It has also been used as a fixative for FSR membranes. Following treatment with 2.5 mM glutaraldehyde, both the extra (calcium dependent) ATPase activity and Ca uptake in sarcoplasmic reticulum membranes is inhibited [29]. Present evidence indicates [30] that glutaraldehyde serves to form cross links between neighboring amino bases. Phosphatidyl serine and phosphatidyl ethanolamine as well as lysine residues are thought to participate in the crosslinking reactions.

Glutaraldehyde fixation (2%) (Fig. 8) resulted in a considerable increase in the negative mobility of FSR membranes. The mobility increased from the unfixed value of -0.67 at pH 7.0 (ionic strength $= 0.10$) to -1.42 at the same pH and ionic strength. The negative mobility was increased correspondingly at all pH's. The relationship between pH and mobility in glutaraldehyde fixed FSR vesicles was

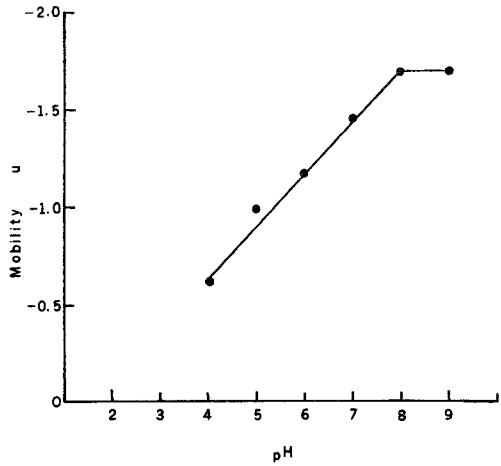

Figure 8. Electrophoretic mobility of adsorbed FSR vesicles following fixation for 10 min in 2% glutaraldehyde. $\Gamma/2 = 0.10$.

almost linear from pH = 4 to pH = 8.0. The region of plateau observed between pH = 5.0 and 7.5 in unfixed vesicles was not observed following glutaraldehyde fixation, i.e. the mobility was a linear function of pH over most of the range examined.

Mobility of FSR Membranes Following Treatment with Triton X-100, Phospholipase C, and Caffeine

The calcium binding and uptake as well as ATPase activity in FSR membranes has been studied following treatment with a variety of agents. Triton X-100, a detergent, is known to alter membrane structure and in the case of FSR vesicles, its action appears to be one of altering calcium uptake. We have measured calcium uptake as a function of Triton X-100 concentration (Fig. 9). Uptake (in the presence of 5 mM

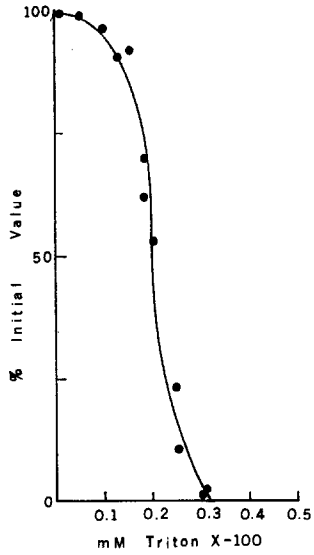

Figure 9. Calcium uptake of Triton X-100 treated FSR vesicles. The reaction mixture contained 10 mM Tricine, 0.12 mM $^{45}CaCl_2$, 5 mM $MgCl_2$, 5 mM potassium oxalate, 5 mM ATP, pH = 7.0. Microsomal protein concentration varied between 0.01 and 0.10 mg/ml. Accumulation time was 10 min. Temp. 24° C. (Vesicles were pre-incubated in Triton X-100 for 10 min.)

oxalate) decreases sharply with increasing Triton X-100 concentration reaching a value of zero at 0.3 mM Triton. Mobility of FSR vesicles following treatment with 1.0 mM Triton for 10 min (25 C) was about the same as for untreated vesicles even though calcium uptake was completely inhibited (Table II).

TABLE II. Effect of various agents on the electrophoretic mobility of adsorbed FSR vesicles. (pH = 7.0, $\Gamma/2 = 0.10$, mobility, μ, in microns/sec/volt/cm)

Normal (no pretreatment)	-0.67 ± 0.10
Glutaraldehyde fixed (10 min at 24°C)	-1.42
Phospholipase C treated (2 h, 0.2 mg/ml, 24°C)	-0.97
Triton X-100 treated (10 min at 24°C, 1 mM)	-0.63
Caffeine treated (10 mM, 30 min at 24°C)	-0.71

Both ATPase activity and calcium transport are inhibited in FSR membranes treated with phospholipase C. The major action of phospholipase C is to hydrolyze membrane lecithin [6, 31]. Vesicles pretreated for 2 h with 0.2 mg/ml phospholipase C show a large increase in negative mobility. From an average value of -0.67 for untreated vesicles, treated vesicles show a value of -0.97 μ/sec/volt/cm. A previous report [6] indicated that both ATPase activity and calcium transport could be restored through the addition of lysolecithin to the treated vesicles. In that investigation it was concluded that the activating effect was accompanied by tight binding of the lysolecithin to the vesicle membrane. We added lysolecithin to FSR vesicles in concentrations ranging from 0.02 to 0.05 mg/ml. In no case were we able to restore the vesicles such that they exhibited their initial (untreated) mobility. The added lysolecithin had no effect on the electrophoretic mobility of phospholipase C treated membranes.

Caffeine has been shown to have a dual effect on isolated FSR vesicles [32]. In concentrations from 1 to 10 mM it decreases the capacity for calcium storage and reduces the rate of calcium uptake. Caffeine was said to act on the mechanism for energy transfer by reducing the coupling. In addition it was postulated that, "The selectivity of caffeine action enforces the view that the transport system exists in two different conformational states comparable to the allosteric modification of enzyme systems [32]."

We have determined the electrophoretic mobility of FSR vesicles following treatment with 10 mM caffeine (30 min at 24 C). Following this treatment the electrophoretic mobility was slightly greater, showing a value of approximately -0.71 μ/sec/volt/cm. It is unlikely, however, that this difference is significant since it is within the standard deviation of the normal (untreated) mean value of -0.67 ± 0.10. If a conformational change in the membrane did occur its effect on the membrane net charge was not large enough to be measured in our system.

Calcium Binding and Uptake in the Presence and Absence of Oxalate: Variation with pH

The pH dependence of calcium binding and uptake has been previously determined in FSR vesicles prepared from vertebrate muscle

[33]. We have studied calcium binding and uptake in lobster FSR vesicles in order to have a direct comparison with the mobility measurements. Our results generally are in agreement with those of Sreter [33]. In the presence of oxalate (Fig. 10) calcium binding following a 10 min incubation is a maximum at pH = 7.0. It declines

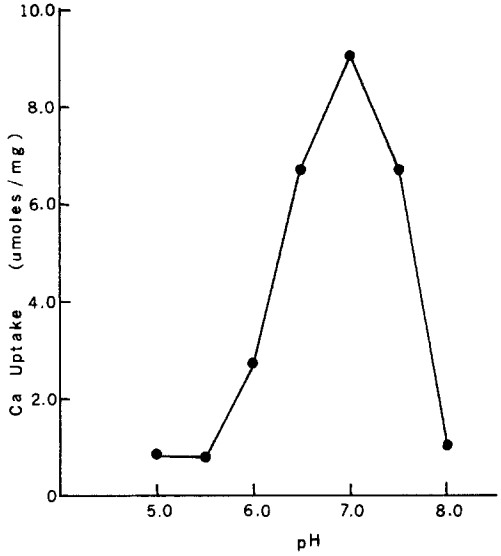

Figure 10. The dependence of calcium uptake on pH. Conditions as in legend to Fig. 9.

from a maximum of 8.9 μmoles/mg FSR protein at pH = 7.0 to 9.5 μmoles/mg at pH = 8.0 and 0.90 μmoles/mg at pH = 5.0. In the absence of oxalate,* calcium binding shows a maximum near pH = 6.0 (Fig. 11). In fresh lobster FSR vesicles a value of 0.675 μmoles/mg was determined for calcium binding following a 10 min incubation at pH = 6.0. Calcium binding declined to a value of 0.090 μmoles/mg at pH = 8.0 and 0.040 μmoles/mg at pH = 5.0. The greater binding in the absence of oxalate found at pH = 6.0, and the greater uptake in the presence of oxalate found at pH = 7.0 are similar to what was found in a previous investigation [33].

Liberation of Bound Calcium: Variation with pH

It has previously been shown that following binding in the presence of ATP and washing off of the ATP in sucrose, approximately 150-200 μeq

* Even though no oxalate was added to the reaction mixture, it is likely that as Pi was liberated during the course of the 10 min reaction, some calcium phosphate was formed inside the vesicles.

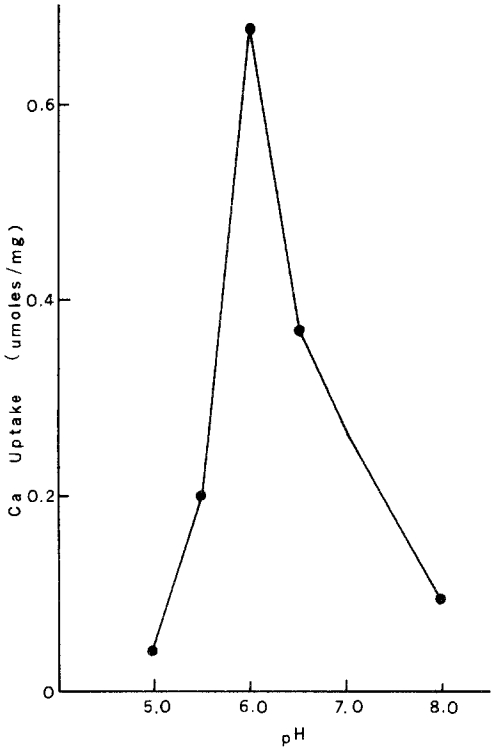

Figure 11. The dependence of calcium uptake, in the absence of oxalate, on pH. Conditions as in the legend to Fig. 9 except the potassium oxalate is omitted.

of calcium per mg of protein remain bound to the FSR membranes. Only 30% of this calcium is exchangeable with added calcium in the absence of ATP, however, in the presence of added ATP, over 95% of this calcium is exchangeable [16]. We have studied the pH dependence of the release of this bound calcium both with and without ATP. FSR vesicles loaded with 104 μeq Ca/gm protein and sucrose washed were placed in a solution containing 100 mM buffer (at the desired pH), 4 mM Mg, and in some experiments, 0.5 mM ATP. The amount of calcium bound to the FSR under these conditions was dependent on the pH (Fig. 12). Maximum binding occurred at pH = 5.0 and was the same in the presence or absence of ATP. At higher pH's the amount of calcium bound was less. In addition, more calcium was bound in the presence of ATP than in vesicles without added ATP. A pH shift from 6.0 to 7.0 would liberate about 25% of the amount bound at pH = 6.0 (in the presence of ATP). In the absence of ATP, a pH shift from 6.0 to 7.0 would liberate almost 30% of the calcium bound at pH = 6.0. (At pH = 4.0 only 3-4 μeq of Ca/gm FSR protein remain bound.)

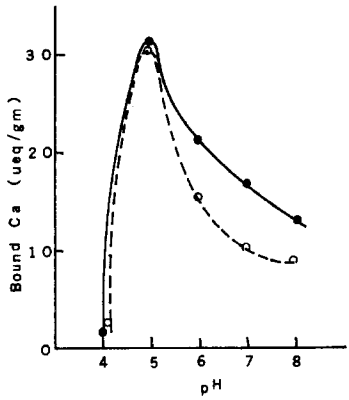

Figure 12. The dependence of vesicle bound calcium on pH. FSR vesicles were incubated as in Fig. 11. Following centrifugation the pellet was resuspended in 0.2 M sucrose. It was again centrifuged and resuspended in buffered solutions containing 100 mM Tris (pH 4 and 5), Tricine (pH 6 and 7), or Acetate (pH 8 and 9), 5 mM $MgCl_2$ (solid circles), and as above but with 5 mM ATP (open circles). Temp. 24° C. Bound ^{45}Ca was determined using the method of Fanburg and Gergely [21].

Discussion

The microscopic method of electrophoresis using latex spheres containing adsorbed FSR vesicles revealed several aspects of the electrophoretic mobility of the membrane. The following results of this study are of particular interest:

(1) No evidence was found which would indicate that the mobility of the vesicles was altered during calcium uptake. That binding and uptake did occur was evidenced by measurements of Ca^{45} transport. Presumably, therefore, no net change in surface charge occurred during this process. Calcium displaced from a given site was immediately replaced by another calcium ion or, when the available calcium is exhausted, by a magnesium ion which since it is not transported can remain bound to the site. Molecular changes resulting in net surface charge alteration were not detectable during the calcium transport process.

(2) At an ionic strength of 0.10, a plateau exists in the mobility versus pH curve. The mobility is independent of the pH in the range from pH = 5.0 to pH = 7.0. Above this pH an increase in negative mobility was seen indicating the presence of dissociating groups.

(3) Divalent cations exerted a strong effect on the membrane mobility. Low concentrations of calcium or magnesium decreased the negative mobility of the vesicles. Also, in the presence of divalent cations the mobility was independent of the pH over a wide range.

(4) Glutaraldehyde fixed vesicles exhibited a greater negative mobility than unfixed vesicles. This is consistent with their amine group binding action since the binding of positive amine groups will result in a greater net negative surface charge. The action of glutaraldehyde on the mobility of FSR vesicles indicates the importance, in the normal vesicle membrane, of amine groups.

(5) A large increase in the FSR vesicles negative mobility was found following hydrolysis of membrane lecithin with phospholipase C. Since lecithin is electrically neutral at pH = 7.3, its removal would not directly effect the net membrane surface charge. The effect on mobility may be due to an action of lecithin postulated by Martonosi [6]. He has suggested that the effect of some phospholipids is to induce conformational changes in membrane proteins. The removal of lecithin would result in rearrangement of membrane proteins causing an alteration in the net surface charge.

Cation Binding Sites Estimated from Lipid and Protein Composition of FSR Membranes

One milligram of FSR protein may be assumed to contain about 20% (0.2 mg) lysine and arginine (positively charged at neutral pH) and 20% glutamic and aspartic acid (negatively charged at neutral pH). Assuming an average molecular weight of 140, the charged amino acids can be estimated to produce a net of 4×10^{17} negative charges per mg of protein. The phospholipid concentration in FSR membranes has been estimated to be approximately 0.3 μmole/mg protein [27]. Assuming an average phospholipid molecular weight of 750, this is equal to 0.23 mg/mg protein. The phospholipid component of the membrane can therefore be estimated to produce 1.8×10^{17} negative charges per g of protein. Estimated in this way the maximum cation binding capacity of the FSR membrane equals 5.8×10^{17} charges or about 1 μeq/mg protein. The cation binding capacity of isolated FSR vesicles has been determined to be approximately 350 μeq/g protein at neutral pH [12]. Assuming both sides of the vesicle membrane have the same capacity, a total capacity for cation binding of 700 μeq/g protein results. Since this is equal to 0.7 μeq/mg, this value is close to the estimate based on the lipid and protein composition.

Number of Cation Binding Sites Estimated from Electrophoretic Mobility

The net surface charge density, estimated from the measurement of the electrophoretic mobility at pH = 7.0 and an ionic strength of 0.150 was 5.8×10^3 esu/cm^2. This is equal to 2×10^{-2} coul/m^2, or about one charge per 10^3 A^2. Assuming a vesicle volume of 2.7×10^8 A^3 and a

surface area of 2×10^6 A^2, one vesicle contains approximately 2×10^3 negative charges. Taking the value of 5.2×10^{-14} mg as the weight of one vesicle [27], an estimate of 0.4×10^{17} charges/mg results. This is equivalent to 0.07 μeq/mg, or considering both sides of the vesicle membrane as being similar, a value of 0.14 μeq/mg of cation binding capacity. This is 20% of the cation binding capacity estimated from direct measurements [12].

The surface charge measured at an ionic strength of 0.150 is determined by ionogenic properties localized within approximately 8 Å of the surface of shear [28]. As a result binding sites located at the physical membrane surface may not be detectable by electrophoretic means. In addition, in making calculations based on the Gouy-Chapman equation, it is assumed that the cell surface is impenetrable to counterions and that surface conductance corrections can be neglected. All of these factors can lead to underestimates of the actual surface charge density.

Estimate of the Number of Calcium Binding Sites

Based on the assumption that the enzyme which binds 1 mole of calcium has a molecular weight of 300,000 Martonosi has estimated that a microsomal vesicle with an average volume of 2.7×10^8 A^3 contains about 100 calcium binding sites. Deamer and Baskin [8], on the assumption that the 80 A particles seen following preparation of FSR vesicles by the method of freeze-etch are the calcium binding and ATPase centers, estimated a molecular weight of 180,000 for the particle. From this estimate about 150 calcium binding sites would be present on the average vesicle.

Hasselbach and Elfvin [34], as a result of ferritin binding to SH groups on the FSR membrane estimated that these groups are separated by about 100 A. A microsomal vesicle with a volume of 2.7×10^8 A^3 has a surface area of 2×10^6 A^2. Assuming a 100 A separation, we estimate that an average vesicle would contain about 170 binding sites. From these estimates we can conclude that an average vesicle would contain between 100 and 200 binding sites.

Comparison Between Number of Cation Binding Sites and Number of ATPase Centers

The net number of cation binding sites was estimated to be about 2×10^3 for an average vesicle. The same vesicle was estimated to contain 100-200 ATP catalyzed calcium binding sites. The "active" calcium binding sites are thus surrounded by at least tenfold more cation binding sites. It is therefore possible that charge alterations occurring at the "active" calcium binding sites during calcium binding and transport

represented too small a fraction of the total membrane charge to be detected by the microelectrophoresis method of determining mobilities.

Calcium Release and Muscle Contraction

Weber [4] has estimated that following excitation, about 0.1 μmole of calcium per g of muscle must be released by the sarcoplasmic reticulum. Our previous calculations have shown that there are approximately 200 calcium binding sites per vesicle. Taking a value of 3×10^{-17} g as the weight per vesicle [27], we can estimate that about 0.1 μmole of calcium per mg protein is bound to the calcium binding sites. Assuming an average vesicle volume of 2.7×10^{8} A^3 [27], 1 mg of FSR would occupy approximately 10 μliters of volume. Peachey [35] has estimated that the sarcoplasmic reticulum occupies about 5% of the fiber volume. On this basis 1 g of muscle would contain 5 mg FSR protein. Thus at least 0.05 μmoles of calcium per g of muscle are available from surface binding sites and could be released to initiate contraction.*

Carvalho [16] has found that 150-200 μeq of calcium/g FSR protein are bound to the membranes after exposing them to ATP and washing of the ATP. This is equivalent to about 0.10 μmoles per mg FSR protein or ten times the amount of calcium estimated on the basis of one calcium ion per binding site. From this consideration, 0.50 μmoles of calcium per g of muscle would be available from these binding sites.

Considering the assumptions involved in the above estimates it appears that muscle contraction initiated solely by release of membrane bound calcium and not involving a membrane permeability change is a distinct possibility. Relaxation could occur simply as a result of the calcium binding to membrane binding sites, actual transport not being required.

Calcium Binding: Variation with pH

It has recently been suggested that calcium release and the initiation of contraction may be triggered by a pH shift accompanying depolarization of the muscle cell membrane [36]. Permeability studies [37] have shown that the FSR membrane is permeable to sucrose and various anions. Larger molecules were permeable at higher values of pH. These permeability characteristics raise a question as to the possible existence of a potential gradient across the FSR membrane. While this question cannot be answered at present, calcium release triggered by a pH shift offers a possible alternative to calcium release caused by depolarization of a polarized membrane. The binding studies reported in this investigation show that the amount of calcium bound to the FSR membrane is a function of pH. A transitory increase in pH would trigger

* This is also based on the assumption that each binding site contains only one calcium ion.

the release of bound calcium. The internal pH of a muscle cell is not known with certainty, however, a number of investigators place the value at about 6.0 [38, 39]. Carter *et al.* indicate that depolarization results in a transitory pH increase of more than one pH unit. We have shown (Fig. 12) that a shift from pH = 6.0 to pH = 7.0 releases 25% of the bound calcium. On the basis of Carvalho's [16] measurements this could amount to more than 0.12 μmoles of calcium per g of muscle. It appears that calcium release from FSR membranes triggered by a pH shift occurring as a result of the depolarization of the cell membrane (and presumably also the *T*-system), must be considered as a possible sequence in excitation-contraction coupling.

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