J. Membrane Biol. 27, 1–20 (1976)


# Localization of Calcium Binding Sites Associated with the Calcium Spike in Barnacle Muscle

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Received 23 May 1975; revised 3 November 1975

*Summary.* La ion behaves as a competitive inhibitor of Ca ions on the calcium spike in the giant muscle fiber of the barnacle, *Balanus nubilus*. La-treated muscle fibers, in which the rate of rise of the spike was diminished to a known degree, have been examined with the electron-microscope. In such fibers dense particles are seen in association with the surface membrane and external lamina of the cell. La particles are not seen in association with fibers that have been allowed to recover from La inhibition before fixation. The number of La particles seen in association with the muscle fiber increases with increasing La concentration when the Ca and Mg concentrations are held constant and decreases with increasing Ca and Mg concentration when the La concentration is held constant. The results suggest that the La visible in the electron-microscope under the conditions of these experiments is bound to a class of sites similar to those involved in the Ca spike.

The giant muscle fibers of the barnacle *Balanus nubilus* normally contract in a graded response to single or summed nonpropagating postsynaptic potentials (Hoyle & Smyth, 1963). When the internal calcium concentration is reduced by injection of a calcium binding agent the fiber membrane produces all-or-none action potentials in response to depolarization. The rising phase of the action potential is due to an increase in the permeability of the membrane to calcium ions, and the action potential is called, therefore, a calcium spike (Hagiwara, Chichibu & Naka, 1964; Hagiwara & Naka, 1964). The behavior of the calcium spike with increasing calcium concentration was studied by Hagiwara and Takahashi (1967). They found that the maximum rate of rise of the spike increased with the calcium concentration in a manner consistent with the ideas that 1) there is in the membrane a finite number of sites that normally bind calcium, and 2) the rate of calcium entry during a spike depends upon the density of calcium bound to these sites when other factors remain constant.

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The existence of calcium binding sites implies that other cations may also be bound by them and that other cations would then behave as competitive inhibitors of the calcium spike. Hagiwara and Takahashi (1967) analyzed the kinetics of the effects of various divalent cations on the calcium spike and found that $Mg^{2+}$, $Ni^{2+}$, $Mn^{2+}$ and $Co^{2+}$ behave as competitive inhibitors. They also mentioned that $UO_2^{2+}$ and $La^{3+}$ as well as several other ions suppress the calcium spike, but they did not study the kinetics of inhibition by the latter ions.

In the present study it is demonstrated that La is also a competitive inhibitor of the calcium spike in the barnacle muscle fiber. This implies that La should be bound by the membrane sites that are normally occupied by Ca ions. We have found that La bound under these conditions can be seen with the electron-microscope. La is found as particles in association with the surface membrane of the muscle fiber and throughout the external lamina (basement membrane) surrounding the fiber. The distribution of lanthanum seen under these conditions may indicate the distribution of calcium binding sites involved in the calcium spike. A preliminary report of this work has been presented (Henkart, 1971).

## Materials and Methods

### *Electrophysiology*

Preparation of the barnacle muscle fiber and recording methods were essentially as described by Hagiwara and Naka (1964) and Hagiwara and Takahashi (1967). A glass pipette 200–500 μm in diameter was introduced longitudinally into the isolated muscle fiber from the cut end. The glass pipette was connected to a syringe with a micrometer advance mechanism through a length of polyethylene tubing into which was inserted a chlorided silver wire. A solution containing a Ca binding agent (*see* Table 1) was injected by hydrostatic pressure, and electrical stimulation was provided by passing current through the internal injection pipette. The action potential was recorded intracellularly by 3 M KCl-filled glass micropipette electrodes. The rate of rise of the action potential was displayed by passing the signal through a differentiating circuit with a time constant of 22.5 μsec. The compositions of external and internal solutions are shown in Table 1. The only alteration in these solutions from those of Hagiwara and Takahashi (1967) was that all experiments were done at pH 7.0 rather than at pH 7.7 because it was found that recovery from lanthanum inhibition was more reproducible at the lower pH. $LaCl_3$ (0.1–1.0 mM) was added to the whole saline. The addition of these small amounts of La resulted in negligible change in the tonicity of the solution.

### *General Procedure*

A muscle was prepared for the physiological experiment, the rate of rise of the spike was recorded, and the external saline was replaced by saline containing a given concentration of

Table 1. Compositions of salines (concentrations in mM)

| *A.* External: | NaCl | KCl | $CaCl_2$ | $MgCl_2$ | Buffer[a] | pH |
|---|---|---|---|---|---|---|
| Normal | 466 | 8 | 20 | 12 | 10 | 7.0 |
| Ca | – | 8 | 340 | – | 10 | 7.0 |
| Mg | – | 8 | – | 340 | 10 | 7.0 |
| Ca | | | | | | |
| Ca-Mg-free | 510 | 8 | – | – | 10 | 7.0 |

| *B.* Internal: | Sucrose | KOH | EGTA[b] | Methane sulfonic acid | Tris-maleate | pH |
|---|---|---|---|---|---|---|
| | 349 | 400 | 100 | 180 | 20 | 6.9 |

[a] Buffers used were tris-maleate-NaOH and HEPES.
[b] Ethylene glycol bis (β-aminoethylether)-N,N′-tetraacetic acid. Solutions with various divalent ion compositions were made by mixing appropriate quantities of Ca, Mg, and Ca-Mg-free salines.

lanthanum. Measurements of the rate of rise of the spike were made at intervals thereafter until a constant value was attained. This usually occurred within 15 min. At that point the muscle fiber was transferred to fixative without addition of further lanthanum.

*Electron Microscopy*

All muscle fibers were fixed in a solution of final concentration: 3 g glutaraldehyde and 20 g sucrose/100 ml of 0.1 M cacodylate buffer at pH 6.7, 7.3, or 7.6 as indicated below. Muscle fibers were rinsed in 0.1 M cacodylate buffer containing 30 g sucrose/100 ml three times for 30 min each. One segment of each fiber was post-fixed in a solution of final concentration: 1 g $OsO_4$/100 ml of 0.1 M cacodylate buffer at the same pH as the glutaraldehyde fixative. Tissues were dehydrated in ethanol and embedded in Epon. Sections were cut on an LKB Ultratome at a thickness setting indicating 750 Å. This was about the middle of the range of settings that produced silver sections. Sections were mounted without support films on 300 mesh copper grids and observed using a Siemens Elmiskop I.

The major portion of each muscle fiber was neither post-fixed in osmium nor stained with other heavy metal stains in order to distinguish electron opaque lanthanum from other dense materials. However, in order to check the quality of the fixation, a segment of each fiber was post-fixed in osmium so that its fine structure as preserved by conventional methods could be examined. As a check of the quality of preservation of fiber segments not treated with osmium, sections of these were stained with uranyl acetate and lead citrate. Because of the mechanical damage to deep portions of muscle fibers caused by the internal injection, studies of structure and of lanthanum binding were confined to the external portions (within about 30 μm of the surface) of the fibers.

Some muscle fibers not injected with calcium binding agent or used for recording were tied to glass rods, immersed in saline to within a few mm of the cut and then immersed in lanthanum-containing saline in the same time sequence as that used with the internally injected fibers. They were then transferred to fixative and treated in the same way as fibers which had been used in recording.

*Quantification of the Lanthanum Binding*

Unstained sections of lanthanum-treated muscles not post-fixed with osmium were scanned until a portion of the surface of the fiber was found. The magnification was then increased to 30,000 × and, beginning at one edge of the visible surface area, a series of micrographs was taken, one adjacent to the next, until the entire surface had been photographed. Then another area of the surface was found, and so on. No process of selection was employed except that the surface had to be recognizable. Enlargements were made on Kodabromide F 5 paper and often prints from several experiments were shuffled before counting was begun. The surface appearing on each print including the surface of clefts but not *t*-tubules (the distinction will be described below) was measured, by laying a moist thread along its contours and then measuring the thread, or by a map measurer. Each distinguishable dense particle located in association with the surface was counted. Since in unstained material there is no sharp dense line as a criterion for the membrane location but rather a change in image density that is more difficult to localize precisely, a reasonable estimate is probably that what we refer to as "the surface" is within 10 nm of the center of the sharp dense line we would have seen in Os-fixed, stained material. It was assumed that any particle present within the thickness of the section would be visible. The number of particles, then, was taken as the number present on an area of surface the length of the measured surface by the thickness of the section. By this method a randomly selected 3–7 $\mu m^2$ of the surface was surveyed for each fiber. No attempt was made to quantify La deposited in the external lamina. Twenty-seven fibers were examined with the electron-microscope and 44 were studied by electrophysiology.

## Results

### *Effects of La on the Calcium Spike*

Fig. 1 shows spike potentials developed in a barnacle muscle fiber injected with EGTA-containing internal solution in the presence and absence of externally applied 0.2 mM La. La decreases the spike height,

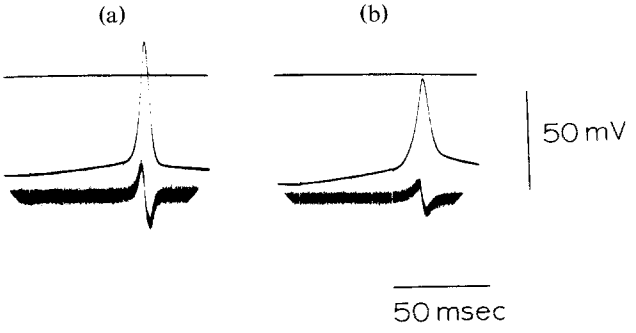


Fig. 1. Action potentials in a barnacle muscle fiber injected with EGTA solution. (*a*) was in normal saline and (*b*) in saline plus 0.2 mM $LaCl_3$. The upper trace is the reference potential level; the middle trace is the membrane potential change in response to a supra-threshold outward current pulse. The lower trace is the derivative of the membrane potential trace. Voltage calibration 50 mV, time calibration 50 msec

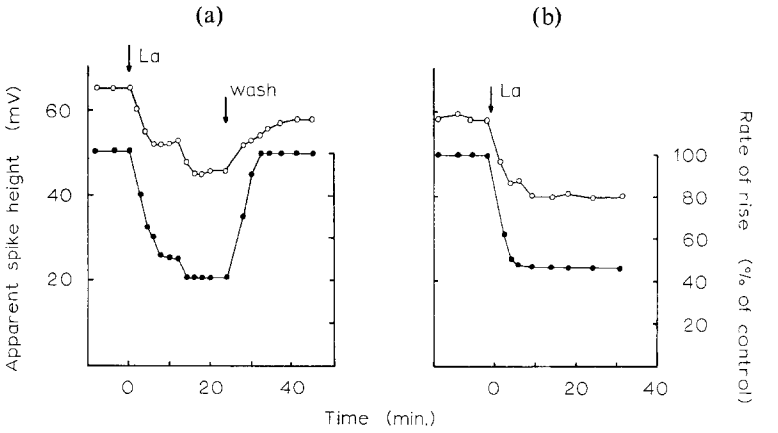


Fig. 2. Time course of the effect of La on the amplitude and maximum rate of rise of the Ca spike. Open circles represent apparent spike height, and closed circles are maximum rate of rise expressed as percent of the value before the addition of lanthanum. The first vertical arrow indicates the addition of La. In (*a*) La was washed away at the second arrow

decreases the rate of rise, increases the depolarization required for spike initiation (threshold), and prolongs the spike. Thus, La acts like high Ca in raising the threshold, but interferes with the current-carrying function of calcium. La at the concentrations used in these experiments had no effect on the resting membrane potential.

Fig. 2*a* and *b* show the time course of the effect of La on the amplitude and maximum rate of rise of the Ca spike. The effect of La reaches equilibrium within 15 min and is reversible by washing with normal saline.

### *Demonstration of Competitive Inhibition by La*

Hagiwara and Takahashi (1967) have shown that the dependence of the maximum rate of rise $T$ of the Ca spike on the Ca concentration [Ca] and inhibitor ion concentration $[M]$ is described by the following equation:

$$\frac{T_{\max}}{T} = 1 + \left(1 + \frac{[M]}{k_M}\right) \frac{k_{\mathrm{Ca}}}{[\mathrm{Ca}]} \tag{1}$$

where $T_{\max}$ is $T$ when [Ca] approaches infinity and $k_M$ and $k_{\mathrm{Ca}}$ are dissociation constants of $M$ and Ca to the membrane sites. This is based on the following assumptions: (1) Ca and $M$ are bound by the same membrane sites *competitively*, (2) the number of sites is finite, (3) changes in either [Ca] or $[M]$ do not alter the membrane potential at which the rate of rise

of the spike becomes maximum, and (4) the maximum rate of rise of the Ca spike is proportional to the density of sites occupied by Ca. Eq. (1) predicts: 1) a plot of the reciprocal of the maximum rate of rise of the spike ($1/T$) *vs.* the inhibitor concentration at a given Ca concentration is a straight line, and 2) $1/T$ *vs.* 1/[Ca] in the presence of varying concentrations of inhibitor are straight lines of different slopes all of which intersect at the $1/T$ axis. Figs. 3 and 4 are the results of experiments designed to test the two predictions. However, it is necessary first to deal with an evident complication.

Since the membrane potential at which the rate of rise of the spike becomes maximal does increase with increasing Ca concentration and with increasing La concentrations, assumption (3) is not fulfilled. Hagiwara and Takahashi (1967) showed that this effect could be minimized by carrying out experiments in the presence of 100 mM Mg rather than 12 mM, the Mg concentration in the normal saline. Under these conditions the observed relation between $1/T$ and [La] can be considered linear as shown by the lower curve of Fig. 3. (The experiment illustrated in the upper curve of Fig. 3 was done in the presence of 12 mM Mg, the normal Mg concentration. In this case the relation between $1/T$ and [La] is not linear.) The apparent dissociation constant for La ($k'_{La}$) calculated from the slope of the lower curve in Fig. 3 is about 1 mM. Here,

$$k'_{La} = k_{La}\left(\frac{40\,\text{mM}}{k_{Ca}} + \frac{100\,\text{mM}}{k_{Mg}} + 1\right). \tag{2}$$

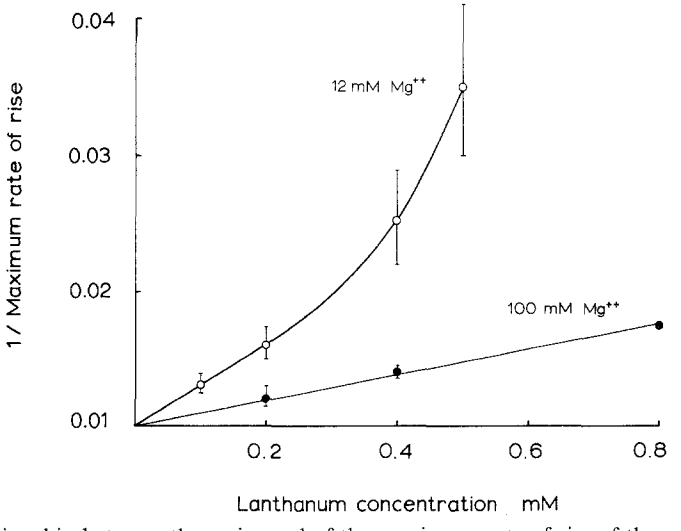


Fig. 3. Relationship between the reciprocal of the maximum rate of rise of the spike and the La concentration in the presence of 12 mM Mg (open circles) and 100 mM Mg (filled circles). Vertical bars through each point represent + and − the standard error of the mean. The maximum rate of rise in each case was normalized to the value in saline containing 40 mM Ca

Therefore, the actual dissociation constant of La ($k_{La}$) should be smaller than 1 mM.

Fig. 4 shows that plots of $1/T$ *vs.* the reciprocal of the Ca concentration in the presence of 0, 0.2, and 0.8 mM La are straight lines of different slopes that intersect at the $1/T$ axis. These results show that both predictions of the binding equation are fulfilled and indicate that lanthanum behaves as a competitive inhibitor of Ca for these membrane sites. This is interesting since La is a trivalent cation whereas the Ca ion is divalent. The ratio between the dissociation constants of Ca and La, $k_{Ca}/k_{La}$ is about 40 as estimated from the data shown in Fig. 4.

*Morphology of the Barnacle Muscle*

Figs. 5 and 6 illustrate some important features of the morphology of the barnacle muscle fiber (*see also* Selverston, 1967; Hoyle, McNeil &

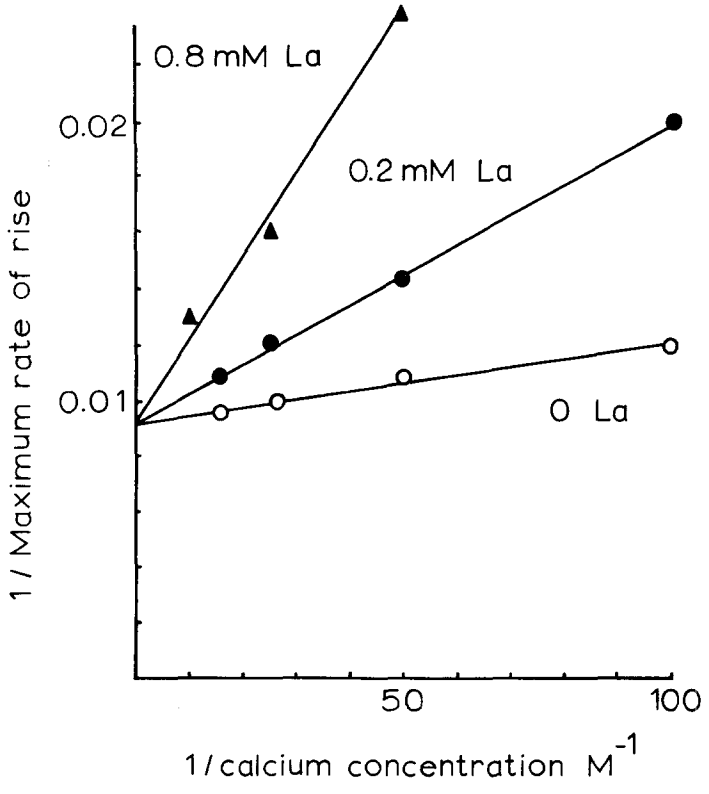


Fig. 4. Relationship between the reciprocal of the maximum rate of rise of the Ca spike and the reciprocal of the Ca concentration in the presence of 0 La (open circles), 0.2 mM La (filled circles) and 0.8 mM La (filled triangles). Curves are from individual experiments in the presence of 100 mM Mg. As in Fig. 3 the maximum rate of rise was normalized to the value in saline containing 40 mM Ca

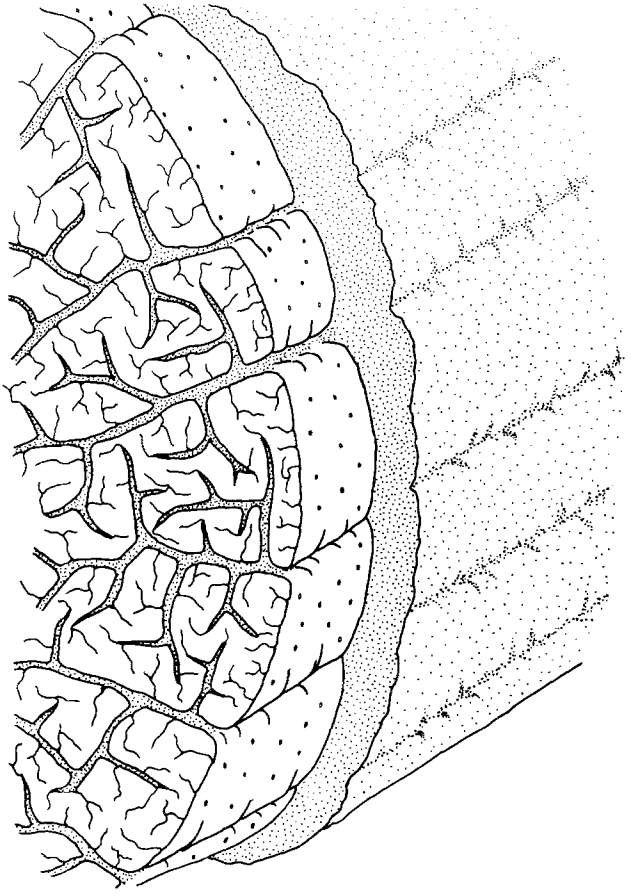

Fig. 5. Drawing illustrating some morphological features of the barnacle muscle fiber. A segment of an annulus from the surface of a fiber is represented in three dimensions. The external lamina (fine stippling) has been removed from a portion of the fiber exposing the surface membrane in which the openings of transverse tubules are represented by larger dots. The surface of the fiber is furrowed by clefts filled with the external lamina material. Crooked and branching lines in the cross-section of the fiber represent transverse tubules. The sarcoplasmic reticulum and its relationship to the surface, cleft, and tubular membranes are not represented. The total diameter of such a fiber would be 1–2 mm

Selverston, 1973). The surface of the fiber is furrowed with invaginations or clefts which ramify throughout the fiber. The surface membrane is invested with a layer of basement membrane (also called external lamina, Fawcett, 1966) 0.5–1.0 μm thick. This external lamina follows the invaginations of the surface membrane, filling the clefts. A "transverse" tubular system also exists. Tubules enter from the surface of the fiber or from clefts and are distinguished from the clefts by their generally smaller diameter (ca. 300 Å) and by the fact that they appear to contain no external lamina-like

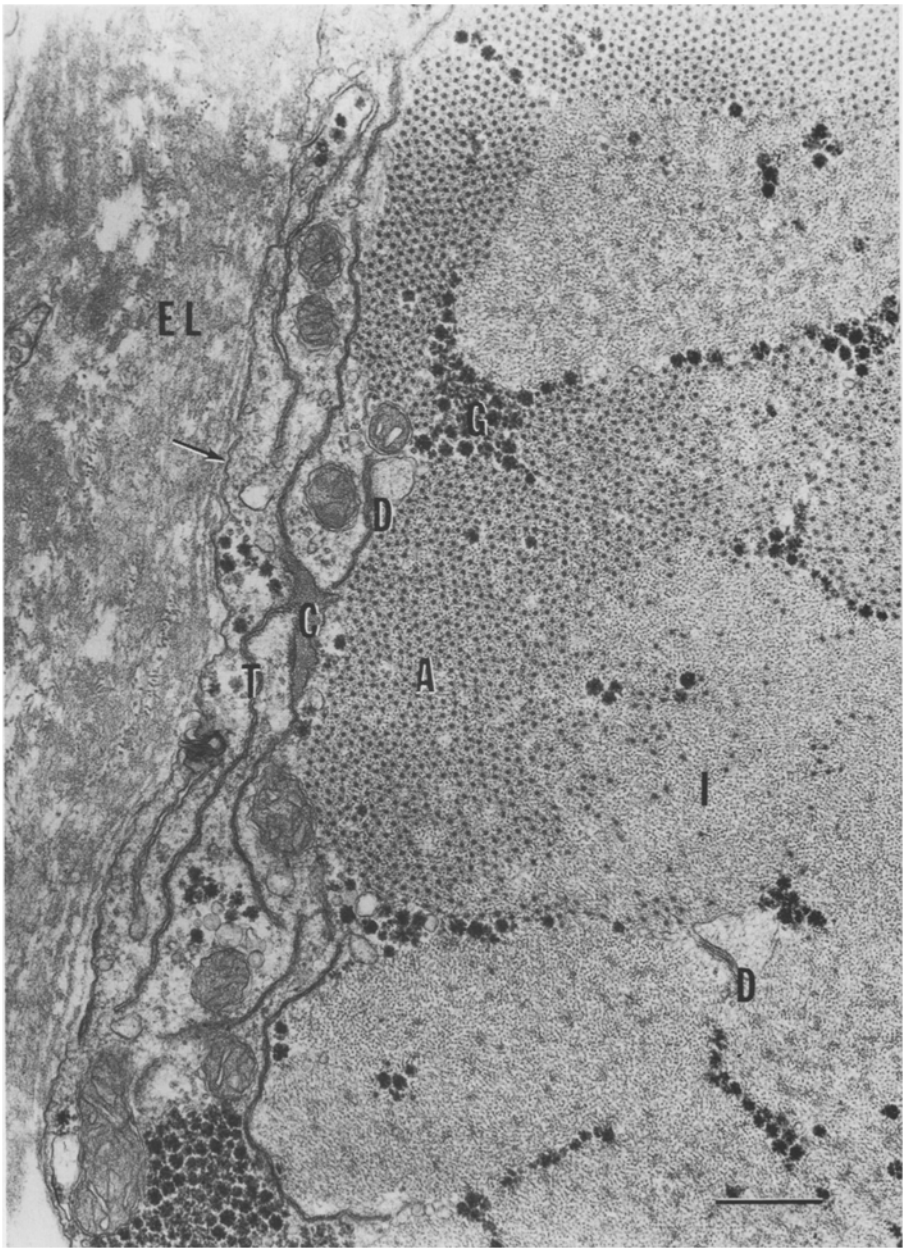


Fig. 6. Electron-micrograph of small portion of surface of single giant barnacle muscle fiber cut in cross-section. Material prepared by standard methods for electron-microscopy: fixed in glutaraldehyde, post-fixed in $OsO_4$, and stained with uranyl and lead salts. The small cleft (*C*) filled with external lamina material is continuous with the extracellular space in another plane of section. The bar represents 0.5 μm. *A* = *A*-band, *D* = dyad, *EL* = external lamina (basement membrane), *I* = *I*-band, *T* = transverse tubule, arrow indicates location of the surface membrane

material, although such material often extends a short distance into the mouths of tubules. These tubules frequently run longitudinally and circularly as well as transversely. Specialized sites of association with cisternae of the sarcoplasmic reticulum (dyads) occur on the surface membrane, on the membrane of clefts and on tubule membranes.

### *Lanthanum Binding*

Fig. 7 shows portions of the surface of a muscle fiber in which the rate of rise of calcium spike in 20 mM Ca was reduced to about 40 % of its original value by 0.5 mM La. Lanthanum is distributed apparently randomly in particles over membranes of the superficial regions of the cell, and throughout the external lamina. Fig. 8*a* is a muscle fiber fixed after exposure to 1.0 mM La for 10 min. The spike in this case was completely blocked. More La is seen in association with the surface than in the case of 0.5 mM La. There seems to be less La deposited in the tubules than on other areas of membrane. Occasionally, localized patches of very dense La binding were encountered. In unstained sections of material not treated with $OSO_4$ these were difficult to associate with any other structure. The size, shape and frequency with which these patches were encountered suggested that they corresponded to dyads. This interpretation was strengthened by observation in a few cases of an increased density of La binding in the areas of dyad junctions that were found in stained material. Because these areas were encountered relatively rarely and because the La-binding properties were so clearly different from those of the surface membrane, generally these patches were excluded from the quantitative studies of La binding.

Lanthanum particles were not found inside cells except when there was reason to believe that there was damage to the membrane, i.e. when there was obvious mechanical damage or when there had been a marked drop in the resting potential.

### *Recovery*

Fig. 8*b* shows a muscle fiber in which the spike had been completely blocked by 1.0 mM La. The bath was then flushed with 15 ml of saline without La. After 20 min when the spike had recovered to within 10 % of its original value, the fiber was fixed and prepared for electron-microscopy. Hardly any La particles are visible.

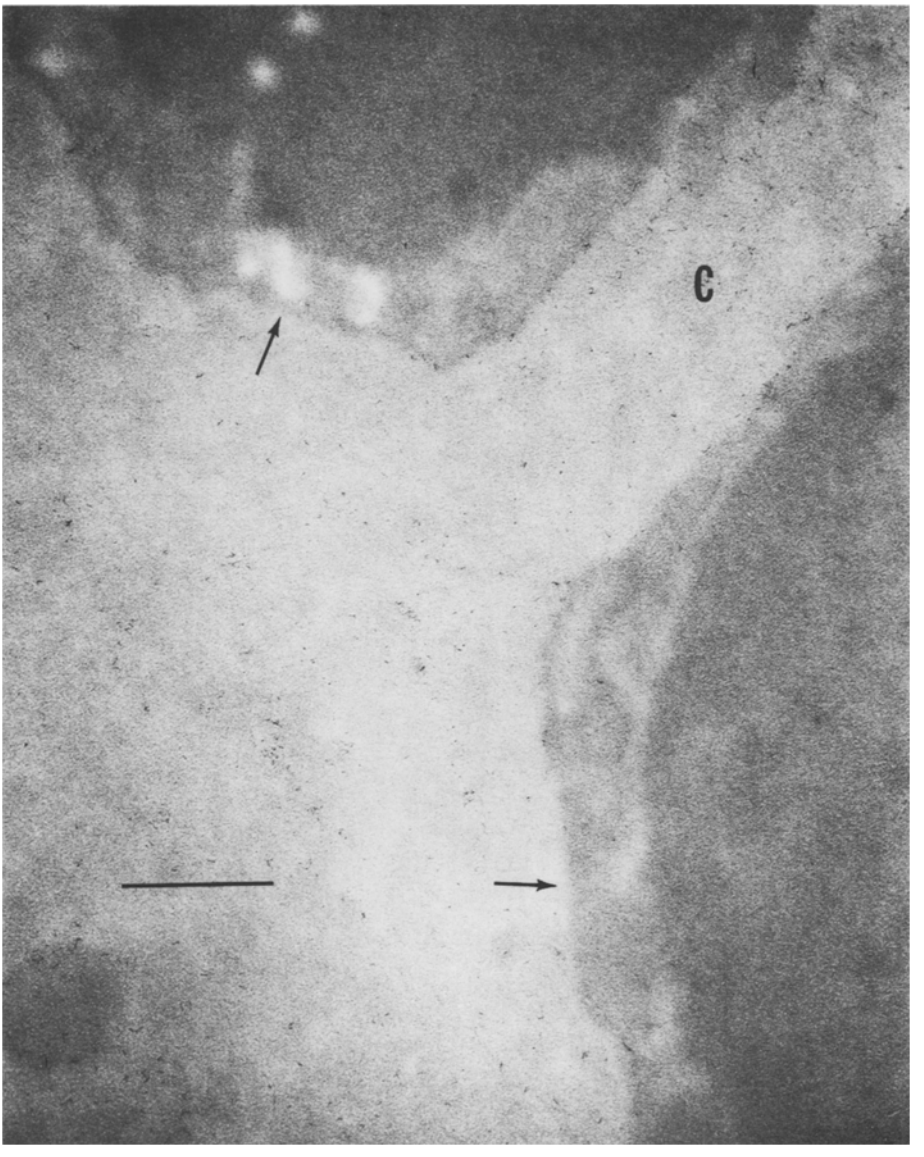


Fig. 7. Portions of the surface of a single giant barnacle muscle fiber which had been incubated for 15 min in saline containing 0.5 mM La, 20 mM Ca, and 100 mM Mg. The rate of rise of the Ca spike was reduced to about 40% of its original value. La particles are distributed over the surface of the fiber and throughout the *EL*. The bar represents 0.25 μm. *C*=cleft; arrows indicate location of the surface membrane

### *Binding vs. pH*

If the La particles were nonspecific deposits of $La(OH)_3$ precipitate, the amount of La deposited should vary with the pH of the fixative. The

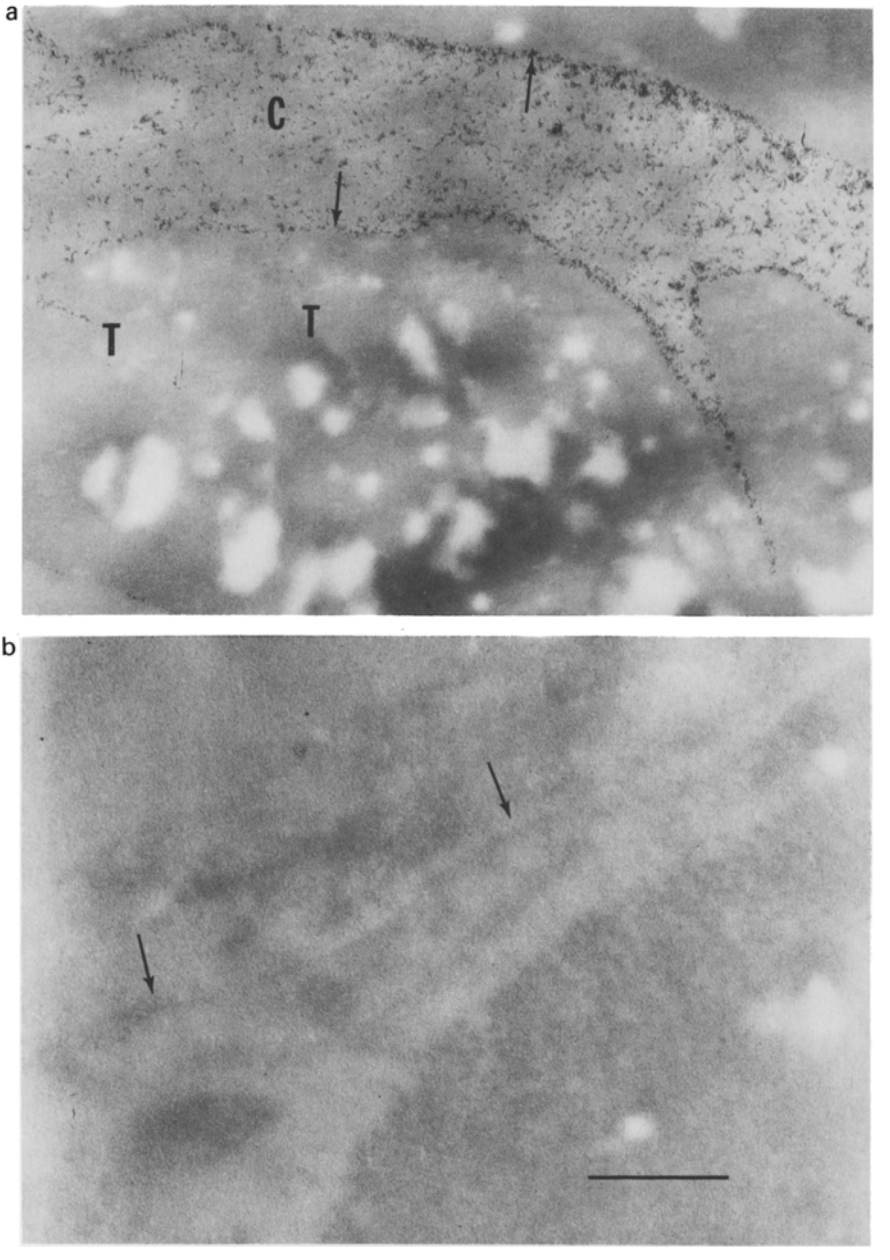


Fig. 8. (*a*) An area of a superficial cleft from a single giant barnacle muscle fiber that had been incubated in 1.0 mM La in the presence of 20 mM Ca and 12 mM Mg. The Ca spike was completely blocked in 10 min. La particles are distributed densely over the membrane and in the *EL* of the cleft. Some *t*-tubules opening from the cleft appear to contain La in their superficial regions while others do not. La particles at this concentration are too closely packed to be counted. (*b*) The surface of a fiber in which the spike had been completely blocked by 1.0 mM La. The fiber was then washed for 20 min in saline and the rate of rise of the spike had recovered to within 10% of its pre-La value before fixation. Calibration line 0.25 μm. *C* = cleft; *T* = transverse tubule; arrows indicate location of the surface membrane

number of La particles found in association with fibers at a given La concentration was not significantly different whether the fibers were fixed at pH 6.7, 7.3 or 7.6.

### *Quantiative Estimates of La Binding and Competition between La and Ca and Mg*

Fig. 9 shows the relationship between the number of La particles counted and the La concentration in the presence of different Ca and Mg concentrations. These results indicate that: 1) the number of visible particles increases with increasing La concentration when the Ca and Mg concentrations are fixed; 2) increasing the Ca concentration at given La and Mg concentration decreases the number of particles significantly; 3) similarly increasing the Mg concentration has a much smaller effect on the number of particles. The ratio of binding constants for La and Ca can be roughly estimated from the number of visible La particles observed in fibers treated with 0.5 mM La in the presence of 20 or 100 mM Ca. Values in the range 30–40 are found which are similar to the ratio of binding constants obtained from physiological measurements of the Ca spike.

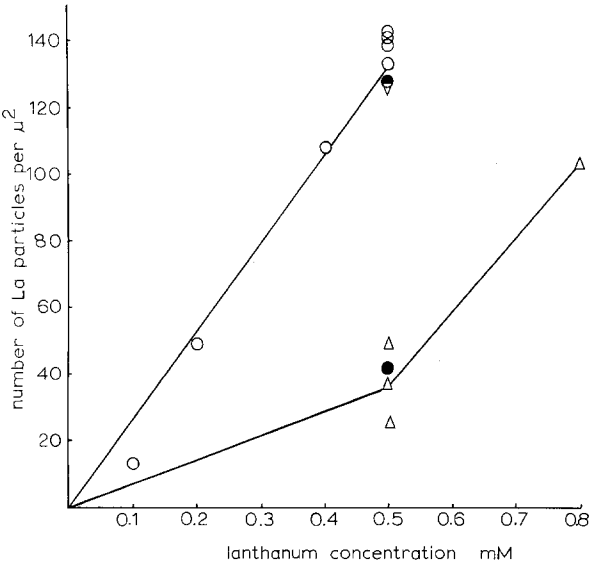


Fig. 9. Relationship between the number of La particles seen in association with the surface of some representative muscle fibers and the La concentration. Different symbols represent various Ca and Mg concentrations as follows: ○ = 20 mM Ca, 12 mM Mg: ◒ = 40 mM Ca, 12 mM Mg; ● = 100 mM Ca, 12 mM Mg; ▽ = 20 mM Ca, 100 mM Mg; △ = 100 mM Ca, 100 mM Mg. The two curves are drawn through points represented by ○ (varying La concentration in the presence of 20 mM Ca and 12 mM Mg) and △ (varying La concentration in the presence of 100 mM Ca and 100 mM Mg)

In one respect, however, the La particles behave differently from the sites involved in the Ca spike. If the La particles represented La bound only to sites involved in competitive inhibition of the Ca spike, the number of particles should show saturation in the range of La concentration which corresponds to blockade of the Ca spike as determined physiologically. This saturation was not demonstrated for the La particles. Apparently there are other classes of sites that bind La at higher La concentrations, and the curves for the number of particles *vs.* La concentration continue upward. This is not surprising since there is extensive evidence in the literature indicating the presence of negatively charged surface coats on all cells. Aside from this understandable discrepancy at higher La concentrations the sites identified by La particles under the conditions of these experiments are very similar to those deduced from the competitive inhibition of the Ca spike.

### *EGTA-injected vs. Uninjected Fibers*

The external lamina, surface membrane and area immediately beneath the surface of muscle fibers that had been injected with a Ca binding agent appeared the same as in uninjected fibers, and the binding of La by the surface membrane region and the external lamina was indistinguishable in the two types of fiber.

## Discussion

### *Specificity of La Binding*

Under the conditions of these experiments La particles are found in association with the external portions of barnacle muscle fibers. Two principle explanations exist for the presence and distribution of the La particles that are found. One possibility is that $La^{3+}$ which is present in interstices of the extracellular space during the physiological experiment encounters a sufficient concentration of $OH^-$ during fixation so that colloidal $La(OH)_3$ particles are formed and these are nonspecifically deposited throughout the extracellular space. The other explanation is that La particles are bound to specific sites that normally bind Ca involved in the Ca spike. We will evaluate these two possibilities in the light of 1) previous uses of La as a stain in electron-microscopy, 2) the literature concerning the

chemistry of $La^{3+}$ in aqueous solution, and 3) the experimental results reported above.

### *Previous Uses of La in Electron-Microscopy*

Lanthanum has been used as a stain for electron-microscopy by a number of workers in recent years, and the details of the methods used have differed considerably. The interpretations of the results in each case have not always taken into account the differences in the methods, and for this reason some confusion exists as to how lanthanum staining works, particularly with respect to the question of the specificity *vs.* nonspecificity of the staining. Some examples will illustrate this.

Doggenweiler and Frenk (1965) fixed tissues in freshly synthesized $La(MnO_4)_3$ or preincubated tissues with $La(NO_3)_3$. They considered La to bind strongly to sites that normally bind Ca, thus implying some specificity of staining. They did not distinguish between their two methods of preparation in the legends to their micrographs, however. It seems likely that fixation in $(MnO_4)^-$ which is a strong oxidizing agent might alter the number or nature of sites which bind lanthanum.

Revel and Karnovsky (1967) added NaOH to a 2–4% $La(NO_3)_3$ solution until an opalescent precipitate appeared at pH 7.6–7.8. When some of this suspension was added during post-fixation in $OsO_4$ they found a dense precipitate generally throughout the extracellular space, particularly in regions where the extracellular space was very narrow. When the suspension was added during initial fixation in paraformaldehyde glutaraldehyde the lanthanum tended to wash away during subsequent handling. Their interpretation of these results was that there was no indication of specific binding, but that colloidal $La(OH)_3$ was deposited nonspecifically throughout the extracellular space.

Some other authors who have used variations on these techniques include Lesseps (1967) who fixed tissues in $KMnO_4$ in the presence of $La(NO_3)_3$ at pH 7.8, and Overton (1968, 1969) who added $La(NO_3)_3$ during fixation in glutaraldehyde at pH 6.9.

### *Chemistry of Lanthanum Ion*

Because of the electronic configuration of the $La^{3+}$ ion it tends to form complexes, and the behavior of complex-forming metal ions in the presence of other compounds in solution and particularly in the presence

of macromolecules on the surfaces of cells is difficult to predict. The literature concerning the chemistry of lanthanum ion in aqueous solution provides some indication of the ionic and molecular species that might be expected to exist at various pH's. Values of equilibrium constants for the hydrolysis of $La^{3+}$ have been obtained by Biedermann and Ciavatta (1961). In the pH range 6.5 to 8.1 the hydrolysis products, $La(OH)^{2+}$, $La_2OH^{5+}$, and $La_5(OH)_9^{6+}$ or $La_6(OH)_{16}^{8+}$ occur but would represent less than 10% of the total dissolved La, and all the hydrolysis products are positively charged. However, the solubility product of $La(OH)_3$ is $10^{-19}$ (Topp, 1965). This means that in a 2% solution of $La(NO_3)_3$, for example, the solubility product of $La(OH)_3$ would be exceeded at pH 7.86. This is the condition used by Revel and Karnovsky (1967) to produce a lanthanum precipitate for use as an extracellular tracer. At the lanthanum concentrations that we have used (below 1.0 mM) the solubility product of $La(OH)_3$ is exceeded only at pH 8.7. Since the pH of the physiological experiments was 7.0 the predominant ionic species should be $La^{3+}$. The pH of the fixative was never higher than 7.6, and since the results were the same whether the fixative was pH 7.6 or 6.7 it seems unlikely that the particles found in our experiments are simply colloidal $La(OH)_3$.

On the other hand, the particles are obviously not single $La^{3+}$ ions. For this reason it is somewhat puzzling that the number of particles visible in the electron-microscope obeys the binding equation as well as it does. A possible explanation is that associations of positively charged La species with specific binding sites in the external lamina and membrane of the cell serve as nuclei for small deposits of lanthanum complexes.

The strongest evidence for the specificity of the La binding is that the number of particles seen with the electron-microscope is related to the effect of lanthanum on the maximum rate of rise of the calcium spike. The number of particles increases with increasing La concentration when the concentrations of Ca and Mg are held constant, and decreases with increasing Ca and/or Mg concentration when the La concentration remains constant. The results indicate that the observed La particles probably represent those bound to membrane sites. Ca and Mg compete with La for the same sites but the affinity of Mg for the sites is much smaller than that of Ca. These properties are very similar to those of the binding sites involved in the Ca spike as analyzed by electrophysiological methods.

As far as can be told from electron-microscopy, external lanthanum is sufficient to produce the observed effects on the calcium spike. There is no evidence that La enters a healthy cell even after many spikes have been fired. The presence of La in the external lamina suggests that this

material may be or contain a polyanionic substance or a substance that bears ligands that normally bind $Ca^{2+}$. This is consistent with numerous observations that such materials stain with cationic dyes and with metal-containing tracers that either are positively charged or are capable of complex formation (e.g., Luft, 1964; Pease, 1966; Rambourg & Leblond, 1967; Behnke, 1968; Howse, Ferrans & Hibbs, 1970; Langer & Frank, 1972).

There seems to be a relative scarcity of La in *t*-tubules. This lack of La is difficult to evaluate because in unstained sections of material not post-fixed with osmium it is not always possible to recognize tubules if they contain no La. It is also difficult to be sure that some of the "tubules" containing La might not be thin clefts. However, errors from both these sources would make an estimate of the fraction of tubules containing some La spuriously high. Although the present results do not justify any firm conclusion on this point, they suggest that calcium binding sites in the tubules are not as directly involved in the calcium spike as sites on the surface and cleft membranes. Howell (1974) has recently reported that ruthenium red generally fails to penetrate the transverse tubular system of frog skeletal muscle. The failure of multivalent cations to enter the tubular system in either barnacle or frog muscle suggests that some property of the tubular system controls the access of cations to the lumen, and that the lumen of the transverse tubular system is not simply an extension of the extracellular space.

The fact that the binding of the La by the external lamina and surface membrane of EGTA-injected and uninjected fibers is qualitatively and quantitatively the same is an indication that the mechanisms of the calcium spike in barnacle muscle injected with a calcium binding agent are probably related to the mechanisms normally functioning in the physiology of the muscle.

### *Function of the Binding Sites*

Lanthanum blocks the calcium spike as a competitive inhibitor. This is consistent with the model that a fixed number of negatively charged membrane sites which normally bind $Ca^{2+}$ may be occupied by $La^{3+}$. Since in these experiments we have shown that visible La binding correlates with the effect of La on the rate of rise of the Ca spike, the sites demonstrated should be primarily those associated with the Ca that is available to carry current. It should be emphasized, however, that the

binding sites demonstrated in this study should not be taken to correspond to calcium "channels" since there is no criterion for relating a number of particles to a number of channels. The value that we estimate for the number of sites that bind La in fibers treated with 1 mM La in the presence of 20 mM Ca and 12 mM Mg (about 300/$\mu m^2$ of membrane to block completely) is greater than the estimate obtained by Moore, Narahashi and Shaw (1967) for the number of Na channels in the nerve from the lobster walking leg (13/$\mu m^2$). On the other hand, our value is about 1000 times smaller than the calculation of Hille (1968) from the data of Chandler, Hodgkin and Meves (1965) for the total negative charge density of the squid giant axon in a medium with the ionic strength of squid saline ($3.5 \times 10^5/\mu m^2$). Our value is about 500 times smaller than the surface charge density calculated by Hille (1968) to explain his data concerning shifts of voltage-dependent parameters of the sodium spike in the frog node of *Ranvier* with changes of Ca concentration and pH ($1.4 \times 10^5/\mu m^2$). These numbers are included simply for comparative purposes since there is no reason to believe that individual La particles represent single negatively charged sites.

As for the function of Ca-binding sites in the external lamina, it seems reasonable to suppose that the external lamina might contain a material with properties similar to an ion exchange resin that acts as a Ca reservoir or buffer in the vicinity of the membrane. A similar function has been proposed for the external lamina in vertebrate heart muscle (Langer & Frank, 1972).

The usefulness of this technique is limited by the fact that it is necessary to avoid using all dense materials that can obscure the location of La particles. This makes the recognition of many structural details extremely difficult. In the present case this turned out to be tolerable because the main morphological features of the muscle (external lamina, surface, clefts) were generally distinguishable. The demonstration that in this case La could apparently be used to distinguish sites that normally bind calcium should not be taken as proof that all La stains are similarly specific. In fact, the amount of visible La continued to increase in the presence of La concentrations higher than that required just to block the Ca spike as shown in Fig. 9. This indicates that when a heavy metal is used as a marker for Ca binding sites associated with a particular function its distribution may be interpreted as specific only when the marker has been shown to be deposited under the precise conditions that are required for the effect of the marker metal on the physiological function in question.

The evidence presented here is consistent with the hypothesis that there is on the membrane of the barnacle muscle a fixed number of sites that normally bind the calcium that acts as a carrier of current during the Ca spike. Lanthanum can bind to the same sites and behaves as a competitive inhibitor of the calcium spike. Using the electron-microscope we have demonstrated lanthanum binding to a set of sites on the surface of the muscle that have properties similar to those of the binding sites involved in the calcium spike.

The authors are indebted to Dr. B.E. Volcani of Scripps Institution of Oceanography and to the Department of Neuroscience of the University of California, San Diego for the use of electron-microscope facilities. This research was supported by NIH postdoctoral fellowship No. FO2 NS 41,652 and NIH grant NS 09012 to Dr. S. Hagiwara.

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