THE CALCIUM BINDING SITES OF SYNAPTIC VESICLES OF THE FROG SARTORIUS NEUROMUSCULAR JUNCTION

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INTRODUCTION

Biochemical and electrophysiological observations have shown that calcium ions (Ca^{++}) play an essential role in many biological processes (1, 2). Experiments involving cell fractionation have demonstrated that certain cell organelles are capable of binding and sequestering Ca^{++}. Therefore, the concentration of free Ca^{++} in the cytoplasm seems to be rather low in physiological conditions (2-5). Mitochondria (6) and sarcoplasmic reticulum (3) are the best known examples of cellular organelles that sequester Ca^{++}. Sampson et al. (7) demonstrated ultrastructurally electron-dense granules associated with the membranes of mitochondria, myelin sheath and associated with synaptic complexes in the brain of the rat. Oschman and Wall showed that fixation of the intestinal epithelium of the cockroach in the presence of Ca^{++} results in the appearance of electron-dense precipitates on some of the cell membranes (8). Electron probe analysis shows that similar precipitates on the surface membranes of squid giant axons contain high concentrations of calcium (9; Hillman and Llinas, personal communication). It has been postulated that these calcium-containing precipitates represent calcium binding.

Depolarization-secretion coupling has been shown to be Ca^{++} dependent in several secretory systems (2; 5, 10, 11). Extracellular Ca^{++} is required for the release of acetylcholine (Ach) at the neuromuscular junction (NMJ); intracellular injection of Ca^{++} causes release of transmitter at the giant synapses of the squid (11), and in addition, the concentration of unbound Ca^{++} in the presynaptic terminal of the squid giant synapse increases in relation to the transmitter release (5). Knowledge of the precise location of the Ca^{++} binding sites at the presynaptic terminal of the NMJ would provide information relevant to the mechanism of depolarization-secretion coupling.

MATERIALS AND METHODS

The methods according to Oschman and Wall (8) were used in preparation for electron microscopy. Frog (*Rana pipiens*) sartorius muscles were dissected in normal Ringer's solution, and then pinned down to the bottom of a Sylgard chamber (Dow Corning Corp., Midland, Mich.) where they were fixed with one of the following solutions: (*a*) control fixative solution—2.5% glutaraldehyde in 80 mM collidine buffer containing 150 mM sucrose, pH 7.15; (*b*) control plus 5 mM EGTA (ethyleneglycolbis-[β-amino-ethyl ether] N,N'-tetra-acetic acid, Sigma Chemical Co., St. Louis, Mo.) (*c*) solutions with $CaCl_2$—2.5% glutaraldehyde in 80 mM collidine buffer containing 5 or 90 mM $CaCl_2$ at pH 7.15 or pH 7.40. The glutaraldehyde and the buffer were obtained from suppliers that assured a minimal Ca^{++} contamination (8). Some preparations were postfixed in buffered 1% OsO_4. The specimens were dehydrated through a series of graded alcohol solutions and embedded in Epon. Some of the specimens were stained on the grid with uranyl acetate.

RESULTS AND DISCUSSION

Fixation in the presence of $CaCl_2$, with or without postfixation in OsO_4, brings about the visualiza-

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tion of electron-dense particles which are (*a*) localized inside synaptic vesicles (Fig. 1), (*b*) associated with the postsynaptic membrane (Fig. 3), (*c*) localized inside mitochondria (Fig. 4), and (*d*) associated with the triad region of the sarcoplasmic reticulum (Figs. 4 and 5). In this report we will describe the differences in the synaptic vesicles after fixation with and without $CaCl_2$. Figs. 1 and 2 are electron micrographs of synaptic vesicles from neuromuscular junctions that were treated identically, except that the tissue in Fig. 1 was fixed in a solution containing 90 mM of $CaCl_2$, and that of Fig. 2 was fixed without $CaCl_2$ in a solution containing 5 mM EGTA. No discernible

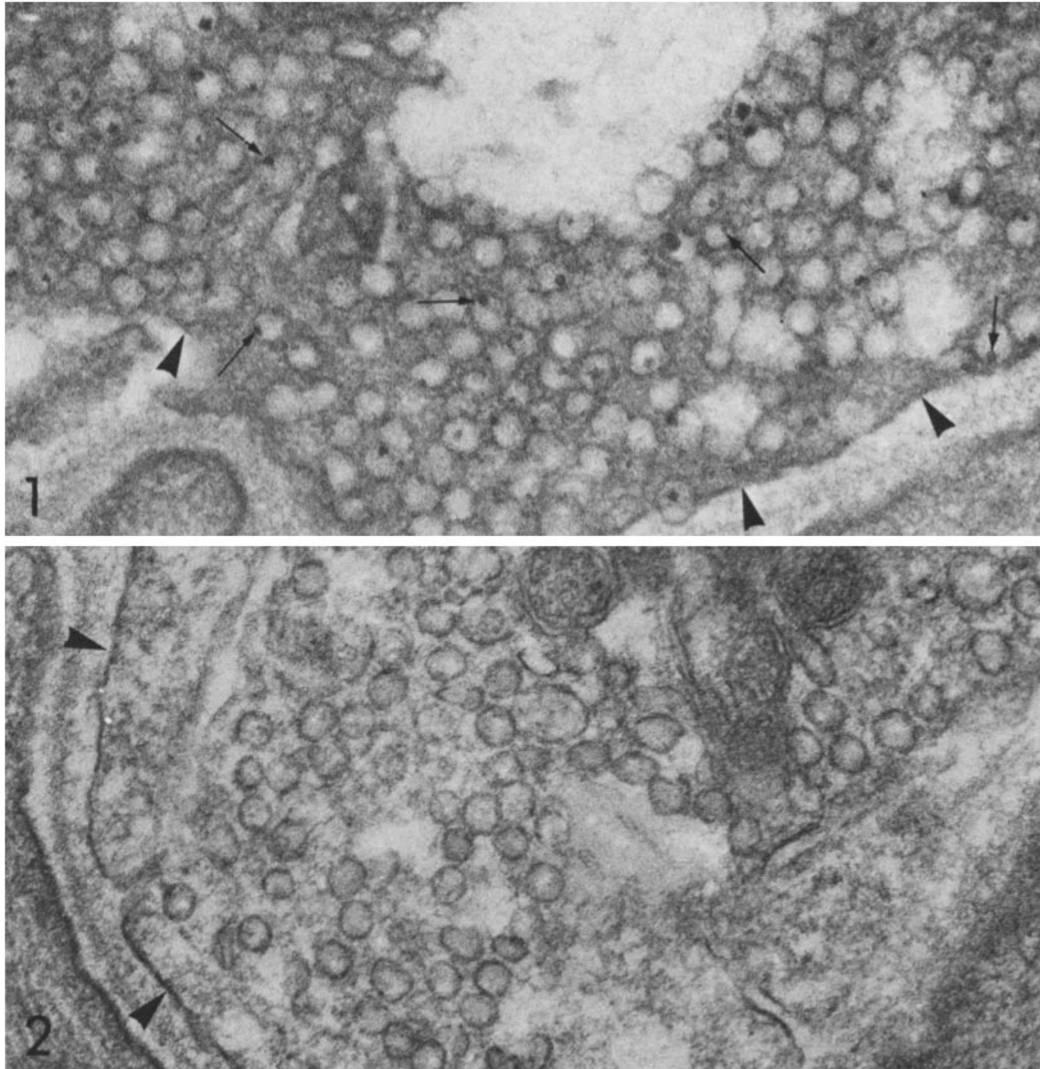
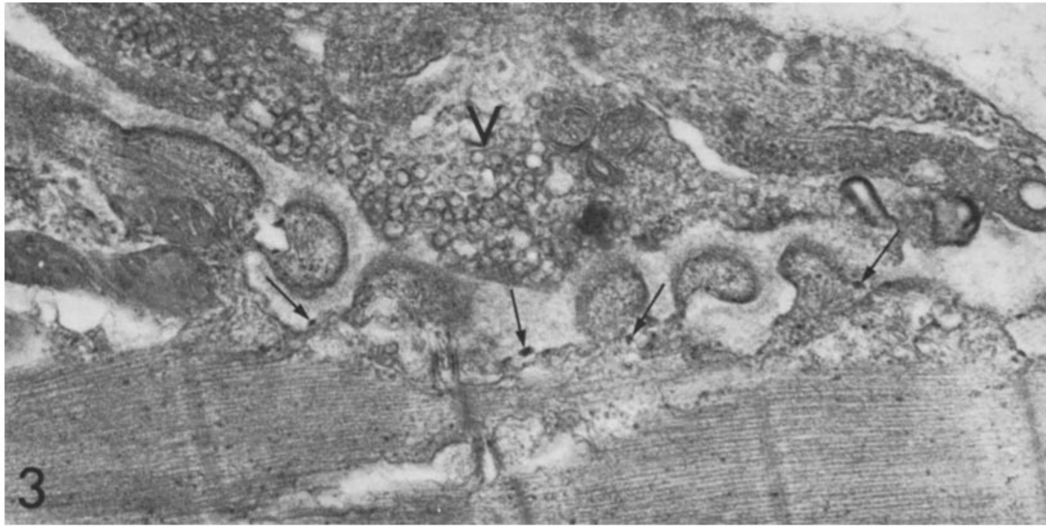


FIGURE 1 Electron micrograph of synaptic vesicles in a frog neuromuscular junction, fixed with glutaraldehyde containing 90 mM $CaCl_2$. Discrete particles (at arrows) can be seen in synaptic vesicles. Arrowheads indicate presynaptic membrane. $\times$ 65,000.

FIGURE 2 Similar preparation as Fig. 1, except fixed in Ca^{++}-free solution containing 5 mM EGTA. No comparable particles are present when the tissue is fixed in a Ca^{++}-free medium. Arrowheads indicate presynaptic membrane. $\times$ 65,000.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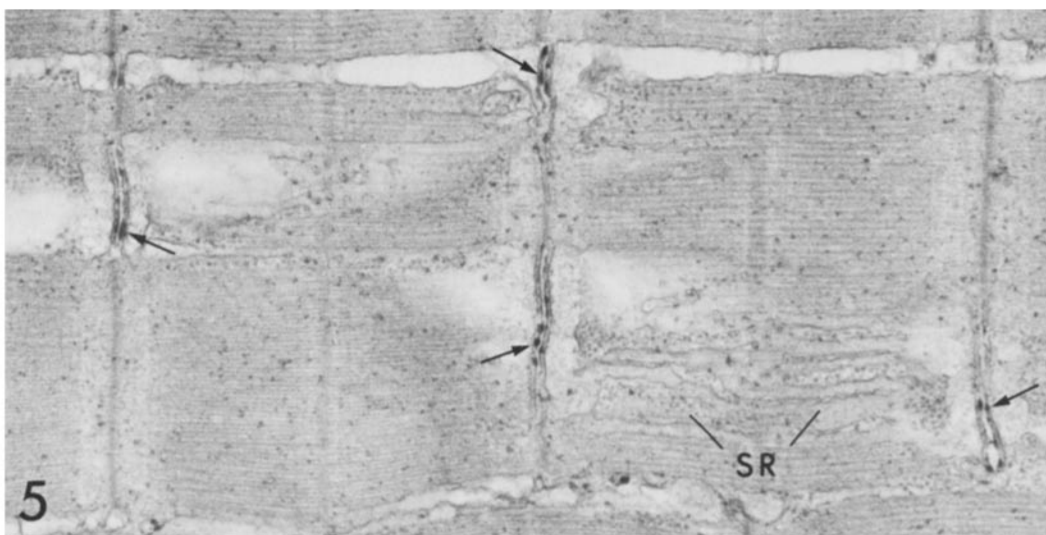
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particles are present in the $CaCl_2$-free, EGTA-containing preparations (Fig. 2).

When 5 or 90 mM $CaCl_2$ were added to the fixative, between 50 and 80% of all the synaptic vesicles showed a dense particle approximately 50–70 Å in diameter. Rarely, two particles were seen inside the profile of one synaptic vesicle. Particles were not seen in pinocytotic vesicles of the muscle fibers or in endothelial cells of the surrounding capillaries. The orientation of the particles with respect to the presynaptic membrane is clearly random, i.e., there seems to be no preferential location of the particle within the vesicle with respect to the axis of the nerve terminal (as seen in transverse and/or longitudinal sections). The particles are located more frequently at the periphery of the vesicles. If the particles were located randomly inside the vesicles, the probability of finding one particle per unit area of projection should increase from the periphery to the center, independent of the thickness of the section. On the other hand, if the particles were attached to the membrane, it should be expected that the probability of finding a particle per unit area of projection should increase from the center to the periphery, again, independent of the thickness of the section. An experimental criterion to distinguish between these two alternatives is to determine the probability of finding particles that seem to be touching the inner surface of the vesicle membrane or are a part of it. Our calculations clearly indicate that the particle is actually touching or is part of the vesicle membrane[1] (12).

In a recent report, Bohan et al. (13) described electron-dense particles in isolated synaptic vesicles of *Narcine* electroplaque. These authors raised the question of whether the cholinergic ves-

[1] If the particle is randomly located inside the vesicle (model 1), the probability of finding it within any given volume element of the vesicle is independent of the location of that element and proportional to its volume. The photographic images of the vesicles are the projection of spherical bodies on a flat surface. Therefore, the probability of seeing a particle within a given area of projection is proportional to the vesicle volume that is projected over such area. Particles seen over the projection area of the vesicle might appear sometimes touching the vesicle membrane because: (*a*) the particle is actually touching the membrane, or (*b*) the projection of the distance between the particle and the vesicle membrane is equal to or smaller than the resolution of the image. A particle that is seen as touching the membrane, then, would have to be contained in a volume (V) that could be defined as the vesicle volume that would remain after drilling out a hole that passes through the center of the vesicle, that is, perpendicular to the plane of the picture, and that has a radius equal to the radius of the vesicle minus the thickness of the membrane (ϕ) and minus the resolution of the electron micrograph (a). If the vesicles are spherical, it can be shown that

$$V = \int_{r-(a+\phi)}^{r-\phi} 4\pi x \sqrt{r^2 - x^2}\, dx \qquad \text{(I)}.$$

where "x" is the radial distance along a diameter that is parallel to the plane of the picture, "r" is the radius of the vesicle, "ϕ" is the thickness of the membrane, and "a" is the image resolution. The ratio between V and the total volume of the vesicle is equal to the conditional probability (P_1) of finding a particle that looks attached to the membrane, given that only vesicles with particles are counted. Then,

$$P_1 = \frac{V}{\frac{4}{3}\pi r^3} = \frac{1}{(r-\phi)^3}\left([2r-2\phi-a]a\right)^{3/2} \qquad \text{(II)}.$$

An alternative model (model 2) is based on the assump-

FIGURE 3 Electron micrograph of an oblique section through a frog neuromuscular junction fixed with glutaraldehyde containing 90 mM $CaCl_2$. Discrete particles (at arrows) can be seen associated with the postsynaptic membrane. As in Fig. 1, particles are present in association with the synaptic vesicles (V), although they are less visible at low magnification. × 38,000.

FIGURE 4 Electron micrograph of a longitudinal section of a muscle fiber fixed with glutaraldehyde containing 90 mM $CaCl_2$. Discrete particles (at arrows) can be seen localized inside a mitochondrion. Also, dense particles can be seen associated with the triad region of the sarcoplasmic reticulum (double arrows). × 29,000.

FIGURE 5 Electron micrograph of a longitudinal section of a muscle fiber, fixed with glutaraldehyde containing 90 mM $CaCl_2$. A profile of the sarcoplasmic reticulum can be seen (*SR*). As in Fig. 4, discrete particles (at arrows) can be seen associated with triad region of the sarcoplasmic reticulum. × 33,000.

icles of the electroplaques are intrinsically different from the vesicles of the neuromuscular junction. Our observations indicate that the isolated synaptic vesicles are not different from those of the NMJ and that their results can be explained by the high Ca^{++} concentration used in their fixatives (90 mM, $CaCl_2$). Similar precipitates can be seen in micrographs of thin sections of intact frog NMJ in a recent publication by Heuser and Reese, who used 20 mM $CaCl_2$ in their fixatives (14).

Synaptic vesicles have been shown to take up various divalent cations, i.e., Pb^{++}, Cd^{++}, Zn^{++} (15–18). The deposits are of two types, one resembling our results with calcium and the other being a more diffuse reaction within the contents of the synaptic vesicles. It is well-known that Mg^{++} blocks synaptic transmission (19). Recently it has been shown that Co^{++} is a competitor to Ca^{++} and at very low concentrations, blocks transmission at the frog sartorius NMJ (20). Our preliminary findings indicate that when $CoCl_2$ is present in the fixative instead of $CaCl_2$, a similar precipitate is present on the synaptic vesicle membrane. Apparently the calcium binding site on the synaptic vesicle membrane shows affinity for other divalent cations.

The basic question of the validity of considering the calcium precipitates as markers of normal, physiologically meaningful calcium binding sites can be raised[2]. It is encouraging to note that similar calcium precipitates are clearly localized in two structures that are already known to bind and take up Ca^{++} under a variety of conditions, i.e., the mitochondria (Fig. 4, reference 6) and the sarcoplasmic reticulum (Fig. 5, reference 3). These calcium precipitates are also absent in our controls. It is known that most soft tissues normally have a few millimolars of bound Ca^{++} (1, 2, 21). It would be expected that some calcium precipitates should be seen, even if the fixation is done in the absence of calcium. However, fixation without calcium fails to show these precipitates, probably because conventional fixation causes a severe loss of normal cytoplasmic ions (21). Because there is no significant difference in particle size and density whether 5 or 90 mM $CaCl_2$ are added to the fixative, fixation with only a few mM $CaCl_2$ seems to saturate the binding sites available.

There are three arguments that suggest that this particle corresponds to a real biological structure that is "stained" by calcium: (*a*) particulate calcium precipitates were not randomly distributed throughout the presynaptic process; (*b*) the particles are attached to or form part of the vesicle membrane; (*c*) there seems to be only one distinct particle per vesicle.

If Ach is contained within the vesicles and if the site marked by these particles plays a role in the Ach release, it would be necessary to consider that the vesicles are polarized, anisotropic structures and that a specific region on their surface has to be in contact with the presynaptic membrane in order to release its contents.

SUMMARY

Fixation of neuromuscular junctions in the presence of 5 or 90 mM of $CaCl_2$ brings about the visualization of an electron-dense, 50 to 70-Å diameter particle that is attached to or forms part of the synaptic vesicle membrane. Electron-dense deposits appeared also in the postsynaptic mem-

tion that the particle is attached to or forms part of the vesicle membrane. In this case, it can be shown that the conditional probability (P_2) of seeing a particle attached to the membrane is equal to the surface area of the vesicle that is projected over the vesicle profile plus its adjacent regions within the limits of resolution, divided by the total vesicle area. Then,

$$P_2 = \frac{1}{r}\sqrt{2\,ar - a^2} \qquad \text{(III)}.$$

If "a" is between 20 and 30 Å, ϕ = 50 Å, and r = 250 Å, P_1 lies between 8.1 and 15.6% and P_2 between 39.2 and 47.5%. The experimental value turned out to be 41.5%. Clearly, this result agrees with the model of the particle actually touching or being part of the membrane (i.e., model 2).

[2] Most histochemical localizations involving the electron microscope present the problem of discerning whether the visible reaction products are localized over the specific reaction sites, or whether these reaction products diffused to neighboring sites. The main reason for this is that these histochemical localizations depend upon intermediate chemical reactions that finally lead to the precipitation of electron-opaque metal salts. In our case, there seems to be no intermediate reaction, and the end product is the complex formed by calcium and the binding substance(s). If the calcium precipitates do not move during or after fixation, their localization may indicate calcium binding sites of the synaptic vesicles. On the other hand, if the fixation and embedding procedures cause or allow the movement of the precipitate, the final localization would be distorted.

brane, mitochondria, and the triad region of sarcoplasmic reticulum.

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