ELECTRON MICROSCOPY AND X-RAY MICROANALYSIS OF CALCium-BINDING SITES ON THE PLASMA MEMBRANE OF BEROE GIANT SMOOTH MUSCLE FIBRE

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SUMMARY

When they are fixed with glutaraldehyde in the presence of calcium ions, the plasma membranes of Beroe giant smooth muscle fibres display micropapillae filled with an electron-dense deposit. After freeze-fracturing of fixed tissue, the micropapillae are still present, therefore their shape and size are determined during or before glutaraldehyde fixation, and are not due to rearrangement during subsequent steps of tissue processing; intramembranous particles are seen at the periphery rather than at the top of micropapillae. In conventional stained sections, the surface of most micropapillae is surrounded by fine fibrils; when the fuzzy coat is separated from the muscle cell by a clear space, this fibrillar material becomes conspicuous and links the micropapillae to the coat. After calcium-free (EGTA) fixation, the plasma membrane is completely free of electron-dense sites but 'empty' micropapillae can be seen. X-ray microanalysis of single electron-dense deposits by wavelength-dispersive spectrometry reveals a high calcium content. A weak osmiophily is suspected, but does not seem to interfere with this analysis of calcium. The highest peak-to-background ratios for calcium were obtained using the smallest aperture of the Wehnelt of the analytical microscope.

In the Discussion, the micropapillae are compared to similar structures described by other authors in a variety of cell types.

INTRODUCTION

Calcium ions (Ca\(^{2+}\)) are involved in many physiological processes through their interaction with cellular membranes; plasma membranes normally separate an intracellular compartment, very poor in free Ca\(^{2+}\), from the extracellular milieu where the concentration of this ion can be 10,000 to 1000,000-fold (see references quoted by Scarpa & Carafoli, 1978). In this connection, the localization of a calcium store on the inner side of the plasma membrane would be of great physiological significance. Such a store has been hypothesized from physiological studies (Bulbring & Tomita, 1970; Chapman, 1971; Hallett, Schneider & Carbone, 1972; Sugi & Yamaguchi, 1976; Frank, 1979; Stolze & Schulz, 1980) as well as from ultrastructural cytochemistry (Oschman & Wall, 1972; Herman, Sato & Hales, 1973; Oschman, Hall, Peters & Wall, 1974; Hillman & Llinas, 1974; Plattner & Fuchs, 1975; Atsumi & Sugi, 1976; Goffinet, 1978; Stockem & Klein 1979; De Araujo Jorge, De Souza & Machado, 1979). The most relevant cytochemical results have been obtained using glutaraldehyde in the presence of Ca\(^{2+}\) (Clawson & Good, 1971; Oschman & Wall, 1972). We have
used this type of fixation, followed by electron microscopy, freeze-fracturing or X-ray microanalysis, on the giant smooth muscle fibre of the ctenophore *Beroe ovata*. The present work is part of a current study of the only giant smooth muscle cell so far known in the animal kingdom (Hernandez-Nicaise, 1976; Nicaise & Hernandez-Nicaise, 1979; Hernandez-Nicaise & Amsellem, 1980; Hernandez-Nicaise, Mackie & Meech, 1980; Nicaise & Hernandez-Nicaise, 1980). Our aim was to start a study of the precise nature of the plasmalemmal calcium-binding sites.

**MATERIALS AND METHODS**

*Beroe ovata* were collected at the Station Zoologique, Université Pierre et Marie Curie, Villefranche-sur-Mer, France. The animals were kept in sea water at 10-15 °C.

**Conventional electron microscopy**

Whole animals (3 to 5 cm long) or pieces of body wall of larger specimens were dipped into cold (4 °C) fixative for 90 min. The fixative was 3 % or 5 % glutaraldehyde in artificial, cacodylate-buffered, sea water (Na-cacodylate, HCl (pH 7.8), 100 mM; NaCl, 400 mM; KCl, 10 mM; CaCl₂, 10 mM; MgCl₂, 58 mM). Distilled water or NaCl was used to adjust the saline solution to the osmolality of sea water (usually 1150 mosM). Two individuals were fixed in a similar solution, but without CaCl₂ to ensure complete elimination of Ca²⁺, 1 mM-EGTA was added to the fixative. The specimens were then rinsed briefly in the buffered isotonic saline. Small pieces were dissected out and post-fixed in 1 % osmium tetroxide in the same saline for 1 h. The tissues were dehydrated subsequently in a graded series of ethanol, followed by three changes of propylene oxide, and embedded in Epoxy resin. Sections were stained with saturated uranyl acetate in methanol (6-10 min) followed by lead citrate (6—10 min) and examined in a Philips 300 or a Hitachi HU 12 A electron microscope at the Centre de Microscopie Electronique Appliquée à la Biologie et la Géologie (University Claude Bernard).

**Freeze-fracturing**

For freeze-fracture studies, pieces of body wall were fixed as above in isosmotic, cacodylate-buffered glutaraldehyde for 3 h. The tissue samples were rinsed subsequently in buffered saline, and infiltrated with 30 % glycerol in the same buffer for 3 h. Tissue blocks were then placed on specimen holders, rapidly frozen at −210 °C in nitrogen slush and transferred to a 'Reichert Jung CF 250' unit. They were then fractured, and the exposed fracture surfaces were shadowed with carbon/platinum at an angle of 45°. The replicas were cleaned with NaClO and mounted on copper grids.

**X-ray microanalysis**

After conventional fixation and embedding (see above), dark-gold sections were collected on aluminum grids with no supporting film. Staining with heavy metals was omitted, sufficient contrast resulting from the post-fixation with osmium tetroxide. The sections were analysed on an analytical electron microscope 'CAMEBAX + TEM' (from Camcena) equipped with two wavelength-dispersive spectrometers (vertical and inclined) at the Centre d'Etudes Nucléaires de Grenoble. The vertical spectrometer was focused on the Kz line of Ca and the inclined spectrometer on the Mø line of O₅; each had pentamerythritol crystals. Pulses corresponding essentially to X-ray photons collected by the spectrometers (plus electronic noise) were counted during 40s for the peak and 40s for the background for each of the spots analysed. Probe currents of 70 or 100 nA were used, under accelerating voltages of 45 or 50 kV. The probe current was not regulated, but the absorbed current was checked from time to time on a grid bar and adjusted manually when necessary. Better brightness of the electron beam was
eventually obtained by reducing the aperture of the Wehnelt piece from 1 mm to 0.6 or 0.5 mm. With a standard tungsten filament, the beam diameter at the level of the specimen can thus be reduced to 300 nm for a probe current of 100 nA and a tension of 50 kV.

RESULTS

After fixation with Ca²⁺-containing solutions, the surface of Beroe smooth muscle fibres is studded with micropapillae (Fig. 1). Their size and shape vary from those of a rather small lenticular swelling to those of a micropapilla with a maximal height of 40 nm and a maximal diameter of 55 nm.

Fig. 1. Periphery of a cross-section of a giant smooth muscle fibre fixed in the presence of 10 mM-Ca²⁺. The electron-dense micropapillae (arrows) are distributed at random at the surface of the cell, either on the clear (filament-free) protrusions, or on the area of the plasma membrane that is adjacent to the myofilaments. me, mesoglea; my, myofilaments. × 58000.

Observation of freeze-fracture replicas confirms the measurements made on conventional preparations and proves that the small lenticular aspects are not merely lateral sections of full-size micropapillae (Fig. 2). These structures appear to be distributed at random on a given muscle cell. Their abundance varies from one muscle cell to another in the same animal, some cells appearing devoid of micro-
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papillae. In conventional preparations, the inner (cytoplasmic) side of a micropapilla is generally filled with electron-opaque material, even when uranyl and lead staining of the sections is omitted.

The fuzzy coat that surrounds the muscle cell is generally seen separated from the cell membrane by a clear space, probably due to the retraction of the cell during histological processing. Most often this separation does not occur at the top of micropapillae; a fibrillar material, radiating from the outer surface of the micropapilla, links this structure and the coat (Figs. 3, 4).

After Ca^{2+}-free fixation, the plasma membrane was completely free of these electron-dense sites. However, in some cells, but not in all, micropapillae with pale contents were present (Fig. 5).

In freeze-fracture, particles are rare or absent at the top of a micropapilla; more often they surround the base (Fig. 2, insert).

The results of X-ray microanalysis are summarized in Table 1. Six series of measurements were made over a period of several months and under variable conditions. Some of these conditions were controlled: probe intensity, accelerating voltage of the electron beam, size of the Wehnelt aperture; other variables such as the spot size and the geometry of the analysing chamber (position of the specimen holder relatively to the beam) were not controlled. In general, no significant difference was found between the series obtained under the different controlled conditions (probe current, voltage, Wehnelt). However, the highest peak-to-background ratios for Ca were obtained in series 5, with the smallest aperture of the Wehnelt, i.e. with the maximum brightness of the beam. A total of 45 areas containing an electron-dense site recognized as a micropapilla was compared to a similar number of regions devoid of electron density, at the same peripheral location.

The peak-to-background ratio of Ca and Os signals from each of these spots is close to, or better than 2; it is therefore safe to assume that the spectrometers detected a Ca and an Os signal from each spot analysed. In each series of analysis, the Ca signal ($P - B$) of regions containing the electron-dense sites was significantly higher than that of the membrane nearby ($P < 0.05$). In two series out of six the Os signal was significantly higher on the electron-dense sites ($P < 0.05$). The background count over the electron-dense sites was never significantly different from that over the membrane nearby ($P > 0.2$). In series 2 and 6, two measurements were made on a pair of electron-dense sites but are not included in Table 1. Although no statistical analysis can be made, it is worth pointing out that in both cases the Ca peak was much higher than the average of the series but lower than twice the mean; the Os peak as

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Fig. 2. Freeze-fractured preparation of prefixed (in the presence of Ca) and glycerinated Beroe body wall showing the shape and size of micropapillae (arrows) on a smooth muscle fibre. On the P face (PF) the micropapillae appear as bulges, on the E face (EF) as pits. me, mesoglea; my, myofilaments; n, nucleus. $\times 33000$. Inset: the particle distribution, at the periphery of the micropapilla (arrows) rather than on its top is seen better at higher magnification. $\times 115000$. 
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well as the background counts were very close to the mean. In short, the fact that two dense sites were analysed in the same spot increased the Ca peak only.

DISCUSSION

The size, location and electron density of the structures presented in the present paper are clearly reminiscent of the electron-opaque deposits described by several authors on the cytoplasmic side of the plasma membrane of various cell types (e.g. Elfvin, 1968; Clawson & Good, 1971; Ouschman & Wall, 1972; Ouschman et al. 1974; Skaer, Peters & Emmines, 1974; Larsen, 1975; Plattner & Fuchs, 1975). Some authors, following Ouschman et al. (1974), consider that the deposits are due to the precipitation of Ca entering the cell during fixation; this pre-supposes the simultaneous presence of extracellular Ca, a precipitating system (eventually, by a phosphate-splitting enzyme), and the opening of a pore. None of these requirements is specific to fixation and it has not been proved that such calcium deposits cannot form in vivo (see, for example, Plattner & Fuchs, 1975).

Whenever X-ray microanalysis was performed on these structures, they appeared to contain calcium (Ouschman et al. 1974; Hillman & Llinas, 1974; Plattner & Fuchs, 1975; Tsuchiya, 1976; Fisher, Kaneshiro & Peters, 1976; Goffinet, 1978; Sobota, Przelecka & Janossy, 1978; Stockem & Klein, 1979; de Chastellier & Ryter, 1981), and this is also the case in Beroe smooth muscle fibres.

Beroe tissues, like those of other planktonic animals, have very poor intrinsic electron opacity, and we had to use osmicated blocks to obtain sufficient contrast. The use of osmium could therefore have introduced a microanalytical bias: firstly, a local increase in mass can raise the general level of noise (with a similar effect on the Ca peak and the background signal); secondly, the electron scattering induced by a local accumulation of Os can excite Ca atoms present in the specimen, lying outside the incident electron beam, and increase the Ca peak above the background signal (see Morlevat & Roussignol, 1972; Galle, 1975; Nicaise & Bilbaut, 1975). In two series of measurements out of six, the Os signal was indeed significantly higher in the areas with Ca-binding sites. However, the background measurements listed in Table 1 are similar in all membrane areas of a given series, regardless of the presence of Ca-binding sites. The influence of Os must therefore be minimal, especially if one takes into account the examples mentioned above, where in the presence of two Ca-binding sites in the same analysed area, the Ca signal almost doubled while the Os signal was unchanged. Conversely, when the beam was focused on a myelin figure, the Os signal was generally much more affected than the Ca signal. It appears safe to

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Figs. 3-5. Micropapillae on the plasma membrane of giant smooth muscle fibres. The arrows point to a fibrillar material, probably glycoprotein, radiating from the top of micropapillae, and linking these structures to the fuzzy coat. Figs. 3 and 4 are taken from tissues that were fixed in the presence of 10 mM-Ca²⁺; for Fig. 5, the specimen was fixed with Ca-free, EGTA-containing fixative. × 250,000.
Table 1. Results of X-ray microanalyses

<table>
<thead>
<tr>
<th>Series no.</th>
<th>Probe I (nA)</th>
<th>Wehnelt $\phi$ (mm)</th>
<th>Calcium</th>
<th>Membrane nearby</th>
<th>Calcium</th>
<th>Membrane nearby</th>
<th>Osmium</th>
<th>Osmium</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$P$</td>
<td>$B$</td>
<td>$P/B$</td>
<td>$P$</td>
<td>$B$</td>
<td>$P/B$</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>50</td>
<td>1</td>
<td>9 75 17 58 2.5 6.1</td>
<td>4 47 16 30 2.0 4.3</td>
<td>9 114 9 105 5.0 17.2</td>
<td>4 70 8 62 6.5 10.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>70</td>
<td>50</td>
<td>0.6</td>
<td>10 100 26 73 2.9 5.5</td>
<td>10 55 23 32 1.8 3.2</td>
<td>10 82 8 74 6.7 21.0</td>
<td>6 69 7 68 6.5 18.8</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>70</td>
<td>50</td>
<td>1</td>
<td>7 125 34 91 2.4 5.2</td>
<td>5 76 26 50 1.8 3.7</td>
<td>3 157 10 147 15.7 16.7</td>
<td>5 157 11 125 8.5 29.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>100</td>
<td>45</td>
<td>1</td>
<td>4 153 37 116 3.5 4.7</td>
<td>3 88 38 51 2.3 2.5</td>
<td>4 277 13 264 12.1 48.0</td>
<td>3 163 11 152 8.9 18.8</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>100</td>
<td>50</td>
<td>0.5</td>
<td>10 221 32 189 4.7 8.5</td>
<td>9 149 34 115 3.0 5.7</td>
<td>7 307 41 265 4.6 10.8</td>
<td>8 267 38 223 3.4 10.6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>100</td>
<td>50</td>
<td>0.5</td>
<td>5 276 60 215 3.4 6.1</td>
<td>5 222 53 169 3.0 5.7</td>
<td>5 285 101 184 2.1 5.3</td>
<td>5 279 107 172 1.9 5.2</td>
<td></td>
</tr>
</tbody>
</table>

$n$, number of spots analysed (the counts affected by high accidental noise, i.e. more than twice the double of the mean, were eliminated).
$P$, mean of the peak counts (standard deviation of the mean in parentheses).
$B$, mean of the background counts ($\sigma$ as for $P$).
$P/B$ (min, max), minimal and maximal values of peak-to-background ratios.
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conclude that the electron-opaque deposits of Beroe smooth muscle fibre are indeed rich in Ca and that this Ca signal is affected very little by the Os, which may also be present at the same sites.

It appears from the literature that the electron-dense sites are not present in every cell type (e.g. they are lacking in the neurosecretory neurones examined by Normann & Hall, 1978). In particular, to our knowledge, no detailed study of such sites has been made on muscles. However, their presence was mentioned by Oschman & Wall (1972) in insect gut muscle, by Politoff, Rose & Pappas (1974) in frog sartorius, and by Twarog (1977) in Mytilus anterior byssus retractor.

Another feature of interest in the different articles published on this subject is that in a given cell type the number of electron-dense sites varies according to the physiological state and/or the experimental conditions (Elfvin, 1968; Sampson, Matthews, Martin & Kunin, 1970; Skaer et al. 1974; Przelecka & Sobota, 1976; Sobota, Hrebenda & Przelecka, 1977; Geyer, Linss & Stibenz, 1978; Stockem & Klein, 1979; de Chastellier & Ryter, 1981). In this respect the Ca-binding sites of Beroe smooth muscle fibre deserve further attention, because all muscle cells do not display equal frequencies of sites in a given individual.

The unequal distribution of the electron-opaque sites in Beroe muscle is paralleled by the uneven presence of 'empty' micropapillae after fixation with EDTA (Fig. 5). But we cannot tell if these empty micropapillae were fixation-induced Ca-binding sites, subsequently depleted of their Ca by EGTA or if they can form in vivo without the fixative. The sea water surrounding the muscle fibre at the time of fixation contains enough Ca$^{2+}$ for the glutaraldehyde to act in the presence of this ion, and the 'Oschman & Wall reaction' could take place before the action of EGTA (this molecule does not necessarily reach the tissue at the same time as the aldehyde).

In paramecia, Plattner (1975) examined the possible correlation between membrane-intercalated particles, as revealed by freeze-cleaving, and membranous Ca-binding sites. If the particles of the ciliary bases are associated with Ca-binding sites, the reverse is not true; that is, other Ca-binding sites could not be associated with particles (Plattner, 1975). In a completely different cell, we found that the micropapillae of Beroe smooth muscle fibre are surrounded by particles, but the bulge itself is generally smooth. This pattern may reflect a mechanical sliding of the particles during the formation of the bulge, and does not necessarily imply that the particles represent pores allowing the Ca influx. But if this were true, the number of particles would be sufficient to explain the extreme multiplication of electron-dense sites observed in certain experimental conditions (unpublished). The freeze-cleaved membranes of Beroe smooth muscle fibres display micropapillae of various sizes, with a maximum diameter of 55 nm. This suggests that the shape of the Ca-binding sites is determined during or before fixation with glutaraldehyde and that the membrane components do not rearrange to form the deposits during subsequent steps of tissue processing (see Oschman et al. 1974, p. 163).

Although not directly demonstrated, a relationship between plasmalemmal Ca-binding sites and contractile proteins has been strongly suggested by several studies: (i) after removal of their spectrin by EDTA, erythrocyte ghosts lose the ability to
form Ca-binding micropapillae (Geyer et al. 1978); (ii) in amoebae, the electron-dense deposits are formed only in areas of the plasma membrane associated with actin filaments, and are affected by cytochalasin D (de Chastellier & Ryter, 1981); and (iii) in the squid giant axon, cobalt-induced electron-dense deposits are associated with thin, actin-like filaments (Metuzals, Tasaki, Terakawa & Clapin, unpublished). We would like to add that the formation of a bulge is in itself suggestive of local activation of the submembranous contractile system; local entry of Ca$^{2+}$ is very likely to trigger contraction of this system, and the precipitation of entering calcium could terminate the micropapilla-forming process. We did not observe distinct microfilaments in empty micropapillae. However, our observation of frequent differentiation of the cell coat at the top of the Ca-binding sites may be related to the activation of the contractile proteins underneath. Published data indicate that in many cells there is a contractile system at the inner side of the plasma membrane, and that this system is connected through the membrane to glycoproteins on the outer side (see references quoted by Emmelot, 1977; Weihing, 1979).

More work is necessary to define precisely the nature of the precipitating system of Beroe Ca-binding sites, as well as the pore mechanism by which Ca eventually enters the cell at these sites. In the present state of knowledge, this last point is more promising; even if the Ca precipitation and the micropapillae are fixation artifacts, these artifacts are discrete naturally quantified signals, possibly indicating a membrane mechanism at the molecular level that would be impossible to visualize by other means. The Ca-binding sites of the plasma membrane may not be a physiological Ca store for the cell but they may well be a tool for the cell physiologist.

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