ULTRASTRUCTURAL LOCALIZATION OF CALCIUM-BINDING SITES IN THE ELECTROCYTE OF ELECTROPHORUS ELECTRICUS (L.)

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SUMMARY
Calcium-binding sites were detected in the electrocyte of Electrophorus electricus (L.) using the Oschman & Wall technique, in which CaCl₂ was added to the fixative and washing solutions. Deposits were seen scattered along the plasma membrane of the electrocyte, inside mitochondria, associated with the post-synaptic membrane and the membrane of synaptic vesicles.

INTRODUCTION
Calcium ions (Ca²⁺) play an essential role in many biological processes such as depolarization-secretion coupling (Del Castilho & Katz, 1954; Baker, Hodgkin & Ridgway, 1971), muscular contraction (Katz & Miledi, 1968; Heuser, Katz & Miledi, 1971), flagellar or ciliary activity (Plattner, 1975; Fisher, Kaneshiro & Peters, 1976), etc. In general these processes are mediated by interactions between Ca²⁺ and cell membranes. The possibility of localizing Ca²⁺-binding sites by ultrastructural cytochemistry was suggested by Oschman & Wall (1972) using CaCl₂ in the fixative solutions. This technique has been widely used in recent years with the aim of localizing Ca²⁺-binding sites in several biological structures.

The electrocytes of the electric organ of Electrophorus electricus are highly specialized syncytial cells able to produce a synchronous electric discharge generating bioelectric potentials similar to those in nerve and muscle (Esquibel, 1970). They present an innervated posterior face which is electrically and chemically excitable and a non-innervated anterior face which is not excitable (Keynes & Martins-Ferreira, 1953) and in both faces there are perpendicularly oriented tubular invaginations of the plasma membrane. Because of the relative scarcity of cytoplasmic organelles, the plasma membrane is the predominant component of the electrocyte. This, therefore, makes it a convenient system for studying membranes and related processes.

It was shown recently (Oliveira, Machado & Chagas, 1978) that isolated electrocyte membranes have high affinity for Ca²⁺. In the present paper we describe the results obtained by applying Oschman & Wall's technique to localize Ca²⁺-binding sites in the cellular structures of intact electrocytes.

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MATERIALS AND METHODS

Fragments of the main electric organ, close to the skin of adult fishes, were removed, dissected in Ringer solution and fixed by immersion in 2.5% glutaraldehyde in 80 mM S-collidine buffer for 2 h at room temperature, washed in the same buffer plus 8.5% sucrose and post-fixed in 1% OsO₄ in 80 mM S-collidine buffer plus 7% sucrose for 1 h at 4 °C. In all solutions CaCl₂ was added at concentrations of 5 or 90 mM. Two controls were used: in one, 1 mM EDTA (ethylenediaminetetra-acetic acid) was added to all solutions; in the second, CaCl₂ was added to the glutaraldehyde solution, but omitted and replaced by 1 mM EDTA in washing and postfixation solutions. Dehydration was carried out in acetone and embedding in Epon. Tissue fragments were oriented so that sections were obtained transverse to the electrocyte; ultrathin sections were obtained with an Ultratome III LKB ultramicrotome, stained with either uranyl acetate for 10 min and lead citrate for 3 min, or only with the latter, and observed either in an AEI EM-6B or in a Philips EM-301 electron microscope. Some sections of purple colour were picked up on carbon-coated, collodion-covered nylon grids and examined in a JEOL 100-CX microscope equipped with a Kevek energy-dispersive X-ray analysis system. A microprobe was used as generated by the STEM accessory.

RESULTS

As previously described (Machado, De Souza, Cotta-Pereira & Oliveira Castro, 1976) the electrocyte has tubular invaginations on the anterior and the posterior faces. At the anterior face they are longer, more numerous and closely packed. At the posterior face many synaptic contacts are observed. They contain a large number of 38.5-nm vesicles, sometimes in contact with the presynaptic membrane. In front of the synaptic contact the plasma membrane of the electrocyte presents a region of increased electron density associated with the inner leaflet of the membrane. Some mitochondria are found mainly surrounding the nuclei. Many microfilaments are seen scattered throughout the cytoplasm.

The Oschman & Wall technique forms electron-dense granules on calcium-binding sites and the presence of CaCl₂ in the fixative and washing solutions was indispensable for their formation and maintenance. When either calcium was omitted or EDTA was added to the solutions no electron-dense deposits were detected (Fig. 1).

In tissue fragments fixed with glutaraldehyde and OsO₄ containing calcium numerous electron-dense deposits were observed at the plasma membrane, including the invaginations, in either the anterior or the posterior faces (Figs. 2 and 3, arrows). The aspect and distribution of the granules were the same when either 5 or 90 mM CaCl₂ was used. The deposits are globular, 45-50 nm in diameter and are scattered

Fig. 1. Surface of the electrocyte of a control preparation in which EDTA was added to the washing solutions. No reaction deposit is observed in the membranes. c, cytoplasm; i, invagination. × 52 500.

Fig. 2. Anterior surface of the electrocyte of a preparation in which CaCl₂ was added to the fixative and washing solutions. Deposits (arrows) are observed associated with the electrocyte's membrane and cytoplasmic microfilaments (mf). × 45 000.

Fig. 3. Posterior surface of the electrocyte in the same preparation as Fig. 2. × 112 500.

Fig. 4. Calcium-binding sites (arrows) in the mitochondrion (m) of the electrocyte. × 75 000.
Calcium-binding sites in *E. electricus*
Figs. 5-8. Posterior face of the electrocyte from the preparation in which CaCl₂ was added to the fixative and washing solutions. Deposits (arrows) are associated with the membrane of the synaptic vesicles (v) (Figs. 5, 7, 8) and post-synaptic membrane (ps). Fig. 5, × 75 000; Fig. 6, × 70 000; Fig. 7, × 171 000; Fig. 8, × 171 000.
Calcium-binding sites in *E. electricus*

along the membrane. Similar deposits were also seen in the cytoplasm, associated with microfilaments (Figs. 2 and 4, arrows) and inside mitochondria (Fig. 4).

At the post-synaptic membranes, however, there was a higher density of granules (Figs. 5, 6), thus suggesting the presence of many Ca$^{2+}$-binding sites at this region. In the synapse, electron-dense deposits were seen associated with the membrane of the synaptic vesicles (Figs. 5, 7, 8). They did not occur in all vesicles and apparently did not present preferential orientation with respect to the axis of the nerve terminal.

Electron-probe X-ray analysis of the dense granules (Fig. 2) on the invaginations of the membranes showed the presence of Ca$^{2+}$ and osmium (Fig. 9). A chloride peak appeared in the substrate and is probably due to the plastic support.

![Fig. 9. Energy-dispersive X-ray spectrum from a dense granule attached to the plasma membrane of the electrocyte. Calcium (CA), osmium (OS) and chloride (CL) peaks are indicated.](image)

**DISCUSSION**

The observation made by Oschman & Wall (1972) that when CaCl$_2$ is added to fixative solutions it is possible to visualize by electron microscopy Ca$^{2+}$-binding sites offers a new possibility for the structural localization of these important sites. The specificity of the technique is confirmed by the observations that the deposits are removed when the tissue is treated with EDTA or EGTA. The use of X-ray micro-analysis also shows the presence of Ca$^{2+}$ as well as phosphorus in the deposits (Oschman, Hall, Peters & Wall, 1974; Hillman & Llínás, 1974; Gambetti, Bulkar, Somlyo & Gonatas, 1975; Fisher *et al.* 1976). In the electrocyte we could also detect by X-ray analysis the presence of Ca$^{2+}$ in the dense granules attached to the plasma membrane, both at the surface and on invaginations. Since the first application of this technique to intestinal cells of the cockroach (Oschman & Wall, 1972) it has been used to detect Ca$^{2+}$-binding sites in different cell types such as the frog sartorius neuromuscular junction (Polittoff, Rose & Pappas, 1974; Pappas & Rose, 1976), the squid giant axon (Hillman & Llínás, 1974; Oschman *et al.* 1974), plasma membrane...
of *Acanthamoeba castellanii* (Sobota, Hrebenda & Przelecka, 1977), insect oocyte (Przelecka & Sobota, 1976), *Paramecium* engaged in secretion (Fisher et al. 1976) and in ciliary movement (Plattner, 1975; Fisher et al. 1976), gap junction membranes (Larsen, 1974) and glial cells (Gambetti et al. 1975). Moreover, the deposits have been found in areas in which physiological and biochemical studies have implicated Ca\(^{2+}\) in the control of cell coupling (Larsen, 1974), excitation-contraction coupling at the transverse tubules of muscle cells (Heuser et al. 1971; Politoff et al. 1974), excitation-secretion coupling at the synapse (Baker et al. 1971; Hillman & Llinás, 1974; Oschman et al. 1974; Pappas & Rose, 1976; Babel-Guerin et al. 1977), platelet release reaction (Skaer, Peters & Emmines, 1974), etc.

In the present work deposits were seen associated with the plasma membrane of either the innervated or the non-innervated face, which raises the question of the function of calcium in the electrocyte. It is possible that calcium deposits may be drawn upon as needed, depending upon the Ca\(^{2+}\) concentration. Although the electric organ originated from muscle cells (Esquibel et al. 1971) it does not contain a structure similar to the sarcoplasmic reticulum which, in muscle cells, plays a fundamental role in the control of the concentration of Ca\(^{2+}\) in the cytoplasm. In the sarcoplasmic reticulum we can see such electron-dense deposits (Politoff et al. 1974; Pappas & Rose, 1976) and this suggests an analogy between this structure and the plasma membrane invaginations of the electrocyte. The simplified interpretation of the invagination as serving only to increase the surface has to be reviewed and clarified by new studies.

The presence of deposits associated with the synaptic vesicles of the nerve-electrocyte synapses has also been observed in other synapses (Heuser et al. 1971; Boyne, Bohan & Williams, 1974; Politoff et al. 1974; Pappas & Rose, 1976). The deposits appear to be associated with the membranes of the vesicles. Probably they represent Ca\(^{2+}\)-binding sites which play a role in the release of acetylcholine (Politoff et al. 1974; Pappas & Rose, 1976). Pappas & Rose (1976) did not observe Ca\(^{2+}\)-binding sites in the synaptic vesicle membrane following intense stimulation of the nerve. However, the deposits seen in the post-synaptic membrane were still present.

As described in other tissues, deposits were also seen in the matrix of mitochondria, a structure which plays a fundamental role in the process of intracellular accumulation of Ca\(^{2+}\) (Carafoli & Lehninger, 1971).

Several mechanisms were discussed by Oschman et al. (1972, 1974) as being possibly implicated in the formation of Ca\(^{2+}\) deposits. Some of them, such as exposure of acidic groups of proteins, tri-complex flocculation of Ca\(^{2+}\), osmium and phospholipid, and coincidence with sites of ATPase activity, seem unlikely in the electrocyte since cytochemical localization of anionic sites and ATPase activity show a uniform distribution of these components throughout the plasma membrane (Benchimol, De Souza & Machado, 1977; Somlö, De Souza, Machado & Hassón-Voloch, 1977), while Ca\(^{2+}\)-binding sites are not similarly localized. Moreover, in several tissues post-fixation with OsO\(_4\) is not necessary for visualization of the electron-dense deposits (Oschman & Wall, 1972; Oschman et al. 1974; Hillman & Llinás, 1974; Politoff et al. 1974; Plattner, 1975; Fisher et al. 1976).
Calcium-binding sites in E. electricus

Recently Oliveira et al. (1978) observed binding of Ca\(^{2+}\) to isolated membranes of the electrocyte of *Electrophorus electricus*. Although a Ca\(^{2+}\)-activated, magnesium-dependent ATPase was detected, experiments with several membrane fractions isolated in a sucrose gradient show that the fraction corresponding to Ca\(^{2+}\)-activated, magnesium-dependent ATPase was not the same as that showing Ca\(^{2+}\)-binding activity. Childers & Siegel (1975) isolated a Ca\(^{2+}\)-binding protein from the electric organ of *Electrophorus electricus*, which was considered to be a major protein component of the electrocyte. Studies with the electric organ of *Torpedo californica*, *Torpedo ocellata* and *Electrophorus electricus* show that Ca\(^{2+}\) has high affinity for acetylcholine receptor proteins (Chang & Neuman, 1976; Rübsamen et al. 1976). Our electron-dense deposits may be the ultrastructural demonstration of this calcium-binding protein.

In view of these results showing the presence of Ca\(^{2+}\)-binding sites in the postsynaptic structures of the neuro-electric junction, new studies are necessary to clarify the role of calcium in the electrocyte’s physiology.

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REFERENCES


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