

CHARACTERIZATION OF CALCIUM OXALATE AND CALCIUM PHOSPHATE DEPOSITS IN SARCOPLASMIC RETICULUM VESICLES

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INTRODUCTION

Sarcoplasmic reticulum vesicles (SRV)¹ isolated from skeletal muscle retain a highly efficient ATP-dependent Ca²⁺ transport system (10, 12). This system plays a key role in the process of excitation-contraction coupling in muscle cells (18).

Oxalate ions strongly enhance active Ca²⁺ transport by the formation of calcium oxalate precipitates within the vesicles (10, 12). In negatively stained preparations, the calcium oxalate deposits formed inside the vesicles are currently identified as discrete areas of low density not penetrated by the staining (1-4, 8-11, 13, 14, 16, 17). However, these deposits are highly sensitive to the illuminating current and decompose after a short exposure to the electron beam (2, 3, 16). The decomposition residues left inside the SRV are difficult to identify. The lability of the calcium oxalate deposits is a serious drawback for the characterization of calcium accumulating vesicles in a given SRV preparation.

Orthophosphate also activates the Ca²⁺ transport of SRV (5, 8, 10, 15, 19). As far as we know, the identification of calcium phosphate deposits inside the SRV has not been attempted yet.

In this paper it is shown that calcium phosphate deposits can also be identified within isolated SRV and that, in contrast to the calcium oxalate deposits, they are quite resistant to the electron bombardment.

MATERIALS AND METHODS

Skeletal muscle SRV were prepared from adult rabbit as previously described (7). For the Ca²⁺ uptake by SRV,

¹ Abbreviations used in this paper: SRV, sarcoplasmic reticulum vesicles.

the incubation medium composition was 10 mM Tris-maleate buffer pH 6.8, 5 mM MgCl₂, 5 mM ATP, 1 mM CaCl₂, 1 mM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid and either 4 mM potassium oxalate, 10 mM potassium phosphate, or 4 mM potassium oxalate plus 10 mM potassium phosphate. The reaction was started by the addition of SRV, total of 0.4 mg of protein per ml. After 10-min incubation at room temperature, a drop of the mixture was used for negative staining. For all the SRV preparation used, control experiments were performed to measure the extent of Ca²⁺ uptake by the SRV fraction, using the same assay medium described above but with ⁴⁵Ca. Ca²⁺ uptake was measured as previously described (6). When oxalate or phosphate was included in the incubation medium, 95-98% of the Ca²⁺ of the assay medium was incorporated by the SRV. In the absence of these anions, the Ca²⁺ uptake was less than 5%.

Calcium oxalate and phosphate crystals were prepared by slowly adding an excess of Ca²⁺ to the assay media used for loading the SRV. The aim of this procedure was to obtain crystals in a manner as similar as possible to that in which crystals form within the SRV.

For negative staining, one drop of the crystal suspension or loaded SRV was placed on a carbon-coated grid and treated with either 1% potassium phosphotungstate or 1% ammonium molybdate. These solutions were previously adjusted to pH 6.8. The grids were examined at direct magnifications ranging from 20,000 × 80,000 ×, in an AEI, EM-6B electron microscope equipped with a decontamination device, double condenser illumination (250 μm Mo aperture) 50 μm Mo objective aperture, with an accelerating voltage of 60 kV and beam current of 200 μA.

RESULTS

The aim of the first set of experiments was to characterize the morphological alterations produced by the electron bombardment on calcium oxalate and calcium phosphate crystals. The crys-

tals of calcium oxalate show growing areas of lower opacity during an exposure of 1–2 min to the electron beam (3). After an exposure of 1–3 min, the crystalline structure is completely destroyed. The residual material derived from neighbor crystals shows a tendency to coalesce and to shrink (Figs. 1 *a* and 1 *b*). The damaging effect of the electron bombardment on a single crystal is shown in Figs. 2 *a–c*. The exposure required for crystal damage varies with the intensity of the electron beam.

The crystals of calcium phosphate (Figs. 3 *a–4 b*) show a granular aspect due to small, circular regions of lower electron density. Upon a prolonged exposure to the electron beam, this granulation becomes less pronounced, and a discrete halo appears surrounding the crystals. No further damage of the crystalline structure was observed even after 30-min exposure to the electron beam.

Figs. 5 *a–6 c* show SRV containing calcium oxalate deposits. In most vesicles these deposits disappear after 1–3 min of exposure to the electron beam (Figs. 5 *a* and 5 *b*). In some vesicles the residual materials derived from the deposit decomposition seem to coalesce, forming a globular mass which is resistant to further exposure to the electron beam (Figs. 6 *a–6 c*).

Fig. 7 *a* shows SRV loaded with calcium phosphate. The material accumulated by the vesicles is electron opaque and has a granular aspect similar to that observed in calcium phosphate crystals (Figs. 3 *a–4 b*). This deposit could be clearly seen even after 30-min exposure to the

electron beam (Fig. 7 *b*). The SRV loaded with calcium phosphate are easily identified in specimens stained with ammonium molybdate. This stain gives a light background contrasting the electron-opaque vesicles (Fig. 7). With the use of uranyl acetate or phosphotungstic acid, it is difficult to distinguish the loaded vesicles from the more electron-opaque background.

Figs. 8–11 show SRV which did accumulate calcium in presence of oxalate and phosphate ions. The loaded vesicles usually contain both calcium phosphate and calcium oxalate deposits. The calcium oxalate deposits are decomposed after 3 to 5-min exposure to the electron beam (Figs. 9–11), and the residual materials coalesce in a way similar to that shown in Figs. 6 *a–6 c*. This could be seen in all the vesicles observed, since now the residual material derived from calcium oxalate is easily visualized due to the contrast given by the surrounding deposits of calcium phosphate.

DISCUSSION

The data presented show that during the process of active Ca^{2+} transport, calcium phosphate deposits are formed inside the SRV. Orthophosphate proved to be a better calcium-precipitating agent than oxalate for the cytochemical identification of the Ca^{2+} -accumulating vesicles since the calcium phosphate precipitates are much more resistant to the electron bombardment than the calcium oxalate precipitates. Probably the calcium oxalate precipitate decomposes by losing water, CO_2 , and CO. The remaining CaO occupies a much smaller

FIGURE 1 Calcium oxalate crystals before (1 *a*) and after (1 *b*) irradiation with the electron beam. In Fig. 1 *a* the crystals show minute areas of lower opacity which are possibly due to the minimum amount of beam exposure necessary for focusing. Fig. 1 *b* shows the aspect of the same crystals severely damaged after 2-min incidence of a full strength 200- μA beam. $\times 60,000$. Calcium oxalate crystals were prepared as described under Materials and Methods.

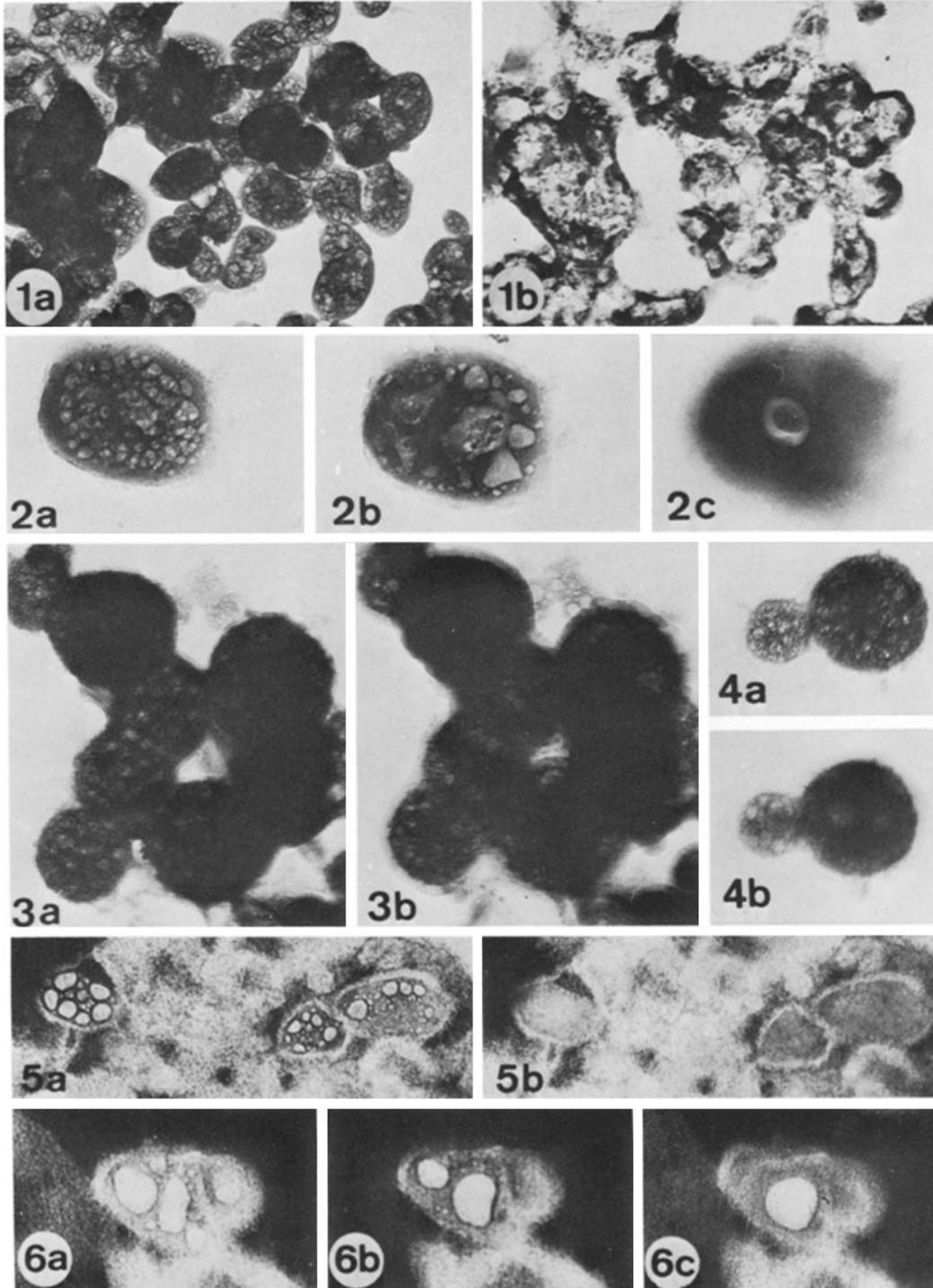
FIGURE 2 An isolated calcium oxalate crystal after 40-s (2 *a*), 80-s (2 *b*), and 120-s (2 *c*) exposure to the electron beam. $\times 120,000$.

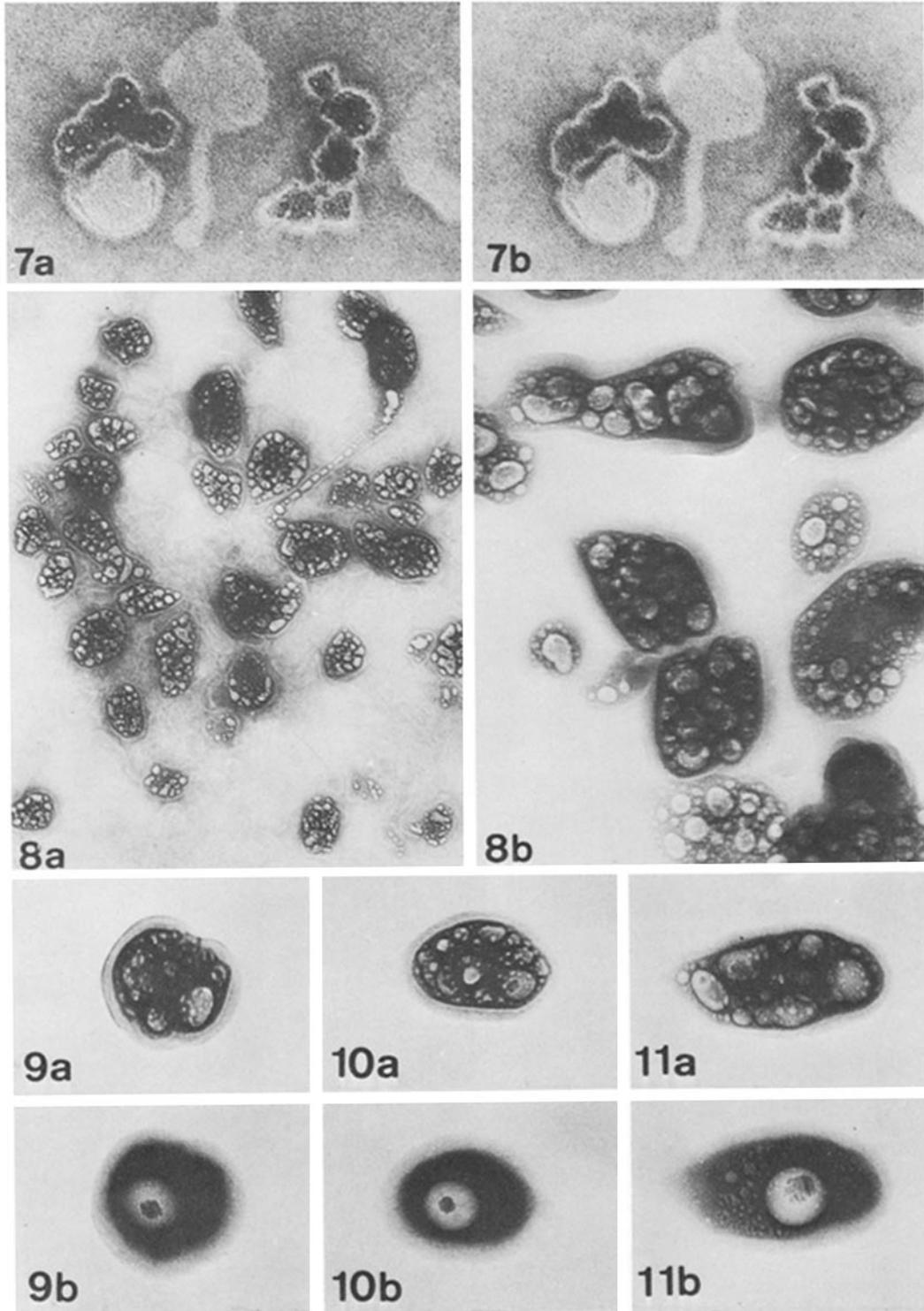
FIGURE 3 Calcium phosphate crystals. The rounded masses containing small regions of lower density (3 *a*) were not noticeably modified after 15-min exposure to the electron beam (3 *b*). $\times 120,000$.

FIGURE 4 Two isolated calcium phosphate crystals. After a prolonged irradiation (4 *b*) the granulation observed at the beginning (4 *a*) becomes less pronounced, but no further change is otherwise noticed besides the appearance of a discrete halo. $\times 120,000$.

FIGURE 5 Sarcoplasmic reticulum vesicles containing initially (5 *a*) calcium oxalate deposits which disappear after exposure to the electron beam (5 *b*). Negative staining with potassium phosphotungstate. $\times 120,000$.

FIGURE 6 SRV containing calcium oxalate deposits. Initial (6 *a*), intermediate (6 *b*), and final (6 *c*) stages of exposure to the electron beam. The residues seems to coalesce forming a globular mass fairly resistant to further exposure to the electron beam. $\times 240,000$.





volume than the crystalline material. Detailed studies of the modification and decomposition of different salt crystals under the electron beam have been described in the literature (20, 21).

Due to the lability of the calcium oxalate deposits, information concerning the shape and distribution of these precipitates "before beam exposure" can be obtained only during a very short observation time, just sufficiently long to find and focus upon the interesting areas.

Fig. 8 *a* shows that, in a given SRV preparation, only part of the vesicles accumulate calcium oxalate and calcium phosphate. With the use of oxalate, this observation has already been reported and carefully discussed by several authors (1-3, 9, 11, 16).

SUMMARY

Sarcoplasmic reticulum vesicles isolated from skeletal muscle show a deposition of calcium oxalate and calcium phosphate inside the vesicles during the process of active Ca^{2+} transport. The damaging effect of electron bombardment on crystals of calcium oxalate and calcium phosphate was investigated and correlated with the appearance of the deposits formed inside the vesicles as seen under the electron microscope using negative staining.

The calcium oxalate deposits formed inside the vesicles are highly sensitive to the illuminating current and decompose after a short exposure to the electron beam. In contrast, the calcium phosphate deposits are quite resistant to the electron bombardment.

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FIGURE 7 SRV loaded with calcium phosphate. The granular aspect of the deposits accumulated by the vesicles (7 *a*) is slightly modified by beam exposure (7 *b*), but the electron-opaque phosphate deposits persist, remaining clearly visible against the lighter background provided by staining with ammonium molybdate. $\times 120,000$.

FIGURE 8 SRV which did accumulate calcium in the presence of both oxalate and phosphate ions. Empty vesicles are seen (8 *a*) in the light background given by ammonium molybdate staining. (8 *a*), 40,000. (8 *b*), $\times 120,000$.

FIGURES 9-11 SRV loaded with calcium oxalate and calcium phosphate (9-11 *a*). After 15 min exposure to the electron beam (9-11 *b*) the oxalate deposits decompose and coalesce, the residual material remaining visible against the dark background given by the calcium phosphate accumulated by the vesicles. $\times 120,000$.