

A ^{45}Ca Autoradiographic and Stereological Study of Freeze-dried Smooth Muscle of the Guinea Pig Vas Deferens

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ABSTRACT In an effort to more clearly elucidate the role of cellular structures as calcium sinks and sources in smooth muscle cells, the intracellular distribution of radioactive calcium was evaluated by a new method based on freeze-drying. The guinea pig vas deferens was exposed to a physiological salt solution that contained ^{45}Ca . The muscle was then freeze-dried and prepared for electron microscope autoradiography. The grain density over the plasma membrane, mitochondria, and sarcoplasmic reticulum (SR) was significantly greater than that over the cytoplasmic matrix. The grain density of the nucleus was only slightly greater than that of the matrix. These results suggest that the plasma membrane, mitochondria and SR have the capacity to accumulate calcium. Which of these structures serve as a source of calcium for contraction remains to be determined. A stereological comparison between freeze-dried and conventionally prepared smooth muscles revealed several differences. The cross-sectional area of freeze-dried cells was about twice that of conventionally prepared cells. Moreover, mitochondria and sub-surface vesicles occupied a significantly smaller percentage of the cell in the freeze-dried tissue than they did in the conventionally prepared tissue.

In smooth muscle, as in other types of muscle, calcium is considered to be the agent that activates the contractile apparatus (12, 23, 38). Two different pools of calcium have been identified (7, 20, 21). One pool is in the extracellular medium or loosely bound to the cell surface; the other is sequestered within the cell or tightly bound to the cell surface. Different types of muscle utilize these two pools differently. In some muscles, such as the pulmonary artery of the rabbit (10) or the rabbit aorta (10, 21), the firmly bound pool is able to support mechanical activity. In others, such as the longitudinal muscle of the guinea pig ileum, the extracellular or loosely bound pool is used (22). Still others, such as the guinea pig vas deferens, appear to fall between these two extremes (27).

A number of attempts have been made to determine the intracellular sites of calcium sequestration. These methods include precipitation of the calcium as electron-opaque calcium salts (9, 18, 25, 33, 34, 50, 52), substitution of a more electron-opaque cation for calcium (31, 42, 45), electron probe x-ray analysis (1, 18, 33, 42, 50, 52), and ^{45}Ca electron microscope autoradiography (EM ARG) (25, 27). These studies have implicated mitochondria, plasma membrane and sarcoplasmic reticulum (SR) as possible sequestration sites for calcium. Unfortunately, all of these studies used tissues that had been

fixed, dehydrated, and embedded. Diffusible substances may have been translocated or removed during these procedures (5, 47, 49).

In an effort to avoid this problem, Somlyo and co-workers (43, 44) have developed a procedure for freezing tissue in super-cooled Freon, then cryosectioning it and freeze-drying the sections. Examining these sections with the electron probe revealed calcium in mitochondria, cytoplasm, nuclei, and some portions of the SR (43). We have developed an alternate method, which involves rapidly freezing tissue on a copper bar chilled in liquid nitrogen, dehydrating the whole tissue under vacuum and then vacuum-embedding it in plastic (28). The distribution of calcium is determined in thin sections, using ^{45}Ca EM ARG. This provides adequate cellular morphology to identify intracellular organelles and plasma membrane (28). In the present paper we demonstrate that this method of tissue preservation maintains the *in vivo* distribution of calcium and is amenable to ^{45}Ca EM ARG. In addition, a stereological comparison is made between conventionally prepared and freeze-dried tissue to determine the effects of these two types of specimen preparation on smooth muscle at the ultrastructural level. A preliminary report of part of this work has been presented elsewhere (29).

MATERIALS AND METHODS

Tissue Preparation

Male guinea pigs (300–500 g) were killed by a blow on the head. The vasa deferentia were removed and immersed in a physiological salt solution (PSS) maintained at room temperature. The PSS had the following composition in millimoles per liter: NaCl, 125; KCl, 2.7; CaCl₂, 1.8; glucose, 11; Tris (Tris[hydroxymethyl]aminomethane, Sigma Trizma Base, Sigma Chemical Co., St. Louis, Mo.) buffer, 23.8. The solution was adjusted to pH 7.5 with 6 N HCl and was saturated with 100% oxygen. Connective tissue was carefully cut away from the exterior of the muscle. Each muscle was then cut into ~1-mm² pieces. Tissues to be studied were selected from the middle third of each vas deferens. The tissues were incubated in fresh, oxygenated PSS until they were frozen.

Freeze-drying, Embedding, and Tissue Evaluation

Several techniques of freezing on a metal bar (6, 19, 53) and dehydrating under vacuum have been described (6, 47, 48). Because we have modified portions of several of these techniques for our purposes, a brief description of the technique used in our laboratory is presented below. A copper bar 7 × 3 in (length × diameter) with a mirror finish on one end was placed in a styrofoam container and was chilled with liquid nitrogen to ~-196°C. Several pieces of tissue were placed on the large end of a No. 3 cork that had previously been covered with aluminum foil. Excess PSS was removed from the tissue. The cork was then inverted and rapidly brought into firm contact with the chilled copper bar. After 20–30 s the cork containing the tissue was rapidly removed from the face of the bar and plunged into a Dewar of liquid nitrogen.

Once the tissue was frozen it was essential that subsequent procedures and manipulations be carried out while the tissue was below -76°C. At temperatures above this, water in the liquid state may be present in the muscle tissue (51). The tissue was prepared for drying as follows: the piece of foil to which the frozen tissue was adhered was rapidly transferred from the storage Dewar to a dissecting

dish containing liquid nitrogen. The tissue was carefully dissected away from the foil and transferred to a chilled aluminum tissue holder. After all the tissues had been loaded into the holder, it was rapidly removed from the dissecting dish and placed inside the precooled, glass specimen container of the freeze-drying apparatus shown in Fig. 1.

Before freezing the tissue, the 5 Å molecular sieve (Union Carbide Corp., Linde Div., New York) contained in the right-hand portion of the apparatus (Fig. 1) was heated to 150°C for a minimum of 12 h in an effort to drive off water and other molecules that may have been trapped in the sieve. The warmed sieve was then evacuated to a pressure of $\sim 2 \times 10^{-2}$ torr to reduce reabsorption of molecules and was sealed off.

The specimen container which was partially immersed in liquid nitrogen was then attached to the main body of the apparatus and was evacuated to a pressure of 2×10^{-2} torr using a mechanical pump. At this point, the side of the system that contained the tissue was opened to the side that contained the sieve, a Dewar of liquid nitrogen was placed around the sieve, and the mechanical pump was removed. In 30 min or less, the pressure within the apparatus fell to $\sim 1 \times 10^{-3}$ torr. The sieve was allowed to pump on the specimen container in this manner for ~4 h.

After the 4-h equilibration period, the Dewar of liquid nitrogen around the specimen container was replaced by a Dewar of dry ice-acetone slush (-80°C). After 36 h the specimen container was raised out of the slush over an 8-h period and was allowed to come to room temperature for ~12 h. The dehydrated tissue was then exposed to the vapor of osmium tetroxide crystals in vacuo to enhance contrast.

At this point the tissue was extremely hydrophilic; consequently it was necessary to break the vacuum by introducing dry nitrogen gas into the system via the port at the top of the apparatus (Fig. 1). The tissue was immediately transferred to a vial containing Spurr embedding medium (Polysciences, Inc., Warrington, Pa.) and was placed under vacuum for 30 min. The vial was then attached to a rotation mixer for 30 min. At the end of that time the tissue was embedded and polymerized according to routine procedures (46).

Thick (500 nm) sections were cut from each tissue block on a Sorvall-MT2B ultramicrotome (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.) and placed on a glass slide. After staining with Azure II-methylene blue, sections were examined under a light microscope. Those tissues that appeared to have maintained adequate cellular integrity were selected and thin 100-nm sections were cut from these blocks. After staining in saturated uranyl acetate in 50% ethanol and Reynolds' lead citrate (35), the tissue was examined in a Hitachi 11-C electron microscope.

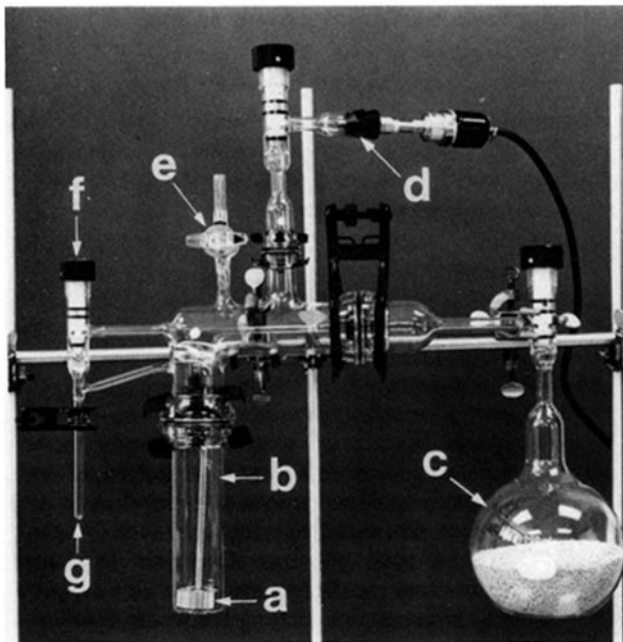


FIGURE 1 After the frozen tissue is loaded into the aluminum tissue holder (a), the holder is placed inside the glass specimen container (b) which is then attached to the remainder of the freeze-drying unit. The right-hand portion of the unit contains the molecular sieve (c). A vacuum gauge (d) is located between chambers b and c. A mechanical pump can be attached to the system via a stopcock (e). After the tissue has been dehydrated, as discussed in the text, valve f is opened and vapor from osmium tetroxide crystals contained in tube g enters the vacuum system. (Note: a solid piece of glass is located between the main body of the apparatus and the underside of valve f to stabilize the valve). The vacuum is broken by introducing dry nitrogen gas through the stopcock (e).

Stereology

Freeze-dried tissues were compared with tissues fixed with osmium, dehydrated with acetone, and embedded in Araldite 506 (27). Thin sections from both preparations were photographed at a constant magnification ($\times 5,840$) and enlarged $\times 2.5$. A total of 163 freeze-dried cells from four animals and a total of 220 conventionally prepared cells from three animals were analyzed. Using a Ladd Graphic Data Digitizer (Ladd Research Industries, Inc., Burlington, Vt.), the total area of each cell was determined as was the area of the nucleus, mitochondria, surface vesicles and clear, membrane-bounded area (presumably SR) that occurred within the cell. By subtracting the area occupied by these structures from the total cell area, an estimate of cytoplasmic matrix area was obtained.

Autoradiography

Smooth muscle of the guinea pig vas deferens was dissected into approximately equal 1×1 mm pieces and was exposed to a PSS that contained ⁴⁵Ca (1 mCi/ml) for 30–56 min. (Note: in connection with another study, we examined tissue that had been incubated in a PSS that contained ⁴⁵Ca for 60–86 min, and there was no significant difference in the relative distribution of ⁴⁵Ca within the cells in the two studies.) The tissue was washed three times with cold PSS in a 6-min period. Thin sections of freeze-dried samples were placed on grids and were coated with a thin carbon film (2). A monolayer of Ilford L-4 emulsion was then applied to the tissue as described by Caro and Van Tubergen (4). Coated tissue was stored at 4°C in black boxes containing silica gel (4). After an appropriate period of exposure, the autoradiograms were developed in D-19 and stained with Reynolds' lead citrate for 20–30 min in an effort to remove unexposed emulsion. They were then stained with uranyl acetate followed by Reynolds' lead citrate to enhance contrast. Micrographs were taken only from grids on which the background grain density in tissue-free areas of plastic was 15% or less than the grain density over the tissue. All photographs were taken at a constant magnification of 9,930 and were enlarged to a final magnification of 24,825. The autoradiograms were then evaluated to determine the intracellular distribution of developed ⁴⁵Ca grains.

RESULTS

Morphology and Stereology

Cells from conventionally prepared vasa deferentia of the guinea pig are shown in Figs. 2 and 3. Each cell is bounded by a plasma membrane and, at distinct points along the membrane, invaginations or surface vesicles are seen. Mitochondria occur throughout the cell as do clear membrane-bounded spaces. It has previously been proposed that the latter can accumulate calcium and therefore should be classified as SR (32, 39). In Figs. 4 and 5, cells from vasa deferentia that were prepared using the freeze-drying technique are shown. These cells are quite similar in appearance to other freeze-dried smooth muscle cells that have been studied (28).

The results of the stereological experiments are shown in Table I. The cytoplasmic matrix, the nucleus, as well as the total mean area occupied by the cells that were prepared using conventional techniques, were found to be approximately one-half that of cells that had been freeze-dried. The SR in the conventional preparation occupied slightly less area than did the SR in the freeze-dried tissue. On the other hand, the mitochondria occupied slightly more area in the conventional preparation than they did in the freeze-dried tissue. Surface vesicles were observed in conventionally prepared cells but were essentially absent in freeze-dried cells.

Autoradiography

Our initial experiments were directed towards comparing the retentions of ^{45}Ca in tissues that had either been freeze-dried or prepared conventionally. The relative loss of ^{45}Ca from the tissue was monitored by liquid scintillation counting of the various media and the solubilized tissue at each step of the preparative procedure. After exposure to ^{45}Ca PSS, both preparations were incubated in nonradioactive PSS. The conventionally prepared tissue was then fixed in osmium tetroxide vapors while the freeze-dried tissue was rapidly frozen. Neither fixation in vapor nor freeze-drying led to a loss of ^{45}Ca . A group of the conventionally prepared tissues was then dehydrated in a graded series of acetones and infiltrated with Spurr embedding medium. These procedures led to a loss of ~23% of the total ^{45}Ca in the tissue. Because the frozen tissue was dehydrated at low temperature in vacuo, there was no loss of ^{45}Ca at this step. However, during infiltration the freeze-dried tissue lost ~4% of its total ^{45}Ca . About 3.5% of the ^{45}Ca was extracted in the first 5 min of infiltration. The remaining 0.5% of the ^{45}Ca was removed over a 55-min period. These observations support the contention that the ^{45}Ca that is lost during infiltration is adhered to the surface of the tissue rather than to deeper sites.

Before autoradiographic experiments were carried out using the freeze-dried tissue, the possibility that ^{45}Ca might be lost during embedding, thin sectioning, coating with nuclear emulsion, and staining the developed autoradiograms had to be evaluated. The possible loss of radioactive label during embedding was explored by examining developed autoradiograms. If ^{45}Ca had been leached from the tissue into the epoxy, the grain density in the plastic adjacent to the edges of the tissue relative to the background grain density in the epoxy farther away from the tissue would increase. However, no significant difference in the grain densities in these two locations was observed. In addition, the background grain density away from the tissue was comparable to the grain density determined for autoradiograms of nonradioactive tissue. Consequently, leaching of

^{45}Ca into the embedding medium did not seem to be a problem in this study.

The next point at which calcium might be lost is during thin sectioning. This possibility was explored as follows. Five thin sections were cut and were allowed to float on the water in the knife boat for 5 min. The sections were then removed from the boat and placed in a vial that contained Soluene-100 (Packard Instrument Co., Downers Grove, Ill.). An aliquot of water from the boat was placed in another vial. The counts per minute in the tissue and in the water were then determined. This procedure was repeated after sections had been floating on water for 15 min. After 5 min the sections lost ~15% of their calcium into the water. After 15 min the loss of calcium did not increase. This finding indicates that there was an initial leaching of radioactivity from the surface of the section in contact with the water. However, deep penetration of the water did not occur (47).

Radioactive label might be leached from the tissue during coating with nuclear emulsion. According to Stirling and Kinter (47), the occurrence of dense halos of silver grains over and around the tissue in developed autoradiograms is indicative of radiation spread. We have not observed this halo effect in any of the autoradiograms of freeze-dried smooth muscle.

After the silver halide crystals in the nuclear emulsion are exposed and subsequently developed it is essential that the silver grains do not shift in relation to the thin section. One point at which the developed grains might shift is during staining with Reynolds' lead citrate and uranyl acetate. It has been reported that alkaline lead staining after exposure and development may leach out grains or cause them to shift (36). This did not prove to be a problem in any of our autoradiograms. We have photographed unstained cells over which grains occur and then stained the same tissue section for 30 min with lead citrate. If the same cells were photographed again, the grains in the two pictures were in an identical position. Similar results were recorded after staining with uranyl acetate.

A major concern with applying ARG techniques to study physiological phenomena is the lack of resolution inherent in the technique. Given the relatively long range (550 nm) of the beta particle emitted by ^{45}Ca (55), the problem of associating a developed grain with its source becomes more important than with a lower energy isotope such as ^3H . However, it can be partially resolved by utilizing the fact that the number of developed grains decreases rapidly as the distance from the source increases. Based on the data presented by Winegrad (55), the probability that a circle of 275-nm radius drawn around a grain should also contain the point source of the radiation is 50%. We, therefore, chose to examine the relationship between grains and sources up to a maximum distance of 275 nm.

Because smooth muscle cells are tapered, cylindrically shaped cells, the diameter of the cells near the ends is much less than the radius (275 nm) of the circle used to score the grains. Identification of the radiation source under these circumstances is virtually impossible. To avoid this problem, grains were included in the study only when the width of the cell or cells adjacent to the grain was three times the radius of the circle, i.e., 825 nm.

The center of the developed grain, i.e., the midpoint between the extremities of the grain, was chosen (37) and a circle of 275-nm radius was placed over the grain. If a structure, i.e., mitochondrion, SR, plasma membrane, or nucleus, lay within

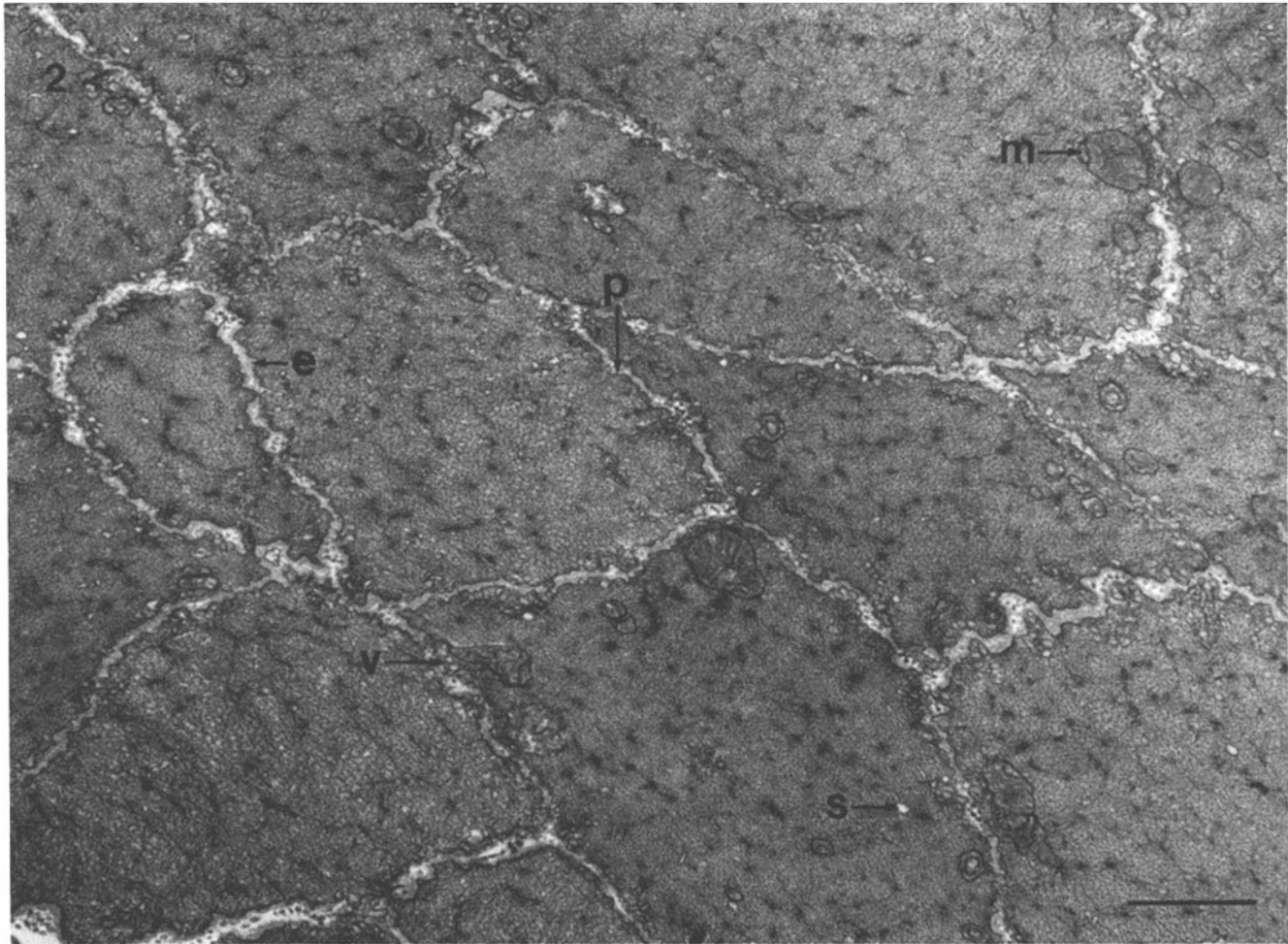
this circle, it was scored as the probable source of the radioactive decay. If no structure occurred within the circle, the cytoplasmic matrix was scored as the probable source. With this method of scoring, the grains in Fig. 6 were analyzed as follows: The upper grain is associated with a mitochondrion. The grain in the center is associated with the plasma membrane. The lower grain is associated with cytoplasmic matrix.

Plasma membrane, SR, mitochondria, and the nucleus are always adjacent to cytoplasmic matrix and, except for the nucleus, all of these structures are smaller than the radius of the circle. Consequently, any circle of 275-nm radius will almost always include some matrix. Therefore, these results must be expressed in terms of a compound item, namely, an organelle and the cytoplasmic matrix within an annulus of 275 nm of the organelle (37, 54). Or, in the case of the plasma membrane, the results must be expressed in terms of the membrane plus matrix plus extracellular space within 275 nm of the membrane.

By employing the probability circle analysis as described by Williams (54) and Salpeter and McHenry (37), it is possible to compute the grain distribution that would be expected among the compound sites and the matrix if the grains are scattered

randomly throughout the cell. The random distribution can then be compared to the observed distribution (Table II). When these two distributions were examined using a χ^2 analysis, it was found that they were significantly different ($\chi^2 = 30.58$, $P < 0.001$). The deviation or lack of deviation from random observed in each item is shown in Table II. The cytoplasmic matrix was observed to have significantly fewer grains associated with it than expected if the distribution were random. The plasma membrane and mitochondria have significantly more grains.

The next step in the analysis of the compound organelle items is to determine the portion of the grains that results from beta decay within the organelle and the portion that results from decay within the adjacent annulus of matrix (54). To carry out this analysis, the assumption must be made that the grain density (grains/unit area) in the cytoplasmic matrix is uniform throughout the cell. That is, the baseline grain density in the matrix away from an organelle is the same as the baseline grain density in the organelle plus annulus region. Therefore, any grains contributed to the organelle plus annulus area by the organelle will be in excess of the baseline grain density. In all of the compound items examined, the grain density is



FIGURES 2 AND 3 Smooth muscle cells of the guinea pig vasa deferentia are shown in both cross section (Fig. 2) and longitudinal section (Fig. 3). The tissue has been prepared using conventional techniques, i.e., fixed in osmium tetroxide vapor, dehydrated in a graded series of acetones, and embedded in Araldite. The cells are separated from one another by extracellular space (e). Within the cell, mitochondria (m) are prominent. Clear, membrane-bounded sarcoplasmic reticulum (s), which varies in size and shape, is present. Clusters of surface vesicles (v) are seen to occur frequently along the plasma membrane (p). Bars, 1 μ m. $\times 17,520$.

greater than the baseline grain density, indicating that the items are more radioactive than the cytoplasmic matrix. To express this enhanced radioactivity as grains/unit area of organelle, the relative area of the organelle in each compound item must be determined. Using the point counting method (54), the mitochondria were observed to occupy 14.9% of the mitochondria/matrix compound item, the SR 16.7% of the SR/matrix item, and the nucleus 88.5% of the nucleus/matrix item. Based on this information, the grain density of the mitochondria is 0.61, the SR is 0.32, and the nucleus is 0.09 (Table III). Therefore, relative to the cytoplasmic matrix (grain density = 0.06), the mitochondria are ~10 times more radioactive, the SR five times more radioactive, and the nucleus two times more radioactive.

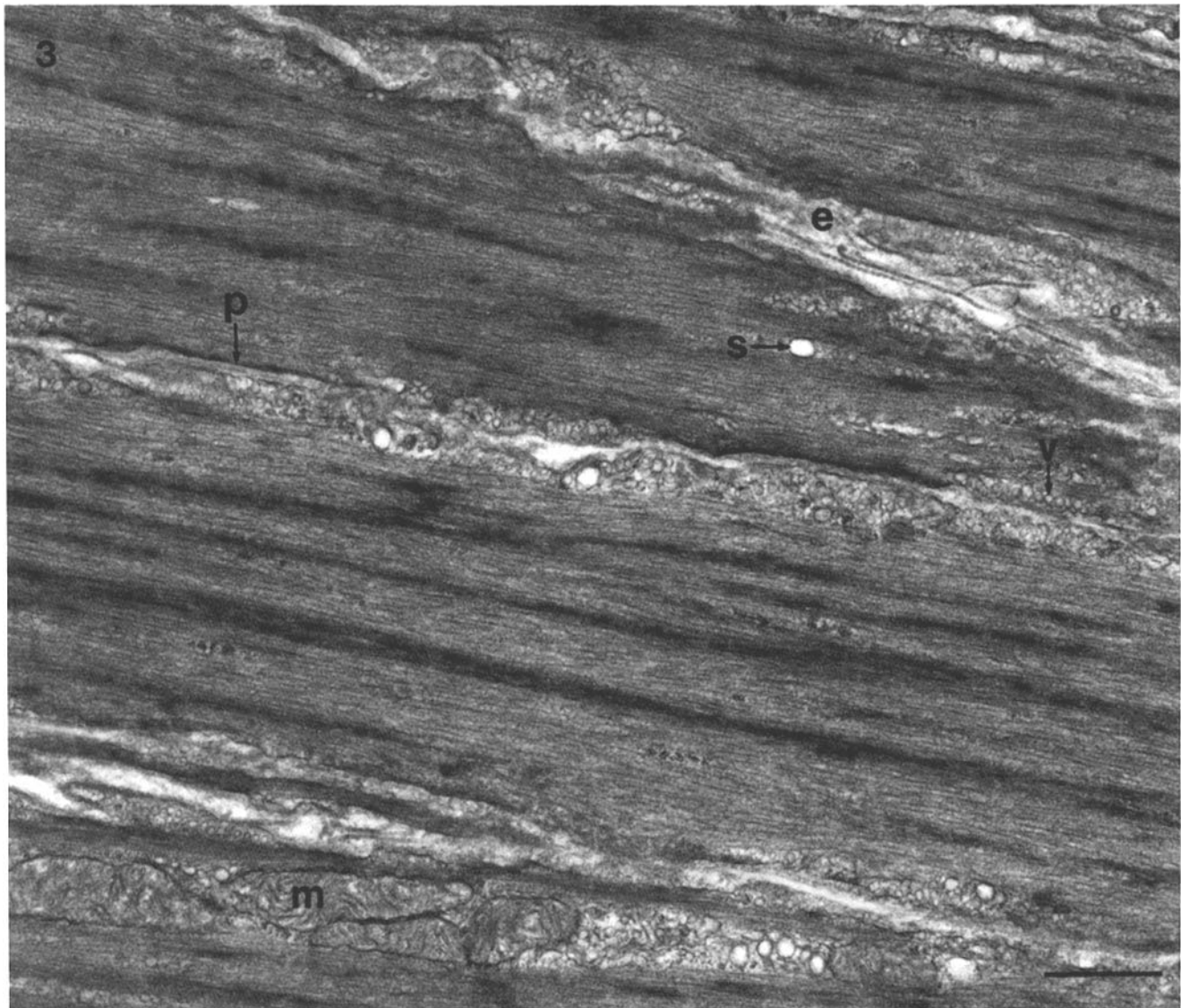
To determine the grain density of the plasma membrane, it must be assumed not only that the cytoplasmic matrix within the membrane plus annulus area contains the same concentration of calcium as does the rest of the matrix but also that the extracellular space within the annulus contains the same concentration of calcium as does the rest of the extracellular space. Based on these assumptions, the grains contributed to the item by the matrix, which occupies 65.1% of the item, and the

extracellular space, which occupies 26.6% of the item can be calculated. After the grains contributed by the matrix and the extracellular space are subtracted, the grains contributed by the plasma membrane remain. From this information and from calculating the portion of the item occupied by the membrane (8.3%), the grain density of the membrane was calculated to be 0.86 or ~14 times higher than the matrix (Table III).

At times a ^{45}Ca grain will be located within a 275-nm radius of plasma membrane and one or more organelles. These situations can not be adequately differentiated by the method described above. Therefore, these grains were omitted from the study. However, because these grains represent a small portion of the total grains observed (<10%), we do not believe that this omission overly restricts the usefulness of the method.

DISCUSSION

By use of stereological techniques, the cell volumes occupied by mitochondria, SR, surface vesicles and/or the nucleus have been estimated in conventionally prepared nonvascular muscles such as the guinea pig taenia coli (15, 16, 27, 34), stomach (30), small intestine (13, 17), and sphincter pupillae (14), the





FIGURES 4 AND 5 Smooth muscle cells of vasa deferentia that have been rapidly frozen, vacuum-dehydrated, and embedded in Spurr resin are shown in transverse section (Fig. 4) and longitudinal section (Fig. 5). In Fig. 4, ice crystal reticulations are evident in cells located more distal to the surface of the tissue that was initially frozen. This results in a less dense cytoplasmic matrix compared to that in more rapidly frozen cells (compare upper left portion of the micrograph with lower portion). In both figures, cells are bounded by a plasma membrane (*p*) and are separated from other cells by extracellular space (*e*). Extremely electron-dense mitochondria (*m*) and electron-translucent sarcoplasmic reticulum (*s*) occur throughout the cells. In Fig. 4, surface vesicles (*v*) are occasionally observed to occur along the plasma membrane. In Fig. 5, a portion of the nucleus (*n*) can be seen in one cell. Bars, 1 μm . $\times 17,520$.

rabbit taenia coli (10) and the turtle oviduct (40). From these studies, it appears that the percent of cell taken-up by a particular structure varies considerably from muscle to muscle. For example, mitochondria have been reported to occupy from 3.1 to 12.0% of the cell (13–15, 17, 27, 34), the SR 1.5 to 4.0% of the cell (10, 17, 27, 30, 34, 40), surface vesicles 2.3 to 3.5% of the cell (27, 34), and the nucleus 12.9 to 33.4% of the cell (16, 34).

In conventionally prepared muscle of the guinea pig vas deferens, it has previously been reported that the SR, mitochondria, and surface vesicles occupy 1.7, 2.2, and 2.0% of the cell, respectively (27). If the area of the nucleus, which was not determined in that study, is excluded in the present study, the percentages of the cell occupied by the SR, mitochondria, and surface vesicles in conventionally prepared muscle are 1.3, 2.5, and 0.4%, respectively. These values are in good agreement except for the surface vesicle measurement. The lack of agreement regarding the relative area of surface vesicles can possibly

be attributed to the different stereological techniques used in the two studies.

The literature contains no data with which the estimates of cell area in freeze-dried smooth muscle can be compared. However, when freeze-dried cells are compared to conventionally prepared cells, several differences are evident (Table I). Freeze-dried cells were observed to be about twice the size of conventionally prepared cells. Several possible explanations for this difference in size were considered. One possibility was that the thin sections in the two preparations did not come from the same relative portion of the cell. A second possibility was that the freeze-dried cells were larger because they were more contracted. A third possibility was that the differences in size were caused by the dissimilar preparative techniques.

The first possibility was explored by counting the number of cells in both preparations that contained a nucleus. If the freeze-dried cells are larger because they were sectioned more towards the center, more nuclei should be visible in this prep-



TABLE I

A Stereological Comparison between Conventionally Prepared and Freeze-dried Cells of the Vas Deferens of the Guinea Pig

| Cellular site | Preparative technique | | | |
|------------------------|--------------------------------------|----------------------|--------------------------------------|----------------------|
| | Conventional <i>n</i> = 220 Cells | | Freeze-dried <i>n</i> = 163 cells | |
| | Area in μm^2 * | % of Total cell area | Area in μm^2 * | % of Total cell area |
| Cytoplasmic matrix | 10.54 \pm 4.61‡ | 92.7 | 19.51 \pm 8.66‡ | 94.4 |
| Nucleus | 0.31 \pm 1.29‡ | 2.7 | 0.69 \pm 1.58‡ | 3.3 |
| Sarcoplasmic reticulum | 0.14 \pm 0.63 | 1.2 | 0.19 \pm 0.19 | 0.9 |
| Mitochondria | 0.34 \pm 0.40 | 3.0‡ | 0.28 \pm 0.28 | 1.4‡ |
| Surface vesicles | 0.04 \pm 0.03 | 0.4‡ | 0.00 \pm 0.00 | 0.0‡ |
| Total Cell | 11.37 \pm 5.33‡ | 100.0 | 20.67 \pm 9.44‡ | 100.0 |

* Mean \pm SD.

‡ $P < 0.01$.

aration. Because only a small portion of the total cells examined in either preparation contained a nucleus (15% of the freeze-dried and 8% of the conventionally prepared cells), it is unlikely that the difference in total cell area between the preparations is caused by this parameter. The second possibility is not likely because contraction induced during fixation by freezing, which occurs in milliseconds, should be much less than the contractile

response that occurs during fixation in osmium tetroxide (1), which takes minutes. The third possibility appears to be the most plausible. However, because of the wide range of effects that conventional fixation and dehydration can have on smooth muscle (26, 41, 56), plus the dearth of information in the literature on the effects of freeze-drying in this type of tissue we are unable to evaluate whether shrinkage occurred in the conven-

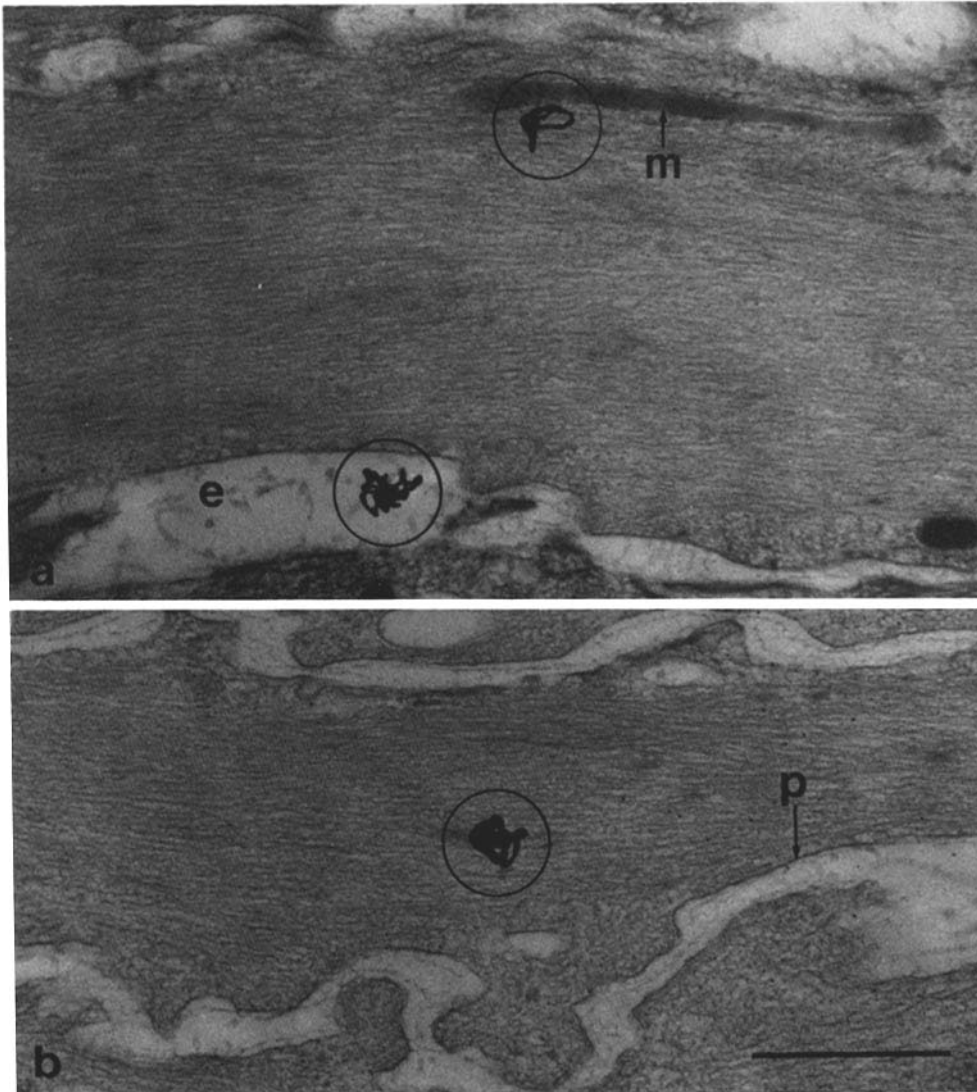


FIGURE 6 Longitudinal sections through guinea pig vasa deferentia which have been incubated with ^{45}Ca , freeze-dried, and prepared for electron microscope autoradiography. The three silver grains in this micrograph were analyzed in the following manner. The center of a developed grain was chosen and a circle of 275-nm radius was drawn around it. In *a*, the upper grain is associated with a mitochondrion (*m*) and the lower grain is associated with the plasma membrane (*p*). The grain in *b* is associated with the cytoplasmic matrix. Bar, 1 μm . $\times 25,818$.

tionally prepared tissue and/or whether swelling occurred in the freeze-dried tissues.

Of the cellular components examined, the difference in the percent of the total cell area occupied by mitochondria in the two preparations is most striking. Upon examination of Figs. 4 and 5, the occurrence of an electron-translucent space around some of the mitochondria is evident, suggesting that mitochondrial shrinkage may have occurred. However, this translucent space was included in the area determination of the organelle. An alternative explanation is that the mitochondria in the conventionally prepared cells are swollen as a result of chemical fixation in an aqueous medium. Although it is tempting to speculate that the latter is more plausible, some shrinkage during freeze-drying cannot be ruled out at the present time.

The absence of surface vesicles in freeze-dried cells is more perplexing. Perhaps the plasma membrane has been damaged during freezing and/or vacuum dehydration, resulting in the disappearance of surface vesicles. Alternatively, the appearance

of surface vesicles may have been induced during conventional preparation. Whether either of these explanations accounts for the observed difference is unclear.

One of the aims of the ^{45}Ca portion of the study was to compare the losses of ^{45}Ca from freeze-dried and conventionally prepared tissue. It was determined that $\sim 23\%$ of the ^{45}Ca is lost from conventionally prepared tissue during dehydration and infiltration while about 4% is lost from freeze-dried tissue during comparable procedures. As a result of this finding, we propose that experiments in which the localization of calcium in smooth muscle is attempted should be carried out in freeze-dried tissue. In addition, we suggest that any subsequent loss of ^{45}Ca from freeze-dried tissue during the preparation of autoradiograms is surface calcium and that leaching of deeper, intracellular calcium does not occur.

As a second important aim in this investigation we sought to evaluate the relative distribution of ^{45}Ca within the cell. Our results showed that ^{45}Ca grains were preferentially associated

TABLE II

Comparison between the Observed and a Predicted Random Distribution of ^{45}Ca Grains in Autoradiograms Prepared from Smooth Muscle of the Guinea Pig Vas Deferens

| | Compound Item* | | | | |
|---|----------------------------|--------------------|---------------|-----------------------------------|--|
| | Cyto- plasmic matrix | Nucleus/ matrix | SR/ matrix | Mito- chon- dria/ matrix | Plasma mem- brane/ matrix/ex- tracellular space |
| Observed grain distribution ($n = 265$) | 38.9 | 4.5 | 8.3 | 8.7 | 39.6 |
| Predicted grain distribution | 54.8 | 4.5 | 6.8 | 5.2 | 28.6 |

* The individual χ^2 values for the compound items are as follows:

| | | |
|---|-------|-------------|
| Cytoplasmic matrix | 12.27 | $P < 0.001$ |
| Nucleus/matrix | 0.00 | $P > 0.05$ |
| SR/matrix | 0.88 | $P > 0.05$ |
| Mitochondria/matrix | 6.17 | $P < 0.05$ |
| Plasma membrane/matrix/extra- cellular space | 11.26 | $P < 0.001$ |
| Total | 30.58 | $P < 0.001$ |

TABLE III

Grain Density and Relative Distribution of ^{45}Ca Grains among Cellular Sites

| | Cellular site | | | | |
|--|----------------------------|---------|-------------------------------------|------------------------|-------------------------|
| | Cyto- plasmic matrix | Nucleus | Sarco- plasmic reticu- lum | Mito- chon- dria | Plasma mem- brane |
| Grain density of cellular site | 0.06 | 0.09 | 0.32 | 0.61 | 0.86 |
| Grain density rela- tive to cytoplas- mic matrix | 1.0 | 1.5 | 5.3 | 10.0 | 14.1 |

with cellular structures in the guinea pig vas deferens. This finding led to an attempt to estimate the relative concentration of grains associated with the various cellular structures.

The observation that slightly less than half of the total ^{45}Ca grains were found to be in association with the cytoplasmic matrix would suggest that the cells are maximally contracted. This is not compatible with the physiological conditions imposed in this study. However, it is not an unexpected finding because of the lack of resolution of the ^{45}Ca ARG technique. That is, a circle of 275-nm radius circumscribed around a radiation source has approximately a 50% probability of containing a given developed ^{45}Ca grain. Conversely, there is also a substantial probability that the ^{45}Ca grain will lie outside of the circle. This means that a portion of the grains that are seen to occur at a distance >275 nm from a radiation source actually resulted from beta decay within that source. When one is dealing with relatively widely spaced sources such as plasma membrane, mitochondria, SR and the nucleus, there is little possibility that a significant portion of the grains attributed to one of these types of sources actually originated from radiation within another of these sources. However, because of the close apposition of cytoplasmic matrix and plasma membrane, mi-

tochondria or SR, a significant portion of the grains attributed to the matrix may have actually originated from radiation within one of the latter cellular components. This would result in an overestimate of the number of grains in association with the matrix. Consequently, using the techniques presented in this paper, the contractile state of the muscle cells cannot be correlated with the number of grains associated with the cytoplasmic matrix. On the other hand, the number of grains in the matrix does provide an overall estimate of the average radiation density that is scattered in areas that are over 275 nm away from plasma membrane, mitochondria, SR, and the nucleus. The average number of grains per area of cytoplasmic matrix, then, provides a common reference point from which relative concentrations of radioactivity associated with various non-matrix components can be estimated.

Of the cellular components examined, the plasma membrane was associated with the highest concentration of ^{45}Ca . Based on previous physiological and morphological data, this was not unexpected. For example, an energy-dependent calcium uptake by a sarcolemma-enriched fraction from smooth muscles has been documented (8, 24). In addition, in morphological studies a high concentration of electron-opaque salts of calcium has been found to accumulate at the plasma membrane (1, 9, 50).

Similar evidence in the literature also has shown that mitochondria can accumulate calcium (1, 3, 9, 11, 18). This is in agreement with the findings in the present study. The mitochondria were second only to the plasma membrane in relative calcium accumulation.

A number of workers have speculated that the SR in smooth muscle cells may function as a storage site of calcium for contraction (17, 32, 39). We observed that the SR had appreciably fewer silver grains associated with it than did the mitochondria or the plasma membrane. However, whether the SR serves as a source of and/or a sink for activator calcium cannot be determined from this study.

In a recent study, Somlyo and co-workers (43) reported that the concentration of calcium in the nucleus and in the cytoplasmic matrix was not significantly different. Our results in the present study tend to support their findings. The nucleus was observed to accumulate much less calcium than the plasma membrane, mitochondria, or SR, and only slightly more calcium than the cytoplasmic matrix. However, it must be emphasized that because of the infrequency with which nuclei were seen to occur, the observed grain density may not be a reliable estimate of the nuclear ^{45}Ca concentration.

In conclusion, in the stereological portion of this study, freeze-dried smooth muscle cells were observed to have a cellular area approximately twice that of conventionally prepared cells. When the relative area of cellular structures was compared, the mitochondria and surface vesicles were found to occupy a significantly larger percentage of the total cell area in the conventionally prepared tissue than they did in the freeze-dried tissue. In the autoradiographic portion of the study, we found that the freeze-drying technique prevented a significant loss of ^{45}Ca from the muscle cells. By combining the freeze-drying technique with ^{45}Ca ARG at the electron microscope level, the intracellular localization of calcium at physiological concentrations was evaluated. From the results of these experiments, the relative distribution of ^{45}Ca among sequestration sites within the nonstimulated cell was determined. The plasma membrane, mitochondria, and SR were shown to accumulate about ~ 14 times, 10 times, and five times, respectively, more calcium than the cytoplasmic matrix. Therefore,

based on the number of grains per unit area, the mitochondria are about one-third less radioactive than the plasma membrane and the SR about two-thirds less radioactive than the membrane. While the physiological function of this calcium cannot be evaluated at the present time, these results represent the first demonstration of the relative distribution of calcium within a smooth muscle cell.

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