# Unusual Lysosomes in Aortic Smooth Muscle Cells: Presence in Living and Rapidly Frozen Cells

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*Abstract.* Unusual tubular structures have been observed in rat aortic smooth muscle cells (SMC) grown in culture. These tubular structures have several characteristics that strongly suggest that they are lysosomes: they are bounded by a single membrane bilayer, contain densely staining material, and acid phosphatase activity. Furthermore, these structures are present in living cells, as demonstrated by their ability to accumulate the membrane-impermeable fluorescent dye lucifer yellow CH. In ultrastructural preparations they are best seen in samples that are cryofixed by rapid freezing and then freeze-substituted in osmium-acetone solutions. Conventional chemical fixation did not appear to preserve these structures to as great an extent as did rapid freezing. Comparison of SMC in vitro to the same cells in situ revealed differences in lysosome number as well as morphological appearance. Thus, the culturing of rat SMC leads to the formation of unusual tubular lysosomes whose ultrastructural appearance is particularly sensitive to the methods employed for examination.

SMOOTH muscle cells are a major cellular component in the arterial wall and contribute to the structural and elastic properties of these vessels. Cultured aortic smooth muscle cells (SMC)[1] have been used as a model system in many studies related to the pathobiology of atherosclerosis. Such in vitro studies have been important in elucidating various factors related to the growth, differentiation, and senescence of SMC (for review, see 21, 25). Comparisons of SMC in vivo and in vitro have been made by several investigators, and differences have been catalogued. Certain biochemical and morphological properties of calf SMC grown in culture have been shown to be different from these cells in situ (8), while morphological differences between SMC in vitro and in vivo have been noted in other species as well (4, 29).

Lysosomes play an important role in catabolic metabolism within cells and have been shown to be important in cellular regulatory processes (e.g., 3). Changes in smooth muscle cell lysosomes have been observed in models for vascular disease (22, 28). In the present study, we have examined the lysosomal system of aortic smooth muscle cells in vitro and in vivo by morphological and cytochemical means. There appear to be more lysosomes in the cultured SMC from the rat than in situ; similar findings have been noted in other species (8, 29). Furthermore, we find the lysosomal system in these cells to be more elaborate than has previously been observed in smooth muscle cells and that the structural appearance of these organelles may be particularly sensitive to the techniques employed for microscopic examination.

1. *Abbreviations used in this paper:* AcPase, acid phosphatase; HH, Hepes-buffered Hanks' balanced salts solution; LY, lucifer yellow CH; SMC, aortic smooth muscle cells.

## Materials and Methods

### Materials

Sprague-Dawley rats[2] (CD strain) were obtained from Charles River Breeding Laboratories, Inc. (Wilmington, MA). Fetal calf serum was purchased from Gibco Laboratories (Grand Island, NY). The tissue culture medium (RPMI) was obtained from KC Biological Inc. (Lenexa, KS); gentamycin and penicillin-streptomycin were obtained from MA Biological Products (Walkersville, MD). Lucifer yellow CH (LY) and β-glycerophosphate (disodium salt, Grade I) were purchased from Sigma Chemical Co. (St. Louis, MO). Cerium chloride, hafnium chloride, and osmium tetroxide were obtained from Alfa Products (Danvers, MA). Glutaraldehyde (EM grade) was obtained from Polysciences, Inc. (Warrington, PA). Epon was obtained from Balzers Union (Hudson, NH). Davison molecular sieves (Type 4A) were purchased from Fisher Scientific Co. (Pittsburgh, PA). All other chemicals were of the highest grade available.

### Cells

SMC were derived from aortic explants from male Sprague-Dawley rats (250–400 g) as previously described (5, 10). The cells were subcultured in RPMI medium supplemented with 20% fetal calf serum, glutamine (4 mM), and antibiotics (penicillin-streptomycin and gentamycin). For the microscopic studies described in this work, cells were grown on either 9- or 13-mm-diam glass coverslips. All experiments were done with cells in the third to sixth passage.

The enzyme cytochemical studies on SMC in situ were carried out on aortae that were freshly isolated from male rats similar to those described above.

2. Animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. [NIH] 78-23, revised 1978).

Fixation and subsequent steps in the procedure are described in the appropriate sections.

## Electron Microscopy: SMC in Vitro

*Chemical Fixation for Morphological Observations.* Cultured cells were examined while in logarithmic growth. Cells on coverslips (13 mm) were rinsed free of serum in several changes of Hepes-buffered Hanks' balanced salts solution (HH) at 37 °C. The coverslips were then placed in glutaraldehyde-containing buffer. Glutaraldehyde was tested at 2 and 3%. The buffer solutions employed were 0.1 M sodium cacodylate (pH 7.2) with 5% sucrose, 0.1 M sodium phosphate (pH 7.2) with 5% sucrose, or phosphate-buffered saline (PBS). Routinely, 2% glutaraldehyde-0.1 M sodium cacodylate was used. Cells were fixed in glutaraldehyde for 30–60 min at room temperature and subsequently washed several times in the same buffer solution. Washed cells were post-fixed in 2% $OsO_4$ in either 0.1 M sodium cacodylate (pH 7.2) or 0.1 M sodium phosphate (pH 7.2) for 60 min at room temperature. Cells were en bloc stained in 0.5% uranyl acetate as previously described (11). In some preparations, the en bloc staining was omitted. The coverslip preparations were dehydrated, infiltrated with Epon, and polymerized as previously described (18).

*Cryofixation (Quick-Freezing) for Morphological Observations.* Coverslips (9 mm) containing SMC were washed as above to remove the serum. The monolayer of cells was then quickly frozen on a liquid nitrogen-cooled copper rod in the device designed by Boyne (2). The coverslips were rapidly transferred to a liquid nitrogen Dewar flask and stored under liquid nitrogen until needed. Freeze-substitution was achieved in acetone containing osmium (1–4% $OsO_4$ was tested). Routinely, 2% $OsO_4$ was employed. Briefly, the acetone-osmium solution was placed in glass liquid-scintillation vials which contained a layer of molecular sieves to keep the acetone dry. The vials were allowed to cool in a large-mouth Dewar flask containing a slush of powdered dry ice and acetone. They were transferred one at a time to a small cup of slush, opened, and a single coverslip was quickly removed from liquid nitrogen and added to the vial. The vials were transferred back to the dry ice-acetone–containing Dewar flask and kept there for ~24 h. They were then placed in a −20°C freezer for ~24 h before going into a +4°C refrigerator for 24 h. After coming to room temperature, the coverslips were transferred to fresh acetone and rinsed two to three times to remove the osmium. The cells were then en bloc stained for 60 min in hafnium chloride in acetone (hafnium chloride was tested at 0.25–1.0%). Routinely, hafnium chloride was used at 0.5%. The coverslips were then rinsed in acetone and embedded in Epon.

Thin sections were cut, stained with lead citrate and uranyl acetate. Cells were sectioned parallel to the substratum on which they grew. Sections were examined, and photographs were taken with a Philips electron microscope (model 200) operated at 60 kV.

## Enzyme Cytochemistry: SMC in Situ and in Vitro

*Fixation.* Rats were initially perfused with normal saline to remove blood; this was followed by perfusion fixation with 1% glutaraldehyde-0.1 M cacodylate (pH 7.2), containing 5% sucrose for 10 min. The aorta were removed, cut longitudinally, and fixed by immersion in the same fixative for an additional 50–60 min on ice. The samples were washed in several changes of cacodylate buffer over a period of ~1 h at 4°C. Longitudinal sections ~50 μm thick were made on a Vibratome (Oxford Laboratories, San Mateo, CA).

Cells grown on coverslips (13 mm) were washed as above to remove serum. They were then fixed in 1% glutaraldehyde-0.1 M cacodylate (pH 7.2) with 5% sucrose for 20 min on ice. The coverslips were then washed three to four times over ~1 h on ice in cacodylate buffer.

*Cytochemical Reactions.* Acid phosphatase (AcPase) activity was localized at the ultrastructural level using the cerium capture method as we have described (19), except cerium was used at 1.25 mM in the present study. There was a further modification in which the cells were permeabilized with Triton X-100 during the cytochemical reaction (20). Triton X-100 was used at 0.0001% for the cultured SMC, while 0.00015% was employed for the sections of aorta. These very low levels of Triton X-100 were determined experimentally by testing several concentrations and selecting one that adequately permeabilized the cells with a minimum of adverse effects on the ultrastructural appearance. The cytochemical incubations were carried out at 37°C with gentle agitation for 40–60 min. The medium was replaced with fresh medium halfway through the incubation period. Controls consisted of incubation medium that lacked substrate. After the cytochemical reaction, the tissue sections or coverslips were washed once in the cytochemical buffer and once in cacodylate buffer. The coverslip preparations, but not the tissue sections, were refixed in 3% glutaraldehyde-0.1 cacodylate for 30 min at room temperature and then washed several times in cacodylate buffer. Coverslips and tissue sections were subsequently processed and embedded as described above concerning chemical fixation.

*Labeling of Living SMC in Vitro with LY.* Living SMC cultured on coverslips (13 mm) were incubated in multiwell culture dishes with LY in HH at 37°C in a $CO_2$ incubator for 15–90 min. LY was used at 0.4–2.0 mg/ml. At various time points, the coverslips were quickly rinsed in five changes of HH and mounted in HH on glass slides ringed with Vaseline. LY is a highly fluorescent dye which is relatively nontoxic to cells; it is hydrophobic and enters cells by endocytic processes (15, 23, 24). Furthermore, LY that is endocytosed can be delivered to lysosomes (15). Thus, LY was used to label endocytic vesicles and lysosomes in living SMC. Cells were examined by epifluorescent illumination with a Zeiss UEM equipped with a 100-W mercury lamp and photographed with a fluorescein filter set.

# Results

The lysosomal system of rat aortic SMC has been examined using several morphological and cytochemical approaches to compare these cells in vitro and in situ. Cultured SMC prepared for electron microscopy by rapid freezing and freeze-substitution display unusual tubular structures which contain densely stained material and are bounded by a single membrane bilayer (Fig. 1). Thin-section profiles of these cells also have circular and oval structures which have similar densely stained contents. These structures are often numerous and can also be quite long (up to several micrometers in length), passing in and out of the plane of a single thin section. More complex branched forms of these structures, while less frequent than the single tubules, can be routinely observed in these preparations. Furthermore, these long tubular structures are not as readily observed in cultured SMC that are prepared for electron microscopy by conventional chemical fixation as are those prepared with cryofixation. Our results indicate that conventional fixation methods do not preserve these structures intact to the same extent as rapid freezing (Fig. 2).

Enzyme cytochemical experiments were carried out to identify and characterize these unusual tubular structures. Cultured SMC were incubated for the localization of AcPase (an enzyme present in lysosomes) with cerium as the capture metal. There are several structures that stain positively for AcPase in these cells: (*a*) small circular and oval structures, and (*b*) long tubular structures (Fig. 3*a*), as well as (*c*) a portion of the Golgi complex (not shown). The long tubular structures that are AcPase positive are approximately the same size, both length and width, as the long (densely stained) tubular structures observed in the cryofixed and freeze-substituted samples. Cells incubated in control cytochemical media lacking substrate did not have reaction product.

These observations strongly suggest that the long tubular structures containing AcPase activity and densely staining material within a single bilayer membrane are lysosomes. Further support for this contention was obtained from living cells. Cultured SMC take up the membrane-impermeable fluorescent dye LY in a time dependent manner. Within 60–90 min, LY fluorescence is present within small round structures which are probably either pinocytotic vesicles or small lysosomes, larger round or oval structures which are probably lysosomes, and long tubular structures (Fig. 4). These LY-labeled-tubular structures are most likely the same structures which were identified as being positive for AcPase by enzyme cytochemistry. Furthermore, unusually shaped structures (e.g., lollipop shaped) were found to stain positively for AcPase in fixed cells and to label with LY in living cells (Fig. 5). To determine the stability of the LY-labeled structures, particu-

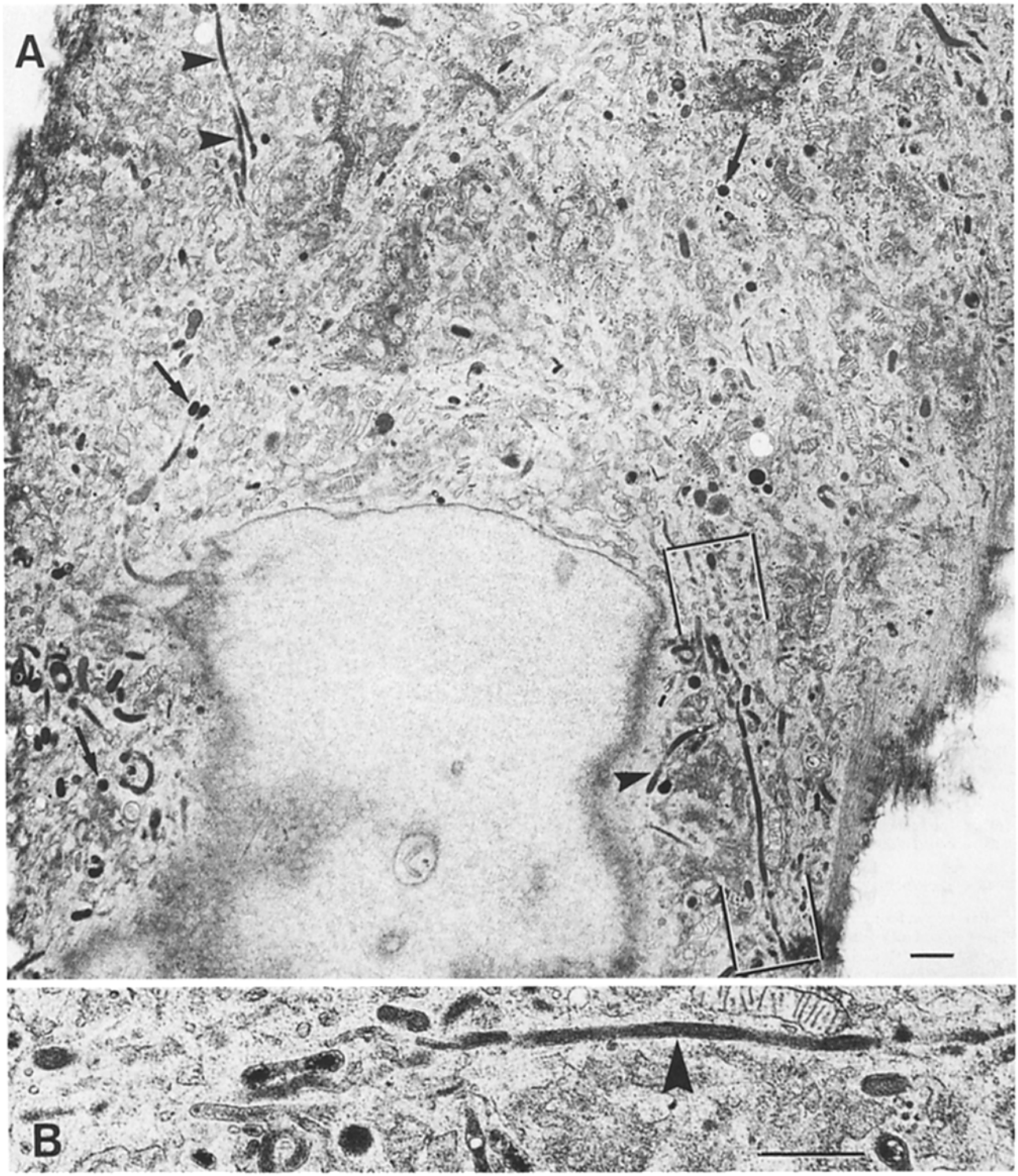


*Figure 1.* Cultured SMC that was quick-frozen and freeze-substituted in osmium-acetone and en bloc stained with hafnium chloride. (*A*) Low magnification illustrating the overall appearance of cells prepared in this manner. Note the presence of structures containing densely stained material, which are tubular (*arrowheads*) and circular or oval (*arrows*) in thin-section profile. Bar, 1.0 μm. (*B*) Higher magnification of portion of cell in brackets in *A*. These densely stained structures are bounded by a single membrane bilayer (*arrowhead*). Bar, 1.0 μm.

larly the long tubules, we examined living, LY-labeled cells that were exposed to epiillumination for different time periods (Fig. 4, *b* and *c*). With prolonged illumination (up to 2.0–3.0 min), these tubular structures often display altered morphology and may even begin to fragment. The nature of this process, whether photochemical or otherwise, is entirely unknown.

For purposes of comparison with cultured SMC, the distribution of lysosomes in SMC in the aorta was determined by enzyme cytochemistry. The cytochemical reaction for AcPase

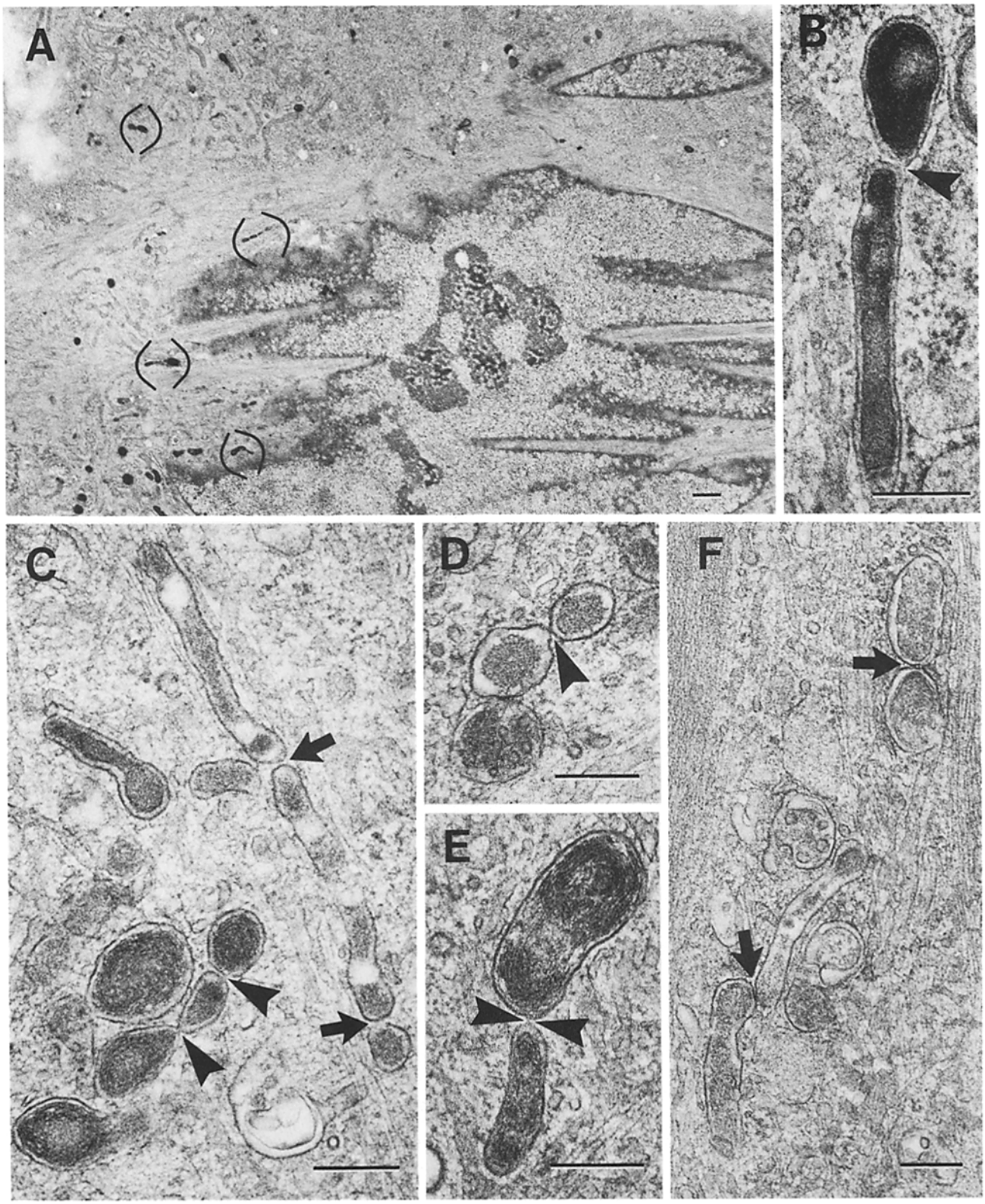


*Figure 2.* Cultured SMC prepared by chemical fixation in 2% glutaraldehyde-0.1 M sodium cacodylate with post-fixation in 2% $OsO_4$-0.1 M sodium cacodylate as described in the Materials and Methods. (*A*) Low-magnification micrograph with numerous densely stained lysosomes. Some of the lysosomes are lined up side by side and give the impression that they may have fragmented during the preparation (*brackets*). Bar, 1.0 μm. (*B–F*) Gallery of higher magnification images of chemically fixed lysosomes which appear to be fragmented. Electron micrographs of lysosomes which appear to be fragmenting but which still retain a connection between two adjacent structures are indicated by the *arrowheads*. Lysosomal structures which are adjacent but which do not display a clear connection in these thin sections are indicated by the *arrows*. Bars, 0.25 μm.

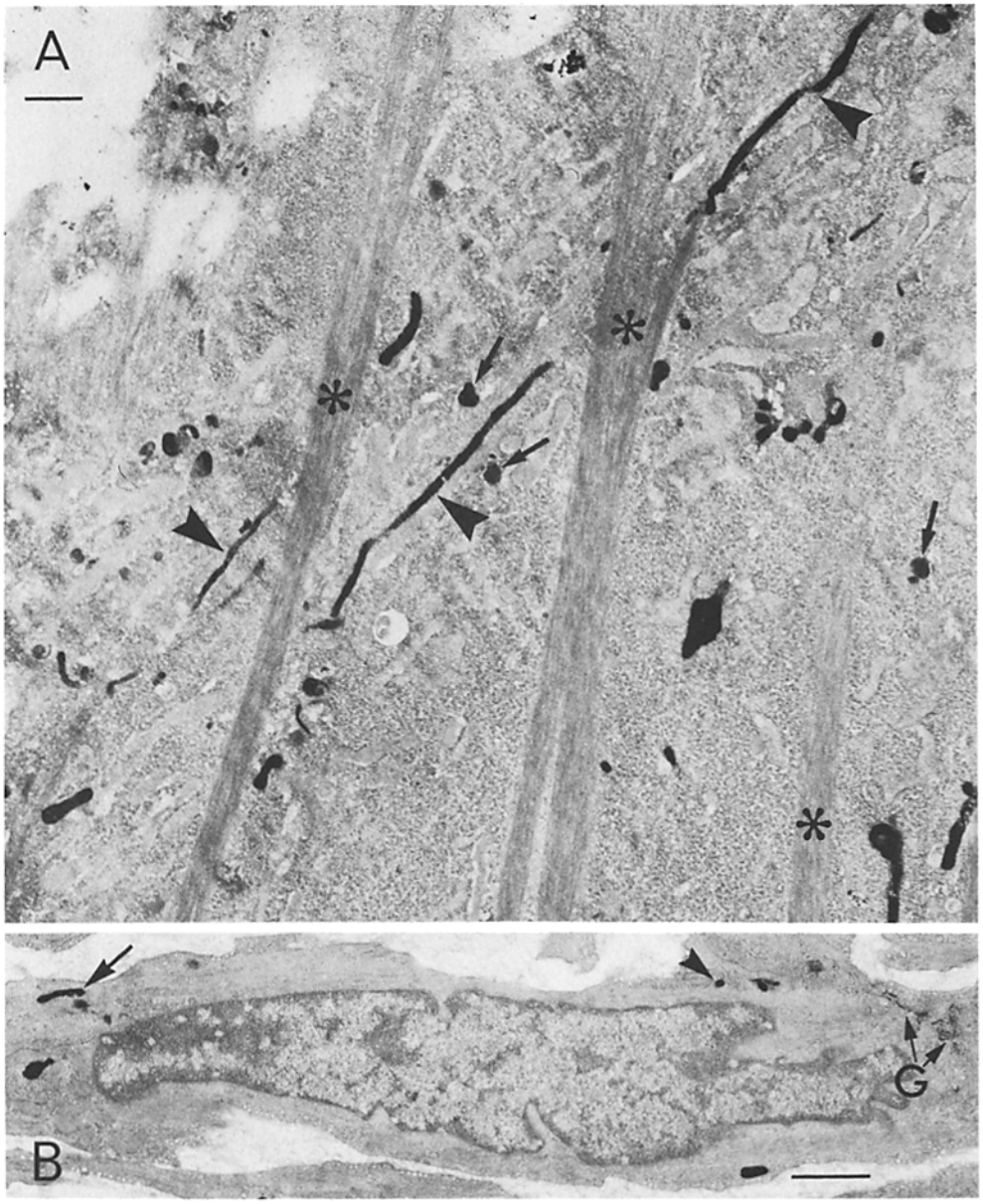


*Figure 3.* Comparison of acid phosphatase localization in smooth muscle cells in culture and in situ. (*A*) Electron micrograph of the periphery of a portion of a cultured SMC which was incubated for the cytochemical detection of AcPase activity. Electron-dense cerium phosphate reaction product is present in long tubular structures (*arrowheads*) or in circular or oval-shaped structures (*arrows*) in thin-section profile. Note that these structures are within cytoplasm bounded by microfilament bundles (*) in this portion of the cell. Bar, 1.0 μm. No en bloc staining. (*B*) Rat SMC in situ which were incubated for the cytochemical localization of AcPase as described in Materials and Methods. Cerium phosphate reaction product is present within a portion of the Golgi complex (*G*), circular and oval lysosomes (*arrowhead*), and short tubular lysosomes (*freeze-substitution*). Bar, 1.0 μm.

was carried out on SMC in situ. Lysosomes as well as portions of the Golgi complexes displayed positive staining (Fig. 3*b*). The lysosomes of SMC in situ are more sparse than in these cells in vitro. The lysosomes of SMC in situ are more "conventional" in appearance but are present in a short tubular form as well as being rounded in appearance.

## Discussion

We have examined the morphology and distribution of lysosomes and SMC in culture and in situ, using rapid-freezing and conventional chemical fixation methods, enzyme cytochemistry, and fluorescence microscopy. The lysosomal population of cultured rat SMC is increased from that of these same cells in situ. In addition to increases in the number of lysosomes, there is a change in lysosome morphology in cultured SMC as compared with SMC in situ. There is a population of lysosomes in cultured SMC which displays an unusual tubular morphology. The long tubular structures which we observe in cultured rat aortic SMC with quick-freezing and freeze-substitution and with cerium-based AcPase cytochemistry have not been previously described at the ultrastructural level. An explanation for this is suggested by our inability to find these structures in significant numbers in conventionally fixed samples. Furthermore, we find electron-microscope images which could be interpreted as representing tubular structures that had become fragmented into smaller structures during preparation. The stage in sample preparation when such alterations might occur is not clear. The quick-freezing followed by freeze-substitution in osmium-acetone results indicate that the initial glutaraldehyde fixation step in conventional preparations may be responsible. However, this does not seem to be the entire reason since these tubular structures are present in the AcPase cytochemical samples which also have an initial glutaraldehyde fixation step. In cells incubated for AcPase cytochemistry the tubular structures become filled with reaction product (cerium phosphate) which may have prevented fragmentation during subsequent steps

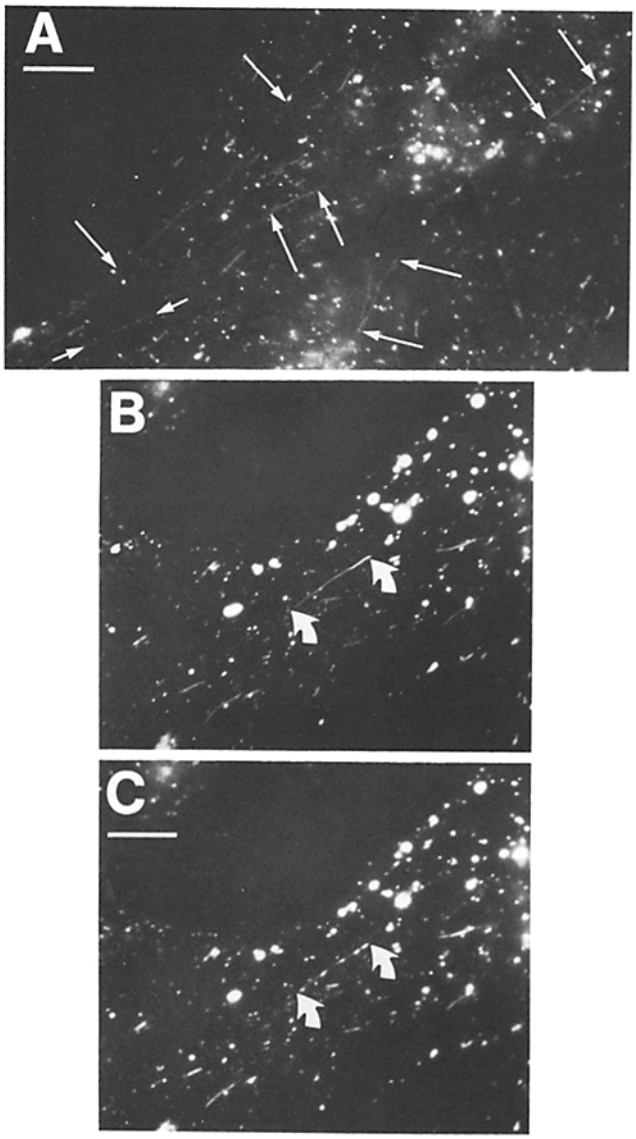


*Figure 4.* Light micrographs of living SMC labeled with LY. (*A*) SMC incubated with LY (2 mg/ml) for 90 min and then observed and immediately photographed with epifluorescent illumination. LY fluorescence is present in punctate structures and in tubular structures, the ends of which are denoted by arrows. Bar, 10.0 μm. (*B*) Portion of cell in which both punctate and tubular-shaped structures are labeled. Note the long LY-positive structure denoted by the arrows. Cell exposed to epifluorescence for a total of 30 s. (*C*) The same cell as in *B* which has been exposed to epifluorescence for 120 s. Note that the tubular structure (between the arrows) appears to be in the process of fragmentation. Bar, 10.0 μm.

in sample preparations. There are other examples where ultrastructural results with samples prepared by quick-freezing are different from the same type of sample prepared by chemical fixation in glutaraldehyde. Membrane fusion events, for example, have been shown to have a different appearance in freeze-fracture preparations when quick-frozen as compared with glutaraldehyde-fixed (6, 17). More recently, differences in the organization of certain cytoplasmic membranes have been noted in rapidly frozen and chemically fixed growth cones from the optic tectum (7). In certain plant material,

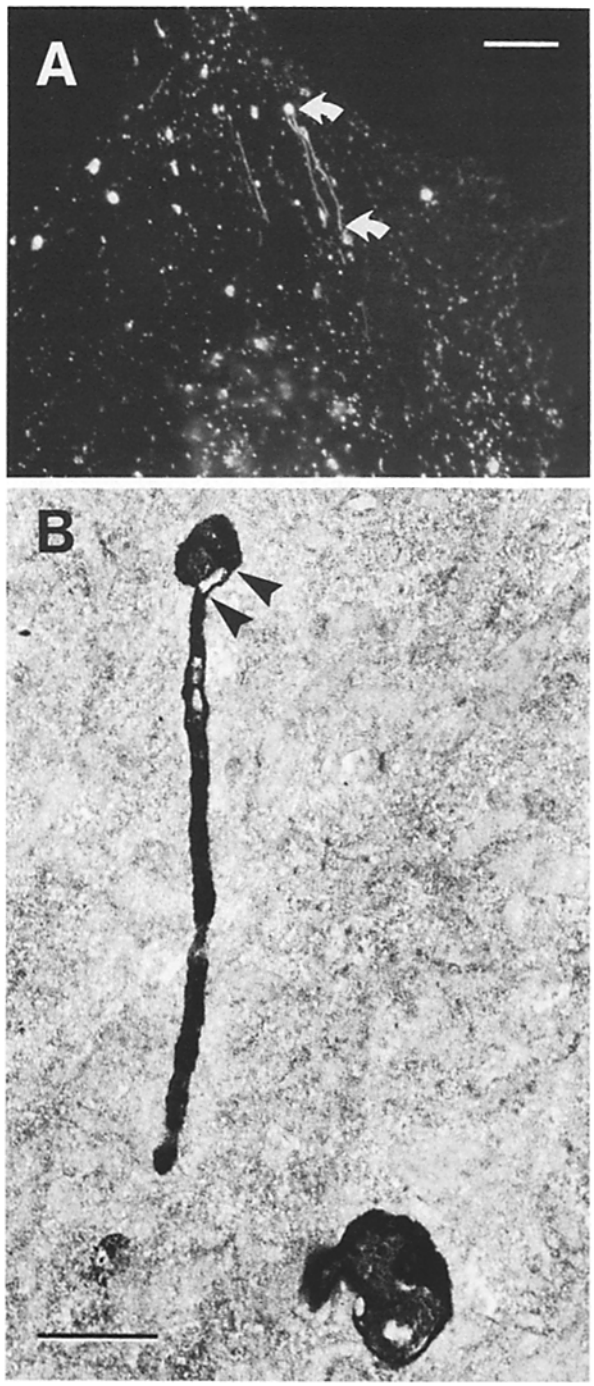


*Figure 5.* Unusually shaped LY- and AcPase-positive structures in cultured SMC. (*A*) Living SMC incubated with LY as in Fig. 4. In this cell, there are punctate and tubular structures which contain LY. The tubular structures sometimes display unusual configurations (the ends of which are denoted by the arrows). The structure denoted by the arrows appears to have a lollipop-like shape. At this level of resolution, it is not possible to state with certainty that this LY-positive structure is indeed lollipop shaped or is merely a tubular structure closely apposed to a punctate structure. Bar, 10.0 μm. (*B*) Electron micrograph of an AcPase-positive structure (prepared as in Fig. 3). In this case, the lollipop-like appearance indicates this is indeed a single structure. The bulbous end is in continuity with the straight tubular portion (*arrowheads*). Bar, 1.0 μm.

chemical fixation has been shown to disrupt a complex tubular membranous system (14). Our findings, as well as those of others, point to the importance of using more than a single method for ultrastructural analysis where possible.

The ability to label long-tubular structures with the membrane-impermeable fluorescent dye LY in living cultured SMC strongly supports the case for such structures being present in these cells. This finding also enforces the importance of correlating morphological and cytochemical results in living as well as fixed cells where possible. It is interesting that LY-labeled tubular structures have also been observed in living macrophages (24). This observation along with the present results suggests that these elaborate tubular lysosomes may be more common of cells than is presently recognized.

Other investigators have found that there are increases in lysosomes (8) and lysosomal enzymes (29) in cultured SMC from calf and rabbit as compared with these cells in situ. Stereological analyses of changes in cellular morphology that occur in cultured SMC have also been presented (13, 26). The unusual tubular lysosomes we describe were not observed in these studies.

There are a few examples from other work where it is known that the configuration of lysosomes are altered from the normal. Monocytes from patients with Chediak-Higashi syndrome have unusually shaped lysosomes (27). Acinar cells from parotid glands of rats with streptozotocin-induced diabetes have crystalloid inclusions which occasionally stain positively for a lysosomal enzyme in cytochemical preparations (9). Unusual basal lysosomes have been described in certain exocrine acinar cells in normal animals. These structures are cytochemically reactive for trimetaphosphatase; however, they are not positive for acid phosphatase (16). More recently, trimetaphosphatase and nicotinamide adenine dinucleotide phosphate phosphohydrolase activities were found to colocalize in structures which have been called "snake-like tubules" in rat exocrine pancreas (1). The function of these basally located structures in exocrine acinar cells, if different from normal lysosomes, is not known.

The subcellular distribution of AcPase in cultured SMC from pig aorta has been examined by subcellular fractionation on sucrose gradients (12). In this study, a large portion of the AcPase activity toward 4-methylumbelliferyl phosphate had an equilibrium density similar to markers for the plasma membrane and the Golgi complex. In the present study, there was no evidence for a plasma membrane AcPase, at least with $\beta$-glycerophosphate, which was the substrate employed in our experiments. The pig SMC were not examined by morphological or cytochemical means (12); therefore, it is not known whether the long tubular AcPase-positive structures found in cultured rat SMC are present in cultured pig SMC. In a preliminary preparation of living cultured rabbit aortic SMC, long tubular LY-positive structures were observed (data not shown), suggesting that these structures may be a common feature of cultured aortic SMC. The behavior of the long tubular AcPase-positive structures in various cell fractionation procedures is not presently known. They may, for example, fragment into small vesicles (smaller than other lysosomes) and thus not migrate with these other lysosomes in a centrifugal field.

It is interesting to speculate on the possible biological significance of these unusually shaped lysosomes in cultured SMC. Far fewer of these structures are present in situ. What are the mechanisms that control their formation in culture, or conversely, prevent their formation in situ? The microenvironment of the vessel wall may influence organelle development in SMC. For example, components of the extracellular matrix may exert such an influence. Another possibility may relate to the geometry of the SMC, which is very different in the vessel wall than in culture. Alternatively, there may be factors in culture (e.g., substrate, serum, growth state) that promote the observed organelle morphology which are different or absent in vivo. This system may be useful in studying the mechanisms influencing lysosome formation and structure.

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