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Topographical difference of cytoskeletal organization in smooth muscle cells of rat duodenum revealed by quick-freezing and deep-etching method

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Summary. The sarcolemmal domain of rat duodenal smooth muscle cells includes caveolae and associated cytoskeletal or filamentous elements. We have used the quick-freezing, deep-etching method to examine the three dimensional relationships between these components. Replica membranes for separated strips of rat duodenal muscle layers were routinely prepared after extraction soluble proteins from cytoplasm and extracellular matrix. As results, 1) cytoskeletal elements in smooth muscle cells consisted mainly of striated thin filaments; 2) thin filaments were connected with some plasma membranes through filaments associated with the sarcolemma, which formed fine network structures beneath the sarcolemma; 3) many bridging structures between the filaments associated with the sarcolemma and the extracellular matrix were frequently detected in the plasma membrane; and 4) compact filaments associated with the sarcolemma almost disappeared near the caveolae, and only thin filaments were anchored to their neck parts. The special arrangement of the cytoskeletal components, which is probably necessary for the intestinal motility, characterizes the topographical difference of the smooth muscle sarcolemma.

Key words: Smooth muscle cells, Duodenum, Myenteric plexus, Quick-freezing, Deep-etching

Introduction

It is well known that musclaris externae of the gut have essential roles for gastrointestinal (GI) motility (Gabella, 1981; Alberts et al., 1994). Such smooth muscle layers are composed of compact and complex structures, containing smooth muscle cells, ganglionic cells, interstitial cells and extracellular matrix (Somlyo and Somlyo, 1975; Somlyo, 1980; Furness and Costa, 1987). In these layers, the smooth muscle cells with some kinds of randomly arranged cytoskeletal elements are basic effectors for GI motility (Devine et al., 1972; Bios, 1973; Geiger et al., 1981; Baulk and Gabella, 1987). Both actin and myosin filaments are suggested to play a major role in contraction and relaxation of the smooth muscle cells. However, higher number ratios, 25-30:1, of actin filament to myosin filament, suggested the existence of a different contractile system than the sliding filaments associated with the sarcolemma (Somlyo, 1980; Tsukita et al., 1982). It is also reported that thin actin filaments are attached to the plasma membrane through dense plaques (Small and Squire, 1972; Small and Sobieszek, 1980; Gabella, 1981). Some studies have demonstrated that several proteins were accumulated in the dense plaques, but ultrastructure of the force-transmitting system thorough the sarcolemma has not clarified it in detail (Gabella, 1981; Alberts et al., 1994).

The smooth muscle cells have many caveolae along their cell surface, which are usually arranged parallel to the cell long axis and keep their high ration of cell-to-volume (Somlyo, 1980; Poulos et al., 1986). However, functional roles of caveolae and their arrangement have not yet been clearly determined in smooth muscle cells (Gabella, 1981). Some cross-linking structure between cytoskeletal elements and membranous organelles has been clarified in neurons (Hirokawa, 1982; Tsukita et al., 1982; Hirokawa et al., 1989), but such corresponding structures have not been demonstrated in smooth muscle cells. As for the extracellular matrix, the GI muscle layers has neither paramecium nor tendon structure. In contrast, narrow septa of the connective tissue between the smooth muscle cells usually form the intramuscular micro tendon (Henderson et al., 1971; Gabella, 1981; Bannerman et al., 1986). The ultrastructural features linking the extracellular matrix with sarcolemma (Takayama et al., 1995, 1999).

A quick-freezing and deep-etching (QF-DE) method has been established over the two past decades (Menco, 1986), and has a great advantage for examining three-dimensional cytoskeletal architectures of various cells, in comparison with two-dimensional views obtained from ultrathin sections by conventional electron

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microscopy (Naramoto et al., 1992; Takayama et al., 1994, 1995). Some ultrastructural studies have been performed on vascular smooth muscle cells by the QF-DE method, but they have not yet been used for examining the GI smooth muscle cells (Somlyo, 1980; Tsukita et al., 1982). It is feasible because well-preserved ultrastructure can usually be obtained within 10 μm of the quickly frozen tissue surface. In the case of intact intestinal walls, which consist of layers of smooth muscle and a neural plexus in connective tissue, the QF-DE method would not provide good preservation of cellular morphology, as previously reported (Geiger et al., 1981; Naramoto et al., 1992; Takayama et al., 1994, 1995). A whole mount preparation technique, however, was used to examine the intramural plexus, and to obtain the histological findings. Previously we used strips of isolated GI smooth muscle layers and reported the three-dimensional ultrastructure of peripheral nerves, as revealed on replica membranes prepared by the QF-DE method (Takayama et al., 1999). In the present study, we examined three-dimensional cytoskeletal architectures of smooth muscle layers, as revealed by the QF-DE method, and we also discuss the special arrangement of the cellular components, which characterize topography of the smooth muscle plasma membrane.

Materials and methods

The present study was performed in accordance with the guidelines for animal experiments in the Yamanashi University School of Medicine. Male Wistar rats, weighing 240-280 g, were purchased from Japan SLC (Shizuoka, Japan) and fed *ad libitum* with a normal diet in air-conditioned rooms. They were anesthetized with ether, and then injected with 0.5 mg/g body per weight of sodium pentobarbital. They were perfused with 2% paraformaldehyde in 0.1M phosphate buffered saline (PBS), pH 7.4 at 4 ºC, from left ventricles. Then the duodenal segment was resected, and mechanically separated into an outer longitudinal smooth muscle layer and the inner circular smooth muscle layer (Takayama et al., 1999). The outer longitudinal smooth muscle layer was relatively flat, and was used for the following examination.

The specimens were washed in PBS (pH 7.4) at 4 ºC, treated with 0.5 % saponin in PBS for 10 min at 4 ºC to remove cytoplasmic soluble proteins (Naramoto et al., 1992; Takayama et al., 1994, 1999), and post fixed with 0.25% glutaraldehyde in PBS for 30 min at 4 ºC. They were washed in PBS, immersed with 10% methanol and quickly frozen on copper blocks (-196 ºC), using a quick-freezing apparatus, JFD-RFA (JEOL, Tokyo, Japan). The frozen specimens were freeze-fractured with a scalpel in liquid nitrogen (Takayama et al., 1999). They were deeply etched in a freeze-fracture apparatus, Eiko FD-3AS (Eiko Co., Ibaraki, Japan) under a vacuum condition of 6-8 x 10^{-5} Pa, at -95 ºC for 15-20 min. The deep-etched specimens were rotary-shadowed with platinum at an angle of 30 º, up to the total thickness of about 2 nm. They were additionally coated with carbon at an angle of 90º.

A drop of 2% collodion in amyl acetate was put onto the replicas as soon as the specimens were taken out of the machine, to prevent the replica membranes from breaking into pieces during the following digestion procedures. The replicas coated with dried collodion were floated on household bleach, for 15-30 min to dissolve the cellular components. The replica membranes were washed in distilled water and cut into small pieces with a pair of scissors. They were mounted on Formvar-filmed copper grids and immersed in amyl acetate solution to dissolve the collodion. Then they were examined in an electron microscope; a Hitachi H-600 or H-8100. Stereo-pictures were taken at tilting angles of ±5º. Replica electron micrographs were printed from the inverted negative films.

Some specimens, before the saponin treatment, were post fixed with 2% osmium tetroxide in PBS for 30 min at 4 ºC for conventional ultrathin sectioning method. They were washed in PBS, dehydrated in a graded series of ethanol, immersed propylene oxide and embedded in Quetol 812. Ultrathin sections were cut with an ultramicrotome, mounted on copper grids and examined by electron microscopy to confirm the conventional ultrastructure.

Results

Figure 1 illustrates regular cytoskeletal organization beneath the sarcolemma of a smooth muscle cell, which was three-dimensionally revealed on a replica electron micrograph. It consisted mainly of numerous thin filaments, while thick filaments were decorated with small granular structures (Fig. 1d). The latter thick filaments were fewer than the former thin filaments. These cytoskeletal filaments ran almost in parallel to the long cell axis, and other short cross-linkers also interconnected to the adjacent thin filaments. Moreover, small granular structures were often accumulated along some cytoskeletal filaments. The parallel cytoskeletal filaments, however, changed their organization near caveolae, showing a relatively irregular arrangement (Fig. 1b). Therefore, they presented a different organization, which would be independent of the contractile system, indicating passively influenced motility of the caveolae during the smooth muscle contraction or relaxation.

The caveolae were heterogeneously localized in the peripheral region of smooth muscle cells (Figs. 1, 2). Few cytoskeletal elements existed around the caveolae, while many were seen in the region without caveolae. Some thin filaments were anchored at neck parts of the caveolae (Fig. 1b). In the extracellular space around smooth muscle cells, network structures existed containing thick striated collagen fibrils and thin filaments. The collagen fibrils mostly ran in parallel to the smooth muscle cell long axis, while the thin filaments formed fine network structures (Fig. 1c).

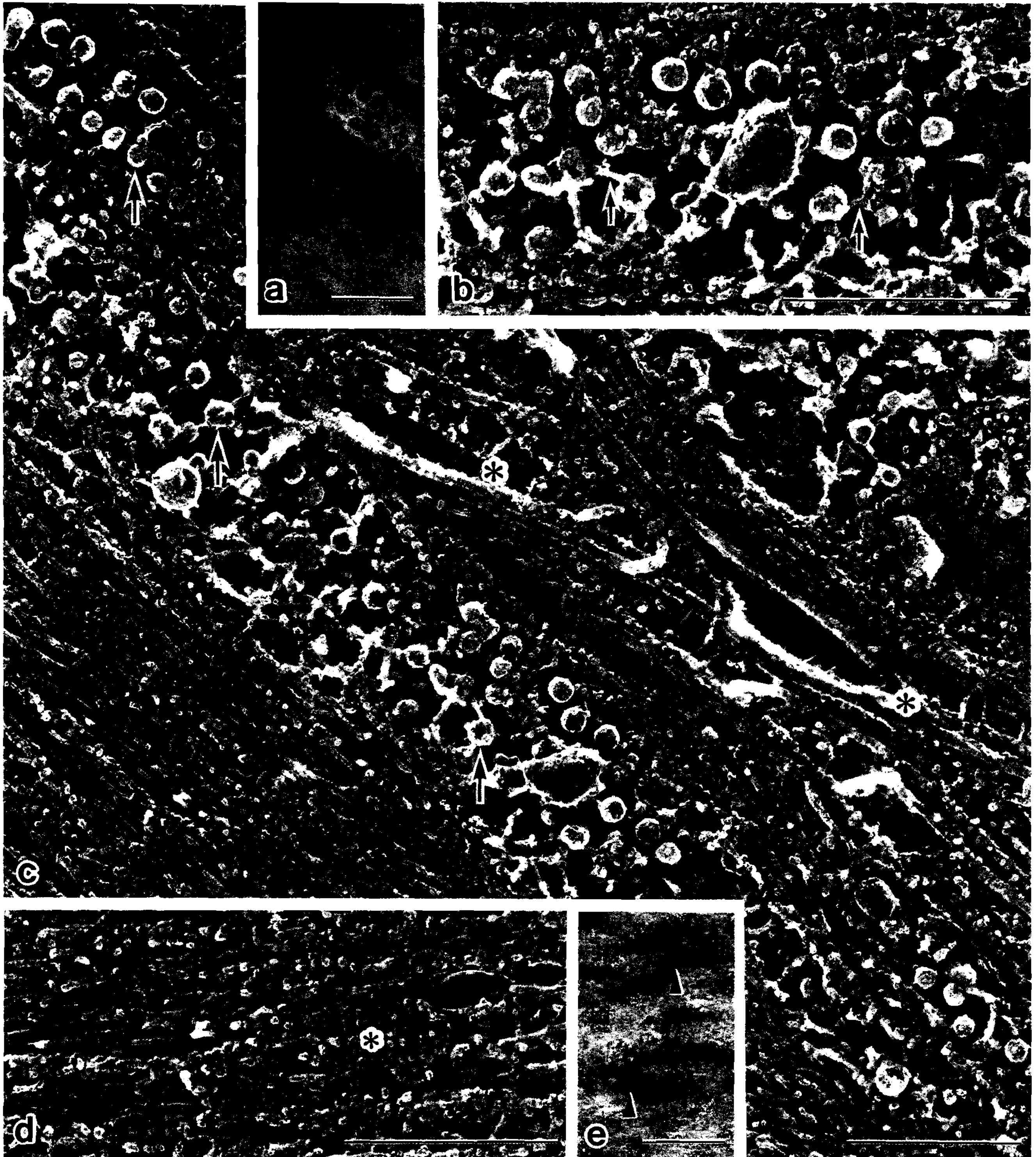

Fig. 1. Replica or conventional micrographs of duodenal smooth muscle layer of rat. **a.** Conventional electron micrograph showing caveolae. x 26,400; bar: 0.5 μm. **b.** replica micrograph showing higher magnified view of caveolae. x 78,100; bar: 0.5 μm. **c.** Replica micrograph demonstrating caveolae (arrows) beneath the plasma membrane and collagen fibrils (asterisks) in the extracellular space. x 57,100; bar: 0.5 μm. **d.** Higher magnified view of cytoskeletal filaments. Asterisk: accumulation of granular structures. Arrow: thick filaments. x 71,400; bar: 0.5 μm. **e.** Conventional view of cytoskeletal filaments. Arrowheads: dense bodies. x 26,400; bar: 0.5 μm.

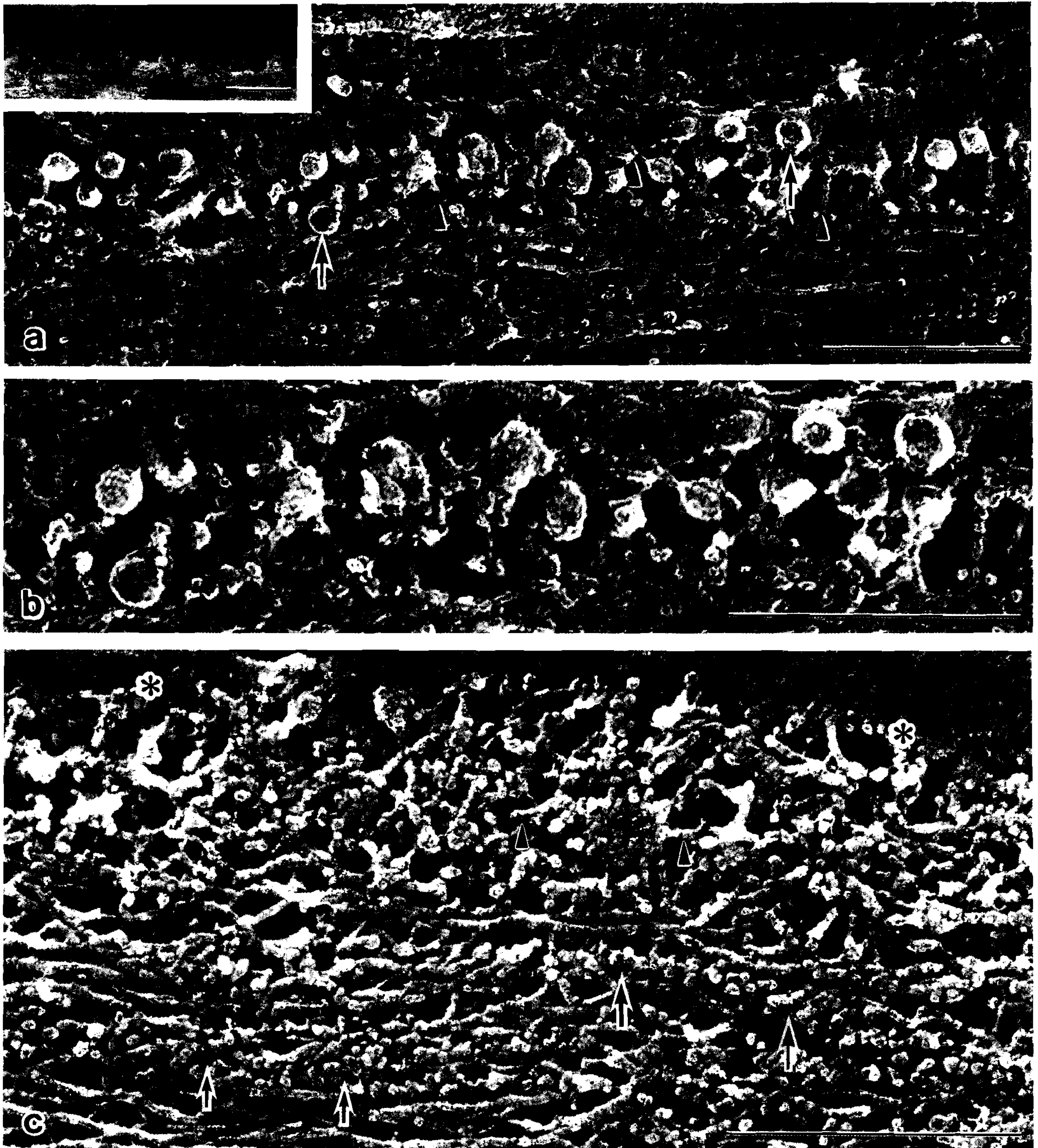

Fig. 2. a. Replica micrograph demonstrating caveolae (arrows) beneath the plasma membrane. Arrowheads: thin filaments. x 65,500; bar: 0.5 μm. Inset: corresponding region on conventional electron micrograph. x 21,600; bar: 0.5 μm. **b.** Higher magnified view of the caveolae. x 96,900; bar: 0.5 μm. **c.** Replica micrograph showing cytoskeletal arrays beneath the plasma membrane. Arrows: thick filaments. Small arrows: granular structures. Asterisks: cytoplasmic side of the plasma membrane. Arrowheads: short thin filaments. x 98,500; bar: 0.5 μm.

Figure 2 illustrates typical regions beneath the plasma membrane of smooth muscle cells. In Figure 2a-b, fewer cytoskeletal elements confirmed to distribute around caveolae on almost cross-sectioned plasma membranes. Some caveolae were connected with cytoskeletal elements or filaments associated with the sarcolemma through short thin filaments (Fig. 2b). As shown in Figure 1, accumulations of granular structures along thin filaments may correspond to dense bodies, which were usually seen on conventional electron

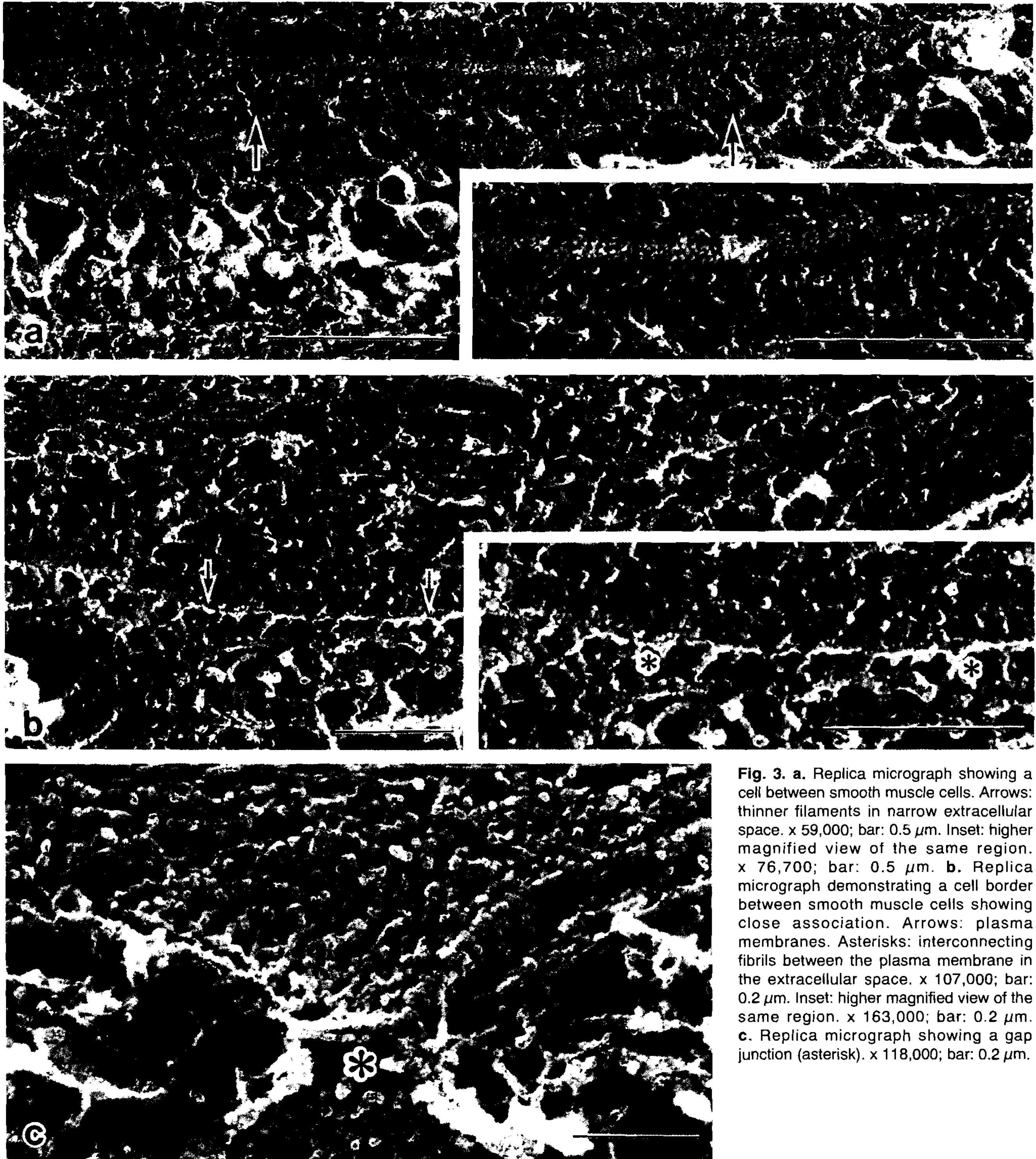

Fig. 3. a. Replica micrograph showing a cell between smooth muscle cells. Arrows: thinner filaments in narrow extracellular space. x 59,000; bar: 0.5 μm. Inset: higher magnified view of the same region. x 76,700; bar: 0.5 μm. **b.** Replica micrograph demonstrating a cell border between smooth muscle cells showing close association. Arrows: plasma membranes. Asterisks: interconnecting fibrils between the plasma membrane in the extracellular space. x 107,000; bar: 0.2 μm. Inset: higher magnified view of the same region. x 163,000; bar: 0.2 μm. **c.** Replica micrograph showing a gap junction (asterisk). x 118,000; bar: 0.2 μm.

micrographs, even in some regions beneath the plasma membrane (Fig. 2a,c).

Figure 3 illustrates the ultrastructural relationships among cytoskeletal elements, sarcolemma and extracellular matrices. The extracellular space appeared to be narrower in Figure 3, in comparison with that seen in Figure 1. In the regions where adjacent smooth muscle cells were closely associated to each other, thick collagen fibrils often disappeared and only thin networks still existed (Fig. 3a). This extracellular matrix showed relatively heterogeneous networks with thinner fibrils, but a compactly organized filament layer, corresponding to the basement membrane seen on conventional electron micrographs, was hardly detected in these areas. Moreover, extracellular networks of thin fibrils appeared to form a direct connection with filaments associated with the sarcolemma (Fig. 3a, inset), which might be suitable for the force transduction of contractility in functioning smooth muscle layers.

In some extracellular regions, the filamentous networks almost disappeared, and both neighboring sarcolemmas were closely associated to each other, together with filaments running nearly perpendicular to the plasma membranes (Fig. 3b). The extracellular space between the plasma membranes completely disappeared at gap junctions (Fig. 3c). Beneath the gap junctions, filaments associated with the sarcolemma seemed to be connected to cytoskeletal filaments, which ran in various directions, suggesting a lower contribution to the smooth muscle contraction (Fig. 3c). Figure 4 illustrates stereo-views of replica micrographs, indicating three-dimensional organization of cytoskeletal elements (Fig. 4a), and cytoplasmic sides of smooth muscle cells (Fig. 4b,c).

Discussion

The present study demonstrated three-dimensional ultrastructure of cytoskeletal elements, filaments associated with the sarcolemma and caveolae in smooth muscle cells by the QF-DE method. Although the QF-DE method has an advantage for such morphological examination, only narrow tissue areas can usually be used by the QF-DE method without an ice crystal artifact (Takayama et al., 1999). To improve such preparation difficulty, we applied a whole mount preparation technique to the QF-DE method (Takayama et al., 1999). Moreover, the paraformaldehyde fixation was useful for removing soluble proteins in the cytoplasm to clarify their cytoskeletal architecture (Takayama et al., 1994, 1999).

We have demonstrated that three-dimensional organization of cytoskeletal elements and caveolae in the smooth muscle cells. They had a regular arrangement of numerous thin filaments, while heterogeneous accumulation of cytoskeletal elements, exactly resembling dense bodies, was not clearly detected in the present study. They had a regular arrangement of numerous actin-like filaments, which were major cytoskeletal elements for the maintenance of cell structures. It is suggested that they have an essential role not only for the contractile movement, but also for the structures of GI walls. Unfortunately, we missed to identify intermediate filaments (Small et al., 1998).

We examined accumulations of granular structures on cytoskeletal filaments. Thus, the small granular structures were heterogeneously linked on the cytoskeletal filaments. Some functional proteins exist which show close association and intercommunication with structural proteins (Somlyo and Somlyo, 1975; Somlyo, 1980; Gabella, 1981; Alberts et al., 1994), and these granules could be compatible with such functional proteins. Furthermore, some granular structures might correspond to dense bodies revealed by conventional electron microscopy, although the dense bodies in smooth muscle cells have been suggested to be associated sites between intermediate filaments and actin filaments, and also to function as Z-lines in the striated muscle cells (Somlyo, 1980; Gabella, 1981; Stormer, 1998; Tsukita et al., 1982). We have speculated that the accumulated granular structures detected in the present study may have some relation to conventional dense bodies.

Short cross-linking filaments between adjacent thin filaments were also detected on the replica micrographs. These structures were different from granular structures as described above, and their origin has not yet been revealed. They could be related to the cooperative movement of abundant actin-like filaments during the smooth muscle contraction. Recent study suggests myosin cross-bridge cycling and actin-associated protein interact to maintain of tension in smooth muscle cells (Small, 1995). Functionally, they could have some roles in smooth muscle contraction. The distribution of the granular structures might correspond to that of dense bodies, as revealed by conventional electron microscopy.

In comparison with the striated muscle cells, it is known that the smooth muscle cells are morphologically flexible, and that they require much less energy for each contraction (Somlyo, 1975; Somlyo and Somlyo, 1980; Gabella, 1981). They have obviously more actin filaments than myosin filaments in their cytoplasm. So, it might be more reasonable that newly formed myosin filaments could have selected proper actin filaments neighboring themselves for each contraction/relaxation.

In the present study, caveolae are detected as small membranous vesicles beneath the sarcolemma, as seen from the cytoplasmic side. Few cytoskeletal elements existed in regions of caveolae. The caveolae have been known to show distinctly different topography from the plasma membrane, especially about the localization of some functional and/or structural molecules (Somlyo, 1980; Alberts et al., 1994). Caveolae may be involved in cholesterol-dependent regulation of specific signal transduction pathways (Kurzchalia and Parton, 1999). The caveolae keep their structures even when the muscle cells are stretched, and their number does not change at different phases of contraction and relaxation (Lee et al., 1983; Poulos et al., 1986). Moreover, during the muscle contraction, their sizes and shapes show no remarkable

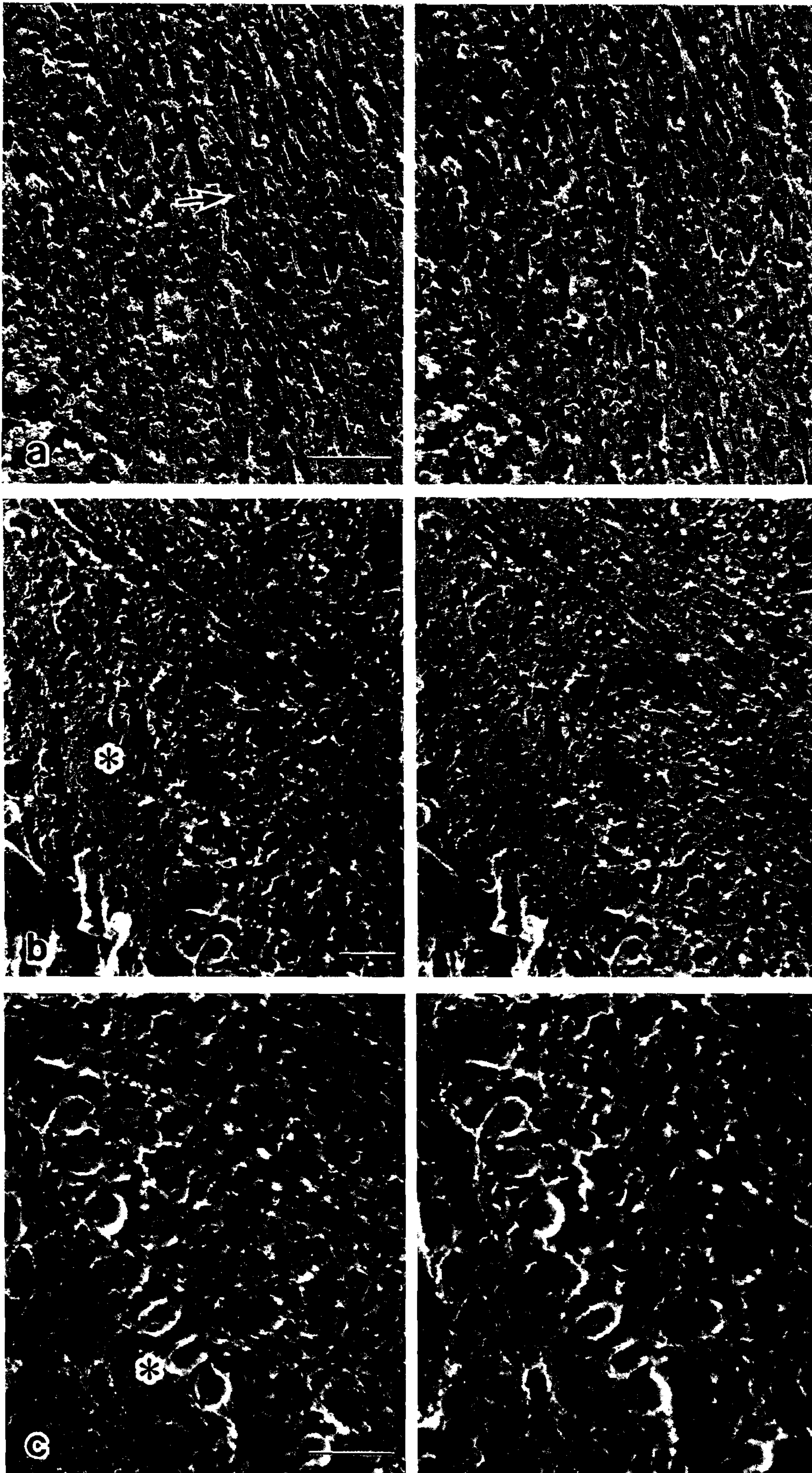

Fig. 4. a. Stereo-paired replica micrograph showing cytoskeletal elements of smooth muscle cells. Arrow; thin filaments. x 65,600; bar: 0.2 μm. **b.** Stereo-paired replica micrograph showing cytoskeletons, plasma membranes and the extracellular matrix of the smooth muscle layers. Asterisk: candidate region for dense plaque. x 42,300; bar: 0.2 μm. **c.** Higher magnified stereo-paired replica micrograph showing cytoskeletons, plasma membranes and the extracellular matrix of the smooth muscle layers. Asterisk: candidate region for dense plaque. x 66,500; bar: 0.2 μm.

changes (Gabella, 1981). Such findings might also suggest some specialized roles of the caveolae. The cytoskeletal arrangement around them, as revealed in the present study, should be highly meaningful for such features. Further investigations are required to clarify the functional roles of caveolae in the smooth muscle cells.

In conventional electron microscopy, morphological artifacts were often detected, especially in the extracellular matrix that was easily extracted and modified because of the lack of membrane protection (Takayama et al., 1994, 1999). Some previous studies with the quick-freezing method demonstrated improved morphology of various cells and tissues without such artifacts that were much reasonable from the viewpoint of physiological functions (Menco, 1986; Naramoto et al., 1992; Takayama et al., 1994, 1999).

The extracellular matrix of endomysium in the GI muscle walls has been reported to have a basement membrane, which consists of an electron-dense layer around smooth muscle cells with electron-dense parts near the cell surface. In contrast, the present study has demonstrated that the extracellular structure, corresponding to the basement membrane, becomes obscure, and that fine network structures in the muscle cells to form the integrated muscle layers (Gabella, 1981; Takayama et al., 1999). They seem to have close association with filaments associated with the sarcolemma and cytoskeletal elements. These morphological features would be reasonable for effective force transmission of the smooth muscle contraction.

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