Caveolae (historical synonyms, before 1990: caveolae intracellulares, surface vesicles, surface microvesicles, plasmalemmal vesicles, micropinocytotic vesicles) are Ω-shaped invaginations (~70 nm) of cell membrane. Since their discovery, more than 50 years ago [1], electron microscopy documented their presence in many tissues and organs. Caveolae are particularly prominent, for instance, in endothelial cells [2], and smooth muscle cells [3–6]. Indeed, their number may appear impressive in visceral smooth muscles: e.g. several tens of thousands/cell, occupying 2–4% of the relative cytoplasmic volume [4, 5].

During the last few years, numerous reviews almost exhausted the body of knowledge on caveolae [7–19]. Interestingly, caveolae have been considered as ‘lipid rafts’ [e.g. 20–24] and three definite caveolins have been identified [24–30].

Caveolae, specialized membrane nanodomains, have a key role in signaling processes, including calcium handling in smooth muscle cells (SMC). We explored the three-dimensional (3D) architecture of peripheral cytoplasmic space at the nanoscale level and the close spatial relationships between caveolae, sarcoplasmic reticulum (SR), and mitochondria, as ultrastructural basis for an excitation-contraction coupling system and, eventually, for excitation - transcription coupling. About 150 electron micrographs of SMC showed that superficial SR and peripheral mitochondria are rigorously located along the caveolar domains of plasma membrane, alternating with plasmalemmal dense plaques. Electron micrographs made on serial ultrathin sections were digitized, then computer-assisted organellar profiles were traced on images, and automatic 3D reconstruction was obtained using the ‘Reconstruct’ software. The reconstruction was made for 1 μm³ in rat stomach (muscularis mucosae) and 10 μm³ in rat urinary bladder (detrusor smooth muscle). The close appositions (about 15 nm distance) of caveolae, peripheral SR, and mitochondria create coherent cytoplasmic nanoscale subdomains. Apparently, 80% of caveolae establish close contacts with SR and about 10% establish close contacts with mitochondria in both types of SMC. Thus, our results show that caveolae and peripheral SR build Ca²⁺ release units in which mitochondria often could play a part. The caveolae–SR couplings occupy 4.19% of the cellular volume in stomach and 3.10% in rat urinary bladder, while caveolae–mitochondria couplings occupy 3.66% and 3.17%, respectively. We conclude that there are strategic caveolae–SR or caveolae–mitochondria contacts at the nanoscale level in the cortical cytoplasm of SMC, presumably responsible for a vectorial control of free Ca²⁺ cytoplasmic concentrations in definite nanospaces. This may account for selective activation of specific Ca²⁺ signaling pathways.

Keywords: caveolae • sarcoplasmic reticulum • mitochondria • nanospace • nanomedicine • Ca²⁺ release unit • Ca²⁺ homeostasis • 3D reconstruction • excitation-contraction coupling • electron microscopy

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Ca\textsuperscript{2+} handling units

- caveolae-SR
- caveolae-mitochondria
- SR-mitochondria

A
Oblique section in SMC (A) reveals three types of interactions between organelles known to be implicated in Ca\(^{2+}\) handling: caveolae-SR (blue circle), caveolae - mitochondria (red rectangle, close contacts - arrowheads), and SR - mitochondria (green rectangle). Infolded plasma membrane carries caveolae into the cortical cytoplasm (arrows).

Cross section (B) shows numerous Ca\(^{2+}\) release units (SR-caveolae) that face each other in the neighboring SMC.

**Fig. 1 A. B** Peripheral SR cisterns and mitochondria (m) are organized in clusters flanking plasma membrane of SMC and rigorously located along the caveolar domains. A - rat stomach muscularis mucosae, B - rat urinary bladder (detrusor).
At present, the attention is focused on the proteomics of caveolae [27–34]. At least 62 proteins are claimed to be located at the level of caveolae from various tissues [33]. Caveolae have been implicated in many cellular processes: transcytosis [35, 36], potocytosis [37, 38], endocytosis [39–41], signal transduction [5, 15, 16, 19, 25, 42, 43], control of cellular growth and proliferation [44, 45], however, their functions are still controversial.

In our opinion calcium handling is one of the most important roles of caveolae in smooth muscle. There is no need to argue that this might be an important “lacking piece” of the physiological and/or pharmacological smooth muscle puzzle. The tools of modern cell biology have begun to provide information in support of our original hypothesis [4, 5] on the function of caveolae in smooth muscle Ca\(^{2+}\) homeostasis, as Isshiki and Anderson [43] pointed out recently.

We propose here an integrating image of the caveolae, SR and mitochondria interactions based on ultrathin serial sections and three-dimensional (3D) reconstruction of caveolar domains in smooth muscle cells using the ‘Reconstruct’ software [46].

**Materials and methods**

**Animals**

The smooth muscles were taken from Wistar rat urinary bladder detrusor and stomach muscularis mucosae for the study of spatial arrangement of caveolae, SR, and mitochondria in SMC.
Fig. 3 A. Higher magnification of the green inset from Fig. 2. Image was rotated by 90° and plasma membrane was removed. Invaginated cellular membrane brings caveolae into the cortical cytoplasm and increases the contact area with SR and mitochondria (mito). Cover illustration. B. Electron micrograph (section 4 from EM series) shows the invaginated plasma membrane with caveolae. Note the close appositions between caveolae and SR (double arrow) and between caveolae and mitochondria (arrowhead). The SR profiles have close contacts on the both sides with plasma membrane (arrow) or with mitochondria (red arrow). Asterisks identify caveolae in the reconstructed volume.
Transmission electron microscopy

Small tissue samples, about 1 mm³, were fixed by immersion for 4 hours in 4% glutaraldehyde and refixed for 1 hour in 1% OsO₄ with 1.5% K₂Fe(CN)₆ (potassium ferrocyanide-reduced osmium) in 0.1M cacodylate buffer at room temperature. Afterwards, the samples were dehydrated and embedded in Epon 812 at 60°C for 48 hours. Routine 60 nm ultrathin sections were cut and mounted on Formvar-coated grids, stained with 1% uranyl acetate and Reynolds's lead citrate. For short series (<10 sections), the ultrathin sections were cut with a diamond knife at 45 nm thickness setting on the ultramicrotome stage (RMC). The transmission electron microscopy (TEM) examination has been performed with a Philips 301 at 60kV.

Reconstruction

Serial electron photomicrographs (EM) were digitized at 1200 dpi by scanning with a BenQ scanner. The images of serial sections were further processed using Adobe Photoshop software. The images were calibrated by drawing traces on an image of a known size scale. Then, the images were imported as tiff documents on RECONSTRUCT software (Reconstruct 1.0.6.0., 1996-2006 John C. Fiala; http://synapses.bu.edu) [46]. Section thickness was set at 0.045 μm.

Fig. 4 a-f. Details (viewing angle rotated with 60 degrees) of the green inset from Fig. 2. All images were saved from the Reconstruct 3D scene at the same magnification. Plasma membrane is represented as traces on serial sections. The reconstructed volume (0.1 μm³) contains 16 caveolae.
The ‘Reconstruct’ software was used for alignment of images with respect to specific structures of interest: caveolae, smooth reticulum, and mitochondria. Three-dimensional reconstruction and measurements were performed on the drawn outlines of the specific cellular structures. Computer-aided tracing of profiles on serial ultrathin aligned sections was followed by automatic 3D surface generation. For the 3D representation of the cellular membranes, mitochondria and SR a Boissonnat surface was selected. For caveolae 3D representation a sphere was used as substitution of the contours traced in a single 45 nm thickness section. The caveolae could be seen in two serial sections, but were traced only on the section in which they had maximum diameter.

**Color code used in reconstructions:** caveolae - blue; SR - yellow; mitochondria - red; plasma membrane - translucent white; nucleus - black.

**Quantitative analysis**

The relative volumes of organelles were obtained using a point-counting morphometric approach [47]. Relative volumes were calculated for complexes formed by caveolae and peripheral SR and for complexes formed by caveolae, peripheral SR and mitochondria as ratio of complex/cell. Mitochondria and SR were considered peripheral if their membranes were located within a distance <150 nm from the sarcolemma.

**Results and discussions**

About 150 electron micrographs of SMC showed that superficial SR and peripheral mitochondria are...
rigorously located along the caveolar domains of the plasma membrane. Typical examples are shown in Fig. 1. Reconstructions of the cortical structures from serial ultrathin sections indicate the close association between caveolae, SR and mitochondria (Figs. 2–6). As a rule, caveolae have close contacts with SR: 78.1% of caveolae interact with SR in 1 \( \mu m^3 \) stomach and 80.8% in about 10 \( \mu m^3 \) detrusor SMC. Mitochondria were seen in close contacts with caveolae (Figs. 2, 5A). About 10% of caveolae established close contacts with mitochondria (7.8% in stomach and 12.8% in urinary bladder). Indeed, in guinea pig taenia coli (counting about 6,000 caveolae from 20 SMC) it was reported that about 90% or 10% of all caveolae 'looked for' peripheral SR or mitochondria, respectively [48]. The distance between caveolae and SR or caveolae and mitochondria was about 15.03±7.08 nm. The inter-membrane spaces are flattened and narrow, usually below 10 nm, and give the impression of the 'synaptic-like' spaces between caveolae, SR, and mitochondria (Figs. 4–7).

Reconstructions showed that the cisterns of SR create sheets with gaps for caveolae that pass through into the free cytoplasm or close to the mitochondria (Figs. 4, 6). In some regions, plasma membrane finger-like protrusions bearing caveolae extend into the cytoplasm in close apposition with the mitochondria (Fig. 3A, 4, 5B) may create a false image of intracellular caveolae (Fig. 3B). Sometimes, caveolae did not appear connected to the plasma membrane because of the plane of the section (Fig. 3B), but their continuity with plasma membrane appears in the next section. SR and mitochondria are supposed to be dynamic organelles [49], but the caveolae movements raise questions until now [37–41, 50]. Our study did not show any caveolae without opening to the plasma membrane suggesting that caveolae are stable structures in SMC.

Our results support the existence of ultrastructural arrangements of caveolae, SR and mitochondria that create three types of junctional nanospaces in a slender cortical sector within the cytoplasm (exoplasm) of the SMC: caveolae-SR, caveolae-mitochondria, and SR-mitochondria. The 3D reconstructions of the cortical cytoplasmic space in SMC are in agreement with previously results [51, 52] and offer structural support for strong interaction of caveolae with SR and mitochondria revealed by the proteome analysis [33].

Privileged communication between different cellular components and their non-random connection may

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Fig. 6 A, B Details of the orange circular area from Fig. 5A - color code preserved.
A. A tilted view of the spatial complex formed by caveolae-SR-mitochondria from cell I. Note that the caveolae directly interact with SR or with mitochondria (arrows). The close association between caveolae-SR-mitochondria in a narrow space could be seen (arrowhead). Moreover, SR has close contacts with mitochondria only (double arrow).
B. Reconstructions showed that the complexes formed by caveolae with peripheral organelles face each other in the adjacent cells.
account for selective activation of specific Ca^{2+} signaling pathway generated by the strategic localization of interaction sites at the subcellular level (positional information). We suggest that there are strategic caveolae-SR-mitochondria contacts at the nanoscale level in the cortical cytoplasm of SMC, presumably responsible for vectorial Ca^{2+} movements. For instance, calmodulin-dependent Ca^{2+}-pump ATPase is located in caveolae of SMC [53–55] as well as the IP_3 receptor [56]. Cyclic ADP-ribose (cADPR) was shown to stimulate Ca^{2+} release from intracellular stores in SMC [57]. The membrane bound enzyme system, which can synthesize and metabolize cADPR, is present in SMC plasma membrane. Further studies are needed to clarify a presumptive relationship between cADPR and caveolae. Last but not least, SMC caveolae may function as sensors for the extracellular free Ca^{2+} concentration, since this was recently supposed to act as an extracellular signaling messenger [58].

Noteworthy, we previously found typical Ca^{2+}-release units in the prolongations of interstitial Cajal-like cells from human fallopian tube [59], uterus [60] and mammary gland stroma [61].

Acknowledgements

Part of this study was supported by a grant VIASAN 391/2004, Ministry of Education and Science, Bucharest, Romania. We thank to Dr. Cretoiu D. for constant help in image processing.

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**Fig. 7** Electron micrograph of a smooth muscle cell showing that caveolae (asterisks), SR and mitochondria (mito) create a composite structural unit in a nanoscale space.
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