COMMENTARY

Caveolae: static inpocketings of the plasma membrane, dynamic vesicles or plain artifact?

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Introduction

The presence of small (50–90 nm diameter) vesicular invaginations (caveolae) in plasma membranes has been known since some of the earliest ultrastructural descriptions of cells, yet the nature and function of these structures continue to stimulate debate and controversy. From their association with subplasmalemmal vesicles of similar dimensions, caveolae were originally assumed to be simple pinocytotic vesicles caught in the act of pinching-off from the plasma membrane. In vascular endothelial cells, this interpretation was refined and elaborated to explain bidirectional transport of substances across the blood vessel wall. In striated and smooth muscle, on the other hand, it was replaced by the concept of specialized, semi-permanent plasma membrane invaginations. The precise mechanism by which the caveola-vesicle system mediates transendothelial transport and the functional significance of muscle caveolae have been extensively discussed, often with conflicting conclusions. One of the central interpretative difficulties in the past has been the dependence on glutaraldehyde fixation to give a faithful representation of membrane organization in vivo. This limitation can now be overcome with ultrarapid freezing techniques, and the nature of caveolae has been re-examined in a number of recent studies using this approach in endothelial cells (McGuire & Twietmeyer, 1983; Robinson et al. 1984; Wagner & Andrews, 1985; Frøkjaer-Jensen & Reese, 1986; Noguchi et al. 1986) and muscle cells (Lee et al. 1983; Frank et al. 1987; Poulos et al. 1986). The purpose of this article is to discuss briefly the impact of these findings and the questions they raise against the background of previously established concepts.

Ultrastructure

The main structural features of caveolae as seen in glutaraldehyde-fixed cardiac endothelial and muscle cells are illustrated in Figs 1 and 2. In planar freeze-fracture fracture views of the plasma membrane, caveolae appear as small circular breaks, which represent their openings to the exterior (Figs 1A, 2A). The diameter, density and distribution of these openings vary in different cell types. In endothelial cells of cardiac capillaries (Fig. 1A), for example, the caveola openings are ≈40 nm in diameter, and occur at densities of 67 μm⁻² on the luminal, and 110 μm⁻² on the abluminal, plasma membrane; in neighbouring ventricular (and papillary) muscle cells, by contrast, they average ≈65 nm in diameter and have a density of just 4–6 μm⁻² (Simionescu et al. 1974; Gabella, 1978; Levin & Page, 1980). In cardiac muscle the caveola openings are usually scattered irregularly over the plasma membrane surface, in some skeletal muscle fibres the distribution pattern is related to underlying sarcomeric organization, and smooth muscle caveolae are ordered into distinct rows and bands that lie parallel to the long axis of the cell (e.g. see Devine et al. 1971; Gabella, 1978; Shafiq et al. 1979; Severs et al. 1985). Fractures that follow the bulb-like membranous neck and body of the caveola (Figs 1B, 2B) reveal few intramembrane particles (IMP) compared with the numbers present in the general plasma membrane, though in some smooth muscle cells a special ring of IMP circumscribes the caveola entrance (e.g. see Gabella & Blundell, 1978; Severs & Simons, 1986). High-resolution scanning electron microscopy reveals that endothelial caveolae and vesicles have a striped bipolar surface structure (Peters et al. 1985), but whether this is also a feature of muscle caveolae is unknown. Linear and branched chains of connected caveolae are often abundant in endothelial cells (Fig. 1B) and, though these may also sometimes occur in muscle (Forbes et al. 1979; Severs et al. 1982), a more common arrangement in cardiac muscle is for bunches of two or more caveolae to be suspended from the same neck (Fig. 2B). The full extent of such...
composite caveolae becomes more readily apparent when extracellular markers are used to stain structures that communicate with the exterior (Figs 1C, 2C).

**Muscle caveolae**

In skeletal, cardiac and smooth muscle, extracellular markers (applied after glutaraldehyde fixation as in Fig. 2C) have repeatedly been shown to penetrate virtually the entire population of subplasmalemmal vesicles of similar dimensions to visibly patent caveolae (e.g. see Forbes & Sperelakis, 1971; Devine et al. 1972; Gabella, 1973; Martinez-Palomo et al. 1973). Those vesicles that appear free-floating are thus in reality caveolae, connected to the plasma membrane.

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**Figs 1, 2.** Comparison of the structural features of caveolae in glutaraldehyde-fixed cardiac capillary endothelial cells (Fig. 1) and muscle cells (Fig. 2) as seen in planar freeze-fracture view of the plasma membrane (A), cross-fracture view (B) and thin-sectioning after Ruthenium Red staining (C). In Figs 1A and 2A, caveolae are fractured across their necks, appearing as circular breaks in the plasma membrane fracture faces (raised rims on the E-face, Fig. 1A; depressions on the P-face, Fig. 2A). Note differences in diameter and numerical density of the caveolar openings in the endothelial cell (Fig. 1A) and the cardiac muscle cell (Fig. 2A). Favourable fractures into the cytoplasm (Figs 1B, 2B) provide side views of caveolae. Arrows indicate caveolae connected linearly in the endothelial cell (Fig. 1B) and pairs of caveolae sharing the same neck in cardiac muscle (Fig. 2B). Apparent vesicles (arrowheads) are abundant in the endothelial cell and can also be seen in cardiac muscle. Ruthenium Red applied after glutaraldehyde fixation (C) stains the surface coat in both cell types intensely and penetrates those structures that open to the exterior, enabling apparent vesicles to be identified as caveolae. p, plasma membrane; cyt, cytoplasm. A, ×81 200; B, ×64 400; C, ×53 000.
out of the section plane. As the idea that all vesicles in all muscle cells/fibres could always be caught simultaneously in the act of fission or fusion is untenable, an endocytotic or exocytotic function was rejected in favour of the concept of static inpocketings of the plasma membrane. The problem then was to assign another function to these specialized semi-permanent cell surface domains. Many ideas have been considered, among them that caveolae serve as membrane reservoirs facilitating plasma membrane expansion, as miniature stretch receptors or special ion transport/storage sites (Essner et al. 1965; Gabella, 1973, 1978; Popescu, 1974; Dulhunty & Franzini-Armstrong, 1975; Prescott & Brightman, 1976; Masson-Pévet et al. 1979; Levin & Page, 1980). Caveolae are, in some instances, intimately associated with sarcoplasmic reticulum, and have been discussed as functional equivalents, precursors and terminal (cell surface) ends of transverse tubules (Devine et al. 1972; Ishikawa & Yamada, 1975; Zampighi et al. 1975; Gabella, 1978; Forbes et al. 1979; Forbes & Sperelakis, 1980). A recent suggestion in studies with atrial myocytes is that caveolae may act as sites for targeted fusion of secretory granule membrane with plasma membrane (Page et al. 1986). Clues have also been sought from the differences in caveola number, size and distribution apparent between different functional classes of cell/fibre and between healthy versus diseased muscle (e.g. see Masson-Pévet et al. 1979; Masson-Pévet & Gross, 1980; Bonilla et al. 1981; Shotton, 1982; Lee et al. 1986). It has not, however, proved possible to integrate the diverse and fragmentary observations into a general theory of caveola function for muscle as a whole. Most of the theories that are amenable to experimental test have been discounted and the remainder are either restricted in their applicability or based more on conjecture than hard fact.

**Endothelial caveolae and vesicles**

A functional role for the caveolae of endothelial cells has, by contrast, long been widely accepted as part of a vesicle system responsible for the exchange of fluid, solutes and especially large hydrophilic molecules, between blood and tissue (e.g. see Wagner & Casley-Smith, 1981). The mainstream theory of endothelial vesicle transport, developed from observations on the fate of macromolecule tracers in cells fixed at intervals after presenting the tracer to the luminal surface, involves the coupling of endocytosis to exocytosis – a process referred to as transcytosis (Simionescu, 1981). In its simplest (and original) form, this involves vesicles pinching-off from the plasma membrane on one side of the cell and discharging their contents by fusion on the opposite side (Palade & Bruns, 1968). Caveolae, according to this view, would represent vesicles that are forming from or becoming incorporated into the plasma membrane. One modification to this direct vesicle transport mechanism incorporates the possibility of transient fusions between cytoplasmic vesicles so that their contents become intermixed (Clough & Michel, 1981). Other refinements to the transcytosis theory were developed from the observation that when the luminal plasma membrane is labelled (e.g. with cationized ferritin) before macromolecules (haempeptides) are presented for transport, the caveolae and vesicles remain unlabelled with the ferritin, even though the haempeptides are taken up and apparently transported to the abluminal side by vesicles (Simionescu, 1981). This, reinforced by cytochemical and structural findings, led to the concept that the endothelial vesicles and caveolae constitute a membrane system that is quite separate and distinct from the plasma membrane. Vesicles are envisaged as repeatedly taking up and discharging their contents by shuttling back and forth across the cell, making transient connections with the plasma membrane on each side, but maintaining their own distinctive composition, structure and shape throughout. In this model, transient vesicle connection may occur at the general plasma membrane or at semi-permanent caveolae, and complete transendothelial channels may form temporarily by extension of a chain of fused vesicles and caveolae across the cell. Endothelial caveolae arc, from this viewpoint, interpreted as (1) transiently connected endothelial vesicles, (2) longer-lived plasma membrane invaginations composed of endothelial vesicle-like membrane, or (3) trans-endothelial channels.

The debate as to which of these proposed transport mechanisms is the predominant or exclusive one, or indeed whether any of them are valid at all, has been fuelled from the results of post-fixation extracellular marking and ultrathin serial section studies (Bundgaard et al. 1979; Frokjaer-Jensen, 1980, 1984; Davies & Kuczera, 1981; Chien et al. 1982; Noguchi et al. 1986). These have consistently shown that the number of truly discrete cytoplasmic vesicles is much lower than was originally assumed from standard sections; many apparently free vesicles in fact belong to long, branching, bead-like chains of fused vesicles (racemose structures), which open to the cell surface via caveolae. From these findings, transport by vesicles if accepted at all, is relegated to a minor role, and diffusion through caveola/vesicle channels favoured as the principal transcellular transport route. In one careful serial section reconstruction in rat heart capillaries (Bundgaard et al. 1983), the entire population of apparently free plasmalemmal vesicles was identified as belonging to racemose invaginations or caveolae that, instead of forming channels across the cell, all terminated within Caveolae 343
it. On these grounds it was argued that there was no evidence whatsoever that endothelial transport is mediated by vesicles, caveolae or channels. The apparent transport of macromolecules from the luminal to abluminal surface, which originally inspired the development of the transcytosis theory, could, it was suggested (Bundgaard, 1980), be plausibly explained by diffusion of the macromolecule tracers initially into the luminal caveolar chains (where in sectional view they would often appear as if in free vesicles), and then, after passage via the paracellular pathway, entry into caveolae on the abluminal side (which would give the impression of vesicle discharge).

Following this idea to its conclusion, endothelial caveolae would be envisaged as sessile structures of undetermined function (Bundgaard et al. 1983; Frøkjær-Jensen, 1984), more elaborate in structure but otherwise similar to those envisaged in muscle. In the same vein, a few studies on muscle have ignored prevailing opinion and continued to discuss muscle caveolae as a dynamic vesicle system rather than as semi-permanent invaginations (Pollack, 1977; Piper et al. 1984; Schwartz et al. 1984). Thus, a minority view for each cell type effectively transposes the established concepts of muscle and endothelial caveolae.

Ultrarapid freezing and the examination of cells without chemical fixation

A central flaw in the formulation of all these interpretations of caveola-vesicle functional relationships has been their dependence on glutaraldehyde to preserve instantly, in its native configuration, a lipid-rich, potentially labile membrane system. Membrane lipids are, however, only poorly stabilized by glutaraldehyde (e.g. see Jost et al. 1973; Glaeber, 1975), and even gross movements of intracellular organelles can continue for many seconds after exposure of cultured cells to the fixative (Buckley, 1973). The advent of ultrarapid freezing techniques (Dempsey & Bullivant, 1976; Heuser et al. 1979; Muller et al. 1980) and their increasing application to the examination of unfixed cells in the 1980s, led to re-appraisal of a series of membrane-related phenomena (e.g. see Chandler & Heuser, 1979; Kachar & Reese, 1982; Green & Severs, 1984), bringing to the forefront fears that glutaraldehyde fixation had often given a false picture of membrane structure and dynamic events in the past. This problem had not surfaced earlier because examination of unfixed specimens had only occasionally been attempted by conventional freezing methods as routinely practised for freeze-fracture throughout the 1970s. With few exceptions, conventional freezing necessitates treatment of the specimen with a cryoprotectant (glycerol) to prevent distortion of cell structure by ice crystals, and this is routinely preceded by treatment with glutaraldehyde to try and minimize cryoprotectant-induced artifacts. The essence of ultrarapid freezing is to improve the cooling rate of the specimen sufficiently for ice-crystal damage to be avoided, even in the absence of prior cryoprotectant treatment, and this then renders the glutaraldehyde fixation step unnecessary. In this way, the hope of capturing cells and their dynamic membrane events by the physical process of freezing, without ice-crystal damage, in a state close to that existing in vivo, becomes a practical possibility.

Caveolae in directly frozen endothelial cells

When directly frozen endothelial cells were first examined by freeze-fracture and thin-section (after freeze-substitution) methods, the existence of both caveolae in the plasma membrane (Fig. 3A) and apparently free vesicles in the cytoplasm was confirmed (Dempsey et al. 1973; Mazzone & Kornblau, 1981; Severs & Green, 1983a). However, by quantitative analysis after ultrarapid freezing in two endothelial cell types (rat thoracic aorta, McGuire & Twietmeyer, 1983; rete mirabile of the eel swim bladder, Wagner & Andrews, 1985), the number of caveolae after glutaraldehyde fixation was found to be about threefold higher than that of unfixed cells. This discrepancy was attributed to glutaraldehyde-induced fusion of free cytoplasmic vesicles with the plasma membrane; an idea first discussed in an earlier investigation of surface vesicles in conventionally frozen (fixed and unfixed) fibroblasts (Bretscher & Whytock, 1977). If, in reality, endothelial cells have significantly fewer caveolae than originally thought, it might be assumed that the number of free vesicles is correspondingly higher, shifting the balance in favour of vesicle transcytosis rather than caveolar channel diffusion as the principal or exclusive mechanism of transendothelial transport. However, the potential for vesicular transport implied by the apparent abundance of vesicles in fixed cells may also be misleading; glutaraldehyde-fixed endothelial cells, according to some reports, contain substantially more vesicles than do unfixed freeze-substituted cells from the same vessel (Mazzone & Kornblau, 1981; Robinson et al. 1984; Wagner & Andrews, 1985). If, as supposed, glutaraldehyde depletes the free vesicle population to increase artifically the number of caveolae, then there must be further mechanisms by which it generates additional vesicles. In the rete mirabile, the explanation seems to be that the fixative causes cisternal membranes to fragment and minivesicles to fuse together (Wagner & Andrews, 1985), but whether this mechanism operates in other endothelia is unknown. Another possibility suggested from a study on cultured endothelial cells...
Fig. 3. Freeze-fracture views of unfixed, directly frozen endothelial plasma membrane (A) and cardiac muscle plasma membrane (B). For A, a spermatic artery was mounted between two thin copper holders in situ in a living (anaesthetized) rat, and frozen within 2 s of excision by plunging into propane (Severs & Green, 1983a). Note variation in the degree of invagination of caveolae, suggesting different stages in vesicle formation or fusion. Fig. 3B is from a heart that was beating in an anaesthetized rat until the instant it was frozen by the impact of a cold copper block (Severs & Green, 1983b). The sparsity of caveolae results in large tracts of membrane in which no caveolar openings are seen (cf. Fig. 2A). EF, E-face; PF, P-face (in contrast to fixed specimens, unfixed endothelial plasma membranes show a higher density of intramembrane particles on the EF than on the PF). X 64 600.
(Robinson et al. 1984) is that glutaraldehyde induces inward blebbing of the plasma membrane – a mechanism that could, at the same time, account for the elevated numbers of caveolae. In view of the lessons learnt from three-dimensional reconstruction in fixed specimens, it is clearly of importance to establish what proportion of the apparently free vesicle population in directly frozen specimens in fact belongs to caveola–vesicle chains. The surprising conclusion reached from examination of rapidly frozen freeze-substituted frog mesentery and rat skeletal muscle capillaries (Frøkjær-Jensen & Reese, 1986; Noguchi et al. 1986) is that, as in fixed specimens, truly free vesicles are rare, but chains of fused vesicles, some of which connect via caveolae to the cell surface, are common. One possible explanation for these seemingly contradictory findings is that owing to intrinsic differences in the proportions of caveolae and free vesicles, not all endothelia respond to chemical fixation in the same way; the native configuration of the caveola–vesicle system may thus be more faithfully preserved in some than in others.

**Caveolae in directly frozen muscle cells**

All these new findings on endothelial vesicle–caveola functional relationships, however incomplete, raise some interesting questions as to the nature and function of muscle caveolae. If, in endothelial cells, glutaraldehyde fixation can induce fusion of free subplasmalemmal vesicles with the plasma membrane, could not the same happen in muscle cells? If it did, the idea of muscle caveolae as part of an endocytotic or exocytotic vesicle system would not, after all, be quite so implausible. Alternatively, if glutaraldehyde can induce inward blebbing of the plasma membrane, most or even the entire population of muscle caveolae could be artifactual. Either way, a fresh look at the status of muscle caveolae as specialized semi-permanent invaginated domains and their reality in vivo is called for.

In skeletal muscle fibres, the first indications were that unfixed ultrarapid-frozen specimens have markedly fewer (and smaller) caveolae than do fixed specimens (Lee et al. 1983). When the same trend was reported in a recent study on cardiac muscle (Frank et al. 1987), with which our own unpublished observations agree (Fig. 3B), a consistent pattern across muscle types seemed to be emerging. This hope was, however, dashed with the publication in 1987 of a careful and detailed study by Poulos et al. (1986). They found that caveola number in intact ultrarapid-frozen skeletal muscle fibres did not differ from that observed after glutaraldehyde fixation, and was, moreover, unchanged by brief or prolonged (neuromuscular blocking agent-induced) rest, brief or prolonged electrical stimulation, stretch (within the normal physiological range) or careful compression cutting. However, the number and size of caveolae were greatly reduced after mechanical damage, i.e. when myofibres were laterally sheared or torn during preparation for ultrarapid freezing, and it is to this that the earlier discordant results on human skeletal muscle reported by the same group (Lee et al. 1983) are now attributed.

The findings of Poulos et al. (1986) bring renewed support to the concept of muscle caveolae as long-lived specialized invaginations of the plasma membrane, but again, further questions are posed. Why, after direct freezing of unfixed cardiac muscle, is a reduced number of caveolae observed? Although mechanical damage and/or time-related changes during specimen preparation (Severs & Green, 1983a; Poulos et al. 1986) might seem the obvious explanation, the study by Frank et al. (1987) minimized these by using entire papillary muscles, which were excised and mounted in a physiological medium and then frozen within 30 s. Moreover, the example illustrated in Fig. 3B comes from a heart that was beating in situ in the living animal until the very instant it was frozen by impact of a cold copper block (Severs & Green, 1983). It seems improbable that caveolae in cardiac muscle could differ fundamentally from those in skeletal muscle, yet that is what these preliminary results suggest. Further work using techniques designed to reduce or eliminate specimen manipulation and preparation time before ultrarapid freezing (e.g. see Severs & Green, 1983a, b) is still needed to confirm these findings. How else can the function and properties of muscle caveolae now be best explored? The isolation of caveolae from plasma membrane does not seem feasible at present but recent advances in cytochemistry offer new opportunities for determining the chemical make-up of the caveolar membrane. Further investigation of some of the physiological conditions that alter caveola number (e.g. anoxia, myocyte dissociation and culture conditions) might also prove worthwhile. For the moment, however, the function of muscle caveolae still seems as elusive as ever.

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**References**


Buckley, I. K. (1973). Studies in fixation for electron...


Masson-Pévet, M., Bleeker, W. K. & GROS, D. (1979). The plasma membrane of leading pacemaker cells in the...


