PLASMALEMMAL VESICLES AND THE EFFECTS OF STEROL-BINDING AGENTS IN RABBIT AORTIC ENDOTHELIUM

H. L. SIMONS AND N. J. SEVERS*
Department of Cardiac Medicine, The Cardiothoracic Institute (University of London), 2 Beaumont Street, London WIN 2DX, UK

SUMMARY
The rims of vesicle openings in capillary endothelium are reported to be highly sensitive to perturbation by the sterol probe filipin when this agent is administered by perfusion at 50 μM concentration in aldehyde fixative for 10 min. If, as supposed, this specific rim response reflects functionally significant aspects of membrane organization at the vesicle-plasmalemma boundary, then it would be expected to be a reproducible and universal feature of endothelial vesicles in general. In the present study, we have investigated in detail the response to filipin of rabbit and rat aortic endothelial plasmalemma. First, the effect of standard filipin treatment (150 μM, 3–20 h) was examined by freeze-fracture. The extensive generalized response observed, taken together with the effects of filipin seen by fluorescence microscopy of stripped aortic endothelial sheets, and the marked corrugation of the plasmalemma observed by freeze-fracture after tomatin treatment, indicate that substantial amounts of cholesterol are present in the membrane. Exposure of rabbit aortic endothelium to 50 μM filipin for 10, 20, 40 and 60 min enabled the progressive appearance of filipin deformations in the plasmalemma to be traced. Even at the very earliest stages of the response, there was no preferential association of filipin deformations with endothelial vesicle rims. Specific sensitivity of endothelial vesicle rims would, therefore, seem to be either non-universal in occurrence or non-reproducible, and so is unlikely to reflect any membrane property of fundamental significance to endothelial vesicle function.

INTRODUCTION
The possibility that sterols play a role in the function of endothelial plasmalemmal vesicles has been discussed after freeze-fracture investigation of the membrane-perturbing effects of filipin in capillary endothelial cells (Simionescu et al. 1983). Filipin is a polyene that interacts with 3β-hydroxysterols, producing deformations that are visible by freeze-fracture (Verkleij et al. 1973; Tillack & Kinsky, 1973), and which were suggested (Andrews & Cohen, 1979; Elias et al. 1979) and subsequently widely used (for a review, see Severs & Robenek, 1983) as markers for localizing sterols in membranes. The question as to how reliable this technique really is for pinpointing sterol distribution in the membrane plane has been given increasingly detailed scrutiny (e.g. see Severs et al. 1981; Feltkamp & van der Waerden, 1982; Karnovsky, 1982; Severs & Robenek, 1983; Severs & Simons, 1983; Miller, 1984; Behnke et al. 1984), and this problem will be considered further in the Discussion.

*Author for correspondence.

Key words: plasmalemmal vesicles, endothelial vesicles, sterols, cholesterol, filipin, tomatin.
In an earlier report from this laboratory, it was noted that after standard (prolonged) filipin treatment of rabbit myocardium, the deformations observed in capillary endothelial plasma membranes were neither preferentially associated with nor excluded from the plasmalemmal vesicles (Severs, 1981). From experiments using very brief exposures to filipin, however, Simionescu et al. (1983) subsequently reported that rings of filipin-sensitive membrane surround the openings to plasmalemmal vesicles in capillary endothelia from a variety of different tissues, including myocardium from mice and rats. It was concluded from these findings that peristomal rings of sterols might play an important role in endothelial vesicle function, e.g. by contributing to local stabilization of the plasma membrane and, or, to phase separation between plasma membrane and vesicle membrane during fusion/fission (Simionescu et al. 1983).

If the specific rim response reported by Simionescu et al. (1983) reflects these or other functionally significant aspects of membrane organization at the vesicle-plasmalemma boundary, then it might be predicted to be a universal feature of endothelial vesicles in general. In the present study, therefore, we set out to discover whether any evidence could be found for the existence of filipin-sensitive ring-like domains at the rims of plasmalemmal vesicles in rabbit and rat aortic endothelium. A preliminary account of part of this study has previously been reported in abstract form (Simons & Severs, 1984).

**Materials and Methods**

**Animal material and tissue preparation**

Adult male New Zealand White rabbits (body weight 2.5 kg) were used for the main series of experiments. Sprague-Dawley rats (250–300 g) were used in a later experimental series to investigate the possibility of species differences in response. The animals were maintained on standard pelleted diets and tap water, available ad libitum.

Aortas were fixed in situ by retrograde perfusion. The animals were anaesthetized with pentobarbitone, and the posterior abdominal aorta was cannulated and perfused for 5 min with heparinized oxygenated phosphate-buffered saline (pH 7.4) at a pressure of 100 mmHg and a temperature of 37°C. Perfusion with Karnovsky (1965) fixative, containing either 3% glutaraldehyde/3% formaldehyde or 1.5% glutaraldehyde/2.5% formaldehyde in 0.1 M-sodium cacodylate (pH 7.4) followed for 15-25 min at 37°C. Aortas were then excised, and fat and adventitial layers removed by dissection in fresh fixative under a binocular microscope. This procedure was carried out thoroughly over a 2-h period of immersion fixation for samples subsequently given standard filipin treatment. Perfusion-fixed vessels destined for brief exposure to filipin received a more rudimentary 'clean' involving a 10–15 min immersion fixation step.

**Standard treatment with filipin**

Samples of fixed aorta were incubated in 150 μM-filipin in cacodylate-buffered (pH 7.4) 2.5% glutaraldehyde for 3, 7 or 20 h under standard conditions (Severs, 1984b). On completion of treatment, three rinses in cacodylate buffer of 5 min duration each were given before further processing for fluorescence microscopy or freeze-fracture electron microscopy.

**Fluorescence microscopy**

En face preparations of aortic endothelial sheets were obtained by the method of Poole et al. (1958) from control and filipin-treated specimens. This involves anchoring the endothelium in a
Endothelial vesicles and filipin

film of nitrocellulose so that the intimal layer can be stripped away from the remainder of the vessel wall, and mounted on a glass slide for viewing in the light microscope. For fluorescence microscopy, a Zeiss Standard 16 light microscope equipped with Zeiss IV Fl epi-fluorescence optics was used.

Tomatin treatment

Tomatin was used at a concentration of 150 µg cm⁻³ in cacodylate-buffered 2.5 % glutaraldehyde (pH 7.4). The samples were treated for 5 h and 20 h using similar conditions to those used in the filipin experiments.

Brief treatment with filipin

Two procedures were used to investigate the time-course of the response of rabbit aortic endothelium to filipin. First, aortas were perfused as described above for 10 min with fixative solution containing 50 µM-filipin or 150 µM-filipin. The second procedure was to take samples of perfusion-fixed aortas and incubate them in cacodylate-buffered glutaraldehyde containing 50 µM-filipin for periods of 10, 20, 40 and 60 min under the same conditions as used for standard filipin treatment. After this precisely timed exposure, the specimens were immediately rinsed in a large volume of cacodylate buffer to remove excess filipin. Similar experiments were later carried out on rat aortic samples treated with 50 µM-filipin for 10, 15, 20 and 40 min.

Freeze-fracture electron microscopy

All samples were placed for 1 h in cacodylate-buffered 25 % glycerol. Specimens given standard filipin or tomatin treatment were mounted by sandwiching between pairs of thin copper supports for subsequent fracturing in a hinged (double replica) device. This procedure was found to be effective only when the adventitial layers of the sample had been removed by extensive teasing and peeling-off. As samples given brief treatments with filipin did not receive a long immersion fixation step in which this delicate and time-consuming procedure could be carried out, they were mounted with a drop of polyvinylalcohol (Brown, 1981) on single holders for microtome fracturing instead. Freezing was by insertion into propane cooled by liquid nitrogen. The specimens were fractured and replicated at −110°C (vacuum, 40 µPa) in a Balzers BAF 400T apparatus. Replicas were cleaned with chromic acid, and examined in a Philips EM301 electron microscope. Quantitative analysis of the numerical density and distribution of filipin deformations was carried out as described by Jones et al. (1983).

RESULTS

Effect of standard (prolonged) filipin treatment

To provide a comparison with our earlier results on myocardial capillaries (Severs, 1981), we first established the response of the rabbit aortic endothelial plasmalemma to standard (prolonged) filipin treatment. After 20 h exposure to the agent, numerous filipin deformations were observed scattered over both the luminal and abluminal aspects of the membrane (Fig. 1). Quantitative analysis gave a mean numerical density of 181.9 ± 5.8 (S.E.M.) filipin deformations µm⁻², a noticeably lower response than that of the myocardial capillary endothelium (Severs, 1981). Samples of aorta treated with filipin for 3 h and 7 h showed a similar response. No obvious pattern to the distribution of filipin deformations was apparent on visual inspection; the deformations were neither preferentially associated with nor excluded from the peripheries of vesicle openings (Fig. 1). Statistical analysis confirmed a random distribution, with coefficients of dispersion ranging from 0.79 to 1.19.
Alternative approaches to sterol detection

Fluorescence microscopy of filipin-treated samples and freeze-fracture of tomatin-treated samples were carried out as independent approaches to check the results obtained by freeze-fracture after standard filipin treatment.

Stripped aortic endothelial sheets treated with filipin for 3 h fluoresced with the ice-blue colour characteristic of filipin–cholesterol complexation, whereas control specimens not exposed to filipin failed to do so. The fluorescence faded rapidly (<3 s) on excitation, making photography difficult. Another technical problem was the preparation of ‘clean’ endothelial sheets, uncontaminated by adhering fragments of smooth muscle cells. The few satisfactory preparations examined, however, indicated extensive binding of filipin to cholesterol in accord with the freeze-fracture findings.

Freeze-fracture of aortic samples given tomatin treatment for 5 h revealed widespread corrugation of the endothelial plasmalemma (Figs 2, 3), often with virtually all the exposed membrane area affected. The hemitubular deformations, formed by complexing of the agent with sterols, were characteristically arranged as multiple

Fig. 1. Effect of filipin on rabbit aortic endothelial plasmalemma (P-face, luminal aspect) after standard treatment (20 h/150 μg-filipin). Filipin deformations are seen as numerous randomly scattered 25 nm ‘bumps’ (fd), quite distinct from the broader circular depressions representing vesicle openings (v). ×48 600. 
large whorls, between which small regions of unaffected membrane sometimes remained. These 'bald' patches bore no observable relationship to the presence or absence of membrane structural specializations, and their occurrence is presumed to be related to the mechanism by which the deformations form and develop within the membrane.

Where furrowing of the membrane was relatively mild, individual corrugations could be seen perturbing both peristomal and intervening membrane areas (Fig. 3). The tomatin-induced corrugation was often so severe as to distort the vesicle openings (Fig. 2), sometimes obscuring them completely. Extension of the tomatin treatment for 20 h exacerbated this problem and led, in many cases, to extensive membrane disruption.

**Time course of filipin response determined from serial brief exposures**

Following 10 min of perfusion of the rabbit aorta *in situ* with 50 μM-filipin — conditions corresponding to those used by Simionescu et al. (1983) in mice and rats — all aortic endothelial cells examined were unaffected. Raising the concentration of filipin to 150 μM or lowering the concentration of fixatives in the perfusion medium, did not lead to any detectable response over the same period of time. It was clear from these results that the large quantities of filipin that would have been required for perfusion of the rabbit aorta *in situ* for longer periods were prohibitive.

A different experimental procedure was therefore adopted in which tissue pieces, prefixed by perfusion, were incubated in the filipin–fixative solution for the required period, with constant rotary mixing to ensure optimal exposure of the endothelial surface to the agent.

Using this procedure for the rabbit aorta, 10 min of incubation in 50 μM-filipin gave no response (Fig. 4), as in the corresponding perfusion experiments. By 20 min, however, filipin deformations had started to appear in the plasmalemmata of most endothelial cells (Figs 5, 6). This effect was confined to the luminal plasmalemma, the abluminal aspect remaining unperturbed (Fig. 7). Careful inspection of micrographs at this stage, when few filipin deformations are present, shows that the formation of the deformations is neither favoured in nor restricted to the vesicle rims, but occurs randomly over the entire endothelial surface (Figs 5, 6). After 40 min of treatment, the response had developed more fully on the luminal plasmalemma and had started to show on the abluminal aspect (Fig. 8). By 60 min, most endothelial cells displayed a maximal response (equivalent to that observed with standard filipin treatment) on both the luminal and abluminal plasmalemma (Fig. 9), and underlying smooth muscle cells were also affected. The rims of caveolae of the smooth muscle plasma membrane were no more sensitive to filipin than neighbouring membrane areas within the caveolae-containing bands, interband (non-caveolar) zones remaining resistant as previously reported under standard treatments (Severs & Simons, 1983).

When the brief-exposure experiments using 50 μM-filipin were repeated on rat aorta, the effects of filipin were seen more rapidly than in the rabbit. Deformations were detected in the luminal plasmalemma after 10 min (Fig. 10), and in the
Endothelial vesicles and filipin

abluminal plasmalemma after 20 min (Fig. 11). As in the rabbit, however, there was no preferential association of the deformations with endothelial vesicle rims.

DISCUSSION

The introduction of a simple cytochemical technique for cholesterol detection based on treatment with a sterol-binding agent (filipin, digitonin or tomatin) followed by freeze-fracture electron microscopy (Elías et al. 1978, 1979) generated considerable interest and research activity, and studies applying this approach with filipin now abound in the literature (e.g. see Severs & Robenek, 1983, and references therein). However, since the first report of a false-negative response to filipin (Warren & Severs, 1980), evidence that there are dangers in interpreting the effects

Fig. 4. Luminal plasmalemma (P-face view) of rabbit aortic endothelial cell after 10 min incubation in 50 μM-filipin. No filipin deformations are present after this brief treatment. ×50 600.

Fig. 2. Effect of tomatin treatment (5 h) on rabbit aortic endothelial plasmalemma (P-face view, luminal aspect). The membrane has become completely covered in tomatin-induced corrugations, and though the vesicle openings (v) have been somewhat distorted, they remain visible. ×59 400.

Fig. 3. Higher magnification view of the effect of tomatin. In this example, the agent has induced milder furrowing, and the vesicle openings (v) have remained clearly visible. No difference in response is apparent between the membrane immediately surrounding vesicle openings, and that further afield. ×97 200.
Endothelial vesicles and filipin

of this agent at face value has steadily accumulated. It is now widely recognized, for example, that densely packed arrays of intramembrane particles and dense aggregations of peripheral proteins or other structures at the membrane's cytoplasmic surface are capable of preventing or inhibiting deformation of cholesterol-rich membrane by filipin (Severs et al. 1981; Feltkamp & van der Waerden, 1982, 1983; Karnovsky, 1982; Brown et al. 1982; Severs & Robenek, 1983; Severs & Simons, 1983; Tamm & Tamm, 1983; Steer et al. 1984). However, the action of digitonin and tomatin is in some (though not necessarily all) such instances unimpaired (e.g. see Severs et al. 1981; Severs & Simons, 1983; Severs, 1984c), and an alternative to freeze-fracture for detecting filipin binding is provided by fluorescence microscopy (e.g. see Tamm & Tamm, 1983; Pumplin & Bloch, 1983; McGookey & Anderson, 1983). The idea thus evolved that other techniques – including tomatin treatment/freeze-fracture and filipin treatment/fluorescence microscopy – might prove useful in backing up results obtained by the filipin/freeze-fracture approach (Severs et al. 1981; Severs & Simons, 1983; Severs, 1984a,fe). In the present study we used both these supplementary approaches in parallel with the standard filipin/freeze-fracture method and, though none of these techniques used alone is infallible (Severs, 1984a), when taken together the consistency of all the findings points to the presence of substantial quantities of cholesterol in the aortic endothelial plasma membrane.

As reported previously (Severs, 1981; Simionescu et al. 1983), standard (prolonged) exposure to filipin gave no hint of any heterogeneity in the distribution of filipin deformations within the plane of the endothelial plasmalemma. However, by using a series of brief exposures to filipin at low concentration to follow the time course of appearance of deformations in rat and mouse capillary endothelial plasmalemma, Simionescu et al. (1983) reported a preferential reaction at the vesicle rim. The first filipin deformations to be detected (10 min treatment/50 μM-filipin) occurred predominantly in rings circumscribing the openings to endothelial vesicles and fenestrae. As the treatment period increased, however, filipin deformations subsequently developed throughout the membrane to give, ultimately, the generalized response pattern characteristic of standard (prolonged) treatment.

In contrast to the findings of Simionescu et al. (1983), when we followed progressively the appearance of filipin deformations in rabbit aortic endothelial plasmalemma
we were unable to find any evidence for a preferential early response at the vesicle rims. Neither were we able to detect a corresponding effect at the rims of caveolae in smooth muscle cells. It might be argued that such a discrepancy could be due to differences between our experimental regime and that used by Simionescu et al. (1983). Our original intention was to follow the methods of these authors as precisely as possible (suitably adapted to the rabbit aorta), but after several trials this was found impracticable owing to the large quantities of filipin that would have been required for multiple aortic perfusions. By incubation of samples in fixative solution containing the same concentration of filipin as that used by Simionescu et al. (1983), however, we were able to follow just as precisely the very early stages in the response; the factor considered crucial to the demonstration of sterol rings. With both the incubation method and our perfusion trials we found a longer lag before the first filipin deformations appeared than that reported by Simionescu et al. (1983). To determine whether a species difference was involved in these differences, we repeated the serial brief-exposure experiment on the rat - one of the species used in the Simionescu study. The results revealed a similar time course to that reported by these authors, with filipin deformations present in the luminal plasmalemma as early as 10 min. However, as in the rabbit, the first filipin deformations to be formed occurred randomly over the membrane, showing no preferential association with the vesicle rims.

On the basis of the present results then, preferential sensitivity to filipin does not appear to be a universal feature of the rims of endothelial vesicles. Even if it were to prove a reproducible finding in vessels other than the aorta, assessment of its precise meaning in terms of cholesterol distribution would not be straightforward. Theoretical considerations of the mobility of cholesterol and primary filipin-cholesterol complexes (Severs & Robenek, 1983; Miller, 1984) question the extent to which it is valid to extrapolate directly from small-scale local variations in filipin-deformation distribution to corresponding planar heterogeneities in cholesterol concentration. In view of this limitation and the transient nature of the reported rim-effect, it might well be argued that positive interpretation of filipin-sensitive vesicle rims solely as membrane domains specifically enriched in cholesterol would lack

Fig. 9. Appearance of luminal (l) and abluminal (a) endothelial plasmalemma after 60 min of exposure of rabbit aorta to 50 μM-filipin. Filipin deformations are abundant on both aspects of the plasmalemma. The response is now maximal, corresponding to that observed after standard (prolonged) filipin treatment (cf. Fig. 1). Note that as the abluminal plasmalemma is seen in, E-face view, its filipin deformations appear predominantly as small pits rather than bumps. cyt, cytoplasm; arrow, possible site of shedding of filipin-affected membrane. ×48 600.

Fig. 10. Rat aortic endothelial plasmalemma (luminal aspect, P-face view) after 10 min exposure to 50 μM-filipin. As in the rabbit, the first filipin deformations to be detected occur randomly over the membrane. ×70 000.

Fig. 11. Abluminal plasmalemma of rat aortic endothelium after 20 min exposure to 50 μM-filipin. By this time the agent has just penetrated across the cell, and filipin deformations (seen as dimples, indicated by arrows in this E-face view) are starting to appear in the membrane. Again no specific association with the endothelial vesicles is apparent. ×68 200.
credibility. Indeed, notwithstanding their speculation on the possible significance of peristomal rings of sterols, Simionescu et al. (1983) also commented that, rather than demonstrating an especially high cholesterol content, their results might indicate greater accessibility of filipin to cholesterol at vesicle rims compared with elsewhere in the membrane.

In conclusion, then, although the reasons for the discrepancy between our results and those of Simionescu et al. (1983) remain unclear, our failure to demonstrate filipin sensitivity at the rims of aortic endothelial vesicles calls into question the universality and, or, reproducibility of this response and casts doubt on the idea that it reflects any membrane property of fundamental significance to endothelial vesicle function.

This work was supported by British Heart Foundation grant no. 81/44. Thanks are due to Dr G. E. Jones and Dr J. A. Witkowski for help with the computer analysis of numerical density and distribution of filipin deformations.

REFERENCES


(Received 15 November 1985 – Accepted 27 January 1986)