Ultrastructural Features of the Innervation and Smooth Muscle of the Rabbit Facial Vein, and Their Relationship to Function

REED A. ROWAN, ROSEMARY D. BEVAN, AND JOHN A. BEVAN

SUMMARY The purpose of this study was to determine to what extent the ultrastructure of the intramural plexus of autonomic nerves and the smooth muscle of the rabbit facial vein could be correlated with the functional properties of this vessel. The mean observed widths of neuromuscular clefts were 250 nm in untreated control vessels, 260 in the dilated vein, and 390 in the contracted vein. Variation in the plane of section and in cell surface contours may lead to overestimation of cleft width, particularly in contracted vessels; the conclusion was reached, therefore, that the actual mean cleft width in this vessel, which may be closer to 200 nm, is relatively narrow in comparison with other blood vessels. There is probably little significant variation in cleft width with changes in vessel diameter. This narrow cleft correlates with the pronounced neurogenic response of this vessel. The smooth muscle cells of the facial vein appear to contain a relatively small amount of sarcoplasmic reticulum, which may be related to the dependence of maintained tone on extracellular calcium. Areas of close apposition of cell surfaces, with gaps of approximately 15 nm, may be related to propagation of electrical activity from one smooth muscle cell to another. Circ Res 49:1140-1151, 1981

STUDIES of the ultrastructure of blood vessels with well-documented functional properties can lead to a better understanding of the relationship of vascular structure to circulatory control. As the number of such correlative studies increases, the functional significance of structural variation becomes more firmly established, with the ultimate possibility that functional behavior might be predicted on the basis of structural observations. This kind of information might be useful in studying the microcirculation in which direct functional studies are difficult.

The buccal segment of the facial vein of the rabbit has an unusual combination of well-defined functional properties, providing a good opportunity for correlation with ultrastructural features. This vessel responds to stretch with a maintained myogenic tone, which is dependent on the constant availability of extracellular calcium (Winquist and Bevan, 1977). The relative dependence of vascular smooth muscle on extracellular as opposed to intracellular sources of calcium may be related to the amount and distribution of sarcoplasmic reticulum within the cells (Devine et al., 1972). The intrinsic myogenic tone of the facial vein is exquisitely sensitive to small changes in temperature, which is consistent with the possible role of this vessel in cranial thermoregulation (Winquist and Bevan, 1980).

A fairly dense plexus of adrenergic nerves is present throughout the full thickness of the tunica media in the buccal segment of the facial vein, and the response of the vessel to stimulation of these nerves, or to exogenous norepinephrine, is a relaxation which is mediated by $\beta$-adrenergic receptors (Pegram et al., 1976). The threshold frequency for the neurogenic response is low, and the vessel responds to a greater degree to changes in nerve stimulation frequency than do most other blood vessels which have been studied in vitro (Bevan, 1978). The tendency toward such a pronounced neurogenic response is often associated with the presence of nerves within the tunica media, but it also suggests the possible existence of a relatively narrow neuromuscular cleft (Bevan, 1977). The narrower the neuromuscular cleft, the more localized and intense is the effect of the neurotransmitter. Whether or not the presence of a three-dimensional, intramural nerve plexus can result in such a large neurogenic response as that of the facial vein without requiring a narrow neuromuscular cleft is unknown. Another unanswered question is whether or not the dimensions of the neuromuscular cleft vary with changes in the diameter of blood vessels with an intramural nerve plexus.

The purpose of this study was to examine the ultrastructural features of the buccal segment of the rabbit facial vein, particularly the dimensions of the adrenergic neuromuscular cleft within vessels in different degrees of contraction, and the features of smooth muscle cells which might be related to sources of activator calcium, in order to determine
whether the conditions proposed as suggested between structure and function pertain to the facial vein.

Methods

For the examination of general ultrastructure, small rings or strips of the buccal segments of facial veins from New Zealand white rabbits were obtained in either a dilated, contracted, or untreated condition, and immersed in a fixative solution containing 2% glutaraldehyde and 1% formaldehyde (freshly prepared from paraformaldehyde) in a 0.1 M sodium phosphate buffer, pH 7.4.

In order to obtain a relaxed and dilated vessel segment, a facial vein was exposed, and an intravenous needle was introduced near the distal origin of the vessel. The vein was perfused in situ for 1 minute with a Krebs bicarbonate solution which contained 5 × 10⁻² M sodium nitrite. The temperature of the solution was approximately 25°C. This was followed by perfusion with a fixative, which also contained sodium nitrite (5 × 10⁻² M). Vein tissue was then removed and immersed in the routine fixative.

To obtain a contracted vessel, a ring was removed from the vein and placed in Krebs bicarbonate solution which was gassed with 95% O₂ and 5% CO₂. The solution contained propranolol (10⁻⁶ M) to block β-receptors and norepinephrine (10⁻⁵ M) to induce a contraction. The contracted vessel segment was then immersed in the routine fixative.

To obtain untreated, control tissue, vein segments or strips were removed and immersed immediately in the routine fixative. All tissues were fixed for approximately 3 hours, followed by a 1- to 2-hour post-fixation in 2% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 7.4.

In order to characterize the innervation of the facial vein, small strips of one vessel were fixed by the chromaffin method for the demonstration of adrenergic neurotransmitter vesicles (Tranzer and Richards, 1976). This method involves an initial 15-minute fixation in a cold solution containing 1% glutaraldehyde and 0.4% formaldehyde in a sodium chromate and potassium dichromate buffer (0.1 M), pH 7.2. This is followed by overnight storage in sodium chromate and potassium dichromate buffer (0.2 M) at pH 6.0, and a subsequent 1-hour post-fixation in 2% osmium tetroxide in sodium chromate and potassium dichromate buffer (0.1 M), pH 7.2.

All tissues were block-stained for 1 hour in 0.5% uranyl acetate in 75% ethanol, and then were dehydrated and embedded in Araldite. Longitudinal and transverse sections were stained with lead citrate, and were examined and photographed in an Hitachi HU 11A electron microscope.

Dimensions such as the width of neuromuscular clefts were determined by electron micrographs. The average distance from the part of the surface of any axon varicosity which was not covered by a Schwann cell to the nearest smooth muscle cell surface was regarded as a neuromuscular cleft. The fraction of the total smooth muscle cell surface represented by surface vesicles (or caveolae) was estimated in 15 randomly selected electron micrographs of transversely sectioned smooth muscle cells in relaxed, contracted, and untreated vessels. A transparent plastic sheet with a test pattern of parallel lines was placed over each micrograph, and the number of intersections of the lines with the plasma membrane in surface vesicles and with the plasma membrane over the remainder of the cell surface were counted separately. After an initial count, each micrograph was turned 90 degrees and a second count was made.

Diameter and wall thickness of contracted, dilated, and untreated facial veins were measured on transverse sections of vessels which were obtained as described previously. These measurements were made with the aid of a Tektronix 4052 computer graphic system. The fractional volume of smooth muscle in the tunica media was determined by a manual point-counting method (Weibel, 1973), using a test grid mounted in a microscope eyepiece.

Results

The tunica media of the buccal segment of the rabbit facial vein was found to consist of smooth muscle cells with an approximately circular orientation and relatively large bundles of collagen (Fig. 1). Smooth muscle cells represented approximately 62 ± 2% (mean ± 1 SD) of the total volume of the tunica media in all facial veins examined. Longitudinally oriented elastic fibers, profiles of fibroblasts, and profiles of unmyelinated autonomic axons and their accompanying Schwann cells were observed throughout the full thickness of the media. Axon varicosities were sometimes found near the endothelium (Rowan et al., in preparation). Profiles of axon varicosities, which are enlarged axon segments containing neurotransmitter vesicles, were usually partially surrounded by Schwann cells, although varicosities without a Schwann cell covering were sometimes observed. Schwann cells, when present, typically covered the surface of the varicosity that faced away from the nearest smooth muscle cell, while a bare area of the varicosity surface was directed toward that smooth muscle cell. Varicosities throughout the vessel wall contained small neurotransmitter vesicles, 40-60 nm in diameter, and large vesicles approximately 60-120 nm in diameter. Large vesicles represented a minority of the total vesicle population. After fixation by the chromaffin method, two different types of varicosity could be distinguished. The one seen most frequently contained small and large vesicles with electron-dense cores (Fig. 2). The second type contained small clear vesicles without dense cores, as well as large vesicles with a central density similar to that seen in large vesicles in routinely fixed tissue.
A transverse section of the buccal segment of an untreated, control facial vein. Part of the lumen is visible at the lower left. The vessel wall contains smooth muscle (SM), which is generally circumferential, and large bundles of collagen (C). Autonomic nerves (N) and fibroblasts (F) can be seen throughout the wall. The surfaces of smooth muscle cells may be closely opposed (arrows).

Figure 4 came from measurements made in vessels fixed in the routine phosphate-buffered aldehyde fixative. An extensive survey of cleft widths was not made in the tissue fixed by the chromaffin method, but clefts associated with each of the two types of varicosity appeared to be of similar width. Clefts associated with five varicosities of the type that contained small clear vesicles ranged from 80 to 300 nm in width.

There were differences in the appearance of the...
walls of the vessels in each of the three different experimental conditions. The tunica media of the contracted vessel was approximately 130 μm thick, and the outlines of the endothelium and smooth muscle cells were folded and corrugated. The tunica media of the dilated vessel was 20 μm thick, and appeared stretched, and the smooth muscle cells had smoother surface contours. The walls of the untreated vessels, including surface contours of smooth muscle cells, were closer in appearance to that of the dilated vessel. The tunica media was approximately 40 μm thick. The lumen diameter of the dilated vessel was approximately 1 mm, and that of the contracted vessel about 0.25 mm.

Smooth muscle cells in the facial vein had the variety of organelles which are common to vascular smooth muscle in general (Devine, 1978). No specialization of the plasma membranes of smooth muscle cells within the neuromuscular cleft were found. Surfaces of adjacent smooth muscle cells were sometimes found in close apposition, separated by a gap of approximately 15 nm (Figs. 1 and

**Figure 2.** An axon varicosity (V), apparently adrenergic in nature, in a facial vein fixed by the chromaffin method. The neurotransmitter vesicles contain electron-dense reaction products (arrows). The cleft between the varicosity and the smooth muscle cell (SM) is about 100 nm.
FIGURE 3  An axon varicosity (V), probably non-adrenergic in nature, in a facial vein fixed by the chromaffin method. The neurotransmitter vesicles (arrows) do not contain electron-dense material. The neuromuscular cleft here averaged 150 nm.

6). The membranes in these areas were occupied either by dense bodies or by areas of the membrane rich in surface vesicles. No typical gap junctions were found. Surface vesicles were numerous in regions of the plasma membrane not occupied by dense bodies (Fig. 6). These vesicles were usually oval in profile, measuring 100 to 150 nm by 60 to 100 nm. They appeared to represent 47 ± 3% of the total smooth muscle cell surface area.

Cisternae of the sarcoplasmic reticulum were seen near the surfaces of smooth muscle cells, often in association with surface vesicles (Fig. 6). There were no accumulations or stacks of cisternae deeper in the cytoplasm. This pattern of distribution was seen consistently in all smooth muscle cells in seven veins, including dilated, contracted, and untreated vessels.

Discussion

The ultrastructure of the autonomic nerves in the tunica media of the rabbit facial vein and their
The measurements of cleft width reported here appear to indicate that the cleft in the facial vein is relatively narrow in comparison to clefts in other vessels (Bevan, 1977); these measurements, however, may be an overestimate. The true mean cleft width may be even narrower than the values indicated in Figure 4. Variations in the orientation of the plane of the tissue section with respect to the general plane of the cleft may result in some degree of error in measurements made on electron micrographs. In a hypothetical, idealized cleft, in which the surfaces of the axon varicosity and the smooth muscle cell are assumed to be parallel over a large area, the apparent width of the cleft on an electron micrograph can be determined for various angles at which a section 80 nm thick may pass through the cleft (Fig. 7). A number of such determinations can be made for hypothetical clefts of different widths, and the relationship between section angle and the apparent cleft width can be demonstrated (Fig. 8). In general, when the plane of section is closer to 90 degrees from the approximate plane of the cleft, there may be an apparent false narrowing, but when the plane of the section approaches the plane of the cleft, there may be a false widening effect. For clefts of approximately 150 nm or wider, the false narrowing effect is relatively small, whereas the false apparent widening may be large. False widening is most significant in the wider clefts. For 100-nm clefts, nearly all section angles will produce a false narrowing effect. Clefts 75 nm or less in true width can only appear narrower, and may even be falsely obliterated.

If the assumption is made that the microtome knife may encounter neuromuscular clefts in the facial vein at all possible angles, and that sections are evenly distributed over the full range of possible angles, then there would be a tendency for the mean apparent width to be greater than the true width for clefts of approximately 150 nm or more. This effect would be offset to some degree by the narrowing effect in clefts of approximately 100 nm. There are probably few clefts narrower than 75 nm in the facial vein, since clefts narrower than 40 nm in apparent width or falsely obliterated clefts were not found. One potential drawback with this approach in interpretation is that sections may not be evenly distributed over the full range of possible angles. If the membranes of varicosities and smooth muscle cells are nearly parallel to each other, then longitudinal sections of relaxed and dilated vessels, which include transverse profiles of relatively smooth-surface smooth muscle cells, might be expected to yield the most accurate results, with the least false widening. This study has not provided
FIGURE 5  Two relatively narrow neuromuscular clefts. Cleft number 1 is about 75 nm in minimum width, and cleft number 2 is about 50 nm. The axon varicosity profiles (v) are filled with neurotransmitter vesicles, mostly of the small type which averages 50 nm in diameter.

enough information to answer this question, although all clefts of 380 nm or more in apparent width in dilated and untreated vessels were observed only in transverse sections. There was no apparent difference in cleft width distribution between longitudinal and transverse sections of contracted vessels. Because the cleft width values displayed in Figure 4 came from both longitudinal and transverse sections, the most likely conclusion seems to be that the false widening effect would be more significant than the false narrowing effect, so that the mean values are an overestimate.

Variations in surface contours of contracted smooth muscle cells, which are more irregular and corrugated than in relaxed and stretched smooth muscle, might be expected to increase the probabili-
Transversely sectioned smooth muscle cells in a longitudinal section of dilated vein. There are numerous surface vesicles (SV), and some cisternae of the smooth (SSR) and rough (RSR) sarcoplasmic reticulum are visible. There is an area of close apposition of the surfaces of two smooth muscle cells (A) with a gap of about 15 nm.

The false widening effect might be the dominant one in both longitudinal and transverse sections, so that the mean cleft width of 390 nm for the contracted facial vein may be an even greater overestimate than the mean values for the dilated and untreated vessels. The cleft width measurements made in the contracted vessel might possibly be exaggerated due to the absence of a resisting force during the contraction in vitro. The degree of contraction might not be as extreme in vivo, since the vessel would be contracting against the pressure of the blood in the lumen. If much or all of the increase in apparent mean width in the contracted vessel is false, then the true mean cleft width probably remains nearly constant despite changes in vessel tone and diameter. This conclusion contrasts with the report that neuromuscular separation distance...
FIGURE 7 This diagram indicates the effect of variation in the angle of the section through an ideal, hypothetical cleft, in which the cell surfaces are parallel. The apparent width of the cleft projected from a section at 70 degrees is slightly narrower than the true width, but the apparent width at 20 degrees is significantly wider than the true width.

in the rabbit ear artery, in which the innervating adrenergic plexus is restricted to the inner adventitia, may vary significantly with changes in vessel diameter (Govyrin, 1976); the latter study, however, appears to include all adventitial axons rather than just varicosities, and so it is difficult to interpret. On the basis of the results presented here for the facial vein, cleft widths, at least in vessels with an intra-medial nerve plexus, should probably be measured in longitudinal sections of dilated vessels with circular smooth muscle.

Because the angles at which clefts were cut in this study are not known, and because the plasma membranes of varicosities and smooth muscle cells which form cleft boundaries are not always parallel over their entire surfaces (Khor’kov, 1978), more accurate determinations of cleft width in the facial vein would require three-dimensional reconstruction from serial sections, or the use of stereo tilt pairs and parallax measurements. Nevertheless, the approximate range of cleft widths may be estimated. If sections are actually distributed randomly over the full range of possible angles, the range of widths observed in this study is consistent with that which might result if the majority of clefts were actually in the approximate range of 100 to 300 nm. Using the data in Figure 8, a mean apparent width can be calculated for the hypothetical clefts in this range. If the assumption is made that clefts of 150 to 200 nm are most numerous, then a mean value of apparent widths at intervals of 5 degrees can be determined for one cleft at each of 100, 250, 300, and 350 nm, and two clefts each at 150 and 200 nm. The mean in this case is approximately 280 nm, which is similar to the means for dilated and untreated facial veins. The most likely conclusion seems to be that most clefts in this vessel are from 100 to 300 nm wide, and the true mean width lies between 150 and 200 nm.

The correlations of cleft width measurements may provide a more nearly accurate estimation of the true range of cleft widths, but whether corrected or not, the measurements indicate that neuromuscular clefts in the rabbit facial vein are relatively narrow among blood vessels. The widths reported here are similar to those that have been reported to occur in arterioles or small arteries (Lever et al., 1965; Devine and Simpson, 1967; Bell, 1969; Beacham et al., 1976; Forbes et al., 1977). Compared to vessels with wide clefts, these narrow clefts are associated with a greater change in the size of the response to changes in nerve stimulation frequency, a larger maximum neurogenic response in comparison to the maximum response of which the vessel is capable, and a greater dependence on neuronal
re-uptake of neurotransmitter as a means of terminating the neurogenic response (Bevan, 1979). Blockade of neuronal re-uptake of norepinephrine in the facial vein causes a significant potentiation of the relaxation response, and there is functional evidence that β-adrenergic receptors have a close spatial relationship with axon varicosities (Winquist and Bevan, 1981). Cleft widths have been measured in other veins having an intra-medial nerve plexus. These include the dog lateral saphenous vein, in which most clefts appear to be 100-300 nm wide, with a few as narrow as 20 nm (Coimbra et al., 1974), and human omental veins, in which most clefts appear to be 100-500 nm wide, with a few as narrow as 30 nm (Thureson-Klein et al., 1976). The increased sensitivity and size of the neurogenic response of blood vessels which have an intra-medial nerve plexus may be due for the most part to the presence of narrow neuromuscular clefts, rather than to an intra-medial accumulation of neurotransmitter.
mitter released from varicosities not associated closely with smooth muscle cells.

Some of the ultrastructural features of the smooth muscle cells which were observed in this study might be related to certain functional properties. Sarcoplasmic reticulum is one of the most likely locations for intracellular storage of activator calcium in smooth muscle, and the ability of a vessel to maintain contraction in the absence of extracellular calcium may be directly proportional to the amount and distribution of this organelle (Devine et al., 1972). The apparently limited amount and distribution of sarcoplasmic reticulum in the rabbit facial vein resembles more closely the pattern in vessels which are more dependent on extracellular calcium. Besides being a possible source of activator calcium, the sarcoplasmic reticulum may have an active role in relaxation, which might involve uptake of free calcium from the cytoplasm; however, the relaxation response of the facial vein would not necessarily require a large volume of sarcoplasmic reticulum if, as has been suggested, the effect of β-receptor activation is to inhibit the influx of extracellular calcium (Collis and Shepherd, 1979).

The level of tone of the facial vein is probably proportional to the net influx of calcium through the plasma membrane of the smooth muscle cells. There does not appear to be any unusual feature associated with cell surfaces which might be related to calcium movements in the facial vein or to their temperature sensitivity. The surface vesicles may be larger, but their contribution to the total cell surface area seems to be similar to the values which have been reported for other smooth muscle, including 45% for the rabbit pulmonary artery (Verity and Bevan, 1966) and 42% for guinea pig taenia coli (Gabella, 1976). Dimensions of surface vesicles are not affected by contraction or relaxation (Gabella and Blundell, 1978). More measurements in different types of smooth muscle, including surface-to-volume ratios, may indicate whether or not there is a relationship between such structural features and calcium movements.

The function of the numerous regions of close apposition between smooth muscle cells, with intervening gaps of approximately 15 nm, is unknown. As discussed by Somlyo (1980), they could conceivably represent points of electrical coupling between cells. In the facial vein, there is some evidence to suggest that smooth muscle cells might be coupled, including the observation of rhythmic, coordinated contractions in vitro during exposure to ouabain or potassium-free solutions (J. Prehn, personal communication), as well as the rapidity of the first phase of the biphasic response to nerve stimulation (Pegram et al., 1976; Bevan, 1979). The relationship between the membrane appositions and cellular coupling is still hypothetical, and the assumption cannot be made that the appositions seen in electron micrographs accurately reflect the cellular relationship in vivo. If such junctions do exist in vivo, the gap may be somewhat greater than 15 nm on the average, but they can be assumed to be relatively narrow because they often appear falsely obliterated due to variation in the plane of section.

In summary, the neuromuscular cleft in the rabbit facial vein is relatively narrow in comparison with other blood vessels, and cleft dimensions probably remain fairly constant during changes in the diameter of the vessel. The functional implications of structural aspects of the neuromuscular relationship are becoming more firmly established, but more work will be required in order to understand fully the role of smooth muscle structure in calcium movements and electrical phenomena.

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