PERIODIC, transversely oriented folds, as well as vesicular invaginations called caveolae, are two prominent structural features that amplify the membrane area of the plasmalemma in mammalian ventricular heart muscle (Rayns et al., 1968; Gabella, 1978). The existence of plasmalemmal folds led us to inquire whether they affect the resting tension of heart muscle over the physiological range of sarcomere lengths. Caveolae are present in both the external plasmalemmal envelope and T-tubular plasmalemma. They show no preferential distribution with respect to underlying myofibrillar striations or membrane folds and are nearly devoid of membrane particles in freeze-fractured material. The surface density of caveolar necks (4.0/μm² apparent plasmalemmal area) is only 16-20% of that reported for frog skeletal muscle. Caveolae augment plasmalemmal area by 21-32%, assuming two or three caveolae per neck, respectively. Caveolar membrane does not serve as a reservoir of membrane to be recruited into external plasmalemma, at least over the physiological range of sarcomere lengths. In heart muscle they do not account for the T-tubular access resistance, and their function in this tissue remains unknown. Circ Res 46: 244-255, 1980

The existence of caveolae raises questions about the extent to which these structures contribute to plasmalemmal membrane area and, in particular, to specific membrane capacity, specific ion conductances, and ion fluxes per unit of membrane area. In this paper we have investigated these problems by means of measurements made on freeze-fractured membrane replicas and transmission electron micrographs of rabbit right ventricular papillary muscle. Our measurements were prompted by an extensive study of plasmalemmal folds and caveolae in frog skeletal muscle recently published by Dulhunty and Franzini-Armstrong (1975).

Portions of this work have previously appeared in abstract form (Levin and Page, 1977).

Methods

Preparative Methods for Transmission Electron Microscopy and Freeze Fracture

Experiments were performed on New Zealand White female rabbits (body weight 3.0-4.5 kg). After
injection with heparin sodium (~300 USP units/kg), the animals were anesthetized by stunning. Their hearts were excised and perfused at 37°C through the coronary circulation on a Langendorff cannula. The first (control) perfusing solution was a salt solution whose composition (in mmol/liter) was: KCl, 5.93; NaCl, 153.4; CaCl2 • 2H2O, 1.4; MgCl2 • 6H2O, 0.56; Tris maleate, 2; acetyl glycinate, 2. This solution was adjusted to pH 7.2 by adding NaOH and was saturated with 100% oxygen (Hutter and Warner, 1967; Page and Page, 1968). After 5 minutes, the perfusing solution was changed to a 3% glutaraldehyde fixative solution containing in mmol/liter) NaCl, 91; KCl, 5.93; CaCl2 • 2H2O, 1.40; MgCl2 • 6H2O, 0.56; and Na cacodylate-HCl 50 (pH 7.2–7.4). The osmolality of the fixative solution without glutaraldehyde was 300 mosmol/kg water.

The fixative was forced through the coronary vessels with a syringe for 20 minutes. The heart then was removed from the cannula; the right ventricular papillary muscles were excised and cut into small pieces, which were immersed in the same fixative for an additional 150 minutes at room temperature. The tissue was rinsed four or more times in cacodylate buffer at 24°C.

In eight hearts, the fixation procedure was modified slightly to stretch the right ventricular papillary muscles in situ before introducing the fixative. Stretching was accomplished by tying off all ventricular outlets and dilating the ventricle with control perfusing solution. In four of these eight experiments, additional stretching was attempted by omitting calcium from the perfusing and fixative solutions.

In selected experiments, glycerol was added to the perfusing solution to dilute the caveolae (Dulhunty and Franzini-Armstrong, 1975). For the purpose of these experiments, the usual 5-minute preperfusion with the control physiological salt solution was followed by a secondary 15-minute perfusion with a solution having the same composition as that of the control except for the addition of the 1000 mm of glycerol. After this secondary perfusion, the heart was reperfused for 10 minutes with the glycerol-free control solution, then perfused as usual with the glutaraldehyde fixative.

In one experiment the extracellular space was occupied by the addition of 8% (by weight) tannic acid to the usual 3% glutaraldehyde-cacodylate fixative (Bonilla, 1977), the pH being adjusted to ~7.2 with NaOH.

Muscles to be processed for transmission electron microscopy (TEM) were postfixed for 90 minutes in a solution containing 32.7 mmol/liter OsO4 in the same cacodylate buffer used throughout (total fixative osmolality 322 mOsmol/kg H2O, pH 7.2). The tissue was then rinsed, dehydrated, embedded in Epon, sectioned, stained with uranyl acetate and lead citrate, and photographed in a Siemens Elmiskop 1A electron microscope as previously described (Stewart and Page, 1978).

For freeze fracture, muscles were equilibrated for 8–12 hours at room temperature with 5, 10, 15, 20, 25, and 30% glycerol solutions in cacodylate buffer. The muscles were rapidly frozen on a liquid-solid Freon interface cooled by liquid nitrogen. They were then transferred either to a Denton model DFE-3 freeze fracture apparatus modified with a Fullam platinum-carbon electrode (as suggested to us by Dr. C. Peracchia) or to a Balzer's model BAF 301 freeze fracture apparatus operated with two electron guns. The tissue was fractured and replicated with platinum-carbon. Freeze fracture was carried out without etching at −110°C under a vacuum of 3 × 10−7 to 5 × 10−7 torr. The replica was cleaned in a Na hypochlorite solution, washed in distilled water, and mounted on a 300 or 400 mesh grid.

Quantitative Analysis of the Replica

All technically satisfactory plasmalemmal areas were photographed at a magnification of 10X and printed to give a final magnification of 30 × 10X. For the quantitative analysis, two samples of 10 prints each or one sample of 20 prints were obtained from different areas of each replica. Both the EF (extracellular) and PF (protoplasmic) fracture faces of the membrane replica were used.

Caveolae usually are not seen in freeze fracture, their presence being inferred from the finding of caveolar necks (Dulhunty and Franzini-Armstrong, 1975). To express the apparent surface density of caveolae necks, the membrane was treated as if it were a flat surface; that is, the contribution of folds to the plasmalemmal membrane area was not taken into account. Since the true membrane area could not be measured, the results were expressed as an "apparent surface density" and referred to as an "apparent membrane area." Apparent membrane area was measured by point counting with a square grid (1.0 cm/side) imprinted on a transparent plastic sheet superimposed on a print of the electron micrograph (Page et al., 1971; Stewart and Page, 1978). Openings of T-tubules were counted and recorded separately with a grid 0.623 cm on a side. Areas of the print in which the replica was technically unsatisfactory for visualizing caveolae were likewise separately counted with the finer square grid (0.623 cm/side). The apparent surface density of caveolar necks (number of caveolar necks per unit of apparent membrane area) was derived by dividing the total number of caveolar necks in all the prints of the sample by the total apparent membrane area.

The estimated area of Caveolar membrane per unit apparent membrane area was calculated by assuming a value for the average number of caveolar necks per caveolar neck cell. This assumed value was multiplied by \( \Pi(d')^2 \), in which \( d' \) is the mean equivalent profile diameter of caveolae as determined by TEM with the Zeiss particle size analyzer.

Sarcomere lengths were determined in each print by measuring the distance between T-tubules or between successive Z-lines, whichever was more
Measurements on Transmission Electron Micrographs

For the morphometric analysis, the block of embedded tissue was oriented on an ultramicrotome in a direction parallel to the longitudinal axis of the papillary muscle. For thin sectioning, the block was then turned and reoriented at a 45° angle with respect to the longitudinal axis.

Technically satisfactory areas were identified at low magnification in the electron microscope. The entire trace of the external plasmalemmal envelope of selected cells was photographed at an original magnification of about 20 x 10^3 X. Thirty micrographs at a final magnification of 60 x 10^3 X were analyzed for each rabbit. On each print, the outline (trace) of the plasmalemma was traced onto tracing paper. The mean orientation of this roughly linear trace was determined and used to draw an orientation axis parallel to the trace. The tracing paper was removed from the print and overlaid with a square grid (0.319 cm per side). One axis of this grid was oriented parallel to the orientation axis on the tracing paper and hence to the mean direction of the plasmalemmal trace. The number of intersections of the plasmalemmal trace with the grid coordinates were counted. Intersections with grid lines parallel and perpendicular to the orientation line (C1 and C") were recorded separately. The length of the trace was calculated from the equation given by Underwood (1970) for the total length per unit area of a partially oriented system of lines in a plane, L = (a/M) (C" + 0.571C1), in which L is the length of the membrane trace, M is the magnification of the print, C" and C1 are the grid spacings and a is the grid spacing of the square grid.

All caveolar outlines ("profiles") on the prints used to determine the length of the plasmalemmal trace were also traced onto tracing paper. When multiple caveolae appeared to attach to each other by a common neck, each caveola was traced as a separate structure. The equivalent diameter, d', of the caveolar profiles was then determined with a Zeiss model TGZ3 particle size analyzer. This instrument measures the diameter of a circle equivalent in area to the area of the not exactly circular caveolar profile. From this equivalent diameter the equivalent circumference of the profile was calculated. The equivalent circumference of the profile is an estimate of the equatorial circumference (see Results). The increase in the surface area of the external plasmalemma due to caveolar membrane was calculated from the ratio: (total length of noncaveolar plasmalemma + total length of caveolar plasmalemma)/(total length of noncaveolar plasmalemma), in which total length of caveolar plasmalemma refers to the sum of the equivalent circumferences of caveolar profiles calculated from all of the measured values of d'.

Procedures similar to those applied to the external plasmalemma were used to obtain the caveolar contribution to the plasmalemmal area in the T-tubules. However, for T-tubular measurements, the muscle cells were sectioned parallel to their longitudinal axis rather than at a 45° angle. Only those tubules exhibiting large areas of membrane were photographed. In the tracings, the T-tubular outlines appeared as elongated or oblong structures whose long dimension was chosen as the orientation axis for the counting grid. In the T-tubular measurements, tangent (blurred) membrane traces were excluded because our previous experience in analysis of the external plasmalemma had shown that exclusion of tangent membrane segments did not affect the measurement. Intersection counts on areas of T-tubular plasmalemma forming diadic junctional contacts with terminal cisterns of the sarcoplasmic reticulum were recorded separately. The fraction of T-tubular plasmalemmal area involved in such junctions was calculated as described by Page and Surdyk-Droske (1979).

Results

Morphology of Caveolae and Necks

Figure 1A displays a "typical" caveola, approximately 90 nm in diameter. The caveola invaginates from the plasmalemma and opens to the extracellular space via a narrow neck. The lumen is filled with a dense substance which is continuous with and indistinguishable from the basement membrane covering the external surface of the plasmalemma. Caveolae frequently appear in clusters with more than one caveola per neck. Figure 1B shows such a cluster of three caveolae in a "pinwheel" configuration. The neck, to which all three caveolae are attached, is visible as a small circular profile in the center of the pinwheel. More complex caveolar shapes with as many as four or five caveolae apparently inserting into one neck are also present. Simpler shapes in which one can unequivocally assign only one or two caveolae to a neck (Fig. 1, A and C) are, however, more frequent. In Figure 1C the basement membrane has been opacified by adding tannic acid to the fixative. The caveola in the micrograph appear opaque, indicating that extracellular tannic acid has penetrated into them, presumably through the caveolar neck. Tannic acid also enters the T-tubules, but it does not penetrate the plasmalemma to opacify the intracellular structures. These findings are similar to those reported for other extracellular tracers (Forssmann and Girardier, 1970; Sommer and Waugh, 1976). Figure 2 illustrates the occurrence of caveolae beneath the T-tubular plasmalemma, a location in which caveolae are absent in the frog skeletal muscles studied.
Figure 1  A–C: Transmission electron micrographs of caveolae. Calibration = 0.5 μm. A: Two caveolar profiles of different size. The upper caveola appears in near-tangential section and therefore seems smaller. A narrow neck connects the lumen of the larger caveola with the interstitial space. The basement membrane is seen to follow the plasmalemmal trace into the caveolar lumen. B: Three caveolar profiles in a “pinwheel” configuration around a common neck. C: Opacification of extracellular space with tannic acid (upper dark area) also results in opacification of the interior of the caveolae, indicating that the caveolar lumina are in continuity with the extracellular space. The neck of the left caveola (arrow) lies outside of the section plane.

Figure 2  Longitudinal section showing many caveolae associated with T-tubular plasmalemma. Note that the caveolae do not occur along T-tubular plasmalemma involved in diadic junctions with terminal cisterns of SR. Calibration = 0.5 μm.
Table 1 Location of Plasmalemmal Caveolar Profiles with Respect to Structures Outside the Myocardial Cells

<table>
<thead>
<tr>
<th>Caveolar location</th>
<th>No. of caveolar profiles</th>
<th>% of total caveolae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Facing other myocardial cells</td>
<td>377</td>
<td>58.1</td>
</tr>
<tr>
<td>Facing a capillary</td>
<td>157</td>
<td>24.4</td>
</tr>
<tr>
<td>Facing a pericyte</td>
<td>39</td>
<td>6.1</td>
</tr>
<tr>
<td>Facing a nerve</td>
<td>14</td>
<td>2.2</td>
</tr>
<tr>
<td>Facing interstitial space*</td>
<td>59</td>
<td>9.2</td>
</tr>
<tr>
<td>Total</td>
<td>646</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* As used here, the term specifically denotes interstitial space not containing a cellular structure (all caveolae technically may be said to face the interstitial space).

Table 1 was designed to examine whether the caveolae seen in electron micrographs of thin sections are located preferentially opposite particular cell types or extracellular structures. The tabulation was undertaken because a preferential localization might provide information about caveolar function. However, the data in Table 1 do not support a preferential localization. Plasmalemmal caveolae appear opposite all the structures that the plasmalemma faces, and we could detect no evidence that they occur with especially high or low frequency opposite a particular structure. A definitive conclusion cannot be made without data about the frequency of each tabulated structure in rabbit heart.

Figure 3 is a low magnification survey of a plasmalemmal replica from rabbit right ventricular papillary muscle showing caveolar necks and T-tubular openings on the EF face of the membrane. Figure 4 is a similar survey from rat left ventricular free wall. The underlying sarcomere pattern is readily distinguishable in both figures. The distribution of caveolar necks is not related in any obvious way either to the sarcomere pattern or to the T-tubular stumps. Figure 5 shows the characteristics of caveolar necks in the EF face at high magnification and permits comparison with their appearance on the PF face. Again, no preferential distribution with respect to the T-tubules or the underlying sarcomere pattern is discernible.

Figure 6A shows a fracture through several caveolae which are exceptional in being retained in the fracture face. Both the PF and EF faces of the caveolar membrane are seen to have either no particles or very few particles, an observation previously made by Gabella (1978) for caveolae from rat heart. Necks connecting some of the caveolae with the external plasmalemma can be distinguished by Dulhunty and Franzini-Armstrong (1975).
readily. In Figure 6B, most of the caveolae have been opened by stretching them by treatment with glycerol until they assume what Dulhunty and Franzini-Armstrong (1975) have called the dome configuration. In the figure the caveolae appear concave when viewed from the direction of the shadowing source. By comparison to the high density of particles on the PF face of the external plasmalemma, the PF face of the caveolae again shows only an occasional particle.

Measurements on Replicas of Freeze-Fractured Plasmalemma

Plasmalemmal folding as a function of sarcomere length: In Figure 7 the percent of apparent plasmalemmal area occupied by T-tubular openings has been plotted against sarcomere length. The apparent surface density of T-tubular openings can be expected to decrease as the membrane unfolds, provided folding does not obscure the openings in such a way as to prevent them from showing up in the replica of the fracture face. Figure 7 confirms this expectation. The surface density of T-tubular openings declines in an approximately linear way ($r = 0.81$) over the entire range of sarcomere lengths examined by us, the slope being $-1.4\%$ per micrometer sarcomere length. Assuming that the decline is entirely attributable to the stretching of folded plasmalemma, this observation suggests that the membrane continues to unfold over the entire observed range of sarcomere lengths from 1.60 to 2.30 μm, and that it remains significantly folded at physiological and greater than physiological sarcomere lengths.

Figures 3 and 4 illustrate the persistence of plasmalemmal folds even at long sarcomere lengths (in this case, 2.34 and 2.44 μm). Like the T-tubules, the folds are at the level of the Z-band of the myofilibrillar striation pattern. In the relaxed and passively stretched muscle shown in these figures, folds appear as double convexities with an intervening concave groove.

Surface density of caveolar necks as a function of sarcomere length: Figure 8 is a graph of the number of caveolar necks per unit of apparent plasmalemmal area over the same range of sarcomere lengths displayed in Figure 7. The points fall into two groups: one group of six observations characterized by low values of the surface density of necks, corresponding to sarcomere lengths ≤1.75 μm (contraction), and a second group of eight observations characterized by a higher surface density of necks, corresponding to sarcomere lengths >1.90 μm (physiological and greater than physiological sar-
comere lengths). Table 2 shows that the means of the two groups differ significantly ($P < 0.005$). Whereas there is some scatter in the second group, the slope of the least squares line through the eight points is effectively zero ($r^2 = 0.0054$).

We assumed that neither the average number of necks per $\mu m^2$ of true plasmalemmal area nor the average number of caveolae per neck varies with the degree of shortening in the range of sarcomere lengths encountered in this study. We therefore concluded that estimation of the contribution of caveolae to plasmalemmal area from freeze-fractured plasmalemma should be based on data obtained at physiological or longer sarcomere lengths. The appropriate figure for caveolar necks/$\mu m^2$ apparent membrane area is therefore about 4.0.

**Morphometric Analysis of Transmission Electron Micrographs**

Table 3 presents the results of measurements made on ultrathin sections of right ventricular papillary muscles from seven rabbits. Although separate records were kept for the clearly delineated external plasmalemma, tangentially sectioned membrane, and T-tubular membrane in continuity with external plasmalemma, Table 3 presents only the results for the sum of the clearly delineated + tangential membranes, since it was found that the amplification in external plasmalemmal area attributable to caveolar membrane was unaffected by the addition of T-tubular or tangential membranes. The plasmalemmal area corrected for caveolar mem-
brane was found to be 1.13 ± 0.01 times that of the plasmalemma without the caveolar correction.

To compare the contributions of caveolae to plasmalemmal area in the external plasmalemmal envelope and T-system, it is necessary to take into account the fact that the surface density of diadic junctional complexes with terminal cisterns of the sarcoplasmic reticulum (SR) is much higher in the T-tubules than in the external plasmalemma (Page and Surdyk-Droske, 1979). Caveolae do not occupy plasmalemmal surface which is involved in diadic junctions with SR. Since diadic junctional complexes occupy 20% of T-tubular plasmalemma and only 3% of external plasmalemma, it seemed appropriate to compare caveolar contributions to plasmalemmal area after subtraction of plasmalemma involved in diadic junctions with SR. When this subtraction is made, the amplification of the corrected surface area is significantly larger in the T-system (1.21X) than in the external plasmalemma (1.13X) (P < 0.01).

Table 3 also gives the mean equivalent diameter, d′, of caveolar profiles as determined from membrane traces with the Zeiss particle size analyzer. Although d′ for T-tubular caveolae is slightly smaller than that for external plasmalemma, the difference is not statistically significant (P < 0.08).

It must be emphasized that the distribution of caveolar profile diameters will not be exactly the same as that of caveolar diameters and that caveolar membrane area will not be exactly the same as that calculated from d′. On the one hand, for spherical caveolae that lie completely within the section, the Holmes effect (Hilliard, 1968) will result in the choice of the largest (i.e., equatorial) diameter. On the other hand, many caveolae that lie partially outside the section will be sectioned nonequatorially, yielding smaller than representative values for d′. In addition, because caveolar profiles usually are more nearly elliptical than circular, the approximation that they can be represented by spheres leads to an underestimation of the areas of their curved surfaces. The overall effect of these various factors may be expected to underestimate significantly the curved surface of the caveola. The magnitude of the error could not be
determined precisely, since the quantities needed for such a determination (Weibel and Paumgartner, 1978) are not available for heart muscle.

The value $d'$ can also be used as follows to obtain a minimal estimate of the number of caveolae per $\mu m^2$ of external plasmalemma. The total area of external plasmalemma in rabbit papillary muscle for animals of the size used in the present experiments is $0.29 \, \mu m^2/\mu m^3$ cell volume (Stewart and Page, 1978). The membrane area of caveolae at the external plasmalemma is therefore $0.0377 \, \mu m^2/\mu m^3$. This area can accommodate $0.0377/[(1.38/\mu m^2)/(1.29/\mu m^3)] = 1.38 \, \mu m^3/\mu m^2$ cell volume. The number of caveolae per $\mu m^2$ external plasmalemma is then given by $(1.38 \, \mu m^3/\mu m^2 \, cell \, volume)/(0.29 + 0.0377)\mu m^2/\mu m^3 \, cell \, volume] = 4.2 \, caveolae/\mu m^2 \, external \, plasmalemma$.

**Discussion**

**Folding of External Plasmalemma**

Although our data do not permit an estimate of the contribution of folds to external plasmalemmal area, the experiments plotted in Figure 7 can be used to determine the upper limit of mean sarcomere length at which the membrane becomes completely unfolded. The fraction of plasmalemmal area contributed by T-tubular openings cannot be less than zero. If the line in Figure 8 is extrapolated to zero percent T-tubular area, the intercept on the abscissa is found to be $2.80 \, \mu m$. This figure is a maximal estimate of what Dulhunty and Franzini-Armstrong have called the critical sarcomere length, i.e., the sarcomere length at which the folds become completely extended. Our extrapolated value of $2.80 \, \mu m$ is similar to that of $3.0 \, \mu m$ measured in frog skeletal muscle by Dulhunty and Franzini-Armstrong.

Evidently, the plasmalemma of heart muscle, like that of skeletal muscle, retains a significant degree of folding even when stretched to sarcomere lengths significantly longer than physiological (Figs. 3 and 4). It follows that heart muscle cells can be stretched passively to sarcomere lengths considerably longer than those at which they normally operate without subjecting the plasmalemma to increased tension. This conclusion suggests that the cardiac plasmalemma does not contribute significantly to resting tension at physiological and supra-physiological sarcomere lengths up to about $2.8 \, \mu m$. Our measurements therefore support the attribution of resting tension to other structures like the myofilaments or the extracellular filament network (Sonnenblick and Skelton, 1974; Fabiato and Fabiato, 1975, 1976; Winegrad, 1974).

**Characteristics of Caveolae in Heart Muscle**

Our experiments indicate that the surface density of caveolar necks in external plasmalemma of rabbit right ventricular myocardial cells is only about 16-20% of that reported for frog sartorius and semitendinosus at a sarcomere length of $2.0 \, \mu m$ (Dulhunty and Franzini-Armstrong, 1975). Moreover, unlike the caveolae and caveolar necks of frog skeletal muscles, those of rabbit papillary muscle showed no
PLASMALEMMAL FOLDS AND CAVEOLAE IN CARDIAC CELLS/Levin and Page 253

TABLE 2 TEM of Plasmalemmal Caveolae

<table>
<thead>
<tr>
<th></th>
<th>External plasmalemma</th>
<th>T-tubules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of membrane trace (nm × 10⁻³)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Noncaveolar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>External plasmalemma + tangent</td>
<td>1954*</td>
<td>—</td>
</tr>
<tr>
<td>T-tubules</td>
<td>—</td>
<td>560**</td>
</tr>
<tr>
<td>Caveolar</td>
<td></td>
<td></td>
</tr>
<tr>
<td>External plasmalemma + tangent</td>
<td>239.6*</td>
<td>—</td>
</tr>
<tr>
<td>T-tubules</td>
<td>—</td>
<td>103.2**</td>
</tr>
<tr>
<td>Area of caveolar + noncaveolar membrane</td>
<td>1.13 ± 0.01</td>
<td>1.18 ± 0.02</td>
</tr>
<tr>
<td>Area of noncaveolar membrane</td>
<td>(7)</td>
<td>(5)</td>
</tr>
<tr>
<td>Fraction of noncaveolar membrane involved in diadic junctions with SR</td>
<td>0.033 ± 0.005***</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Caveolar + noncaveolar area – diad area</td>
<td>1.13 ± 0.01</td>
<td>1.21 ± 0.03</td>
</tr>
<tr>
<td>noncaveolar area – diad area</td>
<td>(7)</td>
<td>(5)</td>
</tr>
<tr>
<td>Equivalent diameter of caveolar profiles (d') in nanometers</td>
<td>93 ± 1</td>
<td>89.7 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>(7,822)***</td>
<td>(5,325)***</td>
</tr>
</tbody>
</table>

Tangent refers to a membrane trace which is not clearly defined because the membrane is tangent to the plane of section. Data for "T-tubules" were obtained on a separate series of micrographs.
* Summation of data from seven rabbits. ** Summation of data from five rabbits. *** From Page and Surdyk (1979). ** First figure in parentheses refers to number of rabbits, second figure to number of caveolae. Mean ± SEM is averaged over the number of rabbits.

discernible preferential distribution with respect to the Z-lines or to longitudinal folds. Caveolae in heart muscle also differ from those in skeletal muscle, in being present in the plasmalemma of the T-system. Like the caveolae of skeletal muscle described by Dulhunty and Franzini-Armstrong (1975), those of heart muscle have relatively few particles on their PF and EF fracture faces both under control conditions and after transformation into the "dome" configuration by treatment with glycerol.

We have shown that the number of caveolar necks per μm² of apparent surface area is low at extremely short sarcomere lengths. This number is both significantly larger and approximately constant at physiological and longer than physiological sarcomere lengths. These observations are consistent with the interpretation either that at very short sarcomere lengths caveolar necks are partially occluded, or that the extreme degree of plasmalemmal folding interferes with visualization of caveolae by the freeze-fracture method. Alternatively, both of these mechanisms may occur. Since the surface density of T-tubular openings decreases as the muscle is stretched, the constant surface density of caveolar necks at physiological and greater sarcomere lengths was unexpected. The more probable explanation is that folding interferes with shadowing and visualization of caveolae. For example, assume that the distribution and surface density of caveolar necks over this range of sarcomere lengths are identical for folded and unfolded plasmalemma. Stretching would then add to the unfolded plasmalemma an increment in membrane area having the same surface density of caveolar necks; hence the number of necks per unit membrane area would remain approximately constant.

The average number of caveolae per neck was reported to be about two for frog skeletal muscle by Dulhunty and Franzini-Armstrong (1975). If our minimal morphometric estimate of 4.2 caveolae/μm² plasmalemma obtained from transmission electron micrographs is divided by the figure of 4.0 caveolar necks/μm² of apparent membrane area obtained by freeze fracture, the resultant figure of 1.05 is a minimal estimate for the number of caveolae per neck. For reasons discussed in the Results section, d', from which the number of caveolae per

TABLE 3 Measurements on Caveolar Necks of Freeze-Fractured Plasmalemma

<table>
<thead>
<tr>
<th>Total</th>
<th>Total</th>
<th>No. of</th>
<th>Mean</th>
<th>Mean % increase in apparent plasmalemmal area due to caveole</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>apparent plasmalemmal area (μm²)</td>
<td>no. of caveolar necks</td>
<td>unit plasmalemmal area (necks/μm²)</td>
<td>sarcomere length (μm)</td>
</tr>
<tr>
<td>All S.L.</td>
<td>(n = 11)</td>
<td>8255</td>
<td>27,279</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>S.L. ≤ 1.75</td>
<td>(n = 6)</td>
<td>3670</td>
<td>9,465</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>S.L. &gt; 1.90</td>
<td>4585</td>
<td>17,814</td>
<td>4.0 ± 0.2</td>
<td>2.11 ± 0.05</td>
</tr>
</tbody>
</table>

Downloaded from http://circres.ahajournals.org/ by guest on August 30, 2017
μm² membrane area was calculated, seriously underestimates caveolar membrane area, and indeed, Figure 1, B and C, which shows examples of more than one caveola per neck, as well as data in the literature (Gabella, 1978), support the notion that the average number per neck should significantly exceed 1.0. In Table 2 we have therefore calculated the percent increase in apparent plasmalemmal area due to caveolae assuming 1, 2, and 3 caveolae/neck, respectively. An exact determination of the average number of caveolae/neck in heart muscle presumably must await high voltage microscopy of appropriately opacified caveolae.

Implications for Cardiac Cellular Electrophysiology

The present observations suggest that the spread of the action potential into the T-system of mammalian ventricular myocardial cells differs in important respects from that in frog skeletal muscle. On the basis of high voltage electron microscopy of frog skeletal muscle, Peachey and Franzini-Armstrong (1977) have suggested that the interstitial space and the T-tubular lumina in this tissue are connected through the caveolae via narrow necks or tubules leading from the caveolae to the interstitial space. Their structural findings imply that the caveolae and the narrow tubules which link them to the interstitial space may represent the access resistance for the spread of the action potential into the T-system of skeletal muscle.

Our observations indicate that in mammalian ventricular heart muscle the access resistance for the spread of the action potential into the T-system is not localized to the caveolae and their connections to the cell surface. As is well known, the diameter of T-tubules in heart muscle is nearly an order of magnitude greater than in skeletal muscle (Pager, 1971; Page and Pazzard, 1973; Page and Upshaw-Earley, 1977; Stewart and Page, 1978). In the membrane replicas of freeze-fractured cardiac plasmalemma, the T-tubular ostia appear open (Figs. 3 and 5; see also Rayns et al., 1968). Moreover, caveolae are present in T-tubular plasma membrane, an observation which is incompatible with the interpretation that their chief function is to regulate the access resistance of the T-tubules.

If the caveolar necks do not present a significant access resistance to the flow of current into caveolar membrane, the inclusion of caveolar membrane in the usual calculations of plasmalemmal membrane capacitance per unit plasmalemmal membrane area for ventricular muscle (Weidmann, 1970; Sakamoto, 1969) would reduce the calculated specific membrane capacity by 21–32%, assuming two or three caveolae per neck, respectively (Table 2). This is a substantially smaller decrease than that of 70% calculated by Dulhunty and Franzini-Armstrong (1975) for the external plasmalemmal envelope of frog skeletal muscle. However, the skeletal and papillary muscle values are not directly comparable because papillary muscle caveolae are distributed over the entire plasmalemma, including both external plasmalemma and T-system, whereas in frog skeletal muscle, caveolae are confined to the external plasmalemmal envelope.

Unlike specific membrane capacitance, membrane ionic conductances or fluxes per unit area need not necessarily be changed by the inclusion of caveolar membrane area. If some or all of the particles seen in the fracture faces of noncaveolar plasmalemma represent channels for ion flow, the paucity of such particles in caveolar membrane would suggest that ion flow through caveolar plasmalemma is absent or small.

Neither the present experiments nor the literature give any clear indication of caveolar function in heart muscle. Our experiments suggest that, as in skeletal muscle (Dulhunty and Franzini-Armstrong, 1975), caveolae in heart muscle do not normally function as a reservoir of membrane to be recruited when the muscles are stretched. Whether and in what ways the absence of the usual plasmalemmal membrane particles from caveolar plasma membrane reflects different membrane properties cannot be answered until the plasmalemmal particles are identified with particular ion-selective channels or other functions. As discussed elsewhere (Page, 1978), such identifications have not yet been made in heart muscle.

Acknowledgments

We thank Dr. Camillo Peracchia for invaluable advice on the use of the Denton freeze fracture apparatus. We are indebted to Mr. Berve Power for help with the fixation and freeze fracture.

References

Dulhunty AP, Franzini-Armstrong C (1975) The relative contributions of the folds and caveolae to the surface membrane of frog skeletal muscle fibres at different sarcomere lengths. J Physiol (London) 250: 513-539
Fabio A, Fabio F (1975) Dependence of calcium release, tension generation and restoring forces on sarcomere length in skinned muscle cells. J Physiol (London) 249: 469-495
Page E, Pazzard HA (1973) Capacitive, resistive, and syncytial properties of heart muscle. Ultrastructural and physiological
PLASMALEMMAL FOLDS AND CAVEOLAE IN CARDIAC CELLS


Sakamoto Y (1969) Membrane characteristics of the canine papillary muscle fiber. J Gen Physiol 54: 765-781


Underwood EE (1970) Quantitative Stereology. Reading, Addison-Wesley, p 56


Quantitative studies on plasmalemmal folds and caveolae of rabbit ventricular myocardial cells.

K R Levin and E Page

*Circ Res.* 1980;46:244-255
doi: 10.1161/01.RES.46.2.244

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1980 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/46/2/244

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/