THE STRUCTURE OF THE ZONULA OCCLUDENS

A Single Fibril Model Based on Freeze-Fracture

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ABSTRACT

Replicas of freeze-fractured toad urinary bladder and gallbladder were analyzed in an attempt to determine the fracturing properties and structure of the zonula occludens (tight junction). Chalcroft and Bullivant have proposed that the junction has a double set of fibrils with one set associated with each of the adjacent cell membranes. However, the fracturing pattern that is observed might also result from only a single set of fibrils which is shared by the adjacent membranes if fracturing occurred around either side of the fibrils. These two models predict quite different structures at regions of the junction where transitions are made between face A and face B. The relative heights of face A and face B and the shape of the transition from face A to face B do not agree with that expected according to the two fibril model but agree exactly with that expected if only a single set of fibrils existed. Further evidence for the single fibril model is derived from fractures of the mucosa membrane which cross the junction to the membrane of the adjacent cell without deflection. Such fractures reveal a single ridge which appears to be identical to the juxtaluminal fibril of the junction. In addition, small ridges are occasionally found in place of the grooves on face B which, although not consistent with the double fibril model, is expected if the single fibril model were correct. Although alternative explanations might account for these observations, we believe that the simplest and most consistent explanation is that the zonula occludens fractures as would be expected of a single set of fibrils shared by adjacent cells.

INTRODUCTION

The zonula occludens (tight junction) of epithelia, as originally described by Farquhar and Palade (13), is characterized structurally in thin sections as a beltlike region completely encircling the cells in which the membranes of the adjacent cells are so closely apposed that the intercellular space between them is occluded by an apparent fusion of the outer leaflets of the membranes. Although numerous papers recently have emphasized the fact that the zonula occludens is to some extent permeable in that it allows the passage of small solutes between the lumen and intercellular space via a completely extracellular pathway (3, 5, 8, 10-12, 15-17, 23, 33, 34, 36, 37), it is clear, nevertheless, that the zonula occludens constitutes a major permeability barrier.

Since the demonstration by Branton (6) and others (26, 32) that in the freeze-fracture tech-
nique the fracture plane exposes an internal region of the membrane, it has been apparent that the fibrils visualized as ridges and grooves in the region of the zonula occludens also represent internal membrane structures. Using double replica techniques, Chalcroft and Bullivant (7) demonstrated that the ridges and grooves first observed by Kreutziger (21) and Staehelin et al. (31) are complementary and therefore that a single fracture produces both of the fracture faces observed. Chalcroft and Bullivant (7) proposed that two sets of fibrils exist within the junctional region with one set associated with the membrane of each adjacent cell. As described in Fig. 1 a, this model further proposes that the fracture plane always passes around the outer side of the fibril, between the fibril and the outer membrane surface, rather than passing on the juxtaacytoplasmic side of the fibril. Such a model leaves unclear exactly what relationship (if any) the intramembranous fibrils may have to the mechanism whereby the zonula occludens attaches adjacent cells together and functions as a permeability seal.

Analysis of certain fractures which appear to be inconsistent with the two fibril hypothesis have led us to propose in this paper that a single fibril is shared by adjacent cells. This model also differs from the two fibril model (Fig. 1 a) in that it proposes that the fracture plane normally passes on the juxtaacytoplasmic side of the single, shared fibril (F, Fig. 1 b). To the extent that fracturing is believed to occur along hydrophobic regions of relative weakness, this model would suggest that there may be a continuity between the hydrophobic interiors of the adjacent membranes. This fusion of the membranes at those points where fibrils are shared could thus account for the role of the zonula occludens in attaching and sealing together adjacent cells.

Although the observations reported here were made on urinary bladder and gallbladder of the toad, observations have been made with mammalian tissue which suggest that the model developed is applicable to the zonulae occludentes of other tissues and species.

MATERIALS AND METHODS

These studies examined tissue obtained from pithed female toads, Bufo marinus (National Reagents, Inc., Bridgeport, Conn.). Urinary bladders were mounted as sacs tied to tubes as previously described (34). Gallbladders were washed in Ringer's solution, everted, and tied to small tubes. Tissue was bathed in an aerated Ringer's solution consisting of 111 mM NaCl, 3.5 mM KCl, 2.5 mM NaHCO3, and 1 mM CaCl2.

Tissue was fixed by immersion for 15 min in 2.5% glutaraldehyde buffered by 0.1 M sodium cacodylate at pH 7.4. After fixation, tissue was washed and stored in 0.2 M cacodylate buffer. Prior to freezing, tissue was soaked in 25% glycerol in 0.1 M cacodylate. Tissue was frozen in liquid Freon 22 (E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.) cooled by liquid nitrogen and fractured in a Balzers freeze-etch unit BA 360M (Balzers High Vacuum, Liechtenstein) at −104°C, and platinum-carbon replicas were prepared, usually without etching.

Surface replicas were made of toad urinary bladders by using methods similar to those introduced by Smith and Revel (30) for high resolution study of the surfaces of cells attached to coverslips. Bladders were fixed as described above for 1 h and stapled mucosal side up to cardboard discs. After dehydration in alcohol they were transferred to amyl acetate and critical point dried (1) with CO2 in a Sorvall no. 49300 critical point drying system (Ivan Sorvall, Inc., Newtown, Conn.). Platinum-carbon replicas were then made in a Edwards model E12E vacuum-coating unit (Edwards High Vacuum, Inc., Bridgeport, Conn.). Replicas were examined with a Philips 200 electron microscope at 60kV.

OBSERVATIONS

Analysis of Fracture Face Transitions

Replicas of freeze-fractured zonulae occludentes display a characteristic meshwork of interconnecting ridges on the face continuous with the A fracture face or inner membrane face while there are complementary grooves on the B fracture face or outer membrane face. It is commonly observed as demonstrated by Fig. 2 from the epithelium of the toad urinary bladder, that the fracture plane jumps back and forth alternately exposing the ridges of face A or the grooves of face B. The presence of such fractures can be explained equally well by either the double fibril model or the single fibril model but the appearance of the replica in the region of transition from one face to the other will be different depending on whether there are two fibrils or a single fibril.

These models are diagrammatically depicted in Fig. 1 as a cross section through the zonula occludens joining cells C1 and C2 at the region where their cell membranes M1 and M2 come into close apposition. The adjacent fibrils of the double fibril model are designated F1 and F2 while the single, shared fibril of the single fibril model is represented
The fracture plane is depicted by the dark solid line (CP). If it is imagined that during fracture the portion to the left of the solid line is removed and a shadowed replica is made of the remaining right-hand portion, it is apparent how with either model a face A (region A, Fig. 1) with ridges may be found on the same replica adjacent to a face B (region B, Fig. 1) with its grooves but with a short transition zone at the point where the fracture plane changes membranes (T, Fig. 1). Careful analysis of the alternative models suggests that for the double fibril model the level of face B at point H₂ should be elevated above the top of the ridges (H₁, Fig. 1 a) by a distance equal to the height of the ridges (F₂, Fig. 1 a). For reasons of symmetry this prediction does not depend upon the precise location of the fracture plane within the membrane or upon the path followed when changing from one face to the other. However, for the single fibril model the relative heights of the
Characteristic fractures of toad urinary bladder (Fig. 2) and toad gallbladder (Figs. 3 and 4) with ridges on face A, and grooves on face B and frequent transitions between them. The height of the ridges ($H_1$) is level with the height of face B ($H_2$). In about half of the face transitions a small ridge is present (arrowheads). Fig. 2, $\times 104,000$; Fig. 3, $\times 118,000$; Fig. 4, $\times 104,000$.

Comparison of ridge heights ($H_1$, Figs. 2 and 3) with the level of face B ($H_2$, Figs. 2 and 3) in replicas from toad urinary bladder and toad gallbladder epithelium shows that the ridges are essentially level with the height of face B which is inconsistent with the double fibril model but in accord with the single fibril model.

An additional prediction of the double fibril model is that the replica at the point of transition from face A to face B (region T, Fig. 1 a) should have a continuously rising contour. There is no reason to expect a dip in the replica after it has reached the level of face B ($H_2$, Fig. 1 a) if the double fibril model is correct. On the other hand, one can imagine that if only a single fibril exists, the fibril may sufficiently deviate the fracture plane from its normal intramembranous position that after fracturing around the fibril the plane would dip as it returns to its more usual position ($H_2$, Fig. 1 b). Such a situation would appear in replicas as a small ridge in the region of transition (T, Fig. 1 b) projecting above the level of face B ($H_2$, Fig. 1 b) but level with the top of the usual ridges ($H_1$, Fig. 1 b). These small ridges are quite frequently observed at fracture face transitions such as those previously illustrated in Fig. 2, and some (but not all) of the small ridges present are indicated by arrowheads (arrowheads, Fig. 2). This formation is further demonstrated for the gallbladder in Fig. 4 (arrowhead, Fig. 4). It can also be seen that the top of the small ridges at face transitions are level.
FIGURE 5  Model of the zonula occcludens as in Fig. 1 b depicting the two types of fracture face transition (T₁ and T₂) which would be expected if the single fibril model were correct. One type (T₁) has a characteristic small ridge while the other (T₂) has a complementary small groove.

FIGURE 6  Freeze-fracture of toad urinary bladder demonstrating the two types of face transition found. The transition characterized by a groove (T₂) is, in this instance, found immediately adjacent to the transition characterized by a small ridge (T₁). × 104,000.

with the top of the usual face A ridges as predicted by the single fibril model.

Although the double fibril model predicts only one type of structural formation at a face transition, the single fibril model predicts that exactly half of the face transitions should have small ridges (T₁, Fig. 5 a) while the other half should have a complementary grooved appearance (T₂, Fig. 5 b). Such a small groove lying directly adjacent and below a face B is easily obscured by shadows and contamination. However, occasionally replicas are found which appear to be consistent with this second type of transition although other explanations are also possible. In Fig. 6 a transition with a groove (T₂, Fig. 6) is directly adjacent to the more common small ridge transition (T₁, Fig. 6). It can be seen that the ridge of T₁ corresponds closely to the position of the groove of T₂ as would be expected according to the single fibril model. When over 200 face transitions from replicas of the urinary bladder were scored as to T₁ (the presence of a small ridge) or T₂ (presence of small groove or clear absence of small ridge), 43% were found to be T₁ and about 44% were found to be T₂ while 13% could not be classified (Table I). A similar result was found with junctions of the gallbladder (Table I). Therefore, of those that could be classified, the ratio is consistent with the single fibril model.

Analysis of Surface Membrane Fractures

Probably because of its relatively flat surface when stretched, the toad urinary bladder epithelium frequently fractures to reveal quite large areas within the mucosal surface membrane. A fracture face A from such a fracture is illustrated by Fig. 7. The toad bladder has a characteristic surface fold (SF, Fig. 7) immediately adjacent to the junction. It is observed that the fracture plane is usually not deflected in the region of the junction but rather smoothly fractures across from the fold of one cell to the fold of the adjacent cell exposing a central ridge (R, Figs. 7 and 8) which in some regions appears to be rather particulate (Rp, Fig. 7). Although much less frequent than in the urinary bladder, similar fractures are also found in the gallbladder (R, Fig. 9). Such a surface fracture across the junction could be explained by the diagrams of (Fig. 10. If the double fibril model were correct, one would expect that the fracture plane would have to leave the plane of the membrane in order to jump to the other side of the membrane at the top of the usual face A ridges as predicted by the single fibril model.

Table I

| Tissue                  | T₁ | T₂ | Could not be classified |
|-------------------------|----|----|-------------------------|
| Toad urinary bladder    | 97 | 98 | 30                      |
| Toad gallbladder        | 111| 103| 34                      |

* See text and Fig. 5 for description of type T₁ and type T₂ fracture face transitions.
FIGURES 7 and 8  Surface membrane fractures of toad urinary bladder. The surface fold (SF) immediately adjacent to the junction is characteristic of the toad urinary bladder. At the point where the fracture face A passes from one cell to the next there is exposed a single ridge (R) which in some regions appears to be rather particulate (R_p) Fig. 7, × 44,000; Fig. 8, × 88,000.
junction. It might also be expected from the double fibril model that the tops of two fibrils would be exposed by such a fracture (Fig. 10a). According to the single fibril model, however, there is a continuous intramembranous pathway predicted so that a surface fracture might spread from one cell to the next without leaving the membrane (Fig. 10b). In that case, the fracture would expose the surface of only a single ridge (Fig. 10b) which is what is observed (R, Figs. 7, 8, and 9). The shape of this ridge would suggest that the fibril has a more rounded contour on its juxtaluminal side than is indicated by Fig. 10b.

However, alternative explanations are also possible, and therefore it is important to establish that the ridge observed in such surface fractures is truly an intramembranous structure and actually the most juxtaluminal ridge of the zonula occludens. For example, it might be imagined that this ridge represents a surface structure which is located just at the position of the junction but actually external to the hydrophobic plane of the membrane. In order to examine this possibility, surface replicas were made of critical point dried toad urinary bladders. Such preparations show in considerable detail the surface of the membrane including some surface particles (P, Fig. 11) about twice the size of the intramembranous particles although the resolution is not equivalent to that of freeze-fractured material. Nevertheless, there is no clear evidence of the ridge within the cleft of the junctional folds (C, Fig. 11). In addition, a fracture which includes a surface fracture right next to face A of a zonula occludens suggests that the ridge seen in surface fractures (R, Fig. 12) is indeed the same as the juxtaluminal ridge of the junction (R, Fig. 12) although unfortunately there is light platinum shadowing at the point where the two meet so that it is not possible to be completely certain that there is a perfect continuity between the two structures.

This evidence, though not conclusive, supports the proposal that surface fractures crossing the junction do expose the juxtaluminal fibril of the junction, and, therefore, that there is continuity between the intramembranous regions of the adjacent cell membranes and that the zonula occludens has only one set of fibrils which is shared by the adjacent cells.

**Reversed Face Ridges and Grooves**

If the single fibril model is correct, there is an additional fracture which would be expected to occur whose appearance contradicts the double fibril model as proposed by Chalcroft and Bullivant (7). Since the fracture plane can pass on either side of the fibril, the single fibril model predicts that there should be instances where the fracture reverses its course and passes around the other side of the fibril resulting in the occurrence of grooves on face A (Fig. 13a) and ridges on face B (Fig. 13b). Although such structures are not commonly evident perhaps because of shadowing and contamination...
FIGURE 11  Replica of the true outer surface of critical point dried toad urinary bladder. Some particles are seen on the surface which are about twice the size of intramembranous particles (P). There is no evidence within the clefts (C) of the ridge seen in surface freeze-fractures. $\times$ 65,000.

FIGURE 12  Freeze-fracture of toad urinary bladder includes a region of surface membrane fracture with the ridge ($R_s$) directly adjacent and continuous with the juxtaluminal ridge ($R$) of the zonula occludens. $\times$ 88,000.

FIGURE 13  Models depicting the reversed face fracturing pattern which might be expected if the single fibril model were correct. In this case the fracture plane (CP) does not cross over to the membrane of the adjacent cell (cf. Fig. I b) but instead continues around the juxtacytoplasmic side of the fibril with the result that there are grooves on face A (a) and ridges on face B (b).

difficulty, such fractures apparently do exist since some regions of face B are found with small ridges in the position of the usual groove (arrow, Figs. 14 and 15). These ridges, although they are in a slight depression as expected from Figure 13 k, are clearly different from the usual grooves of face B (compare with nearby grooves, Figs. 14 and 15). This structure also differs from the small ridges of the face transition as described in Fig. 5 a in that there is no
change of face evident. From the model as depicted in Fig. 13, one would expect to observe grooves on face A (Fig. 13 a) in numbers equal to that of the reversed face ridges demonstrated in Figs. 14 and 15. We have not yet found a clear example of a groove on a face A but Claude and Goodenough (8) have recently published a very good example of this (their Fig. 7) from Necturus proximal tubule.

Possible explanations for the relatively low frequency of this fracture will be considered further in the discussion. The existence of the ridges on face B, even at a low frequency, supports the concept that there is a single fibril which is exposed by fracturing on either side of it.

Three-Cell Junctions

If, as proposed by the single fibril model, the fibrils of the zonula occludens are shared between the two adjacent cells, it must be considered whether a single fibril is shared by three cells simultaneously at those points in the epithelium where three cells are juxtaposed. Such a simultaneous fusion of three cell membranes may well be structurally impossible as judged by the specialized and extended meshwork of ridges observed at such points (Fig. 16). This ladder-like extension of the meshwork has previously been described (14, 31), but it was not appreciated that the relatively straight central strand which extends basally is actually composed of two fibrils (arrows, Fig. 16) which may become obscured if the fracture plane turns too obliquely. The two central fibrils running almost precisely parallel to each other are seen as two grooves on face B (arrows, Fig. 17). Each of the two fibrils appears to be continuous with the juxtaluminal fibril (arrowheads, Fig. 16) which is shared with one of the two neighboring cells. Thus, it is likely that at the three-cell junctions a cell shares one of the two central fibrils with each of its two neighbors. If so, one might predict the presence of an especially permeable pathway between the regions of membrane fusion found at three-cell junctions. However, the extra distance which these closely apposed central fibrils (arrows, Figs. 16 and 17) characteristically extend in the vertical direction may greatly reduce the importance of this region as a potential pathway for transepithelial fluxes.

DISCUSSION

Although the observations reported here cannot be regarded as totally conclusive, they would appear to cast serious doubt on the validity of the double fibril model as proposed by Chalcroft and Bullivant (7). Detailed analysis of the region of transition from one fracture face to the other has been especially helpful since it is only in this region that the models predict really significant differences in the observed fracture face. Clearly, there are limitations in judging the relative heights of structures from shadowed replicas. Since the contour of the cell membrane is frequently changing, the angle of shadowing varies significantly even between re-
regions close together on the same replica and thus the shadow lengths and apparent size may be deceptive. Nevertheless, since platinum is cast uniformly over a given replica, it is difficult to imagine how the layer of platinum could consistently obscure just at the point of face transition a change in height equal to that of the ridge. Actually, it appears that shadowcasting is remarkably accurate and consistent in detecting differences in contour of immediately adjacent structures. Although neither the relative heights found at face transitions nor the contour of the transition agree with that predicted by the double fibril model, they do agree exactly with that expected according to the single fibril model. In some areas of the meshwork, fibrils are found to run very closely associated with each other (24) and it might be argued that the small ridge-groove association described as transition Ti (Fig. 5) results in some way through a change of face between such closely associated fibrils. Such an explanation is most unlikely since the small ridges at face transitions are found much more frequently than the closely associated fibrils in the tissues we have examined. In addition, the small ridges are found in regions where there are no fibrils closely associated and in regions of the meshwork where fibrils do run closely associated there is no indication that a change of face or appearance of a small ridge is any more likely. Also, it was found that the two types of transition structure predicted by the single fibril model exist in exactly the one-to-one frequency expected.

Further evidence in favor of the single fibril model was found by analysis of membrane fractures which follow the mucosal surface membrane of one cell and cross into the adjacent membrane of the neighboring cell. Such fractures reveal a single ridge at the point of the junction. Although alternative explanations are possible, the most likely explanation for these observations is that this ridge arises from a fracture exposing the top of the juxtaluminal fibril of the junction. Although this might be expected to happen occasionally by chance, the relatively high frequency with which they are found in the toad urinary bladder supports the proposal that there is continuity of the hydrophobic intramembranous regions of adjacent cells at the points where the fibrils of the junction are shared.

An additional observation which is consistent with the presence of a single set of fibrils is the finding of small ridges on face B in positions coinciding with the customary grooves although this observation might also reflect simply exceptional resolution of the groove's structure rather than a fracture on
one would expect to obtain fracture transitions with
fractures can occur on either side of the fibrils, then
picted in Fig. 13. If a double set of fibrils exists but
in the frequency of reversed face fractures as de-
ning has been interpreted within the context of
tial is found in the grooves of face B (35). This
face A ridges have been found to be discontinuous
cludentes from unfixed tissue. In such material the
of ridges on face B has been made on zonulae oc-
2Although the term fibril may unfortunately have
 misleading connotations, we have followed the prac-
tice of earlier workers who have used this term
other workers have referred to these junc-
tional elements as chains (18, 21), strands (8), and
ridges (7, 14). We have reserved the term ridge for
use when referring to the appearance of the fibril
in freeze-fracture replicas.

Actually, this observation may be considered in
view of the single fibril model as simply an increase
in the frequency of reversed face fractures as de-
picted in Fig. 13. If a double set of fibrils exists but
fractures can occur on either side of the fibrils, then
one would expect to obtain fracture transitions with
ridges on the face B side elevated above the ridges
on face A. Unfixed material is currently under in-
vestigation in our laboratory in order to clarify
this point. Preliminary observations are consistent
with those reported by Weinstein et al. (35), but
we have not yet found the face transition which
would be expected if there were a double set of
fibrils.

Several considerations may be important to an
explanation for the low frequency of reversed face
ridges and grooves. One possible factor is that the
angle of the membrane at the point where the frac-
ture plane encounters the fibril may favor frac-
turing toward the apposed cell rather than frac-
turing around the juxtacytoplasmic side of the
fibril. Also, differences in the bonding between the
fibril and components within the membrane may
influence the probability of different fracture path-
ways.

Another possible consideration is that, since
the meshwork of fibrils is interconnected, a reverse
face fracture (Fig. 13) or a face transition frac-
ture (T, Fig. 1 b) would usually require breaking
those bonds which cause portions of the fibrils to
adhere together. Although we have used the term
fibril throughout this paper, this is not meant to
imply anything with regard to the biochemistry
of the junctional elements or to imply that they are
necessarily uniform, continuous strands of ma-
terial.

5 There is some evidence that the ridges may
actually be linear aggregates of particles (14, 31).
To the extent that glutaraldehyde fixation may
cross-link the particles of the fibrils together, this
too might explain some of the differences seen be-
tween fractures of fixed and unfixed tissue. Recent
work indicates that in some tissues fixation can
profundly influence the fracture pathway (9).

Perhaps one of the greatest problems provoked
by the presence of a single, shared fibril is that of
understanding the mechanism whereby such a
structure might arise. At present there is little in-
formation available on the formation of zonulae
occludentes, but important clues may possibly be
found in studies of other systems where fusion of
membranes occurs such as the stacked membranes
of chloroplasts (19, 25) and mucocyst secretion
(29).

Several studies have indicated that the intra-
membranous fibrils themselves are directly corre-
lated with the junction's capacity as a permeability
seal between the lumen and intercellular space
(8, 14, 18). Further evidence for the importance
of the fibrils comes from studies of the toad bladder
epithelium in which it has been demonstrated that
under appropriate osmotic conditions the junction
splits into a series of bubble-like chambers (11, 12,
34). Very recently, it has been established that
these chambers reflect distention of the compart-
ments normally found between fibrils and that the
increased permeability of the junction in such in-
stances (34) is due to the presence of breaks in the
fibrils (Wade and Karnovsky, in preparation). The
single fibril model of the zonula occludens suggests
a very clear structural explanation for the role of
the fibrils in restricting permeability and in cell-to-
cell attachment. However, if the single fibril repres-
sented simply a fusion of the adjacent membranes,
one would expect that the permeability of a junc-
tion composed of even a single fibril could be no
greater than the permeability of the plasma mem-
brane. Yet zonulae occludentes in several tissues
(5, 10, 15, 16, 17) appear to be a much more
permeable site for transepithelial fluxes than the
cell membrane. Clearly the fibrils of the junction
are not simple regions of membrane fusion but
highly specialized structures whose chemical
nature and structural arrangement no doubt plays
an important role in the physiology (4, 8, 14, 20,
17, 27) and pathology (2, 22, 28) of epithelia.

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