LOW RESISTANCE JUNCTIONS IN CRAYFISH

II. Structural Details and Further Evidence for Intercellular Channels by Freeze-Fracture and Negative Staining

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ABSTRACT

Low resistance junctions between axons of crayfish ganglia are studied by freeze-fracture and negative staining. In freeze-fracture, fracture planes that go through a junctional membrane expose two faces, both internal, called face A and face B. Face A belongs to the internal membrane leaflet and faces the gap. Face B belongs to the external membrane leaflet and faces the axoplasm. Face A displays pits, 60-100 Å in diameter, arranged in a hexagonal array with a unit cell of ~200 Å. An ~25 Å bump is frequently seen at the center of each pit. Some pits are occupied by a globule ~125 Å in diameter, which displays a central depression ~25 Å in size. Face B contains globules also arranged in a fairly regular hexagonal pattern. The center-to-center distance between adjacent globules is most frequently ~200 Å; however, occasionally certain globules are seen separated by a distance as short as ~125 Å. The top surface of the globules occasionally displays a starlike profile and seems to contain a central depression ~25 Å in diameter. In negatively stained preparations of membranes from the nerve cord, two types of membranes are seen containing a fairly regular pattern. In one, globules ~95 Å in diameter form a hexagonal close packing with a unit cell of ~95 Å. In the other, globules of the same size are organized in a larger hexagonal array with a unit cell of ~155 Å (swollen arrangement). Some of the globules forming the swollen arrangement are seen containing six subunits. The six subunits form a hexagon which is skewed with respect to the main rows of hexagons in such a way that the subunits lie on rows which make an angle of ~37° with the main rows.

INTRODUCTION

The preceding paper (17) reported several new morphological features of crayfish low resistance junctions studied in positively and negatively stained sections. The membranes of the junctions were seen containing electron-opaque globules which seemed to occupy the entire thickness of each membrane and protrude from both membrane surfaces. The globules were seen arranged in two patterns: a hexagonal one of ~200 Å in periodicity, and a less regular one in which the minimum center-to-center distance between adjacent globules was ~125 Å. Each globule, negatively stained with lanthanum, appeared to be composed of six subunits, arranged in a circle, limiting a central region occupied by lanthanum (possibly a pit).

The present study reports a more detailed description of the structure of the same junctions studied by freeze-fracture and negative staining techniques, offering further morphological evi-
dence for the localization and structure of the presumed intercellular channels as well as suggestions for a certain degree of plasticity in the structure of the junctional membranes.

Several studies have been reported on the structure of vertebrate gap junctions in freeze-fractured preparations (4, 8, 10, 12, 19, 20, 22). In most cases the junctions displayed two fractured faces, each containing a fairly regular hexagonal array of ~100 Å in periodicity. This array was seen composed of particles in the fractured face oriented towards the gap (face A) and pits in the face oriented towards the cytoplasm (face B). In most of the cases, particles and pits were reported to measure ~90 Å in diameter. However, a recent study (12) reported that the diameter of the pits would be 35-40 Å and the diameter of the particles 50 Å. In the same study the appearance of the pitted face was found to vary in relation to the shadowing angle in such a way that clear images of pits (30-40 Å in diameter) would be seen only in areas shadowed at a high angle, while areas shadowed at a low angle would reveal closely packed particles (90-100 Å in diameter), with a cobblestone-like shape.

Few reports have been published on invertebrate gap junctions studied by freeze-fracture. In molluscs the junctions appeared roughly similar to those of vertebrates (5, 7). In insects, on the contrary, the junctions were seen to display peculiar characteristics (6). First of all, the particles appeared on face B and the pits on face A. This is opposite to what is usually seen in other gap junctions. Secondly, the particles were not seen hexagonally arranged but were apparently displayed in rows forming an irregular network.

Recent reports (21, 22) on the freeze-fracture appearance of gap junctions in rat intestinal epithelium suggested the presence of three types of junctions, called types I, II, III, their arrays differing in respect to particle size and unit cell dimension. Type I contains particles 80–90 Å in size with a center-to-center spacing of 90–100 Å. Type II, always found in close apposition with type I, contains particles 100–110 Å in diameter arranged in a hexagonal array with a unit cell of 190–200 Å. Type III contains small square arrays of particles at a center-to-center distance of 60–80 Å. Type I and II junctions closely resemble the close packing and the swollen arrangement, respectively, which I have described in crayfish low resistance junctions prepared by various techniques (15, 16, 17), the only difference being that in crayfish junctions the size of the particles (globules) seems to be the same in both patterns.

The structure of gap junctions has also been studied by negative staining (1, 8, 23). Here, the membrane particles were seen as electron-transparent spots organized in a hexagonal array with a unit cell of ~80 Å (1) or 90–100 Å (8) in liver junctions and 80 Å in junctions of goldfish brain (23). The latter junctions were found to shrink to a unit cell dimension of 60–70 Å as a result of glutaraldehyde fixation.

METHODS

Freeze-Fracture

Crayfish (Cambarus clarkii) were sacrificed by decapitation. A 3% glutaraldehyde or glutaraldehyde-H2O2 (18) solution buffered to pH 7.4 with 0.1 M (Na, K) phosphate was injected into the abdomen. The abdominal nerve cord was dissected out and fixed for 15–20 min in a vial at room temperature. After fixation, the cord was immersed in a 5, 10, 20, 40% series of glycerol solutions at 10-min intervals. The specimens were mounted on aluminum holders, rapidly frozen in liquid Freon 22 at ~−150°C, and transferred to a Denton freeze-fracture device mounted on a Kinney (KSE-2A-M) evaporator equipped with a ballast tank. The shroud and the base of the specimen holder were previously cooled with liquid nitrogen. A vacuum of 1 × 10−6 torr was obtained in less than 5 min after the insertion of the specimens into the bell jar. The specimens were warmed to ~−125°C and fractured with a knife cooled on the shroud. Carbon-platinum and carbon replicas were made at a vacuum ranging from 5 × 10−7 to 3 × 10−8 torr. The specimens were removed from the vacuum and rapidly warmed to room temperature. The tissue was digested in Chlorox and the replicas, washed three times in distilled water, were collected on uncoated 400-mesh grids.

Negative Staining

Unfixed nerve cords were dissected from the animals, placed in 15 ml of 0.001 M NaHCO3 at 4°C, and minced with scissors. The material was transferred to a 15 ml glass homogenizer and homogenized at 4°C with 20 strokes. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1,500 g max for 20 min. The pellet was resuspended and negatively stained with 2% Na-phosphotungstate (PTA) at pH 7.2 on carbon-coated 200-mesh grids. All specimens were examined with an AEI EM 801 electron microscope. The magnification was standardized before each photographic exposure. All the magnifications were previously standardized with a carbon grating replica (#1002,
The black and white arrow in freeze-fracture micrographs indicates the direction of platinum shadowing.

**OBSERVATIONS**

Fracture planes across a ganglion of crayfish abdominal nerve cord frequently expose regions of axon surface membranes in which structures with characteristics similar to those previously described in low resistance junctions can be recognized. In particular, surfaces are seen containing patches of hexagonally arranged pits or particles. Using a conventional terminology (12), the fracture face which faces the gap will be called face A, while the fracture face which faces the cytoplasm will be called face B.

Sometimes the junctions are fractured in such a way that only one of the two membranes is included in the fracture plane (Figs. 1, 2). In these cases, either of the two faces is seen. At other times the fracture plane steps from one to the other joined membrane, thus exposing portions of face A from one membrane and portions of face B from the other (Figs. 3, 4). In the latter case (Figs. 3, 4), in regions containing the typical hexagonal arrangements the two membranes are seen closely joined, as suggested by the presence of a very small step between the membranes, while in other junctional regions, in which the pattern is not visible, the membranes are separated by a larger distance.

On some occasions large regions of axon surface membranes are entirely occupied by either the pitted or the particulated pattern (Fig. 2); on others the pattern is seen in small patches (0.1–0.2 µm in diameter) distributed at random and separated by membranous regions with characteristics similar to those of nonjunctional membranes (Figs. 3, 4).

Face A displays a hexagonal array of circular pits at a center-to-center distance of ~200 Å (Figs. 1, 2, and 4). The pits measure 60–100 Å in diameter and contain in the center an ~25 Å bump (Figs. 2, 3, inset b). Some of the pits are occupied by a globule ~125 Å in diameter, 60–70 Å tall (Fig. 2), which frequently displays a central depression ~25 Å in size (Fig. 2, inset a). The areas between the pits are relatively smooth and are continuous, without a step, with the smooth surface of the perijunctional membrane regions (Figs. 2, 4). Each pit is usually surrounded by five pits, forming images of pentagons, can be seen (Fig. 2, inset b).

Face B of junctional membranes is occupied primarily by globules ~125 Å in diameter, 80–100 Å tall (Figs. 3, 4). The globules are arranged in a distorted hexagonal pattern in which the minimum center-to-center distance between adjacent globules is ~125 Å (Fig. 3, inset a). Certain globules contain a central electron-opaque dot ~25 Å in diameter, probably a depression (Fig. 3, insets). The shape of the globules is usually irregular. However, occasionally their surface seems to be roughly shaped as a star (Fig. 3, insets). The areas between the globules appear smooth and are continuous, without step, with the smooth surface of the perijunctional membrane regions (Figs. 3, 4), similar to what is seen on face A.

Vesicles are frequently seen in axoplasmic regions close to the junctions (Figs. 1, 4). The vesicles appear as hemispherical concave or convex surfaces ~600 Å in diameter. Face A and face B of their membrane correspond to concave and convex surfaces, respectively. Both faces are occasionally seen containing globules ~125 Å in size and, less frequently, pits (Fig. 1, inset a).

Scanning through isolated negatively stained membranes from the nerve cord, occasionally one encounters membranes which display a regular pattern. There are primarily two types of these membranes. One (Fig. 5), usually in fragments 0.1–0.2 µm in diameter, contains a hexagonal array of electron-transparent globules ~95 Å in diameter at a center-to-center distance of ~155 Å (swollen pattern). The other (Fig. 6), in larger fragments, contains a hexagonal array of globules similar to the previous ones as far as shape and size, but closely packed at a center-to-center distance of ~95 Å. The globules of both arrays are frequently seen containing a small central dot ~20 Å in size. Some of the globules which form the swollen pattern are occasionally seen containing six main subunits (Figs. 5, 7). The presence of six subunits is confirmed in experiments in which the rotation method according to Markham et al. (11) is applied. By this method, subunits are clearly seen in a six-step rotation, where they form images of six pointed stars (Fig. 9). On the contrary, in a five- (Fig. 8) or seven- (Fig. 10) step rotation clear images of subunits are not observed.

The six subunits form a hexagon which is skewed with respect to the main rows of hexagons.
FIGURE 1 Freeze-fracture replica of crayfish abdominal ganglion. Several axons and Schwann cells are seen fractured in different planes. Two areas of axon surface membrane face A (AFA) are seen displaying a hexagonal array of pits (at higher magnification in insets b, c). They represent junctional membrane regions. Inset a shows junctional vesicles (v), at higher magnification. Face A (concave) and face B (convex) of the vesicles' membrane are exposed. Bumps are seen on faces A and B. A pit is seen on face B (top vesicle). Ax, axoplasm; Sc, Schwann cell cytoplasm; SFB, Schwann cell surface membrane, face B. × 35,000; inset a, × 127,000; insets b and c, × 100,000.
Figure 4 Freeze-fracture replica of a junction. Three small areas of junction are fractured here in a steplike fashion (arrows). Face B (AFB) of these areas shows only a few globules, at the edge of the membrane, while face A (AFA) displays several pits arranged in hexagonal pattern. Other junctional areas are visible on face A (arrowheads). In the axoplasm several vesicles (v) are seen, appearing as large pits or bumps. Both face A (concave) and face B (convex) of the membrane of the vesicles contain globules ~125 Å in diameter. Ax, axoplasm. X 67,000; insets, X 270,000.

Figure 2 Freeze-fracture replica of a junction (face A). Pits 60–100 Å in size are seen arranged in a hexagonal array with a unit cell of ~200 Å. Some pits display a central bump ~25 Å in size. A few pits are occupied by globules ~125 Å in diameter. The globules contain a central depression ~25 Å in size (inset a). Inset b shows, at high magnification, a region in which a pentagonal arrangement of pits occurs. AFA, axon surface membrane, face A. Ax, axoplasm. X 198,000; insets, X 563,000.

Figure 3 Freeze-fracture replica of a junction. The fracture plane here steps from face B of one axon surface membrane (AFB) to face A of the adjacent axon surface membrane (AFA). Junctional characteristics are seen only on face B. Here, two small areas contain globules arranged in a distorted hexagonal pattern. The center-to-center distance between adjacent globules varies, the minimum being ~125 Å (inset a, arrows). Occasionally the top surfaces of certain globules show points and indentations forming irregular starlike images (inset a, right arrow; inset b, arrow). The top surface of the globules occasionally displays a central electron-opaque region ~25 Å in diameter, probably a depression. (inset a, arrowhead). Ax, axoplasm. X 198,000; insets, 587,000.
in such a way that the subunits lie on rows which make an angle of \( \sim 37° \) with the main rows (Figs. 5, 7, and 9).

The diagram shown in Fig. 11 illustrates schematically an interpretation of the structure of these junctions. Fig. 12 illustrates the pathway followed by the fracture plane in junctions fractured in a steplike fashion.

DISCUSSION

Several peculiar features characterize the freeze-fracture appearance of crayfish low resistance junctions. First of all, the membranes of the junctions are fractured preferentially in an opposite way with respect to junctions of vertebrates and certain other invertebrates (molluscs) (5, 7).

In fact, while in vertebrates and in molluscs the fracture plane cleaves the globules close to their outer surface (that facing the gap), revealing particles in face A and pits on face B, in crayfish the fracture plane runs preferentially on the cytoplasmic side of the globules, revealing primarily pits on face A and bumps on face B. Gap junctions with these characteristics may be typical of arthropods, since junctions with similar features have been recently described in insects. These unusual fracture properties have suggested that these junctions be defined “inverted gap junctions” (6).

The reason for this behavior of the fracture plane in these junctions is not clear, nor is it clear why similar junctions in vertebrates and mol-

**FIGURE 5** Isolated junction, negatively stained with PTA. Globules \( \sim 95 \) Å in size are seen forming a regular hexagonal array with a unit cell of \( \sim 135 \) Å (swollen arrangement). Some globules contain a central electron-opaque dot \( \sim 20 \) Å in size. Certain globules (arrow) are seen composed of six main subunits arranged in a circle around the central electron-opaque dot. \( \times 260,000 \).

**FIGURE 6** Isolated junction, negatively stained with PTA. Globules \( \sim 95 \) Å in size are distinguishable in various regions of this membrane fragment. Here the globules are at a center-to-center distance of \( \sim 95 \) Å (close packing). The inset shows, at the same magnification, a small region of a junction in which the globules are organized in the swollen arrangement. \( \times 158,000 \).

**FIGURES 7-10** Photographic rotation method of Markham (11) applied to a globule seen in Fig. 5 (arrow), which appears composed of six subunits. \( \times 685,000 \).

**FIGURE 7** The six subunits composing the globule lie on a hexagon which is skewed in respect to the main rows of hexagons by an angle of \( \sim 37° \).

**FIGURE 8** A five-step rotation of the globule seen in Fig. 7 produces a blurred image. Subunits are not seen.

**FIGURE 9** A six-step rotation of the globule seen in Fig. 7 clearly shows six subunits resulting in the image of a six-pointed star.

**FIGURE 10** A seven-step rotation of the globule seen in Fig. 7 shows, as in Fig. 8, a blurred image in which the subunits are not distinguishable.

**FIGURE 11** This diagram illustrates, very schematically, an interpretation of the structure of crayfish low resistance junctions. A junction is shown here in its cross-sectioned profile. The two membranes run parallel, separated by a narrow gap (g). Each membrane contains globules (here arranged in the swollen pattern) which occupy the entire thickness of the membrane and protrude from both membrane surfaces (slightly more from the cytoplasmic than from the extracellular surface). Opposite globules of the two membranes appear to come in contact with each other at the center of the junction. The center of each globule is occupied by a presumed intercellular channel. The channel is drawn with dotted lines, since its existence, although further suggested by this study, has not yet been definitely proven. The membrane of the vesicles (v) adjacent to the junction also contains globules which are frequently seen in contact with the globules of the junctional membranes, apparently forming intracellular junctions. C1 and C2 indicate cytoplasm of cell 1 and cell 2, respectively. In the upper right corner the presumed face view of a globule is shown, at least at its extracellular end.

**FIGURE 12** This diagram illustrates the fracture properties of crayfish low resistance junctions. Here the junction is fractured in a steplike fashion. The fracture plane cleaves the globules preferentially at or close to their cytoplasmic end. However, occasionally the globules can be cleaved at or close to their extracellular end. In interglobular regions the membrane is fractured presumably near the center. \( FA \), fracture face A; \( FB \), fracture face B.
luscs fracture in opposite ways. A possible explanation is that the hydrophilic components of the globules in vertebrates and molluscs may interact with the hydrophilic regions of the internal membrane leaflet and with the cytoplasmic matrix more strongly than with the external leaflet and the extracellular components. Thus, at fracturing temperatures (> -100°C) the globules would be anchored more firmly to the cytoplasmic than to the outer membrane leaflet, displaying, as a result of the fracture, particles on face A and pits on face B. In the case of crayfish junctions, on the contrary, the fact that the globules can be cleaved on both sides, although more frequently on the cytoplasmic side, may suggest that they are anchored just about equally to either one of the two membrane leaflets or slightly more strongly to the external one (possibly because of interactions between opposite globules of the two joined membranes).

This unusual feature in the fracture properties of these junctions is particularly helpful in understanding the organization of these membranes, since it allows one to study the appearance of the cytoplasmic side of the globules which usually is not revealed. Moreover, the fact that occasionally the fracture plane cleaves some of the globules on the other side, the one facing the gap, offers the unique opportunity to observe the globules from both sides.

The smooth surface from which the globules protrude appears, in both faces A and B, continuous, without a step, with the smooth surface of the surrounding nonjunctional membranes. It is of general agreement with Branton (3) that the smooth surfaces correspond, in nonjunctional areas, to regions close to the center of the membrane, presumably where the methyl group of opposite fatty acid chains come in contact. This suggests, first of all, that the smooth surface between the globules may also correspond to central regions of a lipid bilayer, and secondly that the globules are likely to occupy the entire thickness of the membranes protruding from both surfaces.

Although measurements of the height of particles still represent very rough estimates in freeze-fracture preparations, because of difficulty in controlling the shadowing angle, it seems that the globules may protrude from the membrane surfaces for at least 20-30 Å, and possibly more from the cytoplasmic than from the outer membrane surface. Moreover, this observation is in agreement with what has been deduced by the appearance of globules in sectioned material (17).

One of the primary questions that ultrastructural studies on low resistance junctions usually try to answer concerns the morphological evidence for the hydrophilic channels proposed by physiologists (2, 9, 14, etc.) to account for the unusual permeability of these junctions to ions and larger molecules. In previous studies, various models have been designed in which the channels were suggested to extend through the center of the particles forming the hexagonal array (12, 13, 14). The evidence for this presumed location as well as for the existence of channels themselves was supported, at least from a morphological point of view, by rather limited findings. One was the presence of an electron-opaque dot located at the center of the electron-transparent globules seen in lanthanum preparations and in isolated negatively stained junctions. Another was the presence of a small depression occasionally seen in freeze-fracture at the center of the bumps on face A. The depressions would represent the extracellular end of one half of the channel and would accommodate, in lanthanum-treated specimens, a certain amount of extracellular stain producing the image of an electron-opaque central dot. The presence of lanthanum filling the presumed extracellular end of the channels is, however, rather difficult to explain. In fact, the proposed channels should be excluded from the extracellular medium to account for their function as intercellular pathways, and theoretically should not be reached by an extracellular tracer such as lanthanum. Disruptions in the structure of the junction, occurring as a result of fixation, could be a possible explanation for the phenomenon, since they could result in the penetration of some lanthanum into the lumen of the channels. However, the possibility that the central regions of the globules can actually be reached by certain extracellular components in vivo cannot be discarded yet.

Even taking into account the relevance of the observation of pits and dots on the extracellular pole of each globule as evidence for the existence and location of the channels, it is clear that the previous models (12, 13, 14) were based on several assumptions. (a) It was not known whether the globules, presumably containing the channels in their core, extend deep enough into the membrane to reach the cytoplasmic surface. (b) Even assuming that the globules occupy the entire thick-
ness of the membrane and reach the cytoplasmic surface, it was not known if also the cytoplasmic end of the globules contains a central pit presumably representing the cytoplasmic mouth of the channels. (c) There was no evidence that globules of one membrane come in contact with the globules of the other membrane, providing continuity for the two halves of each intercellular channel.

Several observations reported in this study provide further morphological evidence for the presumed existence and location of the channels, at least in so far as crayfish junctions are concerned. Here, in fact, the globules seem to occupy the entire thickness of the membrane and protrude from both its surfaces. Moreover, they appear to come in contact with those of the opposite membrane at the center of the junction, offering some evidence for the presumed continuity of the channels across the gap. In addition, the cytoplasmic pole of the globules is revealed in freeze-fracture and frequently seems to display a small depression in the center, presumably representing the cytoplasmic mouth of the channel. An interesting element, further confirming the existence of these small depressions, is offered by the observation that they match with similar size bumps located at the center of the pits seen in face A. Of course, the pits and their small central bumps would represent the stamps left on the cytoplasmic leaflet, after fracture, by the globules and their presumed central depressions. The small bumps at the center of the pits could represent liquid components of the cytoplasmic matrix which occupy the inner end of the channels. This hypothesis, however, is not supported by sufficient evidence in this study, since in these preparations no etching was performed, to exclude the nonaqueous nature of these bumps.

Some data regarding a possible subunit composition of the globules have been previously obtained (23). The present study provides some further information in this regard. Evidence for the presence of six subunits is obtained in lanthanum preparations, one being a close packing and the other a swollen arrangement. In isolated membranes, however, all the dimensions are smaller. Here, in fact, the close packing and the swollen arrangement are hexagonal patterns with a unit cell of ~95 Å and ~155 Å, respectively, while the dimensions of the unit cells of the two arrays in lanthanum preparations were, respectively, ~125 Å and ~200 Å. These variations in the dimensions of the unit cell are probably due to shrinkage occurring in negatively stained membranes.

An interesting finding regarding the globules of the swollen pattern negatively stained is represented by the ~37° angle formed between the axis of the globules and the axis of the subunits. In fact, if one assumed that the globules are able to reversibly change their pattern organization from a close packing to a swollen arrangement, this angle may turn out to be an important element in the understanding of the mechanism by which the change in the aggregation of the globules occurs.

In conclusion, from the observations reported in this and the previous study, new morphological features in the structure of intercellular low resistance junctions have been obtained. Further evidence was provided for the presence of intercellular channels as an anatomically definable means of communication, together with suggestions for the occurrence of plastic modifications in the organization of membrane structure. Finally, elements supporting the existence of intracellular junctions between vesicles and junctional membranes were demonstrated, suggesting the possibility that exchange of molecules can also occur between membrane-bound compartments of the two joined cells.

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