Proc. Nat. Acad. Sci. USA
Vol. 69, No. 5, pp. 1318–1321, May 1972

Three Types of Gap Junctions Interconnecting Intestinal Epithelial Cells Visualized by Freeze-Etching

(membranes/rat)

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Communicated by Keith R. Porter, March 13, 1972

ABSTRACT **Gap junctions are specialized membrane regions that seem to mediate intercellular communication. They appear to contain closely packed arrays of equally sized particles all of which, upon freeze-cleaving, remain attached to one membrane leaflet and not to the other. One gap junction cleavage face, therefore, always exhibits a closely packed array of particles, while the other features a corresponding array of pits. By using these morphological criteria, we have been able to distinguish three different types of gap junctions interlinking adjacent epithelial cells of the small intestine. All three types may be found in close proximity to each other, and in all cases, the particles remain attached to the cleavage face of the cytoplasmic membrane leaflet (A-face). The most frequently encountered type-I gap junctions, which have already been observed in many other tissues, possess 8- to 9-nm (80- to 90-Å) particles with a center-to-center spacing of 9–10 nm (90–100 Å) when packed in a hexagonal lattice. Type-II gap junctions are always found in close association with type-I junctions. They can be distinguished from the type-I junctions by the greater size [10–11 nm (100–110 Å) in diameter] and the greater spacing (190–200 Å) of their hexagonally arrayed particles. In contrast, the particles of the type-III gap junctions are arranged in very small rectilinear arrays with a spacing of only 6–8 nm (60–80 Å). Gap junctions may be involved in the control of intercellular flow of different types of regulatory molecules.**

Intercellular communication is one of the basic prerequisites for the development of multicellular organisms that are ordered according to a pattern and can act in a coordinated way. One type of cell-to-cell communication appears to be mediated by gap junctions or nexuses (1-12), specialized intercellular junctions that provide a route for the direct passage of small molecules from cytoplasm to cytoplasm with little loss (8, 11, 13). Although these junctions can be visualized in standard thin sections (14), in thin sections containing special tracers (4), and by negative staining (15), they can be most readily studied by the freeze-cleaving technique (9, 12, 16, 17).

Freeze-cleaving splits plasma membranes (18, 19), producing two new membrane faces that are opposed to each other in the membrane interior: face A, the split inner face of the cytoplasmic membrane leaflet, and face B, the split inner face of the external or luminal leaflet. These faces can be distinguished both by their structural relationship to other cell components and by their characteristic surface features. Nonjunctional plasma membranes contain many scattered particles of various sizes, which appear to be embedded in a lipid bilayer-type matrix. When such membranes are split, the particles become distributed between *both* of the membrane faces (17), although a majority of particles usually remains associated with the cytoplasmic leaflet. Gap junctional areas, on the other hand, contain closely packed arrays of particles of equal size, all of which, upon cleaving, remain attached to *one* leaflet and not to the other. One gap junction face always shows a closely packed array of particles, while the other features a corresponding array of pits. By using these morphological criteria, we have been able to distinguish three different types of gap junctions interlinking adjacent epithelial cells of the small intestine.

MATERIALS AND METHODS

Adult albino rats from the Charles River Breeding Laboratory were anesthetized with chloroform. Isolated ligated loops of the proximal region of the small intestine were injected either with a 30% glycerol–Ringer solution or with a 2% glutaraldehyde solution in phosphate buffer (pH 7.2). Small pieces of the epithelium prefixed with glutaraldehyde were removed after 15 min and then infiltrated with glycerol before they were cut into cubes less than 1 mm^3 and rapidly frozen on copper disks in Freon 12 held at −150°. The specimens treated with glycerol alone were removed after 30 min and processed in the same way as the prefixed tissues for freezing. The freeze-etching was performed according to Moor and Mühlethaler

FIG. 3. Micrograph of the A-face (*A*) of a freeze-cleaved composite gap junction containing both type-I and type-II elements. Notice that the hexagonally arrayed particles of the type-II region that is located in the periphery are not only larger but are also spaced at greater intervals than those of the type-I area. ×63,000.

FIG. 4. High magnification micrograph of a type-I–type-II composite gap junction as shown in Fig. 3. Differences in the site and packing of A-face (*A*) particles are also reflected in differences in diameter and arrangement of B-face (*B*) pits. ×158,000.

FIG. 5. Micrograph illustrating part of a type-I gap junction together with three typical type-III gap junctions. These type-III junctions appear on A-faces as small rectilinear arrays of between 4 and 30 closely packed particles. ×122,000.

FIGS. 6 and 7. Freeze-etch micrographs of (*B*) images of type-III gap junctions (*arrows*). Notice that the tips of the particulate components of the junctions are approximately level with the surface of the surrounding B-face matrix. It is concluded that these "particles" represent the slender stubs of interstitial material left behind when the closely packed A-face particles (Fig. 5) are torn away during the cleaving process. Fig. 6: ×130,000; Fig. 7: ×124,000.

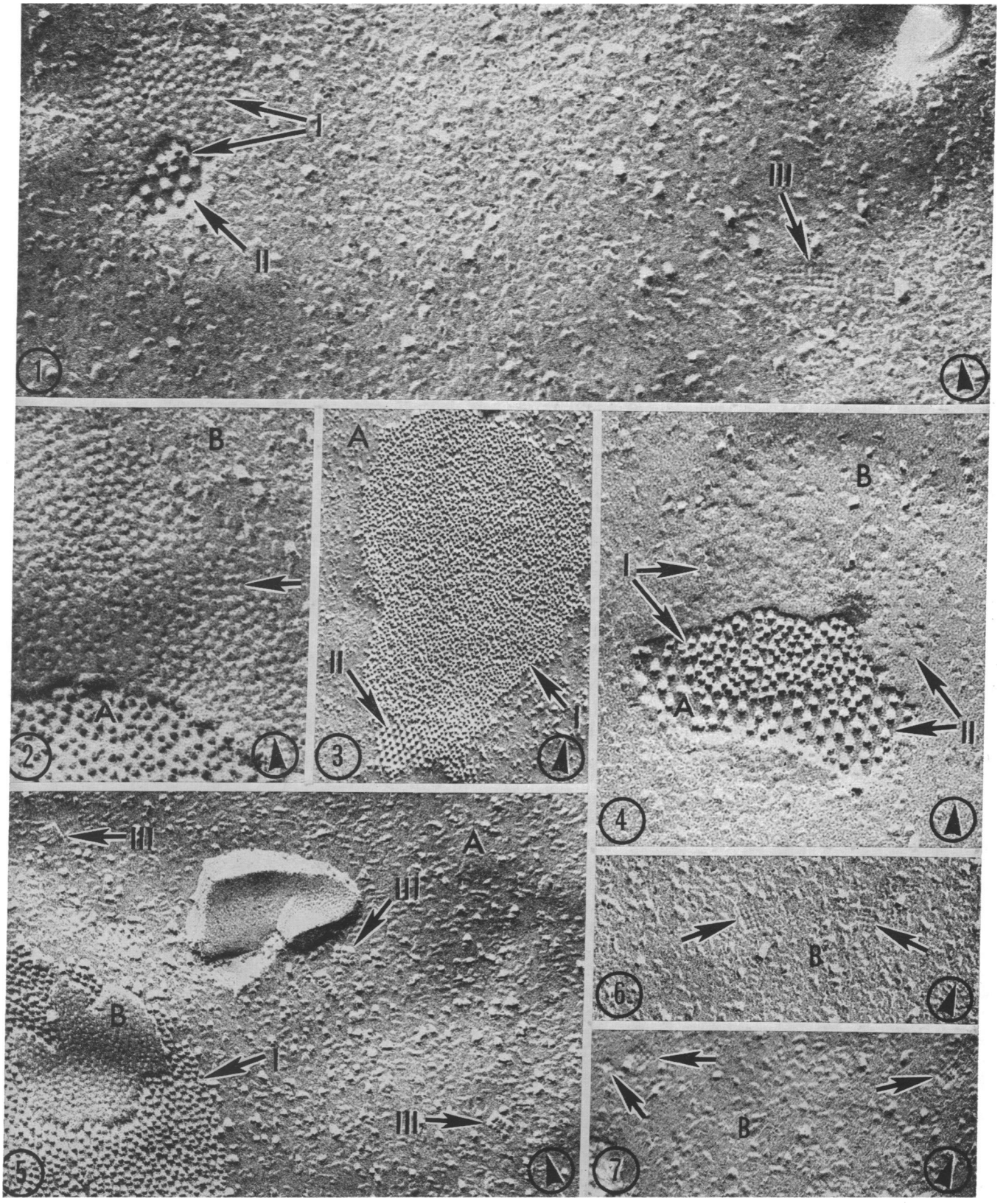

FIG. 1. Replica of freeze-cleaved lateral plasma membranes of intestinal epithelial cells of a rat. Three types of gap junctions (*I*, *II*, and *III*) can be recognized on the exposed membrane faces. They can be distinguished according to the size and arrangement of their characteristic particles and/or pits. ×166,000.

FIG. 2. Micrograph of a freeze-cleaved gap junction (type I) showing that the apparent diameter of the individual pits on the B-face (*B*) varies with the local shadowing angle. Only at a specific intermediate shadowing angle (area marked with *arrow*) is it possible to visualize the pits clearly and to obtain information on their form and size. *A*, A-face. ×195,000

(20) on a freeze-etch apparatus produced by Balzers (Balzers, Liechtenstein). During the shadowing with platinum–carbon, the knife was positioned as indicated by Staehelin and Bertaud (21). Etching of the specimens varied from 5 to 30 sec. The encircled arrow on each micrograph indicates the direction of shadowing.

RESULTS

The three types of gap junctions described in this paper are found in basal regions of lateral plasma membranes of epithelial cells of the small intestine. All three types may be observed in close proximity to each other (Fig. 1), but only rarely are any associated with elements of true tight junctions or with desmosomes as has been reported for other tissues (9, 17).

The most frequently observed type of gap junction (termed here type I) between epithelial cells of the small intestine possesses the same morphological freeze-etch parameters as those already described for mammalian liver (6, 16, 17), vertebrate myocardium (9), cervical epithelial (22), brown fat, and BHK 21 cells (12), as well as for epithelial cells of invertebrates (13, 23–25). With or without chemical fixation the A-face (Figs. 3, 4, and 5) exhibits a closely packed array of particles, 8–9 nm (80–90 Å) in diameter, which have a center-to-center spacing of 9–10 nm (90–100 Å) when arranged in an hexagonal lattice. According to McNutt and Weinstein (9), these particles are probably related to the gap junction channels that have been shown to allow the direct passage of molecules between adjacent cells (8). On the complimentary B-face (Figs. 1, 2, and 4), a similar array of small pits is observed in a smooth background matrix. In contrast to the relatively constant diameter of the arrayed particles (Figs. 3, 4, and 5), the apparent diameter of the individual pits can vary quite dramatically. Fig. 2 shows that this variation is related to the local shadowing angle. At low shadowing angles the outlines of the pits are frequently lost due to overlapping shadows, but at high shadowing angles the small holes become filled with shadowing materials and their apparent size is reduced. Information as to the size, form, and distribution of these pits can therefore only be obtained from regions shadowed at an optimal intermediate angle.

The preservation in freeze-etch specimens of the second type of gap junction (type-II gap junction), which is always found in close association with a type-I gap junction (Figs. 1, 3, and 4), seems to depend on the prefixation of the tissue with an aldehyde. Type-II gap junction areas may be distinguished from type-I areas by the greater size [10–11 nm (100–110 Å) in diameter] and spacing 19–20 nm (190–200 Å) of their A-face particles (Figs. 1, 3, and 4) and the larger diameter and separation of the B-face pits (Fig. 4). Furthermore, these larger particles and pits always seem to be organized into precise hexagonal arrays that adjoin the generally less well-ordered corresponding structures of type-I areas. Most of the type-II regions are located at the periphery of, and are smaller than, the adjacent type-I areas (Figs. 1, 3, and 4).

The third type of gap junction (type III) may be found close to, but never directly associated with, either of the other two types (Figs. 1 and 5). It is characterized by a rectangular arrangement of the particulate components, a closer packing of the particles, and much smaller overall dimensions. The particles, like those of type-I and -II junctions, cleave with the cytoplasmic membrane leaflet and are thus seen on A-faces (Fig. 5). Their center-to-center spacing measures 6–8 nm (60–80 Å). Due to the small size of these rectilinear gap junctions, which contain only 4–30 particulate components (see Figs. 5, 6, and 7), they are frequently missed in the average replica. However, when one such element has been detected, usually many more can be found in the surrounding areas. B-face views of type-III gap junctions also show what appear to be small rectangular arrays of particles (Figs. 6 and 7), but, in contrast to the particles on the A-face, the tips of these are approximately level with the smooth surface of the surrounding matrix material. We conclude, therefore, that these "particles" represent the slender stubs of interstitial material left behind when the closely packed particles are torn away during the cleaving process. The shadowing geometry of these stubs makes it very difficult to demonstrate the presence of the closely spaced intervening pits. Nevertheless, where two, three, or four particles have been pulled away as blocks, the resulting holes can be clearly recognized (Fig. 7). Since the number of gap junctions seems to vary considerably from cell to cell, we have made no attempt to quantify our results.

DISCUSSION

It seems to be now reasonably well established that gap junctions are the sites for the direct exchange of small inorganic ions and larger molecules between animal cells (8, 10–12). Both in developing and in mature tissues such exchanges may be of considerable importance for the coordination and regulation of cellular activities (3, 5). An estimate of the size of molecules that can be passed through gap-junction channels from one cell to another has been obtained by following the spread or nonspread of injected dye molecules of known molecular weight between adjacent, electrically coupled cells. Such studies have demonstrated that molecules up to a molecular weight of about 500 can diffuse freely through the gap-junction channels of adjoining cells (8). Thus, theoretically, many metabolites, as well as other possible types of signal molecules that may regulate cellular growth and differentiation, could be used for intercellular communication.

Gap junctions can be visualized in the electron microscope by thin sectioning (4, 14), negative staining (15), and freeze-etch techniques (9, 16, 17). In all these studies, however, only one type of gap junction has been reported interconnecting cells of the vertebrate myocardium (9), the mammalian liver (16, 17), the cervical epithelium (22), the lateral giant nerve fibers of the crayfish (8, 10), brown fat, and tissue culture cells (11, 12). These results make it difficult to understand exactly how the intercellular flow of different types of regulatory molecules and small ions may be controlled.

Our present investigation has provided evidence that not one but three types of gap junctions probably interconnect epithelial cells of the small intestine (Figs. 1–7). All three types can be readily distinguished in freeze-etch replicas due to the different sizes, spacings, and arrangements of their particles and complementary holes on the split inner membrane faces of the junctional regions. Although type-I and type-II gap junctions are morphologically different according to the above criteria, their close spatial relationship (Figs. 1, 3, and 4) together with the hexagonal patterning of the type-II regions suggests that they might arise from a rearrangement of type-I subunits and *vice versa*. If this were the case, type-I and type-II gap junctions could be envisaged

as representing two different phases of the same basic junctional element. The small type-III gap junctions, on the other hand, seem to be separate entities as far as can be judged from the different geometric (rectangular) arrangement of their particles and the spatial separation from the other two types of gap junctions.

With the demonstration of three different types of gap junctions interlinking intestinal epithelial cells, it seems not unreasonable to suggest that animal cells in general may be interconnected by channels having different sizes and/or different inner surface properties. It is therefore conceivable that the passage of different types of molecules between cells could be regulated simply by controlling the number of different types of channel elements available for transport. Indeed, Loewenstein, Nakas, and Socolar (26) and Rose and Loewenstein (27) have shown that the permeability of gap junctions can be readily controlled by changes in the internal and external concentrations of calcium, and, according to Loewenstein (28), communicating junctions between newt embryo cells can be formed within seconds after such cells have been brought into contact with each other. This notion of gap junctions being dynamic structures, which can be assembled and disassembled as need arises, agrees with our own observations that cells of the same tissue possess different densities and complements of such intercellular connections.

The technical assistance of Stephanie Krah and of Henry V. Webber, III is gratefully acknowledged. This research was supported by the National Institutes of General Medical Sciences under Grant 1 RO1 GM18639.

1. Dewey, M. M. & Barr, L. (1962) "Intercellular connections between smooth muscle cells: The nexus," *Science* **137**, 670–672.
2. Barr, L., Berger, W. & Dewey, M. M. (1968) "Electrical transmission at the nexus between smooth muscle cells," *J. Gen. Physiol.* **51**, 347–368.
3. Loewenstein, W. R. (1966) "Permeability of membrane junctions," *Ann. N.Y. Acad. Sci.* **137**, 441–472.
4. Revel, J. P. & Karnovsky, M. J. (1967) "Hexagonal array of subunits in intercellular junctions of the mouse heart and liver," *J. Cell Biol.* **33**, C7–C12.
5. Furshpan, E. J. & Potter, D. D. (1968) "Low-resistance junctions between cells in embryos and tissue culture," in *Current Topics in Developmental Biology*, eds. Moscona, A. A. & Monroy, A. (Academic Press, Inc., New York), Vol. 3, pp. 95–127.
6. Kreutziger, G. O. (1968) "Freeze-etching of intercellular junctions of mouse liver," in *Proceedings of the 26th Meeting of the Electron Microscopy Society of America* (Claitor's Publishing Division, Baton Rouge, La.), pp. 234–235.
7. Brightman, M. W. & Reese, T. S. (1969) "Junctions between intimately apposed cell membranes in the vertebrate brain," *J. Cell Biol.* **40**, 648–677.
8. Payton, B. W., Bennett, M. V. L. & Pappas, G. D. (1969) "Permeability and structure of junctional membranes at an electronic synapse," *Science* **166**, 1641–1643.
9. McNutt, N. S.. & Weinstein, R. S. (1970) "The ultrastructure of the nexus," *J. Cell Biol.* **47**, 666–688.
10. Pappas, G. D., Asada Y. & Bennett, M. V. L. (1971) "Morphological correlates of increased coupling resistance at an electronic synapse," *J. Cell Biol.* **49**, 173–188.
11. Johnson, R. G. & Sheridan, J. D. (1971) "Junctions between cancer cells in culture: Ultrastructure and permeability," *Science* **174**, 717–719.
12. Revel, J. P., Yee, A. G. & Hudspeth, A. J. (1971) "Gap junctions between electronically coupled cells in tissue culture and in brown fat," *Proc. Nat. Acad. Sci. USA* **68**, 2924–2927.
13. Rose, B. (1971) "Intercellular communication and some structural aspects of membrane junctions in a simple cell system," *J. Membrane Biol.* **5**, 1–19.
14. Robertson, J. D. (1963) "The occurrence of a subunit pattern in the unit membranes of club endings in Mauthner cell synapses in goldfish brains," *J. Cell Biol.* **19**, 201–221.
15. Benedetti, E. L. & Emmelot, P. (1968) "Hexagonal array of subunits in tight junctions separated from isolated rat liver plasma membranes," *J. Cell Biol.* **38**, 15–24.
16. Goodenough, D. A. & Revel, J. P. (1970) "A fine structural analysis of intercellular junctions in the mouse liver," *J. Cell Biol.* **45**, 272–290.
17. Chalcroft, J. P. & Bullivant, S. (1970) "An interpretation of liver cell membrane and junction structure based on observation of freeze-fracture replicas of both sides of the fracture," *J. Cell Biol.* **47**, 49–60.
18. Branton, D. (1966) "Fracture faces of frozen membranes," *Proc. Nat. Acad. Sci. USA* **55**, 1048–1056.
19. Pinto da Silva, P. & Branton, D. (1970) "Membrane splitting in freeze-etching," *J. Cell Biol.* **45**, 598–605.
20. Moor, H. & Mühlethaler, K. (1963) "Fine structure of frozen-etched yeast cells," *J. Cell Biol.* **17**, 609–628.
21. Staehelin, L. A. & Bertaud, W. S. (1971) "Temperature and contamination dependent images of frozen water and glycerol solutions," *J. Ultrastruct. Res.* **37**, 146–168.
22. McNutt, N. S., Hershberg, R. A. & Weinstein, R. S. (1971) "Further observations on the occurrence of nexuses in benign and malignant human cervical epithelium," *J. Cell Biol.* **51**, 805–825.
23. Hudspeth, A. J. & Revel, J. P. (1971) "Coexistence of gap and septate junctions in an invertebrate epithelium," *J. Cell Biol.* **50**, 92–101.
24. Flower, N. E. (1971) "Septate and gap junctions between the epithelial cells of an invertebrate, the mollusc *Cominella maculosa*," *J. Ultrastruct. Res.* **37**, 259–268.
25. Gilula, N. B. & Satir, P. (1971) "Septate and gap junctions in molluscan gill epithelium," *J. Cell Biol.* **51**, 869–872.
26. Loewenstein, W. R., Nakas, M. & Socolar, S. J. (1967) "Junctional membrane uncoupling," *J. Gen. Physiol.* **50**, 1865–1891.
27. Rose, B. & Loewenstein, W. R. (1969) "Depression of junctional membrane permeability by substitution of lithium for extracellular sodium," *Biochim. Biophys. Acta* **173**, 146–148.
28. Loewenstein, W. R. (1970) "Intercellular communication," *Sci. Amer.* **222** (5), 79–86.