Ca++-DEPENDENT DISASSEMBLY AND REASSEMBLY
OF OCCLUDING JUNCTIONS
IN GUINEA PIG PANCREATIC ACINAR CELLS

Effect of Drugs

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ABSTRACT
Incubation of guinea pig pancreatic lobules in Ca++-free Krebs-Ringer bicarbonate solution (KRB) containing 0.5 mM ethylene glycol-bis(β-aminoethyl ether)N,N,N',N'-tetraacetate (EGTA) results in the progressive fragmentation of the occluding zonulae (ZO) with formation of multiple discrete junctions (fasciae occludentes) localized in the lateral and lumenal plasmalemma. After 1-2 h of such incubation, most ZO appear completely disassembled. This results in the disappearance of the heterogeneity in density of intramembrane particles on the P-fracture faces of the basolateral and lumenal plasmalemma. If Ca++ ions are reintroduced into the incubation fluid at this point, continous zonulae reform around the apices of the cells; in contrast, the density of intramembrane particles (imp) at the lumenal plasmalemma remains the same as in the basolateral region, at least for 3 h after Ca++ reintroduction. When added to the incubation fluid, cycloheximide (at a dose known to inhibit protein synthesis >95%) and cytochalasin B (at doses which disrupt microfilaments and modify the cell shape) had no effect on the organization of ZO, on their disassembly in Ca++-free, EGTA medium, or on their Ca++-dependent reformation. Likewise, the organization and disassembly of ZO were unaffected by colchicine; however, after treatment with the latter drug the reassembly was defective, with formation of strand networks on the lateral surface and incomplete segregation of the lumenal region. Antimycin A, on the other hand, when added to the Ca++-EGTA medium, induced a large proliferation of long, infrequently anastomosed junctional strands, usually arranged to form ribbons, festoons, and other bizarre arrays. The possible relationship of these in vitro findings to the in vivo biogenesis and turnover of occluding junctions is discussed. It is suggested that the impairment of reassembly of zonulae by colchicine might be correlated with the disorder induced by the
drug on the general organization of pancreatic exocrine cells. Moreover, antimycin A could act by promoting the aggregation of a pool of free junctional strand components (or precursors) that might exist normally in pancreatic exocrine cells.

KEY WORDS exocrine pancreas \cdot freeze-fracture \cdot zonulae and fasciae occcludentes \cdot cell surface topology \cdot colchicine \cdot antimycin A

Since the introduction of freeze-fracture in biological studies, considerable information has accumulated on occluding (or tight) junctions. These structures, originally described in thin sections by Farquhar and Palade (19), are now known to be composed of elaborated arrays of interconnected strands, which appear as complementary ridges and furrows, respectively, in P- and E-fracture faces (see references 34 and 57 for reviews). Both the geometry of the arrays and the organization of individual strands can vary widely in different cell systems. Thus, in epithelium lining the lumen of cavity organs, occluding junctions are arranged mostly as continuous beltlike structures, localized at the apical margins of cells, that are designated as zonulae occcludentes (ZO); whereas in other epithelia (for instance, in most endocrine glands as well as in other tissues) they form discontinuous arrays of smaller size, called maculae or fasciae occcludentes (34, 57). On the other hand, individual junctional strands appear mostly continuous in some systems (11, 21, 57); in others they are composed of discrete, closely adjacent bars, aligned in rows (11, 13, 21, 39, 55), while in others they consist of rows of smaller bars and particles (23, 47, 54, 64).

Studies carried out on embryonic and adult tissues, as well as on cultured tissues, have yielded information on the dynamics of occluding junctions. It has been shown that these structures are not always stable but can assemble, grow, and even disassemble depending on cell differentiation as well as on a variety of other physiological and experimental conditions. Assembly seems to involve, first, the aggregation of linear chains of intramembrane particles (imp), which then fuse to yield discontinuous junctional strands. Ultimately, the strands merge into continuous zonulae or fasciae occcludentes (13, 14, 18, 39, 48, 49, 59).

It has been suggested that disassembly of occluding junctions might take place by processes analogous to those mentioned above for junction assembly, but occurring in the reverse sequence (13, 48, 49). However, other studies indicate that, at least in some systems, the elimination of the arrays originated by the fragmentation of ZO might take place by endocytosis and lysosomal digestion of the plasmalemma patches bearing junctional strands (1, 14, 46, 57).

In most previous studies on occluding junction biogenesis and turnover, the experimental conditions used were insufficiently controlled to permit a detailed characterization of the processes (for instance, in terms of timetable, metabolic requirements, etc.) or an investigation of the underlying cellular mechanisms. Thus, in many embryonic and regenerating systems, emergence, displacement, and decay of occluding junctions occur concomitantly, making difficult the identification of whether a specific image represents assembly or degeneration (13, 14, 24, 25, 39, 48, 49, 59). Moreover, some of the changes in junctional size and complexity, which can be induced experimentally, are slow processes that take days to develop (18, 40, 45, 60); others are elicited by rough, unspecific treatments, such as exposure to proteolytic enzymes (14, 36, 43, 53).

In the present report, we will describe a convenient experimental model for studying in vitro the disassembly and reassembly of occluding junctions under controlled experimental conditions. We found that, in guinea pig pancreatic tissue lobules incubated without Ca+++, the ZO are progressively disarranged and finally disrupted to yield discrete fasciae occcludentes; the process is rapidly reversed on reintroduction of Ca++ into the incubation fluid. Information on the role of ZO in maintaining the surface topology of pancreatic exocrine cells was also obtained. Moreover, since this Ca++-dependent disassembly and reassembly of ZO is a well-reproducible process, it was possible to investigate whether it is interfered with by drugs, such as cycloheximide, cytochalasin B, colchicine, and antimycin A, which have known effects on individual cell structure and function. The initial part of this work has already been reported elsewhere in preliminary form (22).
MATERIALS AND METHODS

Male albino guinea pigs were starved overnight, then stunned by a blow over the head. Pancreas tissue lobules were prepared as described by Scheele and Palade (51) and incubated in vitro at 37°C under 95% O2-5% CO2 in 100-ml Erlenmeyer flasks oscillating at 60 cycles/min in a water bath. Incubation fluids (10 ml) were the following: Krebs-Ringer bicarbonate solution (51) supplemented with an equilibrated mixture of aminoacids, glucose (14 mM), and soybean trypsin inhibitor (0.1 mg/ml) (KRB); Ca+2-free KRB containing 0.5 mM ethylene glycol-bis(β-aminopropyl ether) N,N',N''-tetraacetate (EGTA). Drugs were added to the incubation fluids at times specified in the text and figure legends and at the following concentrations: colchicine: 10^-6, 10^-5, 2 x 10^-4 and 10^-3 M; cytochalasin B: 10^-4, 2.5 x 10^-4, and 5 x 10^-4 M; cycloheximide: 10^-4 M; antimycin A: 5 x 10^-5 M. In some experiments the following secretagogues were used: caerulein (10^-8 M); urecholine, (4 x 10^-5 M); A23187 (2 μg/ml). The in vitro release of secretory proteins was estimated by the radiochemical procedure of Jamieson and Palade (29).

At the end of the incubations, the lobules were fixed with 1.5% glutaraldehyde + 1% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed in 1% OsO4 in 0.12 M phosphate buffer, pH 7.3, then infiltrated with glycol (at concentrations increasing from 10 to 30%) in the same buffer. Samples were frozen in Freon 22, cooled at -150°C in liquid nitrogen, then freeze-fractured according to Moor and Mühlethaler (41) in a Balzers freeze-etching device (Balzers AG, Balzers, Liechtenstein). The fracturing temperature was -100°C. Platinum-carbon replicas were washed in Na hypochlorite solution to remove organic material, then in distilled water, and finally recovered on 200-mesh copper grids.

Other lobules were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, postfixed in 1% OsO4, in the same buffer, stained in block with uranyl acetate, and embedded in Epon 812. Thin sections, cut with C. Reichert (Buffalo, N.Y.) and LKB ultramicrotomes (LKB Produkter, Bromma, Sweden), were doubly stained with uranyl acetate and lead citrate. Freeze-fracture replicas and thin sections were examined in Philips EM 200, EM 300, and EM 400 electron microscopes.

To measure the density of imp, small cardboard sheets bearing square or rectangular holes (sides between 2 and 5 cm, areas between 8 and 14 cm²) were placed on top of well-resolved replicas printed at a final magnification of 100,000 and moved around until the image appearing through the hole was entirely accounted for by a flat portion (P-fracture face) of either the luminal or the lateral plasmalemma of well-identified pancreatic acinar cells. Particles were then counted and the density was calculated by dividing their number by the area of the analyzed membrane portion (0.08-1.14 μm²). The use of holes of different size and shape enabled us to carry out the counting on sufficiently large, flat areas of both the lateral and luminal plasmalemma, even if the latter is quite irregular due to the presence of microvilli.

MATERIALS: The materials used were obtained from the sources specified below: soybean trypsin inhibitor, colchicine and cycloheximide: Sigma Chemical Co., St. Louis, Mo.; cytochalasin B: Ega Chemie, Steinheim bei Heidelberg/Brenz, Germany; antimycin A: Nutritional Biochemicals, Cleveland, Ohio; urecholine: Merck, Sharp & Dohme Canada Ltd., Montreal, Quebec, Canada. Caerulein and A23187 were the kind gifts of Farmitalia Labs for Basic Research, Milan, Italy and Eli Lilly and Co., Indianapolis, Ind., respectively. All other chemicals were reagent grade.

RESULTS

Control Cells

The appearance of normal exocrine cells of the guinea pig pancreas, both in thin sections and in freeze-fracture electron microscopy, has been described in detail previously (1, 9, 35). Here, only two points will be emphasized, since they are essential for the understanding of the rest of our work: (a) In both acinar and duct cells almost all junctional strands are integrated into well-organized ZO, which delineate circumferentially the luminal portion of the plasmalemma (12, 35). Fasciae occidentes, localized on the lateral surface, were very rarely seen. The organization of the ZO is quite elaborate (Fig. 1) and usually includes continuous parallel strands (two to five in acinar cells), running close to one another perpendicular to the major axis of the cell, surrounded by a looser network of variable orientation. The abluminal strand, which is also perpendicular to the cell axis, is interrupted from time to time by open-ended spurs. Communicating junctions (gap junctions [54]) of variable size are common on the lateral plasmalemma. (b) The P-fracture faces of the flat surfaces of the basolateral and luminal plasmalemma are characterized by a different density of imp. In particular, in acinar cells this difference is quite remarkable (12, 35): 2,768 ± 156 and 768 ± 52 imp/μm², respectively (Fig. 1).

Both the ZO organization and the imp density of the various plasmalemma regions are not significantly modified in tissue lobules incubated in complete KRB for periods of time up to 5 h.

Ca-Dependent Disassembly and Reassembly of ZO

The changes in internal structure that we ob-
served in pancreatic acinar cells incubated in KRB-EGTA medium are analogous to those described previously by Amsterdam and Jamieson (1) after treatment with EDTA. The distribution of the chromatin in nuclei, the parallel arrangement of endoplasmic reticulum (ER) cisternae, and the structure of mitochondria and zymogen granules were essentially unchanged. In contrast, the Golgi complex was clearly altered; it was enlarged and showed many swollen condensing vacuoles containing loosely packed, segregated material (see Fig. 13). Alterations of the junctional complex (such as disarrangement of desmosomes, appearance of punctuate occluding junctions between the lateral plasmalemmas of adjacent cells and, in a few cases, also opening of ZO were detected also in thin sections (not shown). However, a full appreciation of the changes induced in occluding junctions was revealed only by freeze-fracture (Figs. 2-6).

During the first 30 min of incubation in KRB-EGTA, the changes in the ZO were usually confined to the ablumenal seals. Their meshwork was progressively loosened and disarranged; after 10-15 min, long strands began to detach from the ZO body and to move ablumenally (Fig. 2). Eventually (15-60 min), these strands were progressively fragmented into pieces, which were dispersed in the lateral region of the plasmalemma and were often seen wound around communicating junctions (Fig. 3). The strand fragments retained their junctional function, as demonstrated by the continuity of ridges and furrows in adjacent P- and E-fracture faces (Fig. 3, see also Figs. 4-6).

In the majority of cells the arrangement of the luminal parallel strands of the ZO remained unaffected for at least 40 min of KRB-EGTA incubation. However, with longer times these strands also were disarranged and fragmented, so that after 2 h most ZO were transformed into multiple, discrete fasciae occidentes. Of the latter, some remained around the cell apex in the ablumenal regions originally occupied by the ZO, while others were displaced to the lateral and also to the luminal region, where they appeared intermingled with the microvilli (Figs. 4, 5, and 6). As a consequence, some acinar lumena become multichambered, partially obliterated, and distorted. At this stage many fasciae occidentes, especially those that had moved far away, were small in size. Some were composed of only one or a few short strands, either continuous or interrupted by short rows of imp. Continuity with rows of imp was seen sometimes also at the tips of the strands.

In strict correlation with the interruption of the ZO continuity, we observed a disappearance of the surface heterogeneity in imp density of plasmalemma P-fracture faces. Thus, in cells that, even after prolonged incubation in KRB-EGTA, still maintained at least one continuous ZO junctional strand, the density of imp at the luminal P-fracture face remained as low as in the controls. In contrast, in all cells with interrupted ZO, approximately the same high density of imp that in control cells occurs only at the basolateral plasmalemma, was found both at the basolateral and at the luminal plasmalemma regions.

In lobules first incubated for various time intervals in KRB-EGTA, major changes in the occluding junctions were provoked by transfer to the complete KRB medium. In brief, the lateral and luminal fasciae occidentes were drastically reduced and the ZO reassembled. These processes developed rapidly: reassembled ZO were observed in some cells already after 5 min and nearly in all cells by 30 min on (Figs. 7-10). By that time, only a few small fasciae occidentes, formed by short strands and rows of particles, remained in the lateral surface. On the other hand, the increase in imp density on the P face of the luminal plasmalemma occurring after ZO fragmentation was not reversed, at least within 3 h. Thus, most acinar cells of lobules first incubated for 2 h or more in KRB-EGTA and then transferred to plain KRB were characterized by a luminal plasmalemma P face rich in particles (3,103 ± 226 imp/μm^2)^3 surrounded by continuous ZO (Figs. 7-10). The latter were not identical to those described in control cells. In particular, the ordered and peculiar geometry (parallel strands towards the lumen surrounded by a looser meshwork) was no longer evident and was replaced by an even, tighter network. The thickness of this network was often variable, with alternation of deep (10-12 strands) and shallow (two to three strands) portions (Figs. 7-10).

Effect of Drugs

The aim of these experiments was to obtain some indirect information about the cellular mechanisms which might be involved in the regu-
FIGURE 3 Pancreatic lobules incubated for 40 min in KRB-EGTA. A more advanced stage of ZO disorganization is shown. Note the numerous individual strands and small arrays present in the lateral plasmalemma. These structures still maintain a junctional function as indicated by the continuity of grooves and furrows in adjacent P- and E-fracture faces (arrows). Junctional strands wound around five small communicating junctions located in the lateral plasmalemma of a pancreatic acinar cell are shown in the inset. Circled double asterisk and circled single asterisk label the lateral and lumenal plasmalemma regions, E-fracture faces. × 14,000; inset: ×25,200.

In each series of experiments the possibility was first investigated that the drug used, when added to plain KRB medium, had an effect on the structure of pancreatic exocrine cells (in particular on the organization of the ZO) within 4 h of in vitro incubation. In further experiments the drugs were added to the KRB-EGTA medium in order to test
FIGURES 4-6  Pancreatic lobules incubated in KRB-EGTA for 2 h. At this time point of the experiment, the ZO of most acinar cells appear disassembled to yield discrete strands and small strand arrays. As a consequence, the lateral (double asterisk) and the lumenal (single asterisk) regions of the plasmalemma (the latter identified by the presence of microvilli) become directly continuous. Note that the density of imp on the P-fracture face is approximately the same in the two plasmalemma regions. L = acinar lumen. Fig. 4: × 38,500; Fig. 5: × 34,500; Fig. 6: × 50,000.
Figures 7-10  Pancreatic lobules incubated in KRB-EGTA for 2 h and then transferred to complete KRB medium and reincubated for 10 (Figs. 7 and 8), 20 (Fig. 9), and 60 (Fig. 10) min. The acinar cells of these preparations are characterized (a) by the high imp density on the P-fracture face of their luminal plasmalemma (single asterisk) (as in the lobules incubated for 2 h in KRB-EGTA, see Figs. 4-6); and (b) by continuous ZO, which, however, do not exhibit the ordered, elaborate pattern visible in control cells (see Fig. 1), but are arranged more disorderly. L = acinar lumen; double asterisk and circled double asterisk = lateral plasmalemma; P and E faces. Fig. 7: × 32,000; Fig. 8: × 60,000; Fig. 9: × 24,500; Fig. 10: × 63,000.
their effect on the ZO disassembly induced by Ca++ withdrawal. Finally, the effect on ZO reformation was investigated in other lobules, incubated first in KRB-EGTA (usually for 120 min) and then in KRB (usually for 60 min). In many experiments the drugs were added to the second incubation fluid. In some cases, however, they were added to the first incubation or were even present throughout the whole incubation period. Here we will report the results obtained with four different drugs: cycloheximide (an inhibitor of polypeptide elongation); cytochalasin B (a mold metabolite that disrupts cytoplasmic microfilaments and also exerts a variety of other effects in the cells, especially at the level of the plasmalemma [2, 31, 65]); colchicine (which binds to tubulin-containing structures and thus causes microtubule depolymerization); and antimycin A (an irreversible inhibitor of the mitochondrial electron-transport chain). With each of these drugs, at least three complete experiments of ZO disassembly and reassembly were carried out.

The results obtained can be summarized as follows: Cycloheximide, at doses known to inhibit pancreatic protein synthesis by >95% (27), was without clear effect on the general ultrastructure of the cells (as already reported by Jamieson and Palade [27]) or on the organization of ZO. Moreover, no interference with the disassembly and reassembly of ZO was detected (not shown). Analogously, no effect on the ZO structure and dynamics was observed with cytochalasin B (Figs. 11 and 12), which, however, induced profound alterations in the organization of acinar cells, especially at their secretory pole (for details, see references 3 and 62).

**Figures 11 and 12** Effects of cytochalasin B (2.5 × 10⁻⁶ M). Fig. 11 refers to a lobule incubated for 60 min in plain KRB containing the drug. Note that cytochalasin B had no significant effect on the organization of the ZO. In contrast, the luminal surface of the cell (P-fracture face, asterisk) was clearly altered, with formation of large infoldings (I) and bulges. Fig. 12 is from a lobule first incubated in KRB-EGTA for 2 h and then in plain KRB for 60 min. Both incubation fluids contained cytochalasin B. The picture is analogous to those shown in Figs. 7-10. The high imp density on the P-fracture face of the luminal plasmalemma (asterisk) and the continuity of the ZO indicate that the drug did not interfere with the disassembly of the junctions occurring in Ca++-free medium or with the reassembly elicited by Ca++ reintroduction. Fig. 11: × 60,000; Fig. 12: × 68,500.
The results of the colchicine studies were more complex. At doses between $10^{-6}$ and $10^{-4}$ M, this drug induced the appearance of some pseudocrystalline structures and of images of autophagocytosis (63), while at least some microtubules were still evident. Moreover, the organization of the Golgi complex was clearly affected: clusters of vesicles and condensing vacuoles were scattered apparently at random in the whole cytoplasm (Fig. 13). These changes in the internal structure of acinar cells developed slowly (in about 60 min) and were not reversed (at least within 3 h) on drug withdrawal. By itself, colchicine did not induce detectable alterations in the ZO or interfere with the disassembly of this junction occurring in KRB-EGTA. In contrast, the reassembly of the ZO provoked by Ca$^{++}$ reintroduction was clearly impaired. The degree of the impairment was variable. In a few cells, no sign of redistribution or fusion of the discrete fasciae occludentes could be detected even after 2 h of reincubation in complete KRB. In others, the reintroduction of Ca$^{++}$ did result in a clustering of the fasciae and in the consequent formation of tightly packed junctional arrays both at the apical margins of the cells (Fig. 14) and in the lateral plasmalemma (Fig. 15); however, the coalescence of these arrays was only partial and therefore continuous ZO were not reestablished (Fig. 14). Finally, in some cells the junctional strands aligned themselves around the cell apex, but the resulting meshwork was very disordered and focal discontinuities were often observed (Fig. 16).

The effect of antimycin A was even more surprising. When added to plain KRB, this drug induced in acinar cells a vesiculation of the rough-
surfaced ER and swelling of some mitochondria (8, 28). The Golgi complex was also modified, with an increase in stacked cisternae, a decrease in the vesicles, and accumulation of fibrillar material. In most cells, the organization of the occluding junctions was unchanged. In others, the drug induced the appearance of small, bizarre arrays composed of junctional strands, which were localized in the lateral plasmalemma and were often continuous with the ZO (not shown). When added to the KRB-EGTA incubation fluid, antimycin A induced the formation of similar arrays which, however, were much more numerous, large, and elaborate (Fig. 17). In these acinar cells, the lateral surface was often covered by long, infrequently anastomosed junctional strands. Often, two to ten of these strands were arranged in parallel to form ribbons, festoons, whorls, and other bizarre figures, sometimes enclosing entrapped communicating junctions (Fig. 19). Less tightly packed arrays were formed as well (Fig. 18). Also, in lobules first incubated in KRB-EGTA for 2 h, and then in complete KRB containing antimycin A, we observed an apparent increase in junctional strands. Usually, however, the latter were organized to form elaborated, reformed ZO (not shown) rather than the peculiar arrays that developed when the drug was applied under Ca++-free conditions.

DISCUSSION

Previous studies carried out in a variety of cellular systems, especially in embryonic tissues, support the idea that occluding junctions are dynamic structures that can adjust their organization in response to specific and unspecific stimuli. So far, however, the information concerning these junctions is still limited and fragmentary. This is due, on the one hand, to the fact that, in contrast to the situation which exists today for other junctions, such as the communicating junctions (15, 16, 30), little is known about the chemical composition of occluding junctions and about their relationships to the other components of the plasmalemma. Subcellular fractions enriched specifically in junctional strands have not yet been isolated, while the peculiar localization of the strands has prevented detailed cytochemical studies. On the other hand, in many morphological investigations the changes in occluding junctions have been insufficiently characterized in terms of timetable, metabolic requirements, and functional significance, and their correlation with underlying cellular events has not been taken into consideration or sufficiently investigated.

In these respects, the experimental model used in this work (i.e., guinea pig pancreatic tissue lobules incubated in vitro in salt solutions with or without Ca++ and containing different drugs) offers some distinct advantages. This depends in part on the surface morphology of pancreatic acinar cells. In these cells, in contrast to the situation existing in many other epithelia lining a lumen (49, 57), almost all the junctional strands are integrated in continuous, well-organized ZO, while discrete fasciae oculidentes are very rare. This structural feature permitted us to establish that at least most of the fasciae oculidentes that appear on the lateral and luminal surfaces on incubation in KRB-EGTA do not exist before the treatment, but arise as a consequence of ZO fragmentation. Moreover, with respect to the ba-

Figures 14–16 Effect of colchicine (10^-5 M) on the Ca++-dependent reassembly of ZO. Pancreatic lobules were first incubated in KRB-EGTA for 2 h and then in plain KRB for 1 more h. Colchicine was present in both incubation fluids. In the cell shown in Fig. 14 the junctional strands have reassembled to form large meshworks located at the apical margins; however, a continuous, beltlike ZO has not reformed. Clear continuities between the lateral and luminal plasmalemma (E-fracture faces, circled double asterisk and circled single asterisk, respectively) are indicated by arrows. The luminal plasmalemma, identified by the characteristic craters corresponding to the bases of microvilli, is partially occupied by discrete arrays of junctional strands (arrowheads). L = acinar lumen; ER = cross fracture of parallel cisternae of the endoplasmic reticulum. Fig. 15 shows a large array of tightly packed strands localized in the lateral plasmalemma of an acinar cell. Fig. 16 shows a cell in which the Ca++-dependent reassembly has yielded a continuous ZO. The latter, however, is abnormal because (a) it is very thin and disordered and (b) it encircles a very large luminal surface (LS), part of which bears microvilli (and therefore probably corresponds to the original luminal plasmalemma [single asterisk]), while the rest is smooth and is probably accounted for by plasma membrane originally belonging to the lateral region [triple asterisk]. The density of imp at the luminal P-fracture face is as high as in the other cells with reassembled ZO (Figs. 7–10 and 12). Fig. 14: x 32,000; Fig. 15: x 35,000; Fig. 16: x 27,500.
Figures 17-19  Effect of antimycin A ($5 \times 10^{-5}$ M). Pancreatic tissue lobules were incubated in KRB-EGTA for 2 h. Antimycin A was present either during the whole incubation (Fig. 17) or during the 2nd hour only (Figs. 18 and 19). Long, infrequently anastomosed junctional strands have proliferated in the lateral plasmalemma, yielding large, elaborate arrays of various shapes and configurations which sometimes enclose communicating junctions (Fig. 19, Arrow). Circled double asterisk: lateral plasmalemma, E- and P-fracture faces. Fig. 17: $\times$ 56,500; Fig. 18: $\times$ 25,000; Fig. 19: $\times$ 40,000.
solateral region of the plasmalemma, the P-frac-
ture face of the luminal region of acinar cells is
characterized by a much lower density of imp (12)
and this density was markedly increased after the
interruption of ZO continuity by prolonged incu-
bation in KRB-EGTA. This provided us with a
valuable criterion for distinguishing the cells in
which the ZO opening had really occurred from
those which had resisted the KRB-EGTA treat-
ment. This identification was necessary in order to
investigate the dynamics of ZO reassembly which
takes place after readmission of Ca++ into the
incubation fluid. Finally, the reproducibility of the
extracellular Ca+§ changes of occlud-
ing junctions was quite good, especially in terms
of evolution with time. This was a necessary
prerequisite to the experiments in which the effect
of drugs on the disassembly and reassembly of the
ZO was investigated.

On the other hand, it should be acknowledged
that our model also suffers distinct limitations. For
instance, in our experiments it was impossible to
investigate the correlation between ZO organiza-
tion and paracellular ion permeability, measured
by biophysical and physiological techniques, as
done by others in different systems (4, 10, 11, 32,
38). Moreover, our system proved inadequate to
investigate the effect of ZO disassembly (and of
the consequent loss of heterogeneity of P-face imp
density in the plasmalemma) on the localization of
secretion granule discharge by exocytosis. In fact,
in parallel biochemical studies we found that
prolonged incubation in KRB-EGTA inhibits the
stimulated enzyme release in pancreatic lobules
(by 60–80%), even after reincubation in complete
KRB for times up to 2 h (not shown). Analogous
results were obtained recently in another labora-
tory.4 As a consequence, even if we exposed these
lobules to a variety of secretagogue drugs (caeru-
lein, urecholine, and the Ca++ ionophore
A23187), we were unable to observe convincing
images of exocytosis, which, on the contrary, were
quite common at the luminal surface of stimulated
control preparations.

Effect of ZO Disassembly and
Reassembly on Cellular
Surface Topology

The strict correlation between interruption of
ZO continuity and loss of heterogeneity of P-face
imp density at the surface of pancreatic exocrine
cells confirms the hypothesis (which is also sup-
ported by results in another system [44]) that ZO
are not only responsible for the segregation of the
extracellular space, but also act as mechanical
barriers which prevent the intermixing of mem-
brane components of the luminal and lateral
regions of the plasmalemma. When the ZO is
dismantled, the intermixing of these components
is probably very rapid, as indicated by the fact
that intermediate stages (such as gradients of imp
across the continuities of the two plasmalemma
regions) were never observed. It is possible that
the function of the ZO would be in maintaining
and not in establishing in the two regions the
heterogeneous distribution of particles, since the
latter did not reappear (at least within 3 h) after
the continuity of the ZO was reestablished by
reintroduction of Ca++ into the incubation fluid.
The rapid intermixing of the plasmalemma com-
ponents might explain why, in pancreatic acinar
cells dissociated from the tissue, the distribution
of various sugar residues (revealed by the binding
of specific lectins) was found to be uniform over
the entire cell surface (33).

The observation that after opening of the ZO
the imp density of both the basolateral and lu-
menal plasmalemma was not significantly different
from that found in the basolateral region of con-
trol cells strongly suggests that the total com-
plement of plasmalemma imp is conserved through-
out our experiments. In fact, in the acinar cells of
the guinea pig pancreas, the luminal plasmalemma
accounts for only 5.1% of the total surface area
(9). Therefore, the net relocation of basolateral
imp to the luminal plasmalemma which is needed
in order to make the density homogeneous in the
two compartments is small (<4% of the total
basolateral imp) and is not expected to modify
significantly the density of the basolateral region.

On the other hand, there is no reason to believe
that the intermixing of the molecular components
of the two plasmalemma regions was necessarily
complete. On the contrary, some degree of sur-
face heterogeneity, not detectable by freeze-fracture,
might have remained since recent evidence
demonstrates that, in membranes, while the bulk
of the lipids and many proteins are free to diffuse
laterally, other proteins move little or are immo-
bile (20, 52).

Biogenesis and Turnover of
Occluding Junctions

Two phenomena that we have demonstrated

G. A. Scheele, personal communication.

4 G. A. Scheele, personal communication.
might be relevant in relation to the biogenesis and turnover of occluding junctions: the progressive fragmentation of the ZO into smaller arrays on treatment with KRB-EGTA and its reformation when Ca++ is reintroduced into the incubation fluid. The first process is slow (1–2 h), whereas the second occurs rapidly. The time needed for ZO disassembly might be prolonged by the fact that the central strands are probably protected by those located ablumenally, as suggested by the observation that all strands were never affected concomitantly but always in sequence, from the periphery to the lumen.

The occluding strands of the lateral plasmalemma were often seen wound around communicating junctions. Similar images were observed previously in a series of different tissues, (13, 14, 18, 34, 36, 48, 49, 55). In particular, in embryonic tissues it was suggested that interaction with strands might favor the association of communicating junction monomers. We believe that in our cells the process occurs in the opposite direction: junctional strands, while moving in the plane of the membrane, might remain caught by the communicating junctions, probably because the latter are sites of low fluidity with respect to the rest of the plasmalemma. The association of the two junctions is not stable since it is rapidly dismantled when Ca++ is reintroduced into the incubation fluid.

Due to the rapidity of the phenomenon, we were unable to identify clearly the intermediate stages in ZO reassembly. However, the concomitant disappearance of most lateral fasciae occludentes strongly suggests that at least part of the reformed ZO results from the clustering and coalescence of the former structures rather than through complete de novo assembly of junctional strands. Reassembly always occurs around the luminal surface, i.e., at the place occupied by ZO in intact cells.

Our images suggest that the disassembly of ZO might be accompanied by a net decrease in the amount of junctional strands in the cells and that the opposite phenomenon occurs during reassembly. However, the attempts we made to check this point in quantitative terms yielded inconclusive results, as a consequence of the geometrical problems imposed by the displacement of the junctional strands over the cell surface. The suggestion that a partial disassembly and reassembly of junctional strands occurred in our experiments is also supported by the observation that the arrays located in the lateral plasmalemma are often constituted by short bars alternating or continuous with short rows of imp. In other systems, clear evidence indicates that junctional strands arise by alignment of imp followed by fusion of the resulting rows (13, 14, 18, 26, 39, 48, 49, 59). The reverse process has been suggested to take place when strands are disassembled (13, 48, 49). In contrast, we were unable to confirm another process that has been proposed to account for the disposition of occluding junctions, i.e., endocytosis and lysosomal digestion of plasmalemma fragments bearing junctional strands (11, 14, 46, 57). In this respect, it should be emphasized that endocytosis of junctional strands has been demonstrated clearly only in cells detached from their neighbors for various reasons (for instance, in experimental tissue dissociation and during cell differentiation). In all these cases, fragments of adjacent cells remain attached to the fasciae occludentes. Thus, the subsequent endocytosis might be related to the disposition of surface interactions no longer useful rather than to the physiological turnover of occluding junctions.

Taken together, the results discussed so far support the idea that zonulae and fasciae occludentes are not separate entities. Rather, all junctional strands might constitute a common pool. In pancreatic exocrine cells, the integration of these strands into continuous ZO appears greatly favored over ZO disassembly. This might explain why fasciae occludentes appear only when Ca++ is withdrawn from the extracellular fluid. Possibly, a difference in the balance between assembly and disassembly could explain why, in many epithelia lining a lumen, zonulae and maculae occludentes coexist also at physiological Ca++ concentrations and even why, in other systems, continuous zonulae are never assembled. The importance of Ca++ in the assembly of occluding junctions has been demonstrated also in other systems (10, 46) and might be of general occurrence, although it is not yet clear whether Ca++ acts directly (on junctional components) or indirectly (on the organization of the plasmalemma [42, 50] or even on the intracellular environment).

**Effects of Drugs**

We will now consider the effects of drugs on the disassembly and reassembly of occluding junctions. In this respect it should be emphasized that, due to the poor specificity of the drugs used, the interpretation is far from straightforward. How-

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ever, some interesting data have emerged. First of all, we found that cytochalasin B, at doses which disrupt microfilaments and produce clear changes in cell shape, especially at the luminal surface, has no effect on the organization and dynamic changes of the ZO. This result is surprising in view of the previous proposal that the development and function of occluding junctions might be controlled by cellular microfilaments (4, 40).

The effects of colchicine were more complex. Neither the organization nor the disassembly of ZO was influenced by this drug. Moreover, after reintroduction of Ca++ the junctional strands did reassemble to yield large arrays. However, in most cases the arrays either reformed away from the right place or failed to coalesce entirely to yield complete ZO. Thus, in colchicine-treated cells, the defect seems to involve not the ZO reassembly per se, but its ordered localization at the cell surface. This effect of the drug correlates nicely with that elicited on the Golgi complex, which is fragmented and dispersed throughout the cytoplasm. The importance of the Golgi complex in inducing the site of assembly of ZO has been suggested recently by other authors (61). The effect of colchicine on the Golgi complex is not limited to the exocrine pancreas but has been observed also in a number of other systems (17, 37, 58) and attributed to the disruption of the microtubules localized in the controsphere region of the cell (58). Alternatively, colchicine might act directly on the plasmalemma, since this membrane is known to contain large amounts of tubulin and to be profoundly affected by the drug, both in its physicochemical features and in its function (5-7, 56).

Finally, the results with cycloheximide demonstrate that the disassembly and reassembly of ZO does not depend on de novo protein synthesis. Moreover, antimycin A, especially when added to KRB-EGTA, the changes in occluding junction permeability were not integrated in assembled strands, which might exist normally in these cell types.

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