PLASMA MEMBRANES, CELL JUNCTIONS AND CUTICLE OF THE RECTAL CHLORIDE EPITHELIA OF THE LARVAL DRAGONFLY AESHNA CYANEA

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SUMMARY

The cell membranes and cell junctions of the rectal chloride epithelia of the larval dragonfly Aeshna cyanea were examined in thin sections and by freeze-fracture. These epithelia function in active ion absorption and maintain a high concentration gradient between the haemolymph and the fresh-water environment. Freeze-fracturing reveals fine-structural differences in the intramembranous particles of the luminal and contraluminal plasma membranes of these epithelia, reflecting the functional diversity of the two membranes, which are separated by the junctional complex. The particle frequency of the basolateral plasma membranes is reduced after transfer of the larvae into high concentrations of environmental salinity.

The junctional complex is located in the apical region and composed of three types of cell junctions: the zonula adhaerens, seen in freeze-fracture as a nearly particle-free zone; the extended and highly convoluted pleated septate junction and randomly interspersed gap junctions of the inverted type. Gap junctions also occur between the basolateral plasma membranes. They provide short-cuts in the diffusion pathway for direct and rapid co-ordination of the interconnected cell processes.

Colloidal and ionic lanthanum tracer solutions applied in vivo from the luminal side penetrate through the cuticle via epicuticular depressions, but invade only the apical portion of the junctional complex. This indicates that the pleated septate junction constitutes a structural control of the paracellular pathway across the chloride epithelia, which are devoid of tight junctions. The structure of the pleated septate junctions is interpreted as a device for the extension of the diffusion distance, which is inversely related to the net diffusion. A conservative estimate of the total length of the junction, and the number and extension of septa reveals that the paracellular route exceeds the transcellular route by a factor of 50.

INTRODUCTION

Apart from other functions, the larval rectum of dragonflies is involved in respiration and osmoregulation. Hence, two types of epithelium line the rectal wall: the branchial epithelium serving respiratory oxygen uptake and the chloride epithelium serving osmoregulatory salt uptake (for a review, see Komnick, 1978, 1982). Since the larvae, which normally live in freshwater habitats, are hyperosmotic regulators the rectal epithelium separates two aqueous solutions of different ionic compositions and concentrations. The basal side of the rectal epithelium is exposed to the haemolymph, which, in larval Aeshna cyanea under freshwater conditions, has a sodium chloride concentration of about 140 mM and total osmolarity of 330 mosM (Moens, 1975; Herzog, 1979), whereas the apical side is facing the environmental solution ingested into the branchial chamber. The respective external concentrations are lower by about
two orders of magnitude, depending on the type of freshwater habitat. Even when the external concentrations differ by four to five orders of magnitude, as in experiments using demineralized water, the nominal haemolymph concentration remains constant for up to 3 days before it slowly declines to about 50% after 9 days (Moens, 1975). The maintenance of these large differences in concentration calls for a barrier function in the rectal epithelium. The postulated barrier property is of particular interest with respect to the chloride epithelium, which is involved in establishing and restoring the concentration gradient by active absorption of sodium chloride from the external solution into the haemolymph.

The major part of the chloride epithelium is traversed by intercellular clefts resulting from basolateral cell interdigitations. These clefts are in open communication with the haemocoel via the basal lamina, thus providing numerous paracellular pathways deep into the epithelium for blood constituents that are able to pass through the basal lamina. The autoradiographic localization of sodium-pumping sites in the basolateral plasma membranes (Komnick & Achenbach, 1979) indicates that the intercellular spaces also serve as paracellular pathways in the basal direction, at least for sodium secreted by the cells into the clefts. In the apical region the intercellular clefts run into the terminal bars representing the junctional complexes between the epithelial cells. Consequently, with respect to the paracellular route the high concentration gradients mentioned before are presumably maintained over a distance of only a few μm, which is the extent of the terminal bars in the apicobasal direction.

The present investigation of the rectal chloride epithelia was carried out using various electron-microscopic preparation techniques, including tracer experiments, in order to test the presumptive sealing function of the junctional complex and to look for an eventual structural correlate of cuticular permeability, because the cuticle must be permeable at least to the ions absorbed through the rectal chloride epithelia.

MATERIALS AND METHODS

The study was performed on the rectal branchial chamber of the larval dragonfly A. cyanea (Odonata, Aeshnidae) collected from small ponds of the Kottenforst near Bonn and maintained in aerated freshwater aquaria on a Tubifex regimen for up to several months. Larvae of a relatively narrow range of body lengths (2.5-3 cm) were selected for the experiments.

Conventional fixation

Recta were quickly dissected without the use of artificial saline solutions, fixed at room temperature in 3% glutaraldehyde for 6-12 h and postfixed in 1% osmium tetroxide for 1 h. The fixatives and washing solution contained 100 mM-sodium cacodylate buffer (pH 7.4) and 100 mM-sucrose. Thin sections of Araldite-embedded tissues were stained with uranyl acetate and lead citrate, and examined in Philips EM 200 and EM 301 electron microscopes.

Lanthanum staining

Infiltration of isolated tissues was performed by the addition of either colloidal (1% lanthanum hydroxide; according to Revel & Karnovsky, 1967) or ionic lanthanum (1% LaCl₃) to the fixatives and washings described above; duration of fixation with glutaraldehyde was 12-24 h and with osmium tetroxide, 1 h. Thin sections of the styrene methacrylate- or Araldite-embedded tissues were examined without further staining.
Freeze-fracturing

Before freezing the freshly isolated recta were treated in three ways: (1) fixation at room temperature in 2.5% glutaraldehyde containing 100 mM-sodium cacodylate buffer (pH 7.2) for 0.5–1 h and subsequent infiltration with graded aqueous glycerol solutions (10, 20 and 30%, 15–30 min each) as cryoprotectant; (2) glycerination (as above) of unfixed tissue; (3) direct freezing of fresh tissue without fixation and glycerination.

For shock-freezing small tissue pieces were mounted between brass specimen holders and rapidly introduced into liquid propane cooled with liquid nitrogen. Freeze-fracture replicas were obtained with the Bioetch 2005 apparatus (Leybold-Hereaus) under the following conditions: fracturing temperature, −100°C; vacuum pressure <1.33 × 10⁻⁴ Nm⁻² (10⁻⁶ Torr); etching time, 30 s for glycerinated specimens and 5–10 min for freshly frozen specimens; shadowing with platinum/carbon at 45° followed by carbon at 90°; replica cleaning with 30–40% chromic acid. The micrographs are mostly oriented so that shadowing comes from the bottom. Nomenclature of fracture faces and membrane surfaces follows that of Branton et al. (1975).

The frequencies of intramembraneous particles exceeding 7 nm in diameter were studied on the PF and EF of two types of plasma membranes that are in close topographical relation but differ in the Na/K-ATPase equipment: the basolateral plasma membranes of the chloride epithelia and the interdigitated plasma membranes of the subepithelial fat-body cells. Freeze-fracture replicas for comparative particle counting were made from each of six larvae of three groups exposed to different external salt concentration: (1) 5 days in 0.5 mosM sea-salt solution (control); (2) 3 h and (3) 24 h after the transfer from 0.5 mosM into 500 mosM sea-salt solution. The particles were counted in areas of 0.25 or 1 µm² on positive prints of ×100,000 final magnification.

Tracer experiments

Utilizing the normal ventilation of the branchial chamber, the tracer was applied directly and exclusively to the luminal side of the rectum without any dissection or injection, by simply dipping the anal opening of the living and appropriately immobilized larvae into the experimental solutions (Fig. 1). The three consisted of 1.5% aqueous lanthanum hydroxide, lanthanum nitrate or lanthanum chloride. After exposure times ranging from 3 h to 8 days the larvae were allowed to swim by rectal jet-propulsion in large volumes of lanthanum-free water, in order to remove the experimental solution from the rectal lumen prior to dissection and conventional fixation. The distribution of the tracer was examined in thin sections of the recta stained with alcoholic uranyl acetate, or without further staining. Also thin sections and 10 µl samples of haemolymph, withdrawn from the lanthanum-exposed larvae and dried on carbon specimen holders, were analysed for the presence

Fig. 1. Diagram illustrating ventilative tracer introduction into the rectal lumen via the anal opening of the living larva. ps, perforated plastic syringe as a device to immobilize the animal; ts, tracer solution; at, air tube.
of lanthanum with the EDAX-analyser attached to the Philips EM 300 transmission electron microscope and scanning device. Control samples made up by diluting $5\mu\text{l}$ haemolymph samples from untreated larvae with $5\mu\text{l}$ aqueous lanthanum chloride of defined concentrations served to test the detection limit for lanthanum under these conditions.

RESULTS

Plasma membranes

The plasma membranes of the rectal chloride epithelia are greatly amplified on both the luminal and contraluminal sides by densely packed, irregular apical infoldings and by extensive basolateral cell interdigitations, respectively (Greven & Rudolph, 1973; Wichard & Komnick, 1974; Leader & Green, 1978). The apical and basolateral membrane regions are separated by highly tortuous zones of the lateral plasma membranes in the sub-apical region, which represent the junctional complexes of the epithelial cells (Fig. 2A). Freeze-fracture of the apical and basolateral plasma membranes exhibits a relatively homogeneous and dense population of intramembranous particles (IMPs) prevailing on the PF. However, the two membranes differ in several details. The IMPs on the apical PF are of nearly uniform size (10 nm) and show high complementarity of pits on the EF (Fig. 2B). The IMPs on the basolateral PF vary from 6 nm to 9 nm in size, and there are only sporadic pits on the EF. IMPs on the basolateral EF are more frequently encountered (Fig. 2D) when compared with the apical EF, which is extremely poor in particles.

In addition, the particle frequency on the basolateral plasma membranes, which are rich in Na/K-ATPase (Komnick & Achenbach, 1979), changes with different external salinities. As seen in Fig. 3 the 1000-fold increase in the external salinity leads to an average decrease of IMP frequency on the PF of the basolateral plasma membranes to two-thirds and half of the control level after 3 and 24 h, respectively. In contrast, particle frequencies on the PF of the interdigitated plasma membranes of the subepithelial fat-body cells, which are not involved in osmoregulatory ion uptake, remain relatively constant as do the low particle frequencies on the EF of all membranes tested (Fig. 3). Particle frequencies on the apical plasma membranes were not determined because the small fracture faces normally obtained from the apical folds were insufficient for quantitative estimation.

Cell junctions

The convoluted junctional complexes extend from the zone of apical plasma...
Cell junctions of dragonfly rectum

Fig. 2
membrane infoldings in the basal direction for, at most, about one-fifth of the epithelial thickness. Each complex is composed of three different types of junctions: (1) a zonula adhaerens occupying the most apical position of the complex; (2) a septate junction constituting the major part of the complex; (3) gap junctions interspersed in the septate junctional area. Additional gap junctions are present underneath the junctional complex and between the basolateral plasma membranes of the interdigitated cell processes (Fig. 2A, D). Tight junctions are not present in the chloride epithelia.

Freeze-fracture shows a nearly particle-free stripe (Fig. 2c), which corresponds in location and width to the zonula adhaerens seen in thin sections (Fig. 2A). This clear demarcation from the adjacent membrane areas by the sharp reduction of particle

Fig. 3. IMP frequencies on the PF (striated columns) and EF (open columns) of the basolateral plasma membranes of rectal chloride epithelia and the plasma membranes of subepithelial fat-body cells. A. control larvae after 5 days in 0.5 mosm sea-salt solution (equivalent to fresh water of very low mineral concentration). Experimental larvae 3 h (b) and 24 h (c) after transfer from 0.5 mosm into 500 mosm sea-salt solution. Under steady-state conditions the osmotic concentration of the haemolymph amounts to 300 mosm at 0.5 mosm, and to 550 mosm at 500 mosm external concentration. Mean ± standard deviation. PF countings, \( n = 20 \); EF countings, \( n = 5 \).

Fig. 4. A. Pleated septate junction in cross-section (conventional fixation). Arrowheads label the coat on the PS of the junctional membranes. \( \times 150\,000 \). B–F. Sections of lanthanum-stained cell junctions. B. Part of pleated septate junction in cross-section. The arrowheads point to septa with clearly visible central thickenings. gj, gap junction. \( \times 150\,000 \). C. Parts of pleated septate junction in tangential and cross-sections. The arrows label the terminations of two septa. \( \times 150\,000 \). D. Gap junction in cross-section. Arrows label electron-translucent crossings of the lanthanum-filled gap. \( \times 300\,000 \). E, F. Parts of gap junction and pleated septate junction both in tangential section. \( \times 120\,000 \). F. Higher magnification of a tangential section through a gap junction showing junctional particles with central dots of lanthanum stain. \( \times 300\,000 \). G. Freeze-fracture showing the EF and PF of a gap junction located in the region of pleated septate junction (fixed and glycerinated). \( \times 100\,000 \).
Cell junctions of dragonfly rectum

Fig. 4
density (Fig. 2c) resembles the vertebrate zonula adhaerens in intramembranous structure (Staehelin, 1974), but is somewhat different from zonulae adhaerentes of other arthropod tissues, which are devoid of specialized intramembranous features in the junctional region (Lane & Skaer, 1980).

Cross-sections of the septate junction show the characteristic ladder-like structure. After conventional fixation the junctional membranes and the intercellular septa are relatively electron-dense (Fig. 4A). The spacing of the two membranes is about 15 nm, the septa being about 8 nm wide and periodically arranged at centre-to-centre distances of about 16 nm. The junctional membranes take an undulating course with respect to the septa resulting from slight constrictions of the intercellular space at the interseptal regions (Fig. 4A). Occasionally a fuzzy, more or less particulate, coat is visible on the cytoplasmic side of the junctional membranes (Fig. 4A).

The addition of either colloidal or ionic lanthanum to the fixatives leads to heavy staining of the interseptal spaces so that the septa are now delineated in negative contrast. In negative contrast the septa measure only about 3 nm compared to the 8 nm thickness in positive contrast, and show a knob-like thickening in the middle (Fig. 4B). Cross-sections showing a more or less diffuse staining of the intercellular space, with some indications of periodic striations (Fig. 4C), are assumed to run parallel to the septa. In tangential sections the septa appear as electron-transparent zigzag lines against the dark background of the lanthanum stain, with a fairly regular pleating periodicity of about 20 nm (Fig. 4C). Accordingly, the septate junctions of the chloride epithelia belong to the pleated type. (For nomenclature of septate junctions, see reviews by Noirot-Timothee & Noirot (1980) and Lane & Skaer (1980).) The pleated septa run more or less parallel to each other, following a partly straight and partly curved course. At some places, terminations or interruptions of individual septa are clearly visible (Fig. 4C). This indicates that some or perhaps even all septa are not continuous around the whole cell.

Freeze-fracture replicas of glycerinated tissue samples of both fixed and unfixed recta show rows of about 8 nm particles with centre-to-centre distances of about 20 nm on the PF of the junctional membranes, while the EF appears relatively smooth, with only a few particles (Fig. 5A). Favourable local fracturing and shadowing conditions reveal narrow, shallow furrows on the EF, which are continuous with the particle rows on the PF (Fig. 5B). The rows take various courses, but parallel arrangements running at spacings of 15–20 nm appear to predominate. Randomly distributed terminations of individual rows are clearly visible.

Freezing of native recta caused varying degrees of structural damage, particularly...
Cell junctions of dragonfly rectum

Fig. 5
of the groundplasm, as a consequence of ice-crystal formation. Nevertheless, numerous clear membrane-fracture faces of sufficient quality were found for structural analysis of the pleated septate junction.

In agreement with the results from glycerinated fixed and unfixed tissues, the replicas of native frozen recta also show rows of particles on the PF. However, at suitable places these strings of particles are seen to be inserted into grooves of the PF (Fig. 5d), whereas the EF shows elevated strands that are about 15 nm wide and randomly terminated (Fig. 5c, d). Serial thickening of these strands produces the impression of a zigzag pattern (Fig. 5c). Individual particles occurring on the EF are located on top of the strands (Fig. 5d, e). At places where the fracture plane changes from the EF of one junctional membrane to the PF of the opposite one, the strands on the EF are seen to be continuous with the rows of particles in the grooves of the PF on the one hand (Fig. 5c), and with slight protrusions of the PS on the other (Fig. 5e).

Gap junctions are integrated regularly into the septate junctional area (Fig. 4a, c, e, f) and also frequently occur between the interdigitating cell processes in the basolateral region (Fig. 2d). The gap is heavily stained by lanthanum (Fig. 4a, c) and traversed by translucent structures (Fig. 4d). Tangential sections show arrays of electron-transparent intercellular particles (Fig. 4e). The black dot visible in the middle of the particles at high magnification (Fig. 4f) apparently results from lanthanum penetration into the central hydrophilic channel (Lane & Skaer, 1980).

Freeze-fracture replicas reveal that the gap junctions are of the inverted type (Flower, 1972), showing the clusters of junctional IMPs predominantly on the EF (Figs 2d, 4c) and the corresponding pits together with a few particles on the PF (Fig. 4e). The size of the gap-junctional area and hence the number of particles per junction is highly variable. As a rule, the largest gap junctions (approx. 90–120 particles per junction) are present in the junctional complex (Fig. 4e), while the smaller ones (approx. 6–40 particles per junction) occur between the basolateral membranes (Fig. 2d).

Permeability studies

Unlike lanthanum staining of the junctional complex performed during fixation, the permeability experiments are based on the application of lanthanum as a vital tracer before fixation. The varied modes of lanthanum administration allow different conclusions to be drawn, regardless of whether or not the underlying binding and staining mechanism for the electron microscopic demonstration of lanthanum is the same in both cases. The rationale for this kind of approach is that the cuticle provides access to the junctional complex for the vital tracer ingested into the rectal lumen.

Cuticle

Particulate lanthanum deposits are present in the endocuticle and clearly indicate that the lanthanum tracer is able to penetrate through the rectal cuticle (Fig. 6a, b, c). This holds true exclusively for the cuticle of the chloride epithelia; the cuticle of the adjacent branchial epithelium is not labelled with particulate lanthanum (Fig. 6d, e).
Cell junctions of dragonfly rectum

The result of vital lanthanum labelling correlates well with the selective PAS-staining of the cuticle overlying the rectal chloride epithelia (Komnick, 1982). Selective PAS-staining is not unique to the cuticle of dragonfly rectal chloride epithelia, but has been reported for the cuticle of rectal transporting epithelia of various insects (Noirot & Noirot-Timothé, 1969). The presence of label in the endocuticle of the chloride epithelia and its absence from the branchial cuticle raise the question as to whether different lanthanum binding or different lanthanum permeability of the two cuticles is involved. In contrast to the vital tracer experiments, the endocuticles of both the branchial and chloride epithelium stain similarly after lanthanum application during and after fixation. Consequently, the absence of label from the branchial endocuticle in the vital experiments results from the impeded penetration of tracer through the branchial epicuticle. The clear-cut termination of endocuticular lanthanum distribution at the border of the chloride epithelia further shows that the tracer is unable to diffuse laterally within or underneath the cuticle from the chloride epithelium towards the branchial epithelium. This impeded lateral diffusion of tracer is structurally correlated with the absence of the endocuticle over the transitional cells (Fig. 6B) and with their presumptive tight contact with the overlying epicuticle. Accordingly, it is the epicuticle that must be regarded as responsible for the control of cuticular tracer penetration. This fits well with the occurrence of epicuticular depressions, which are restricted to the chloride epithelia and apparently provide preferred transcuticular pathways at least for the substances transported through the chloride epithelia. Their absence from the epicuticle of the branchial epithelium indicates that they are not necessary for the transcuticular diffusion of oxygen. Similar epicuticular depressions were observed in a large variety of insects, predominantly in the cuticle of rectal transporting epithelia and considered as permeable structures (Noirot & Bayon, 1969; Noirot & Noirot-Timothé, 1969, 1971, 1976; Flower & Walker, 1979). However, Noirot & Bayon (1969) overlooked these depressions in the rectum of A. cyanea larvae in their search with the scanning electron microscope.

The depressions are visible in freeze-fracture replicas of the cuticle surface as funnel-shaped structures, randomly distributed at a density of 1–3 per μm² (Fig. 7A). Their bottoms measure 60–80 nm across and are studded with particles that produce the impression of a sieve-like substructure (Fig. 7A). Vital tracer administration results in heavy accumulations of lanthanum deposited directly underneath the depressions (Fig. 7B). A distinct layer of lanthanum label visible at the bottom of the depressions shows faint indications of tiny pores (Fig. 7B, C).

The depressions result from the local 80–90% reduction in thickness of the epicuticle (Fig. 7D). Median cross-sections clearly showing the details and relationships of the cuticular layers in conventionally fixed preparations are extremely difficult to obtain, because the depression diameter (60–80 nm) falls into the range of the section thickness. Following the terminology of Wigglesworth (1975) who simply differentiates between the inner and the outer epicuticle, the reduction in thickness concerns the inner epicuticle exclusively (synonymous with the dense layer), whereas the outer epicuticle appears to be of the same thickness and triple-layered, as it is outside the depressions (Fig. 7D). The three layers of the outer epicuticle are interpreted as
Cell junctions of dragonfly rectum

Cuticulin, wax and cement, according to Locke (1964). There are faint indications that these layers may be slightly different in the depressions. The outer cement layer often shows tufts of fuzzy extensions into the lumen. The intermediate wax layer, if present at all, is hard to distinguish in fine structure and contrast from the cement layer (Fig. 7D). The inner cuticulin layer is a little thinner or less electron-dense and seems to correspond to the thin layer heavily stained with lanthanum (compare Fig. 7C, D). It is not clear whether the light cross-striations in the lanthanum staining of this layer are indicative of pores or merely due to the grains of the stain. They are definitely smaller than the hexagonally arranged pores in the cuticulin layer of the chloride cells in ephemeropteran larvae (Komnick & Stockem, 1973). Also, the question cannot be answered at present as to whether the particulate structure seen in freeze-fracture (Fig. 7A) reflects porosity of the cuticulin layer or just an artifact, e.g. formation of wax crystals or ice crystals in the cement layer.

Junctional complex

Since lanthanum diffuses across the cuticle of the rectal chloride epithelia, it is available on the apical surface to test the presumptive barrier function of the junctional complex. The presence of electron-dense deposits in the septate junction (Fig. 6B, C, D) demonstrates that lanthanum is able to pass through the zonula adherens, which is generally believed to form a continuous girdle around the cell (Staehelin, 1974), and to invade further the intercellular cleft of the pleated septate junction. However, the tracer is always found restricted to the apically oriented part of the septate junction, extending for a maximum of three-quarters of the total junctional length (Fig. 6C, D). Even after continuous perfusion of the rectum with lanthanum for up to 8 days and irrespective of the colloidal or ionic lanthanum solutions used, the last quarters facing the basal side and the labyrinthine basolateral intercellular clefts are regularly devoid of tracer.

Energy dispersive X-ray spectra obtained from droplets of the external solution (1.5 % LaCl₃) clearly show the presence of chlorine and lanthanum (Fig. 8B), whereas the spectra of haemolymph droplets from experimental larvae reveal only the blood constituents sodium, chlorine, potassium and calcium, but no lanthanum (Fig. 8C). The lack of a lanthanum signal in connection with the analysis data of control

Fig. 6. Cross-sections of rectal chloride epithelia fixed after luminal in vivo exposure to aqueous 1.5 % lanthanum chloride. A. After 2 days of exposure. be, branchial epithelium; ce, chloride epithelium; tr, tracheole; l, narrow cleft of the rectal lumen between adjacent gill lamellae with particulate remnants of tracer. ×25 000. B. After 1 day of exposure. Margin of chloride epithelium (ce) followed by two transitional cells (t) and the branchial epithelium. 1. Lanthanum-labelled cuticle of chloride epithelium composed of epicuticle and endocuticle. 2. Unlabelled cuticle of transitional cells lacking endocuticle. 3. Unlabelled branchial cuticle consisting of epi- and endocuticle. Small arrows point to tracer particles between apical infoldings and in the apical portion of junctional complex. ×25 000. C. After 4 days of exposure. Particulate tracer is visible in the endocuticle and apical portion of the junctional complex. The arrow labels the end of the junctional complex in the basal direction. ×30 000. D. Higher magnification of c. Arrow points to the most basally located tracer particle detectable in this section of the junction complex. ×75 000.
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Fig. 8. A. Basal region of rectal chloride epithelium showing lysosome-like granules with particulate deposits (4 days in 1-5% LaCl₃). ×15 000. B. X-ray spectrum of a dried 10 µl droplet of the external tracer solution (1-5% LaCl₃) showing peaks of silicon (1) (artificial), chlorine (2), and lanthanum (3) (Laα; Laβ₁; Laβ₂). C. Diagram of a dried 10 µl haemolymph droplet withdrawn from larval A. cyanea after 4 days in external tracer solution (1-5% LaCl₃) showing peaks of sodium (1), chlorine (2), potassium (3), and calcium (4). D. Diagram of a spot analysis of a lysosomal granule as shown in A. Peaks 1, phosphorous; 2, lanthanum (Laα and Laβ); 3, chromium (Kα and Kβ); and 4, iron (Kα and Kβ). Chromium and iron are artifacts.

haemolymph droplets containing defined concentrations of LaCl₃ means that the haemolymph concentration of lanthanum — if present at all — is two orders of magnitude below the external concentration. On the other hand, lanthanum is identified

Fig. 7. Cuticle of rectal chloride epithelia showing epicuticular depressions. A. Freeze-fracture along the surface of the cuticle showing six epicuticular depressions in en face view. ×100 000. B. Cross-section of cuticle after 4 days of exposure to lanthanum tracer in the rectal lumen, showing four epicuticular depressions. f, microvillous tips of apical folds; epicuticle is labelled by a solid line, endocuticle by a broken line. Note the tracer accumulation in the bottom of and underneath the epicuticular depressions. ×150 000. C. High magnification of the epicuticular depression labelled with an arrow in B. ×400 000. D. High magnification of an epicuticular depression showing the outer (a) and inner (b) epicuticle after conventional fixation. ×400 000.
by elemental analysis inside the chloride epithelial cells in the form of electron-dense deposits confined in lysosome-like granules (Fig. 8A, D).

The results of the vital tracer experiments justify the conclusion that lanthanum ingested into the rectal lumen can pass through the cuticle of the chloride epithelia, preferably via epicuticular depressions. The tracer passes the zonula adhaerens and penetrates into the pleated septate junctions, but does not succeed in passing the entire length of the junction and in reaching the haemocoel at detectable concentrations. Lanthanum penetrating into chloride epithelial cells is segregated and stored in lysosome-like granules.

DISCUSSION

Plasma membranes

Ion absorption across the cells of the rectal chloride epithelia includes two kinds of membrane transport: absorption through the apical and excretion through the basal membrane. These locational and directional differences in membrane transport require a structural polarity of the chloride epithelia with respect to the two membranes involved. The differences so far demonstrated in the geometrical disposition, the enzymic complement, and the intramembraneous structure of the apical and basolateral plasma membranes, as well as the pericellular girdle of the pleated septate junction that demarcates the two functionally different plasma membrane areas, are undoubtedly manifestations of epithelial polarity.

Lane (1979) conjectured that the IMPs of the apical plasma membrane of the cockroach rectal pads act as transmembrane pathways for ions. It is premature to speculate about the function of the apical IMPs of the rectal chloride epithelia, because the proteins and transport mechanisms of the apical plasma membrane are still obscure. As to the basolateral IMPs, it has been shown that the basolateral plasma membranes of the chloride epithelia are rich in Na/K-ATPase (Komnick & Achenbach, 1979). Since the Na/K-ATPases of the mammalian kidney (Deguchi, Jørgensen & Maunsbach, 1977) and the avian salt gland (Gassner, 1982) have been identified as IMPs measuring 8–10 nm in diameter, it appears reasonable to assume that the particles of this size range that are present in the basolateral plasma membranes represent amongst others the Na/K-ATPase. Hence, the progressive numerical reduction of the basolateral IMPs following the transfer of the larvae from hypo- to hyperosmotic sea-salt solutions, in contrast to the unaltered particle density of the likewise interdigitated, but Na/K-ATPase-deficient plasma membranes of the adjacent fat-body cells (Komnick & Achenbach, 1979), is possibly indicative of a removal of Na/K-ATPase molecules from the sodium-pumping plasma membranes under these conditions. Such transfer into hyperosmotic salt solution leads to a rapid rise of the haemolymph concentration within 1 day. After 2 days the hyperosmotic regulation is re-established at an elevated concentration level but a low concentration gradient, the internal concentration exceeding the external by only about 10%. A similar effect of environmental salinity on IMP frequency was reported for nerve cells of an osmoconforming bivalve (Willmer, Skaer & Treherne, 1979). Hossler, Sarras & Allen (1978a)
and Hossler, Sarras & Barnett (1978b) concluded from the reversible and parallel increase in ouabain binding and Na/K-ATPase activity of the avian salt gland that ATPase molecules were inserted into the plasma membrane during acclimatization to osmotic stress and removed during de-stressing.

**Cell junctions**

The joint occurrence of the zonula adhaerens, pleated septate junction and gap junctions in the junctional complex of the rectal chloride epithelia is a further example for the characteristic combination of these three types of cellular junction in insect epithelia of ectodermal origin (Noirot-Timothée & Noirot, 1980). Our investigation confirms and contributes to the present general knowledge of the structure and function of these junctions.

It is widely accepted that the zonula adhaerens or belt desmosome is an adhesive structure providing mechanical cohesion of neighbouring cells (Farquhar & Palade, 1963), whereas the gap junction is a communicating structure coupling adjacent cells via the so-called connexons (Caspar, Goodenough, Makowski & Phillips, 1977; Makowski, Caspar, Phillips & Goodenough, 1977). These represent connexin hexamers and are visible in freeze-fracture as IMPs. The communicating channels are probably provided by the hydrophilic centres of the connexons, which appear as central depressions after rotary-shadowing (Sikerwar, Tewari & Malhotra, 1981) or as black dots after lanthanum infiltration (Revel & Karnovsky, 1967; Staehelin, 1974; Lane & Skaer, 1980).

The additional occurrence of numerous gap junctions interspersed on

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*Fig. 9. Perspective diagram of pleated septate junction of rectal chloride epithelium based on the correlative results of the different preparation techniques used.*
Fig. 10. A. Schematic cross-section of rectal chloride epithelium showing basolateral interdigitation of two epithelial cells (dark dots and light dots) and the topographical distribution of cellular junctions (zonula adherens, pleated septate junction, and gap junctions). The gap junctions in the basal region of the epithelium are interpreted as devices for direct co-ordination of the interdigitated cell processes. B and C. Schematic surface views on the apical (B) and basal (C) sides of rectal chloride epithelium illustrating (as a consequence of basal interdigitation) the different outline of the epithelial cells and the different sizes of the intercellular clefts available for paracellular diffusion at the apical and basal surface.

the basolateral plasma membranes of the interdigitated cell processes reflects a high degree of cell coupling of this transporting epithelium. These basolateral gap junctions appear to represent devices for the reduction of the homo- and heterocellular diffusion distances (Fig. 10A). This possibility of rapid exchange of substances through serial cross-connections between the processes of different cells, and between different processes of the same cell, presumably supports the efficient and simultaneous operation of sodium pumps facing the same intercellular space but located in the plasma membranes of different cells.

In spite of the instructive diagrams existing in the literature (e.g., see Noirot-Timothée & Noirot, 1973; Staehelin, 1974; Flower & Filshie, 1975; Lane & Skaer, 1980; Kukulies, 1982), the complicated structure and mode of function of the pleated septate junction are not fully understood. The similar arrangements of the pleated septa in tangential sections and the particle rows in freeze-fractures led to the suggestion that the beaded IMPs may serve as serial anchorage points for the septa in the opposing plasma membranes. However, the direct correlation of structural details and morphometric data obtained from thin sections and freeze-fracture replicas also depends on preparative parameters (Noirot-Timothée, Smith, Cayer & Noirot,
Most of the freeze-fracture studies of pleated septate junctions are based on glycerinated tissues. To our knowledge, only Flower (1971) presents an image of a pleated septate junction obtained from an unglycerinated native mollusc epithelium and points to structural differences in comparison to glycerinated preparations. Glycerol treatment, which is known for its artefactual effects on membranes (e.g. see Buckingham & Staehelin, 1969; Fineran, 1970; Niedermeyer & Moor, 1976), appears also to influence the structure of the pleated septate junctions in the dragonfly rectal chloride epithelia.

The diagram presented in Fig. 9 includes the observations made on fresh, frozen chloride epithelia. The bulging lines of the EF are considered to result from linear indentations of the ES of the junctional membranes at the insertion sites of the intercellular septa. These external indentations extend over the entire membrane thickness and consequently, in addition to the EF bulges, lead: (1) to linear elevations on the PS; (2) to corresponding linear depressions on the PF, which contain the stringed IMPs; (3) to the location of individual IMPs retained in the EF on top of the linear EF bulges; and (4) to the undulation of the junctional membranes seen in cross-section, which is probably preserved by the osmium fixative. The undulation of the junctional membranes caused by the intercellular septa reflects an intimate and tight connection between the two structures. The chemical nature of the septa, formerly conceived as membrane protuberances by some authors (Locke, 1965; Gouranton, 1967; Flower, 1971), is assumed to differ from that of the junctional membranes (Noirot-Timothee & Noirot, 1973).

The physiological role of pleated septate junctions is still a matter of debate (for a review, see Staehelin, 1974; Noirot-Timothee & Noirot, 1980; Lane & Skaer, 1980). All the functions exerted by the different types of cellular junctions have been attributed to them, being conceived as communicating, adhesive and occlusive structures. The first conception appears to be generally dismissed because of the joint occurrence of septate and gap junctions (Hagopian, 1970; Flower, 1971; Lane & Skaer, 1980). This holds also for the chloride epithelia, which contain numerous communicating gap junctions separated from and associated with the pleated septate junctions. A similar association is also realized with the adhesive-belt desmosomes. Admittedly, the intimate cell interlockings resulting from the highly convoluted junctional membranes probably also serve to give mechanical cohesion to the epithelial cells. However, we regard this adhesive function as a secondary effect of a primary occlusive function. Since the belt-desmosomes appear to be readily permeable to the lanthanum tracer, the gap junctions are restricted in their extent and tight junctions are absent, no other junctional structures but the pleated septate junctions remain for the maintenance of the large difference in concentration.

This conclusion is supported by the finding that the tracer is able to penetrate into the septate junction, but is unable to pass through its entire length, although the result obtained with lanthanum cannot be taken as direct evidence for the junctional tightness against Na⁺, Cl⁻ and water, which represent the most important osmoregulatory freight. Different lanthanum-staining properties along the junctional extension, rather than limited lanthanum penetration, can be excluded, because such specific
lack of lanthanum staining in the basally oriented portion of the junction was not observed when lanthanum was applied during fixation. The attribution of an occlusive role to the pleated septate junction of the rectal chloride epithelia is in accordance with the findings of numerous authors (e.g., see Flower & Filshie, 1975; Lord & DiBona, 1976; Szollosi & Marcillou, 1977; Noirot-Timothée et al. 1978; Green & Bergquist, 1982), but in contrast to views of others, who denied such a role (Lane, 1978; Lane & Chandler, 1980). Following the interpretation of Flower & Filshie (1975) and Noirot-Timothée & Noirot (1980), the intercellular septa appear to represent impermeable obstacles on the paracellular route. Their irregular interruptions force diffusing substances to make a detour but allow a further invasion of the junction. This kind of labyrinthine detour implies an extension of the diffusion distance depending on the number, length and orientation of the septa and the number, width and position of the septal interruptions.

Without going into morphometric and statistical analyses, an estimate of the septate junction depicted in Fig. 2A is presented for rough orientation about these distances. The tortuous septate junction in Fig. 2A extends over an idealized stretch of 2-4 μm in the apicobasal direction. Unfolding of the convoluted junctional membranes into a straight course leads to an approximately tenfold extension, the straightened septate junction measuring 21 μm in length. Since at this position the total thickness of the chloride epithelium amounts to 26 μm, the paracellular diffusion route across the epithelium is nearly twice as long as the transcellular route. The total number of 695 septa identifiable in this cross-section is nearly half the theoretical maximum value (1300) estimated on the basis of junctional extension (21 μm) and septal spacing (16 nm). Although the average length of the septa is not known, continuities for up to 2-4 μm were measured in tangential thin sections and freeze-fractures before the septa 'ran out' of the preparations. Therefore, their actual length can be expected to exceed this value. Assuming that the average detour per septum counted is 2 μm, the paracellular route would exceed the transcellular route by a factor of 50. In other words, the diffusion pathway implicated in this septate junction exceeds the 2-4 μm wide zone into which the convoluted junction is confined, by a factor of approximately 600. This factor impressively illustrates the immense, labyrinthine detour required for the complete penetration of the pleated septate junction. According to Fick's (1855) diffusion law,

$$\frac{dm}{dt} = -DF \frac{dc}{dx},$$

the net flux $\frac{dm}{dt}$ depends on (in addition to the diffusion constant ($D$) and the concentration difference ($dc$)) two structural parameters, being directly related to the surface area ($F$) available for diffusion and inversely related to the diffusion distance ($dx$). Consequently, the great extension of the diffusion distance inherent in the pleated septate junction causes a substantial reduction of paracellular net diffusion across the chloride epithelium.

As to the other structural parameter, the paracellular diffusion area, provided by the more or less hexagonal pattern of intercellular clefts at the apical surface, is only
a small fraction of the area that is made available for diffusion at the basal surface by
the labyrinthine intercellular spaces resulting from cellular interdigititation (Fig. 10b, c).
Consequently, paracellular net diffusion of solutes concentrated in the inter-
cellular spaces by the pump in the basolateral plasma membranes is favoured towards
the haemocoel, whereas little (if any) net leakage into the rectal lumen is expected.
According to the principle of leak and pump (Wilbrandt, 1960), some apical leakage
is tolerable, provided that the efficiency of the pump is high enough or, as formulated
by Lane & Skaer (1980, p. 71), 'the maintenance of a transepithelial potential need
not depend on sealed intercellular spaces but is a function of the relationship between
the electrical resistance of the epithelium and the rate of ion transport'. Therefore, the
pleated septate junction need not necessarily be absolutely tight for the successful
operation of the basolateral pumping sites in transepithelial sodium absorption.
As with the tightness and leakiness of vertebrate tight junctions and their possible
correlation with the number of junctional strands (Claude & Goodenough, 1973;
Schiller, Forssmann & Taugu, 1980), the occlusive function of the pleated septate
junction comes about by structural control of the paracellular route, rather than by
formation of a structural seal. Stressing this functional aspect, Lord & DiBona (1976)
proposed the designation 'limiting junctions' for both tight and septate junctions.
Thus the relative tightness of the septate junction is equivalent to the reduction of net
diffusion, resulting – apart from possible chemical interactions – from the extension
of the diffusion distance, which in turn is caused by the collective detour effect of the
intercellular septa and the convolution of the junction.
Using the method of Machen, Erlij & Wooding (1972) of unidirectional in vivo
application of lanthanum, numerous authors have tested the possible tightness of
pleated septate junctions in a variety of organs and reported, in some cases, the limited
tracer penetration, as found with chloride epithelia, and in others the complete tracer
perfusion of the junction (e.g., see Lane, 1972; Treherne, Schofield & Lane, 1973;
Leslie, 1975; Szollosi & Marchaillou, 1977; Ryder & Bowen, 1977). Since these so-
called in vivo applications included preceding tissue dissections or even in vitro
incubation of the isolated tissue, preparative differences may be responsible for the
discrepancy, as well as differences in the relative tightness of septate junctions of
various origins. In some epithelial barriers of arthropods, tight junctions resembling
the vertebrate tight junctions, and representing the tight barrier against lanthanum
infiltration occur either alone or in association with pleated septate junctions (e.g., see
Lane, Skaer & Swales, 1977; Lane, 1979a, b; Lane & Chandler, 1980). The isolated
reports on the occurrence of vertebrate-type tight junctions in invertebrates do not
justify the generalization of the suggestion that the pleated septate junctions are
permeable and the occlusive function is exerted by vertebrate-type junctions (Lane,
1978). This suggestion is not valid for the dragonfly rectal chloride epithelia, which
are devoid of vertebrate-type tight junctions.

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Cell junctions of dragonfly rectum


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