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Osmotic Reversal Induces Assembly of Tight Junction Strands at the Basal Pole of Toad Bladder Epithelial Cells but Does Not Reverse Cell Polarity

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Summary. This paper reports the effect of reversing the osmotic environment between luminal and serosal compartments of a toad urinary bladder on the polarity of assembly of tight junction strands. Toad bladders were filled with Ringer's solution (220 mOsm) and were immersed in distilled water at room temperature or at 37°C. Within two minutes, new tight junction strands are assembled. The new tight junctional strands unite the basal pole of epithelial cells with the apical side of basal cells. Physiological studies show that oxytocin, a synthetic analog of antidiuretic hormone, is still capable of inducing increases in water transport in epithelia which were osmotically reversed. This capacity decreases significantly for longer periods of osmotic reversal. Osmotic reversal does not alter the original polarity of epithelial cells: 1) the apical tight junction belt, at the apical pole, is not displaced; 2) the freeze-fracture morphology typical of apical plasma membrane (particle-rich E faces; particle-poor P faces) is not altered; 3) oxytocin and cyclic AMP induce aggregates which are observed only at the apical plasma membrane. Massive assembly of junctional elements occurs even in epithelia preincubated in the presence of cycloheximide (an inhibitor of protein synthesis) or of cytoskeleton perturbers. Our experiments show that the polarity of assembly of tight junction strands depends on the vectorial orientation of the osmotic environment of the epithelium.

Key Words tight junctions · freeze fracture · transporting epithelia · water permeability · membrane structure

Introduction

Epithelial cells are linked, always at their apical pole, by tight junctions—a continuous belt of anastomosed strands [10, 11, 24, 30]. The tight junction creates two separate environments thereby assuring the topological definition of the spaces (outside *vs.* inside) that characterizes most epithelia.

The assembly and/or reassembly of tight junction elements can be induced experimentally either *in vivo* [18, 26–28] or *in vitro*, both in cell or organ culture [9, 12, 16, 25, 33] or by incubation of specimens in non-nutrient solutions [2, 14, 29]. Assembly of new strands can occur within a few minutes [14, 25] and may occur spontaneously upon excision of the tissue in situ, postmortem or even during fixation by glutaraldehyde perfusion *in vivo* [14]. In prostrate epithelial cells, assembly of the new strands proceeds in the absence of oxygen, dinitrophenol (a metabolic uncoupler) or of cycloheximide at concentrations that inhibit significant synthesis of proteins [14]. Both the structural stability and the physiologic integrity of the tight junction band appear to depend directly or indirectly on the organization of cytoskeletal elements, in particular microfilaments [2, 5, 17].

The single unifying characteristic in the instances of assembly described above is the brutal challenge of the cellular and tissue environment. We reasoned that one way to challenge an epithelium is to reverse its osmotic environment. In the prostrate, as in most other tissues, this is not easily achieved. In contrast, reversal of osmotic conditions is easily done in the amphibian urinary bladder, an elective model for the study of the transport properties of epithelia [15]. Here, we show that osmotic reversal leads to massive and rapid assembly of new tight junction strands. The new junctional strands unite the *basal* pole of luminal epithelial cells with the *apical* side of basal cells. While, at first, our results might indicate a reversal of cell polarity, physiological measurements and comparison of the freeze-fracture morphology of apical and basal areas of cell membrane show that the polarity of the epithelial cells is retained.

Materials and Methods

Toads (*Bufo marinus*) were obtained from National Reagents (Bridgeport, Conn.) or Carolina Biological Supply Company (Burlington, N.C.). Bladders were removed from pithed toads and each of the two lobes ("hemibladders") was mounted as a sac at the tip of a glass canula [1]. The hemibladders were then

Table. Effect of osmotic reversal on the capacity of the tissues to respond to oxytocin[a]

Series	Variations of net water fluxes ($\mu l \cdot min^{-1} \cdot cm^{-2}$)				
	1	2	3	4	5
Control ($n = 11$)	2.19 ± 0.22	2.13 ± 0.19	2.02 ± 0.22	1.79 ± 0.15	1.85 ± 0.27
2 min osmotic reversal ($n = 6$)	2.40 ± 0.16	1.6 ± 0.16[c]	2.24 ± 0.32[b]	2.0 ± 0.32	1.92 ± 0.32
5 min osmotic reversal ($n = 6$)	2.80 ± 0.32	0.80 ± 0.08[d]	1.69 ± 0.20[c]	1.76 ± 0.30[b]	1.68 ± 0.32[b]
10 min osmotic reversal ($n = 6$)	2.80 ± 0.30	0.56 ± 0.20[d]	1.12 ± 0.20[d]	1.28 ± 0.21[c]	1.28 ± 0.21[c]
30 min osmotic reversal ($n = 6$)	2.54 ± 0.25	0.18 ± 0.07[d]	0.56 ± 0.14[d]	0.53 ± 0.12[d]	0.59 ± 0.17[d]

[a] Five successive stimulations with oxytocin (10^{-8} M) were performed on the same preparation according to the protocol explained in Fig. 13. With increasing periods of osmotic reversal (in particular after short recuperation time) the variation of net water fluxes (Δ_1, to Δ_4) differs significantly from the value measured before osmotic reversal (Δ max).
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] $P < 0.001$.

kept in amphibian Ringer's solution ("Ringers") on both sides so that no net transepithelial water flow could take place (mM: NaCl 112, $CaCl_2$ 1, KCl 5, and $NaHCO_3$ 2.5, pH 8.1 when bubbled with air; + glucose 20) [3–5, 7, 15].

To reverse the normal osmotic environment, toad hemibladders filled with amphibian Ringer's solution were immersed in distilled water. The bladders were incubated under these conditions for 2 to 60 min, either at room temperature (RT) or at 37°C. Controls consisted of paired hemibladders filled with and immersed in Ringer's, incubated at the same temperature and for the same period of time as experimental hemibladders. At the end of the incubation period each hemibladder was sliced in a solution of 2% glutaraldehyde in Ringer's at RT and fixed overnight at 4°C. In other experiments, the epithelia were scraped off [31] from the connective tissue and immediately fixed in glutaraldehyde, as above. Long periods of exposure to osmotic reversal did not affect the viability of epithelial cells. This was shown by: 1) exclusion of Trypan blue; 2) staining of nuclei with Acridine orange (a vital stain); and 3) absence of staining by Eosin (a nonvital stain).

To test the influence of protein synthesis on the assembly of tight junction strands, hemibladders were incubated with cycloheximide (Sigma, 200 μg/ml in Ringer's; *see* ref. 22) for 30 min at RT and 5 min at 37°C before immersion in triple-distilled water. Paired control experiments were incubated in Ringer's solution.

To test the effect of cytoskeleton disruptors, hemibladders were first incubated for 2 hr at RT with colchicine (Sigma, 10^{-3} M, in Ringer's) and then incubated with colchicine (10^{-3} M) plus cytochalasin B (Sigma, 100 μg/ml in Ringer's) for 2 hr at RT before osmotic reversal. Paired controls were incubated first in Ringer's then with 1% DMSO, the solvent used for cytochalasin B.

For freeze fracture, fixed tissues were impregnated in 30% glycerol in Ringer's solution, frozen in Freon 22 (partially frozen by liquid nitrogen), freeze fractured and the fracture faces replicated by evaporation of platinum/carbon. To assess the frequency of fracture faces of plasma membrane displaying tight junction strands, each replica was coded and at least 100 plasma membrane fracture faces scanned. The results were analyzed by Student *t*-test.

To test whether epithelia subjected to osmotic reversal retained the capacity to increase their water permeability in response to ADH, hemibladders were mounted horizontally between two Lucite® chambers, the serosal border facing the upper solution. The net water flux was measured according to the technique of Bourguet and Jard [5]: water was automatically injected into the lower chamber to maintain a constant volume and the magnitude of this fluid movement, equivalent to the net flux, was recorded every minute (Fig. 13). The serosal face of this tissue was bathed with Ringer's, while the NaCl concentration was reduced to 5.6 mM on the mucosal side, making the solution largely hypotonic and creating a driving force for the water movements through the preparation. A typical experiment is described in Fig. 13. Preparations were pretested by oxytocin (10^{-8} M), then submitted to osmotic reversal, the serosal compartment being filled with distilled water for periods of 2, 5, 10 or 30 min. Normal osmotic conditions were then reestablished and after 10 min, 1, 2 and 3 hr, preparations were again stimulated by oxytocin.

For each experimental condition, the bladders of six animals were studied. Variations in net fluxes of water (expressed in $\mu l \cdot min^{-1} \cdot cm^{-2}$) were obtained by subtracting basal levels of water transport from the asymptotic values obtained after stabilization of the response.

These values were averaged and treated statistically (*see* Table). For each bladder, the effect of osmotic reversal on water permeability was given by the ratio of net water flux variation before and after osmotic reversal (Fig. 14).

The previous experiments were designed to establish with physiological measurements the effects of osmotic reversal on water permeability. In addition, we searched for the presence of ADH-induced aggregates of intramembranous particles [3, 4, 7, 13] in preparations that were exposed to osmotic reversal, allowed to recover for 30 min, challenged either by oxytocin [19] (10^{-8} M) or cAMP (10^{-2} M) for 30 min at room temperature, fixed in 2% glutaraldehyde and processed for freeze fracture.

Results

Fine Structure

The fine structure of the amphibian urinary bladder has been described [8, 20, 21]. Briefly, the bladder consists of a complex epithelium, a submucosa and a serosa. The epithelium (Figs. 1, 2) is mainly comprised of a continuous layer of granular and mitochondria-rich cells (approximately 90 and 10%, respectively), lined by basal cells. Granular and mitochondria-rich cells are surrounded by and linked together by a well-developed tight junction band, approximately 0.5 μm wide (Figs. 1 and 3). Laterally, epithelial cells display long infoldings, are mutually imbricated, and are united by sparse desmosomes (Figs. 1, 2; arrowheads). Gap junctions are rare. Basal epithelial cells (Figs. 1, 2) have simpler, less infolded, contours and are in frequent apposition to the basal regions of the luminal epithelial cell layer. The plasma membranes of basal cells display numerous pinocytotic vesicles (Fig. 2, arrows). In freeze-fracture replicas, the presence of these pinocytotic openings serves as a morphological marker in the identification of basal cells (Figs. 4–6, 8 and 9).

Assembly of Tight Junction Strands upon Osmotic Reversal

When toad urinary bladders filled with Ringer's solution were immersed in distilled water for 10 to 60 min at room temperature or at 37°C, massive assembly of junctional strands occurred (Figs. 4–7). The frequency of fracture faces displaying new junctional strands is shown in Fig. 12. New strands were observed over fractures at the basal level of both granular and mitochondria-rich cells (recognized by typical rod-shaped particles; *see* Fig. 7 and Refs. 3, 34) and the apical face of the plasma membrane of basal cells (Figs. 4–6). The strands were morphologically identical to those of the apical tight junctions in imperturbed control epithelia: they consisted of interconnected cylindrical structures that generally partitioned with the protoplasmic (P) faces of the plasma membrane, leaving complementary linear grooves over the exoplasmic (E) faces (Fig. 6). Frequently, the course of fracture exposed areas of the plasma membranes in apposed cells. Here, the cylindrical strands on the P face of one cell were always both contiguous and continuous with the linear grooves observed on the E faces of the apposed cell membrane (Fig. 6). We concluded, therefore, that the junctional strands and grooves represented sites of specialized intercellular contacts. Similar aspects were observed in isolated epithelia that, before fixation, had been scraped from the submucosa.

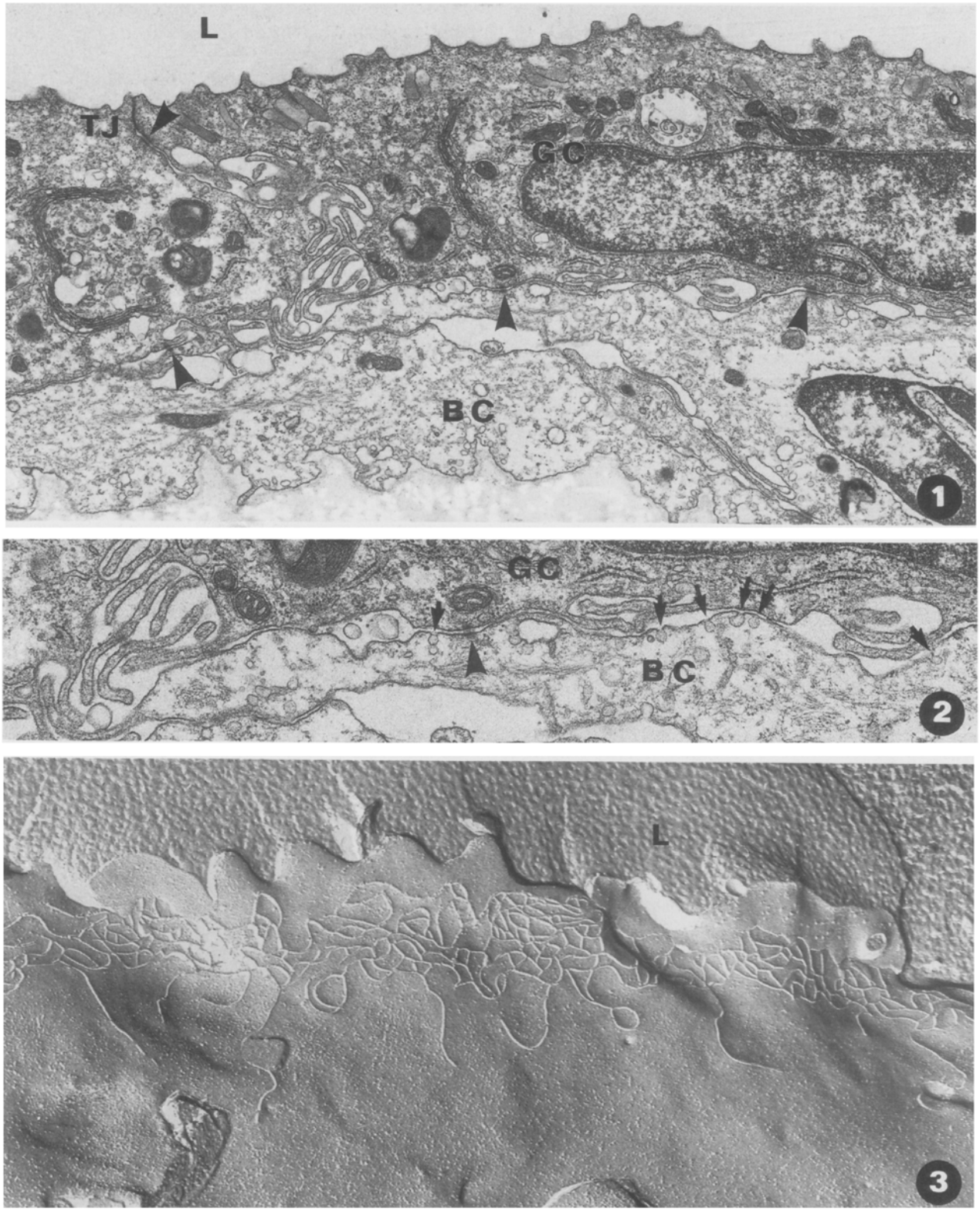
Shorter exposures of the toad bladder to osmotic reversal showed that the process of assembly of tight junction is fast, with 42% of the fracture faces displaying tight junction strands after only 2 min at 37°C (Figs. 8, 11). The complexity of the junctional network appeared to increase with the duration of osmotic reversal, with shorter, loose strands commonly seen in specimens incubated for 10 min or less (Figs. 4, 8). However, due to intense variations—even within a single fracture face—statistical analysis of the length of the junctional strands per unit area was not appropriate.

The junctional strands that were assembled in response to osmotic reversal appeared stable: in hemibladders that, after osmotic reversal (10 to 60 min at RT or 37°C), were returned to normal osmotic conditions for 1 to 3 hr, the frequency of membranes displaying tight junction strands did not increase (*not illustrated*).

Osmotic reversal had no noticeable effect on the ultrastructure and position of the apical tight junction band, except for occasional short prolongations of apical strands along adjacent areas of the lateral plasma membrane (Fig. 3). No additional junctional strands were observed over contiguous regions of lateral membranes. We examined also the freeze-fracture morphology of apical membranes of bladders that had been subjected to osmotic reversal. Even after 1 hr of osmotic reversal, the freeze-fracture appearance of both P and E faces remained unaltered with the E faces displaying the high density of large particles that is typical of the exoplasmic faces of apical membranes (*compare* Figs. 11 and 10).

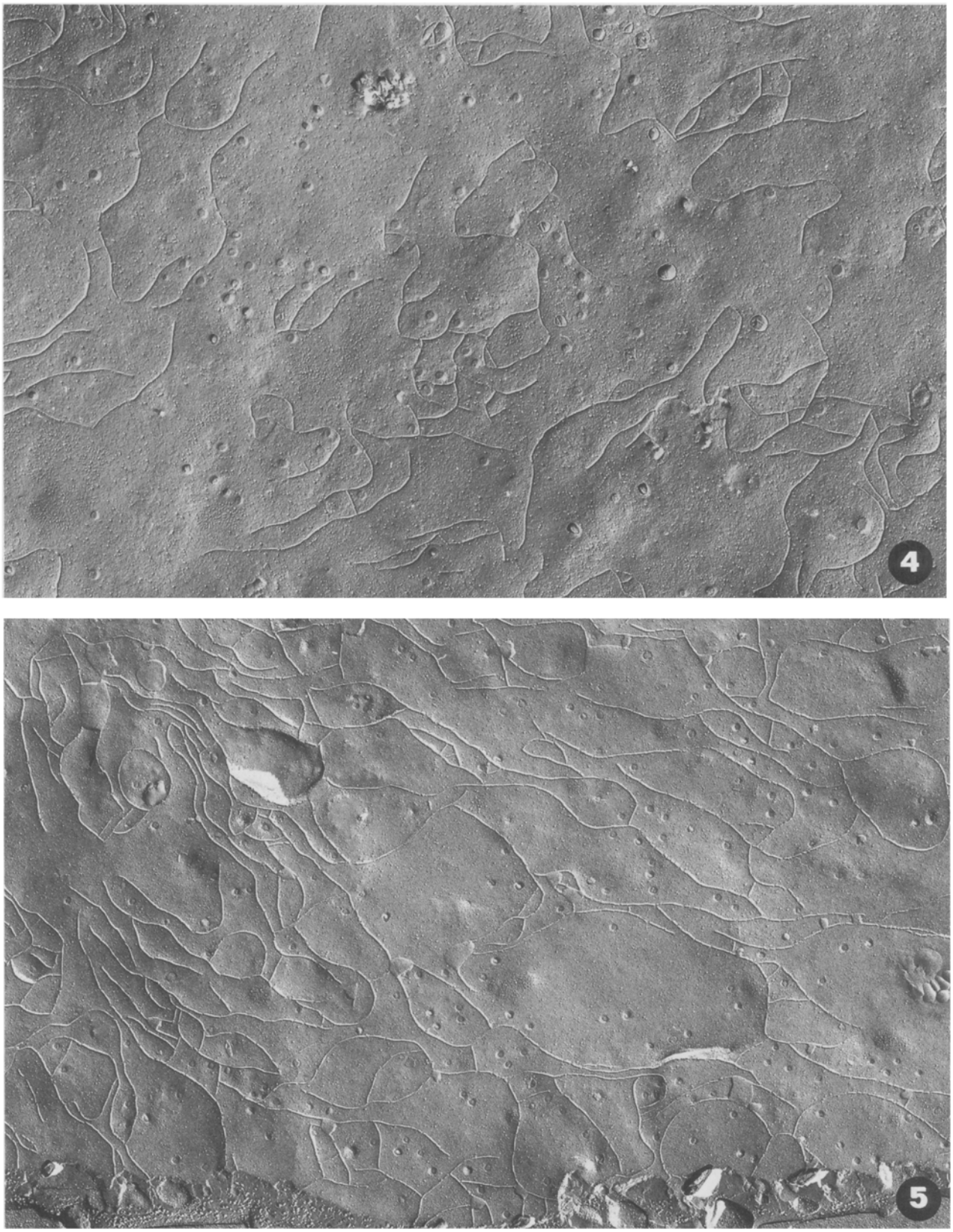
To investigate whether ongoing synthesis of protein molecules was necessary for the assembly of junctional strands we incubated hemibladders in cycloheximide before osmotic reversal. Freeze fracture revealed that neither frequency, apparent intensity, nor the pattern of assembly of junctional strands was altered (Fig. 9, 12). Observation of control hemibladders (no osmotic reversal, presence of cycloheximide) showed that the inhibitor had no apparent effect on the ultrastructure of the apical tight junction band and did not induce assembly of new junctional strands.

Paired hemibladders were also incubated with cytoskeleton perturbers before osmotic reversal. For these experiments, one hemibladder was incubated in colchicine then in colchicine + cytochalasin B. Both hemibladders were then subjected to osmotic reversal (30 min at 37°C). In both cases, identical massive assembly of tight junction strands occurred.



Figs. 1 and 2. Toad bladder epithelium. *GC*, granular epithelial luminal cell; *TJ*, apical tight junction band; *BC*, basal cell; *L*, lumen; arrowheads, desmosomes; arrows, pynocytotic vesicles in basal cells; Fig. 1, 15,000×; Fig. 2, 30,000×

Fig. 3. Appearance of the apical tight junction band of a granular cell after osmotic reversal (30 min at RT). While the typical network remains unaltered, short prolongations of strands into the lateral membranes can be observed. 45,000×



Figs. 4 and 5. Formation of tight junction strands after 10 min (Fig. 4) and 60 min (Fig. 5) of osmotic reversal at RT. The P faces of basal cells (recognized by numerous pynocytotic openings; *see* Fig. 2, arrows) are covered by numerous interconnected strands. Fig. 4, 40,000×; Fig. 5, 30,000×

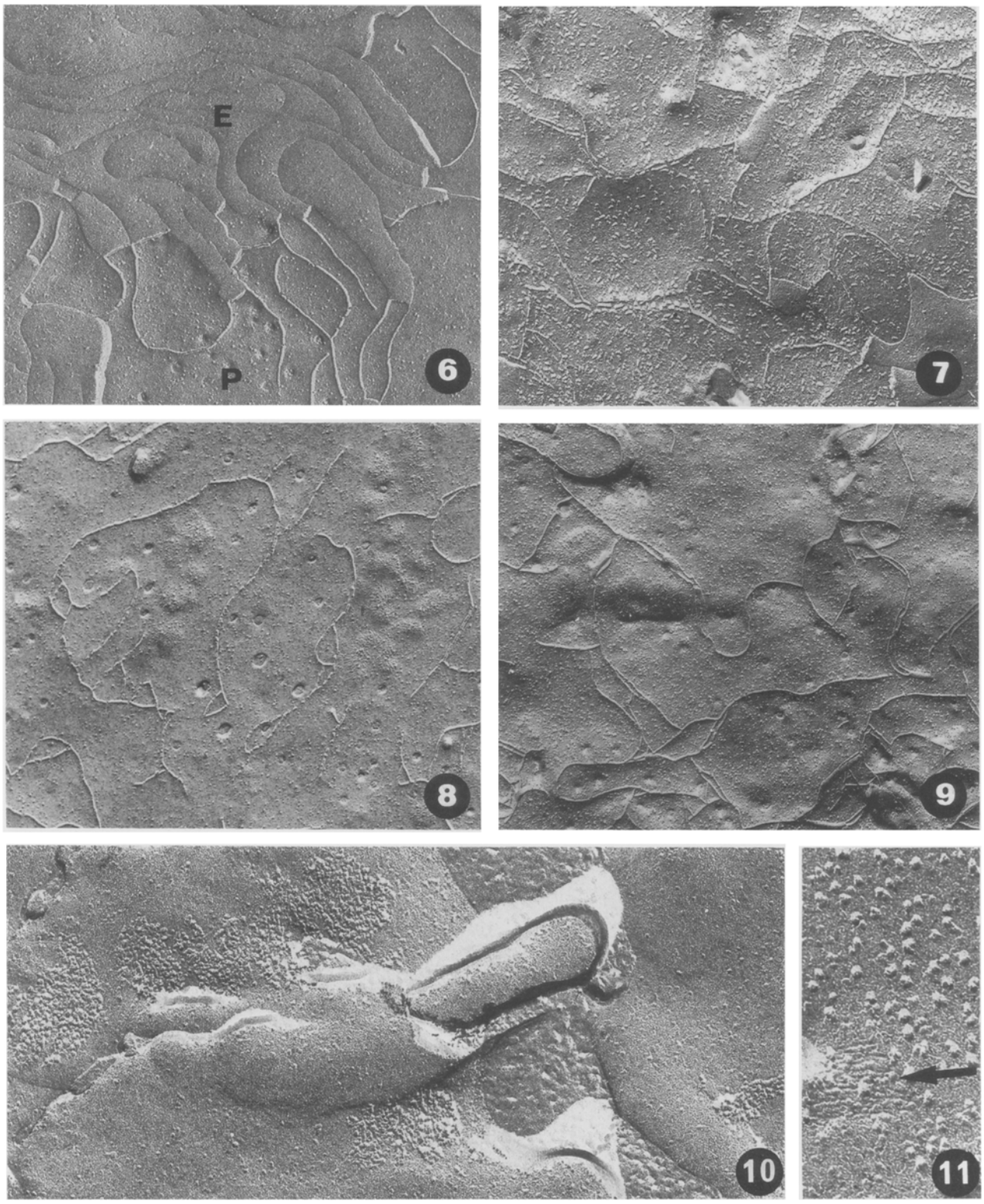


Fig. 6. The strands correspond to intercellular junctional structures: at fracture transitions, contiguous aspects of face E/face P show that the junctional strands on face P are always followed by complementary linear grooves on face E. The micrograph shows the E face of the basal area of the plasma membrane of a luminal epithelial cell overlaying the P face of the apical side of a basal cell (identified by pynocytotic openings; *see* Figs. 2, 4, 5). 43,000×

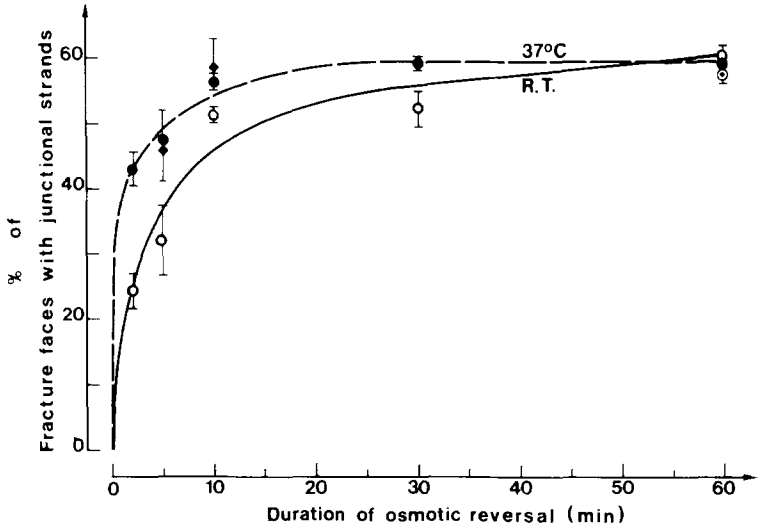


Fig. 12. Frequency of tight junction strands after different periods of osmotic reversal at room temperature (○) and at 37°C (●). Preincubation in cycloheximide at 37°C (◆) or at room temperature (⊙) had no effect on the frequency of fracture faces with junctional strands

Osmotic Reversal, Recovery and Water Permeability

Examination of the thin section ultrastructure of osmotic reversed epithelia showed that, as expected, cells were swollen (*not illustrated*). Upon reestablishment of normal osmotic equilibrium the morphology of epithelial cells regained a normal appearance.

Measurement of the water permeability increases in response to ADH showed that osmotic shock had impaired the capacity of the tissues to respond to oxytocin. Tracings of paired hemibladders showed that osmotic reversal for periods of 2, 5, 10 or 30 min decreased the response to oxytocin by 30 to 90%. Figure 13 illustrates a typical time-course study. While increasing periods of osmotic reversal caused progressive impairment of the capacity of the bladder to respond to the hormone, upon reestablishment of normal osmotic conditions for 1 hr or more, the epithelia appeared to recover partially (Table, Fig. 13). Few, but typical particle aggregates were observed at the apical pole of epithelial cells that after an osmotic reversal of 10 to 30 min were allowed to recover for 30 min and were challenged either by oxytocin or by cAMP. After 1 hr of osmotic reversal, we no longer observed aggregates in oxytocin-treated cells but we still found a few intramembranous particle aggregates after stimulation by cAMP (Figs. 10, 11).

Discussion

We show here that the experimental reversal of the osmotic environment of a toad urinary bladder causes rapid and massive assembly of tight junction strands. Analysis of freeze-fracture images shows that, structurally, the strands correspond to actual intercellular tight junction elements which unite the basal areas of the plasma membrane of luminal epithelial cells with the plasma membranes of basal cells. The new junctional elements occur in membrane areas and in cells where tight junctions have never been reported. Osmotic reversal induces the formation of new junctional strands even if the toad bladders are preincubated in cycloheximide, under these conditions that prevent significant synthesis of protein [22, 25]. The assembly of new strands is not affected by disruptors of the cytoskeleton. The new tight junction elements appear irregularly distributed with striking differences even between

Fig. 7. (*facing page*) Tight junction strands in a mitochondria-rich cell (recognized by their rod-shaped particles; *see* Refs. 3, 31). 46,000×

Fig. 8. Assembly of tight junction strands after 2 min of osmotic reversal at 37°C. 35,000×

Fig. 9. Assembly of tight junction strands in tissues incubated in cycloheximide (200 μg/ml; 30 min at RT and 5 min at 37°C) before osmotic reversal (10 min at 37°C). 35,000×

Figs. 10 and 11. Protoplasmic (Fig. 10) and exoplasmic (Fig. 11) fracture faces of toad bladder epithelial cell submitted for 1 hr to osmotic reversal, allowed to recover and subsequently challenged by cyclic AMP (10^{-2} M). Arrow: typical groove network of ADH-induced aggregates. 60,000×

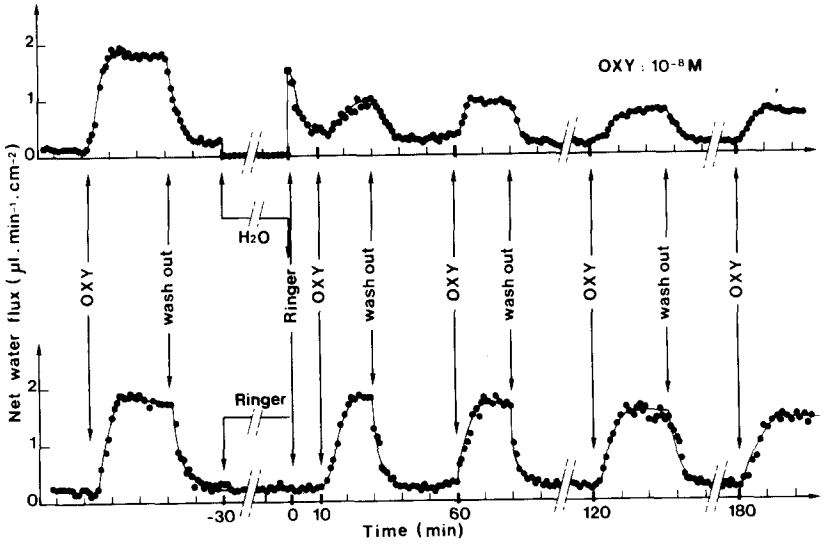


Fig. 13. Time course of the net water flux in a control hemibladder (lower graph) and a hemibladder submitted to osmotic reversal for 30 min (upper graph). Both hemibladders were pretested with oxytocin. One of the hemibladders was then submitted to osmotic reversal with the serosal compartment filled with distilled water for 30 min. Normal osmotic conditions were reestablished and, after 10 min, 1, 2 and 3 hr, preparations were again challenged by oxytocin

neighboring cells and frequently as patches or "macculae." Therefore, a new continuous tight junction belt is not established at the basal region of epithelial cells. This discontinuous nature prevented the use of electron-dense tracers to test any occluding properties of the junctional elements.

Our experiments provide initial evidence for the kinetics of appearance of newly assembled tight junction strands. Either at room temperature or at 37°C, the assembly process has a rapid onset: half of the maximum frequency of fracture faces with tight junction strands is reached within a few minutes. Maximum frequencies are reached within 30 min; after that, both frequency and the apparent complexity of the network appear stabilized. The ultrastructure of the apical tight junction band is not significantly affected by osmotic reversal. Conversely, reconstitution of a normal osmotic environment after osmotic reversal does not cause apparent disassembly of the new basal tight junction strands.

Our results indicate that, similarly to prostrate epithelial cells [14], the assembly of intercellular tight junction strands does not require the synthesis of new proteins and must represent a reorganization of preexisting membrane components. Fast, massive and rapid assembly of junctional strands, even in the presence of inhibitors of protein synthesis, indicates that assembly proceeds from preexisting lipid and/or protein components of the basolateral regions of the plasma membrane of apical epithelial cells as well as of the plasma membrane of basal cells. Alternatively, but in our opinion less likely, assembly could proceed from a preexisting cytoplasmic pool of junctional components.

Our results fail to show that strands assembly proceeds from the lateral adhesion of prelinearly arranged components. Assembly appears to proceed from a linear progression from fewer initial sites. This, and the speed of assembly lend circumstantial support to our previous proposal (24) that tight junctions represent sites of linear intercellular fusion where the intercellular continuity of the outer leaflets of the apposing membranes is established, with the junctional strands representing inverted lipidic cylindrical micelles, with a varying complement of integral membrane proteins. While the present results indicate that the synthesis of new proteins is not essential to the assembly of tight junction strands, they do not imply a secondary role for proteins in the definition of the structural and physiological properties of tight junctions. Membrane proteins—in particular integral membrane proteins—are likely components of tight junctions [12, 24]. But are they necessary components of the junctional strands that are assembled under our experimental conditions? In other words, it is not possible that many, most or even all of these newly assembled strands have little or no protein? To account for the image of the strands that we observed all that may be required is the existence of a linear topological differentiation along the bilayer. Such differentiation could, *a priori*, be provided by an essentially lipidic structure that, by freeze fracture, would appear identical to the strands observed in apical tight junction bands. At present, however, the molecular mechanisms for the initiation and growth of tight junction elements are unknown. Even if it is assumed that the junctional strands represent offset doublets of inverted cylindrical micellar membranous structures, the participation of proteins is not only plausible as it may prove crucial to the mechanisms of intercellular recognition that

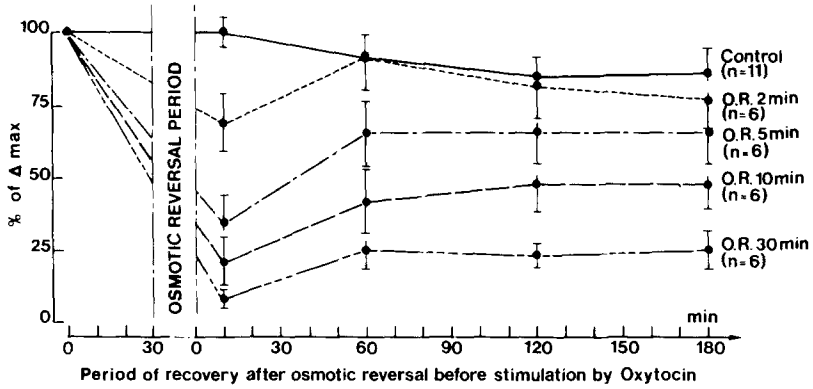


Fig. 14. Effect of osmotic reversal on the hydroosmotic response to oxytocin. Long period of osmotic reversal significantly impaired the physiological response of the tissue to oxytocin (*see* Table). All results are expressed as % variation of the net flux of water in each hemibladder before osmotic reversal (Δ max)

probably mediate initiation and growth of the doublet strands. At this point it is important to reemphasize the intrinsic membrane nature of the tight junction: just as biological membranes are comprised of a bilayered topological continuum interrupted by integral membrane proteins, so can tight junctions be envisaged as including a topologically differentiated linear micellar lipidic phase, modulated by varying complements of integral proteins [24]. This view would account for the structural and physiological diversity of tight junctions in different epithelia.

The formation of tight junction strands uniting the basal pole of toad bladder epithelial cells with apical side of basal cells would at face value indicate that reversal of osmotic conditions led to reversal of the polarity of assembly of tight junction strands. In other words, the inversion of the vectorial orientation of the osmotic relation in the epithelium would lead to inversion of the polarity of assembly of tight junction strands and, by extension, to inversion of the polarity of epithelial cells. However, a deeper analysis indicates that this is not the case. As we worked with intact tissue (as opposed to isolated epithelia) it was easy to ascertain the orientation of the specimens, with the microvilli-rich apical membrane facing an unobstructed lumen and the basal areas of the plasma membrane apposed to the apical portion of basal cells in the contiguous tissue. We could, therefore, ascertain that the freeze-fracture morphology typical of the apical membrane of epithelial cells remains invariant: the E face remains a particle-rich face and the P face particle-poor. Except for the appearance of tight junction strands, the freeze-fracture morphology of the basal area of epithelial cells also remains invariant, with no reversal of the partition of intramembranous particles (i.e., the appearance of large particles typical of the apical E membrane face). Moreover, oxytocin and cAMP result in the formation of typical aggregates of particles over the P face of *apical* membranes of granular cells but never over the *basal* membranes. Our physiological experiments show that the epithelium remains structurally intact without major leaks and retains an albeit limited capacity to increase permeability to water upon treatment with the antidiuretic hormone.

All reported instances of abnormal, massive, assembly of tight junction strands appear to involve conditions leading to unusual cellular stress [14]. Gap junctions can also be easily and rapidly assembled *in vitro*, provided that the tissues are preincubated in the cold and/or with cytoskeletal disruptors [32]. Because of their crucial, specialized functions, and also because of their elaborate morphology, intercellular junctions are generally perceived as higher states of membrane organization [23], thereby requiring special mechanisms for their assembly and structural maintenance. However, as discussed above, junctional assembly occurs spontaneously under conditions that are likely to involve the loss or impairment of cellular control mechanisms. Therefore it may prove more fruitful to investigate the cellular controls that must be retained in order to prevent—rather than promote—the spontaneous assembly of intercellular junctions.

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