Cell Adhesion, Polarity, and Epithelia in the Dawn of Metazoans

M. CEREIJIDO, R. G. CONTRERAS, AND L. SHOSHANI

Center for Research and Advanced Studies (CINVESTAV), México, Mexico

I. Introduction
A. For almost a century transepithelial transport was a hard-to-believe hypothesis
B. The puzzle of the first metazoan
II. Model Systems of Transporting Epithelia
III. The Na⁺/H⁻-ATPase
A. The α-subunit
B. The β-subunit
C. α/β-Subunit interactions
D. The γ-subunit
E. Assembly and delivery of the Na⁺-K⁺-ATPase
F. The triggering effect of Ca²⁺
G. The polarized distribution of Na⁺-K⁺-ATPase
IV. Tight Junctions
A. Structure
B. Transmembrane proteins
C. Membrane-associated proteins
D. Lipids
E. Diversity arising from processing TJ proteins as well as by the expression of several isoforms
F. Proteins that can localize to the nucleus and adhesion complexes
G. Phosphorylation in TJ assembly and function
H. Biogenesis
V. Septate Junctions
VI. Adherens Junctions
A. Structure and function
B. Relationships between adherens and occluding junctions
VII. Polarity
A. The transporting epithelial phenotype
B. Polarity and the TJ
C. Na⁺-K⁺-ATPase, cell attachment, and the position of the TJ
VIII. Evolution of the Epithelial Vectorially Transporting Phenotype
A. Evolution of the Na⁺-K⁺-ATPase
B. Evolution of junction proteins
IX. The Dawn of Metazoans and Transporting Epithelia
A. The “thrifty sponge”
B. Very flat organisms
C. The “mare nostrum” metazoans
X. Na⁺-K⁺-ATPase and Cell Adhesion
A. Role of the β-subunit in cell attachment
B. (P→A), a pump (P) adhesion (A) mechanism

Cereijido, M., R. G. Contreras, and L. Shoshani. Cell Adhesion, Polarity, and Epithelia in the Dawn of Metazoans. Physiol Rev 84: 1229–1262, 2004; 10.1152/physrev.00001.2004.—Transporting epithelia posed formidable conundrums right from the moment that Du Bois Raymond discovered their asymmetric behavior, a century and a half ago. It took a century and a half to start unraveling the mechanisms of occluding junctions and polarity, but we now face another puzzle: lest its cells died in minutes, the first high metazoa (i.e., higher than a sponge) needed a transporting epithelium, but a transporting epithelium is an incredibly improbable combination of occluding junctions and cell polarity. How could these coincide in the same individual organism and within minutes? We review occluding...
I. INTRODUCTION

The study of epithelia always posed formidable conundrums. The first one, briefly described below, arose a century and a half ago when it was discovered that these tissues have a spontaneous electrical potential between the outer and the inner side. Today, even when biological asymmetries (referred to as “polarity”) are still subject to intense research, we are no longer stuck in a puzzling situation nor foresee conflicting aspects. The second riddle is posed by the origin of epithelia as part of the emergence of metazoans. Since its cells perish for lack of nutrients or excess of metabolic wastes, a metazoan higher than a sponge exchanges nutrients and wastes with an internal milieu, which in turn exchanges them through transporting epithelia, but a transporting epithelium is in itself a highly elaborated combination of occluding junctions and polarized mechanisms. Therefore, the origin of the first metazoan would have depended on the incredibly improbable coincidence, in the same individual and within minutes, of an elaborated epithelium with occluding junctions and polarity. We propose that the somewhat baffling episode consisted of a combination of different species of attaching molecules and even fundamental polarizing processes that were already available in unicellulars. This article is therefore devoted to update some aspects of cell attachment and polarity that might have participated in the dawn of transporting epithelia and metazoans.

A. For Almost a Century Transepithelial Transport Was a Hard-To-Believe Hypothesis

In the second half of the 19th century Emile Du Bois Raymond demonstrated that a frog skin separating two saline solutions exhibits a spontaneous electrical potential difference across it. G. Galeotti (166, 167) proposed that this potential could be explained by assuming that the epithelium has a higher Na⁺ permeability in the inward than in the outward direction. This explanation was not accepted because it would be in violation of the first and second laws of thermodynamics (66, 72). A second argument, seeking a source of energy, proposed that it would be provided by the metabolism of the cells, but was also refuted because, according to Curie’s principle, phenomena of different tensorial order cannot be coupled, i.e., Na⁺ transport is a vectorial phenomenon (it occurs in the outside to inside direction) and cannot be driven by a scalar phenomenon (in those days chemical reactions were assumed to be scalar and therefore could not be expected to proceed in a given direction of the space). Half a century later, Hans Ussing devised electric and tracer methods to unambiguously demonstrate that the frog skin can actually transport a net amount of Na⁺ in the inward direction and in the absence of an external electrochemical potential gradient (456). Of course, this demonstration, confirmed thereafter in hundreds of laboratories, prompted a closer look at the arguments that had stood in the way of accepting that metabolism can drive Na⁺ transport. To start with, a protein like Na⁺-K⁺-ATPase cannot interact with Mg²⁺, Na⁺, K⁺, ATP, and ouabain just anywhere on its surface, but at very specific sites located either on the outer or on the inner side of the cell membrane, but not both, i.e., this enzyme is vectorial at the microscopic level. Yet, when studied in a solution where millions of molecules point in all directions, vectoriality is lost. But in the orderly molecular orientation of a cell membrane, Na⁺-K⁺-ATPase is anisotropic both at the microscopic and at the macroscopic level. In turn, De Donder and van Rysselbergh (117) proved that chemical affinity can constitute a driving force, Onsager (340, 341) demonstrated that all fluxes and all forces present in a system can be, in principle, coupled, and Kedem (245) demonstrated that the flux of a given ion can be coupled to metabolism, so the split of ATP into ADP plus P₁ can, in fact, drive the net flux of Na⁺. In keeping with the idea that gradients and fluxes of different substances can be coupled, it was later found that the Na⁺ gradient originated by the Na⁺-K⁺-ATPase can also propel the unidirectional fluxes of sugars, amino acids, Cl⁻, Ca²⁺, H⁺, etc., via co- and countertransporters.

B. The Puzzle of the First Metazoan

Life depends on an intense and highly selective exchange of substances between cells and their environment. In the case of single cells living in the sea, this environment behaves as an infinite reservoir. When cells belong instead to a higher metazoan organism, they exchange with a very thin layer of interstitial milieu that would be quickly spoiled were it not for a circulatory apparatus that constantly restores nutrients and takes away waste products by carrying them to and from large...
areas of epithelia (intestinal, renal, gills, etc.) where they are finally exchanged with the external environment. Even when a primeval metazoan might have been a small organism contained within a single type of epithelium, it required some sort of sealing between its cells, and these cells should have had vectorial transport. Therefore, the emergence of first metazoans higher than a sponge poses a paradox, because somatic cells gathered in a restricted space required a transporting epithelium to exchange substances with the external environment, but the first transporting epithelium depended in turn on the highly improbable coincidence, within a few minutes and in the same organism, of three novelties: 1) assembly of the multicellular organism itself, 2) the establishment of occluding junctions between epithelial cells, and 3) a polarized distribution of transport mechanisms in the membrane of these cells.

In this review we explore the possibility that metazoans, occluding junctions, and vectorial transport appeared as manifestations of specific types of binding between certain molecular species, as well as between epithelial cells. The argument requires a brief description of the preparations developed to investigate the development of the so-called “epithelial transporting phenotype,” as well as a review of Na+/K+-ATPase and cell junctions, concentrating on those properties that involve specific bindings between molecules as well as between cells.

II. MODEL SYSTEMS OF TRANSPORTING EPITHELIA

Most studies to evaluate unidirectional fluxes across the apical and the basolateral membrane of epithelial cells (Fig. 1) followed black box approaches (Fig. 2) (60, 64, 113, 211), but in spite of affording valuable information on overall epithelial processes (46, 65, 67, 68, 71, 73, 233, 318, 367, 369, 386), these approaches lacked the necessary resolution to study their cellular and molecular basis and, more specifically, junctions and polarity. Furthermore, black box methods could only be applied to mature epithelia where junctions and asymmetry are already established. Therefore, entirely different methods were required.

Oxender and Christensen (343) attempted to assemble an artificial epithelium by sandwiching single Ehrlich ascites cells between Millipore filters separating two chambers. However, these cells lacked an intrinsic asymmetry, remained oriented at random, did not attach, and no asymmetry of the whole preparation was detected. Further attempts used epithelial cells dislodged from epithelia through treatment with Ca2+ chelants and hydrolyzing enzymes. The first step was to separate the epithelium of a frog skin (387) and show that it preserves the overall properties of dissected epithelia. Therefore, the second step was to mince the epithelium, dislodge the cells, and investigate whether these maintain a satisfac-

![Figure 1](https://www.prv.org)
tory ionic steady state and responded to ouabain, amiloride, changes in temperature, or have other evidences that cells retained the basic properties of natural epithelia (500, 501). Finally, the third step was to seed the cells on glass or Millipore filters to form a sort of artificial epithelium. Unfortunately, cells performed poorly in culture, detached easily, died soon, and prompted us to try established cell lines. We chose the MDCK line derived by Madin and Darby (288) from canine kidney, which was generally used to grow viruses, because it retained sufficient differentiation as to secrete fluid (276). We cultured these cells on translucent supports (a nylon cloth coated with collagen) on which cells can easily be observed (69, 70, 72, 75), or nitrocellulose filters. A similar preparation was developed by Misfeldt et al. (315). This approach gave us an opportunity to study the development of the transporting epithelia phenotype. Thus we used it to study the synthesis, assembly, and sealing of tight junctions (TJs) (69, 74, 75, 78, 143, 183, 297), as well as the polarity of the Na\(^+\)/H\(^+\)-ATPase (61, 62, 96, 102); Enrique Rodriguez-Boulan introduced the use of viruses that bud either apically (e.g., Flu) or basolateraly (e.g., stomatitis) to track the fate of specific proteins during apical or basolateral polarity (283, 284, 379–382), and Carlos A. Rabito analyzed the onset of polarized cotransporters (368, 370, 371). M. Taub perfected the approach by developing totally defined culture media (440–442), and complementary procedures were refined to open and reseal the TJs by removing and restoring Ca\(^{2+}\) (69, 97, 101, 184, 186), follow the cascades of phosphorylation involved (20, 21, 85), the role of the cytoskeleton (311, 312), the participation of protein synthesis and sorting (196, 376, 379, 381), the polarized distribution of ion channels (317, 359, 360, 361, 421, 439), the involvement of E-cadherin (199–201), the effect of agents that induce differentiation (280), and the vectorial movement of receptors (302). A distinct advantage of cultured monolayers is derived from the possibility of labeling beforehand a given cell type, and then mixing them with other types from different epithelia and even different animal species (98, 103, 185), and other characteristics illustrated below and reviewed in Cereijido (56) and Cereijido and Anderson (58).

In addition to the preparation just described, the information discussed in this review was obtained with suspended clumps and cysts of epithelial cells (465), yeasts, Caenorhabditis elegans and Drosophila (479), fertilized eggs, and the first stages of development from egg to embryo (151).

III. THE Na\(^+\)/K\(^+\)-ATPase

The Na\(^+\)/K\(^+\)-ATPase is not the only pump known today, yet for many years it was the almost exclusive focus of attention, and today is recognized to act as the star in the net transfer of a wide variety of substances across epithelia. The Na\(^+\)/K\(^+\)-ATPase is a member of the family of P-type ATPases. ATP hydrolysis by these ATPases includes a step of transfer of the terminal phosphate to the product (10). For a vignette on the development of this model system, see Current Contents 32: 46, 1989 and Physiologist 46: 114–115, 2003.
phoryl group of ATP onto the carboxyl group of an aspartic acid residue that is located in the active site of the enzyme (42). Among the ions transported by these type of pumps are protons, calcium, sodium, potassium, and heavy metals such as manganese, iron, copper, and zinc (418). The formation of a phosphorylated intermediate during the catalytic cycle is a characteristic of P-type ATPases that distinguishes them from V-ATPases and F-ATPases (347, 348). The Na\(^{+}\)-K\(^{+}\)-ATPase is a heteromultimeric membrane enzyme constituted by \(\alpha\), \(\beta\), and \(\gamma\)-subunits.

### A. The \(\alpha\)-Subunit

The 112-kDa \(\alpha\)-subunit, often called the catalytic subunit, bears the site for ATP hydrolysis; has the binding sites for Na\(^{+}\), K\(^{+}\) and cardiac glycosides; and is homologous to the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum (SERCA) whose 2.6-A resolution structure was recently reported by Toyoshima et al. (449).

Since the first cloning and sequencing of a Na\(^{+}\)-K\(^{+}\)-ATPase \(\alpha\)-subunit (413), hydropathy analyses have deduced 10 transmembrane crossings (241). A combination of heterologous expression and site-directed chemical labeling of cysteine mutants found five exposed extracellular loops and showed that both termini are intracellular (217). The high-resolution structure of SERCA was compared in detail to a Na\(^{+}\)-ATPase structure (277). There are three main intracellular structures: a large central loop between M4 and M5 (210) composed of ~430 amino acid residues, a long NH\(_2\)-terminal tail of ~90 amino acids, and an intracellular loop of ~120 residues between M2 and M3. These form the N or nucleotide-binding domain, the P or phosphorylation domain, and the A or actuator domain (449).

#### 1. \(\alpha\)-Subunit isoforms

Four isoforms of the \(\alpha\)-subunit occur in mammals: \(\alpha_1\), \(\alpha_2\), \(\alpha_3\), and \(\alpha_4\), that exhibit somewhat different properties in terms of cation binding (231) and are expressed in a tissue-specific and developmentally regulated manner. While the \(\alpha_1\)-isofrom is expressed in every tissue, \(\alpha_2\) is limited largely to skeletal muscle, heart, and brain. The \(\alpha_3\)-isofrom is expressed in brain and ovary, and \(\alpha_4\) is exclusive of sperm and its precursor cells (41, 406, 481). The \(\alpha_{2c}\) and \(\alpha_{2r}\)-isoforms appear early in brain development, and around birth in heart and skeletal muscle. Na\(^{+}\)-K\(^{+}\)-ATPase isoform expression can also be differentially regulated by hormones (214, 342), and \(\alpha\)-isoform genes contain 59 specific sequences that are potential trans-acting factors and hormone binding sites (385). Animals lacking the \(\alpha_1\)-isofrom gene do not develop past the blastocyst stage (282). Yet, \(\alpha_2\)-isofrom-deficient animals develop to term, as expected, since it is not expressed in early embryonic development. Interestingly, these animals were either born dead or died within the first few minutes after birth due to failure in the neuronal activity of the breathing center in the neonate (321).

### B. The \(\beta\)-Subunit

This subunit is part of the Na\(^{+}\)-K\(^{+}\)- and H\(^{+}\)-K\(^{+}\)-ATPases but is not included in other P-type ATPases; for example, Ca\(^{2+}\)-ATPase of both plasma membrane and sarcoplasmic reticulum do not have it. It is composed of ~370 amino acids, with the first 30 exposed to the cytosol, and 300-fold to form the extracellular portion that has 3 disulfide bonds. There are 3 N-glycosylation consensus sequences (NXX or NXT) in the extracellular domain (249, 400). The polypeptide chain of the \(\beta\)-subunit weighs 32–35 kDa, and when fully glycosylated can reach an overall apparent molecular mass of 55–60 kDa. Analysis of \(\beta\)-subunits in which the disulfide bonds were removed through substitution in baculovirus-infected insect cells, reveals that the S-S bridges are not important for assembling the heterodimer, yet they are required for membrane targeting (171, 272). Recent studies using heterologous expression suggest that substitution of the essential asparagine residues prevents glycosylation, but this has little if any effect on catalytic activity (30).

The \(\beta\)-subunit has been identified as a factor responsible for cell adhesion in nervous tissue (180). The activity of Na\(^{+}\)-K\(^{+}\)-ATPase can be influenced by lectins, in particular, concanavalin A (428), and galectins (various lectins of animal origin), some of which affect cell adhesion (335) and provide in this way signal transmission to the catalytic subunit.

#### 1. \(\beta\)-Subunit isoforms

Three \(\beta\)-isoforms can be attributed to Na\(^{+}\)-K\(^{+}\)-ATPase in mammals (\(\beta_1\), \(\beta_2\), and \(\beta_3\)), and a fourth to gastric H\(^{+}\)-K\(^{+}\)-ATPase (\(\beta\)HK). Significantly, for the non-gastric H\(^{+}\)-K\(^{+}\)-ATPase, no specific \(\beta\)-subunit has been identified. X-K-ATPase \(\beta\)-isoforms exhibit only ~20–30% overall sequence identity but share several structural features. \(\beta\)-Isoforms exhibit a tissue-specific distribution with \(\beta_1\) of Na\(^{+}\)-K\(^{+}\)-ATPase being expressed ubiquitously, \(\beta_2\) mainly in the heart, skeletal muscles, and glial cells, and \(\beta_3\) in many tissues (for review, see Ref. 42).

Recently, a novel member of the \(\beta\)-subunit family has been identified (\(\beta_m\)) which shares common structural features and signature motifs with X-K-ATPase \(\beta\)-isoforms (355). Despite the similarities with X-K-ATPase \(\beta\)-isoforms, \(\beta_m\) has also some atypical characteristics. First, it contains two long glutamate-rich regions in the cytoplasmic NH\(_2\) terminus that are not present in X-K-
ATPase β-subunits (357). Second, based on results of cell fractionation (356) and on its state of glycosylation (357), \( \beta_m \) appears to be concentrated in the sarcoplasmic reticulum; in contrast, X-K-ATPase \( \beta_m \)-subunits are predominantly found at the plasma membrane. Recent studies reveal that \( \beta_m \) is a resident of the endoplasmic reticulum, which does not act as a chaperone for the maturation of any of the known X-K-ATPase \( \beta \)-subunits, thus representing a protein functionally distinct from other members of the \( \beta \)-subunit family (108).

The \( \beta_2 \)-isoform is strongly expressed in the brain and moderately in the spleen (181). It was identified as an adhesion molecule on glia (AMOG) mediating adhesion between neurons and astrocytes (8, 9). Sequence analysis of AMOG identified it as a homolog of the \( \beta_1 \)-subunit (180). The dual function of the \( \beta_2 \)-subunit in cell recognition and ion transport has been hypothesized to couple cell recognition with regulation of the ionic milieu (180).

In a recent work Okamura et al. (339) analyzed a complete list of the P-type ATPase genes in Caenorhabditis elegans and Drosophila melanogaster. The branching points and the inferred evolutionary rates of the invertebrate \( \beta \)-subunits suggest that D. melanogaster possesses two distinct types of \( \beta \)-subunits, one type (\( \beta_1, \beta_2, \) and \( \beta_3 \)) closer to the vertebrate \( \beta \)-subunits and the other type (\( \beta_4, \beta_5, \) and \( \beta_6 \)) sharing more homology with C. elegans \( \beta \)-subunits. The tissue-specific expression patterns of two of the Drosophila melanogaster genes for the \( \beta \)-subunit have been studied (Nervana 1 and 2, Nrv). Nrv1 produces a single \( \beta \)-subunit isoform expressed primarily in muscle tissue, whereas Nrv2 codes for two different isoforms (2.1 and 2.2) expressed in the nervous system. The tissue-specific expression of each Nrv gene is independently regulated by the cis-elements present in the 5'-flanking region. The Nrv2 5'-flanking DNA directs expression exclusively to the nervous system, whereas Nrv1 5'-flanking DNA directs expression primarily in muscle tissue (490).

C. \( \alpha/\beta \)-Subunit Interactions

The subunits of Na\(^{+}\)-K\(^{+}\)-ATPase are synthesized independently in the endoplasmic reticulum and assembled in this organelle. Detergent solubilized \( \alpha/\beta \)-heterodimers of Na\(^{+}\)-K\(^{+}\)-ATPase are able to carry out normal Na\(^{+}\)-K\(^{+}\)-ATPase activity (136, 137). However, without the \( \beta \)-subunit the \( \alpha \)-subunit does not exhibit a detectable activity (438) and is rapidly degraded (2, 174). The \( \beta \)-subunit may be involved in stabilizing the correct transmembrane folding of the \( \alpha \)-subunit (2, 172, 173, 399). Amino acid residues located in the proximity to the COOH-terminal fragment of the \( \beta \)-subunit appear to participate in the association between the \( \alpha \)-subunit and the \( \beta \)-subunit (29, 95), as does its transmembrane fragment, which interacts with transmembrane fragments M9-M10 of the \( \alpha \)-subunit (396).

In the extracellular M7M8 loop of the \( \alpha \)-subunit, a sequence of 26 amino acid residues between N894 and A919 interacts to produce the associated \( \alpha/\beta \)-complexes (277), yet in insect cells, which lack endogenous subunits, infection with baculovirus particles with only \( \beta \)-subunits results in the expression of \( \beta \), which traffics readily to the plasma membrane (171).

The expression of \( \alpha \)- and \( \beta \)-subunit isoforms follows a tissue-specific pattern (291, 296, 402). While genes encoding \( \alpha \)-subunits have a similarity above 90% (412, 413), the ones encoding \( \beta \)-isoforms only share 39–48% sequence identity (291, 400). The \( \alpha_x \) and \( \beta_x \)-isoforms are exclusive for the axon, and \( \alpha_2 \)- and \( \beta_2 \)-isoforms are exclusive for the Schwann cell, although axonal contacts regulate \( \alpha_x \) and \( \beta_x \)-isoform expressions (244). However, multiple \( \alpha \)- and \( \beta \)-isoforms can be expressed simultaneously in the same cell type (52, 306, 471), suggesting that they have distinct functions. Conversely, in heterologous expression systems, all Na\(^{+}\)-K\(^{+}\)-ATPase \( \beta \)-isoforms can associate with all Na\(^{+}\)-K\(^{+}\)-ATPase \( \alpha \)-isoforms and produce functionally active \( \alpha/\beta \)-complexes with slightly different transport and pharmacological properties (109, 229). Moreover, Na\(^{+}\)-K\(^{+}\)-ATPase \( \alpha \)-subunits can produce stable complexes with H\(^{+}\)-K\(^{+}\)-ATPase \( \beta \)-subunit which are, however, partially inactive (131, 209, 213). Then again, the H\(^{+}\)-K\(^{+}\)-ATPase \( \alpha \)-subunit does not associate with the Na\(^{+}\)-K\(^{+}\)-ATPase \( \beta_1 \)-isoform (189, 466). So far, little is known about the determinants that govern the specificity of X-K-ATPase \( \alpha/\beta \)-interactions in cells expressing multiple \( \alpha \)- and \( \beta \)-isoforms.

D. The \( \gamma \)-Subunit

This subunit is a small membrane protein that associates with Na\(^{+}\)-K\(^{+}\)-ATPase in the kidney and potentially in the placenta and the mammary gland (11, 31, 430). It belongs to the FXYD family, a group of small (7.5–19 kDa) single-span membrane proteins, characterized by the presence of the FXYD motif in the extracellular domain, and that includes phospholemman, channel-inducing factor (CHIF), and other peptides (430). The \( \gamma \)-subunit is not required for catalytic activity (207) but modulates Na\(^{+}\)-pump function by changing the affinity for ATP, Na\(^{+}\), and/or K\(^+\) (10, 366, 446, 447). It is synthesized from a gene with two different promoter regions that provide differential expression of the two splice variants known (264, 430). The \( \gamma \)-subunit or other proteins of the FXYD family, namely, phospholemman, CHIF, PLMS (a dogfish shark FXYD family member), and FXYD7, interact with Na\(^{+}\)-K\(^{+}\)-ATPase and/or modulate its activity, modifying the transport capacity of epithelia in kidney, shark rectal gland, and choroid plexus, as well as the function of other tissues in the central nervous system (31, 32, 108, 146, 289, 445). The \( \gamma \)-subunit and homologous proteins may potentially increase the variety of the Na\(^{+}\) pumps.
E. Assembly and Delivery of the Na\(^{+}\)-K\(^{+}\)-ATPase

Occluding junctions and apical/basolateral polarity are lost upon harvesting with trypsin and EDTA, but regained in a few hours of reseeding at confluence and in the presence of Ca\(^{2+}\). If cells are instead deprived of this ion and maintained in suspension with a stirrer, the transporting phenotype does not develop. The addition of soluble collagen prompts cells to expose more specific binding sites, but cells still remain unpolarized and fail to form TJs (395). Ca\(^{2+}\) promotes the formation of clumps, in which suspended cells take each other as substrate, form TJs and polarize, orienting the apical domain towards the outer side of the clump (465). Collagen added under this condition binds to the apical membrane and causes an inversion of polarity and a relocation of TJs that, by the way, show the dynamic nature of these structures (25, 303).

When cells are plated in the presence of Ca\(^{2+}\) but this ion is removed after allowing 20–40 min for attachment to the substrate, the relatively small fraction of Na\(^{+}\)-K\(^{+}\)-ATPase and ion channels expressed at the plasma membrane cells is not polarized, and these molecules, as well as TJ-associated molecules, remain trapped in intracellular membrane vesicles (96, 286, 361, 395). Ca\(^{2+}\) added at this time triggers polarization and junction formation in a few hours, a maneuver called “Ca\(^{2+}\) switch” (184). However, the presence of this ion cannot trigger by itself the development of the transporting epithelial phenotype, as cell-cell contacts are also required (317, 411, 439).

F. The Triggering Effect of Ca\(^{2+}\)

1. Extracellular events

Ca\(^{2+}\) acts primarily on the extracellular side (97, 100, 101, 186) as indicated by 1) an extracellular Ca\(^{2+}\) concentration of 0.1 mM, which suffices to trigger junction formation and polarization, does not increase the level of cytosolic Ca\(^{2+}\) (14 ± 8 nM) that was reduced as a consequence of the preincubation without this ion (20 ± 8 nM); 2) La\(^{3+}\) blocks Ca\(^{2+}\) penetration, but because of its low affinity for the extracellular repeats of E-cadherin (see below), does not prevent this ion from triggering from the outside, either junction formation or polarization; 3) Cd\(^{2+}\) instead blocks both Ca\(^{2+}\) influx and the development of the transporting phenotype (100). The observation that extracellular Ca\(^{2+}\) is sufficient to trigger polarization and junction formation does not exclude that the subsequent penetration of the ion into the cytoplasm would produce a series of additional phenomena (327). Likewise, E-cadherin appears to have a large number of cellular roles besides that of promoting and maintaining TJs (495).

2. Intracellular events

Once Ca\(^{2+}\) binds to the extracellular repeats of E-cadherin, a signal is transduced via two different G proteins, a protein kinase C (PKC), a phospholipase C (PLC), calmodulin, and mitogen-activated protein kinase (MAPK), triggering the development of the epithelial transporting phenotype (20, 21, 79, 184). The assembly and sealing of TJs occurs so quickly that a fraction of the Na\(^{-}\)-K\(^{-}\)-ATPase is trapped on the apical side but is thereafter removed from this location (96). The polarized insertion of Na\(^{-}\)-K\(^{-}\)-ATPase triggered by Ca\(^{2+}\) can be blocked with inhibitors of protein synthesis, yet the proteins whose synthesis is required are neither the α- nor the β-subunits of Na\(^{-}\)-K\(^{-}\)-ATPase (96).

G. The Polarized Distribution of Na\(^{+}\)-K\(^{+}\)-ATPase

The Na\(^{+}\)-K\(^{+}\)-ATPase of epithelial cells serves two different but integrated roles. The first is the translocation of ions across the plasma membrane as in other cell types (417, 418). The second stems from its expression in only one pole of the cells, in such a way as to carry the translocation of Na\(^{+}\) across the whole epithelium as proposed by Koeffoed-Johnsen and Ussing (252). In turn, a combination between the polarized distribution of Na\(^{+}\)-K\(^{+}\)-ATPase, specific ionic permeabilities, and the polarized expression of co- and countertransporters drives the net transport of other solutes across the whole epithelium (111, 112, 405). In keeping with these roles, Na\(^{+}\)-K\(^{+}\)-ATPase is found to reside on the basolateral surface in most epithelial cells (134, 135, 139, 242). In a few other epithelial cells such as those of the choroid plexus (484), retinal pigment epithelium (204, 422), and cockroach salivary gland (236), this enzyme is expressed on the opposite side of the cells, but always in a polarized manner.

Koeffoed-Johnsen and Ussing (252) conceived their model on the basis of the macroscopic asymmetries of the frog skin (electrical potential, currents, and net ion transport) and represented epithelial cells as simple rectangles where the pump was assumed to be on the inner facing barrier, that would correspond to the basal side of a more realistic picture of the cell (Fig. 1). Yet in model systems of MDCK, either as monolayer (61, 62, 96, 98, 102) or as cysts (465), Na\(^{+}\)-K\(^{+}\)-ATPase is not expressed on the basal side, but restricted to the lateral plasma membrane facing the intercellular spaces. For the macroscopic parameters mentioned above, it is irrelevant whether the pump is located at the basal or at the lateral side, but as we shall discuss below, the expression on the lateral side may be crucial for the polarized distribution of the pump, which is the basis of the whole asymmetric behavior of epithelial cells.

 Newly synthesized Na\(^{+}\)-K\(^{+}\)-ATPase is directly addressed to the basolateral membrane domain in MDCK.
cells (54, 188, 189). This targeting seems to be determined by the impossibility of this enzyme to board the glyco-
sphingolipid (GSL)-rich rafts that assemble in the Golgi
complex and form vesicles that carry proteins towards
the apical domain. This exclusion may be overcome by
endowing the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase with a sequence signal
from the fourth transmembrane segment of the \(\alpha\)-subunit
of H\textsuperscript{+}-K\textsuperscript{+}-ATPase (TM4 signal) that suffices to readdress
the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase towards the apical domain. However,
there are nongastric H\textsuperscript{+}-K\textsuperscript{+}-ATPases that, in spite of lack-
ning the TM4 signal, are nevertheless addressed towards
the apical domain. On the basis of their work with chimeras,
Dunbar and Caplan (130) convincingly suggest that these
differences in the polarized expression of ATPases can be explained as follows: 1) gastric H\textsuperscript{+}-K\textsuperscript{+}-
ATPase has the TM4 apical addressing signal already
formed; 2) nongastric H\textsuperscript{+}-K\textsuperscript{+}-ATPases lack the TM4 sig-
nal, but would be able to form it through a particular
arrangement of the molecule that would join two sepa-
rated segments and thereby complete the signal sequence;
3) also Na\textsuperscript{+}-K\textsuperscript{+}-ATPase lacks a TM4 but, at variance with
nongastric H\textsuperscript{+}-K\textsuperscript{+}-ATPases, it is unable to form one by
reconfiguring the \(\alpha\)-subunit in space; only the insertion
of a TM4 signal by molecular engineering achieves its
apical expression (130, 131).

Based on studies of cultured MDCK cells, three mod-
els have been proposed for the development and polar-
ized expression of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. One of these models
involves intracellular sorting of newly synthesized pro-
teins at the Golgi apparatus, followed by a vectorial
delivery of the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase to the apical mem-
brane (44, 51, 204, 422). Early studies on rat RPE mono-
layers (204) suggested that an entire membrane-cytoskel-
eton complex is assembled with opposite polarity. Recent
studies have shown that other polarized membrane pro-
teins such as viral envelope membrane proteins do pre-
serve their polarity in RPE cells (43, 44). Furthermore,
Rizzolo and Zhou (377) used the RPE of chicken embryos
and observed that the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and \(\alpha\)-spectrin seg-
erate into different regions of the cell. Despite its segre-
gation from \(\alpha\)-spectrin, the Na\textsuperscript{+}-K\textsuperscript{+}-ATPase appears to
associate with a macromolecular complex in microvilli,
suggesting that these properties prevent the Na\textsuperscript{+}-K\textsuperscript{+}-
ATPase from complexing with the \(\alpha\)-spectrin-based cytoskel-
eton by sequestering the enzyme into the compart-
ment where its activity is required. Discussion of the
polarized distribution of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase will be pursued
below.

IV. TIGHT JUNCTIONS

Epithelia separate biological compartments with dif-
cerent composition, a fundamental role that depends on
the establishment of occluding junctions. It is generally
assumed that while TJs play this role in vertebrates, sep-
tate junctions (SJs) play it in invertebrates. Yet, as dis-
cussed below, SJs are transiently present in certain or-
gans of vertebrates during development and permanently
expressed in their nervous system.

TJs were so belittled that more than a century after
they were first described, they were not even represented
in the model of Koeboed-Johnsen and Ussing (252).
The fact that preparations of “tight epithelia”, with a transep-
ithelial electrical resistance (TER) in the order of thou-
sands of ohms per square centimeter, gradually decrease
this parameter after several hours of being mounted be-
tween two chambers, led one to assume that epithelia like
the intestinal mucosa or the gall bladder, whose TER only
amounts to <100 \(\Omega \cdot \text{cm}^2\) from the outset, did not with-
stand either dissection or harsh in vitro conditions, yet
the observation that despite their low TER, these epithelia
did transport substances vigorously (see, for instance,
Refs. 47, 121, 122, 123, 373, 374) and many physiologically
important substances flow mainly through a paracellular
route limited by the TJ, led to a reassessment of the
biological role of the thereafter called “leaky epithelia.”
But even then, given its poor ability to discriminate be-
tween diverse permeating species, compared with the
plasma membrane, it was expected that the TJ would
consist of a couple of rather inconspicuous molecules placed in neighboring cells and bridged by a mere Ca\(^{2+}\)/H\(^{1+}\) salt link (57).

The scope has since drastically changed, due to a series of observations: 1) TER ranges from 10 (e.g., proximal kidney tubule) to \(>10,000\ \Omega \cdot \text{cm}^2\) (e.g., urinary bladder), indicating that TJs can adjust the degree of tightness to physiological needs (91, 92). 2) The TJ has been found to consist of a cluster of protein species (ZO-1, ZO-2, ZO-3, cingulin, occludin, claudins, etc.; see Fig. 3A and Table 1) that stretches from its membrane lips to the cytoskeleton (425, 451). 3) Some of these proteins contain nuclear addressing and nuclear export signals (76, 187, 224). 4) TJ molecules change their degree of phosphorylation in response to physiological conditions and pharmacological challenge (21, 86). 5) In a given moment, TJs may relax their tightness to allow the passage of macrophages toward an infected site, or spermatozoa in their travel from the Sertoli cell to the lumen of the seminiferous tube (49, 81, 351). 6) There is an increasing number of autoimmune diseases associated with faulty TJs that allow the passage of peptides from the intestinal flora. The immune system develops antibodies that not only attack the microbial antigen, but neurons, \(\beta\)-cells from the pancreas, the thyroid gland, and other targets of the host, whose proteins have sufficient homology with the intruder molecule (58). 7) Some TJ-associated molecules contain consensual segments with tumor suppressor proteins (182, 483, 488). 8) TJs act as a barrier that prevents lipids in the apical and the basolateral membrane from mixing (126, 127, 460, 461). 9) Lipids are themselves part of the structure of the TJ (50, 334). Physiologists have traditionally described cell functions such as the action potential, exchange diffusion, and cotransport decades before the specific molecules that performed these roles could be found. The picture is now totally reversed, as we know of dozens of proteins (e.g., the ones mentioned in the section above) whose function is mostly unknown. Some mutations in claudin genes severely impair the TJs (e.g., deletion of claudin-16/paracellin suppresses the ability of kidney nephrons to reabsorb Mg\(^{2+}\) and Ca\(^{2+}\)) (414), and mutations that result in the absence of claudin 14 are responsible for autosomal recessive deafness (39, 474). Knockouts of claudins 1, 5, and 11 produce severe damage of the epidermal, blood-brain, and myelin barriers, causing dehydration in the

![FIG. 3. Effect of ouabain on nucleus and adhesion complexes (NACos)](http://physrev.physiology.org/...)
Functional classification of molecules in the different types of cell junctions

<table>
<thead>
<tr>
<th>Junction</th>
<th>Transmembrane Protein</th>
<th>NACos</th>
<th>Signaling Proteins</th>
<th>Cytoplasmic Plaque Proteins*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adherens</td>
<td>Nectin</td>
<td>ARVCF</td>
<td>SRC family</td>
<td>AF6/AFADIN</td>
</tr>
<tr>
<td></td>
<td>E-cadherin</td>
<td>β-Catenin</td>
<td></td>
<td>Vinculin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>α-Catenin</td>
<td></td>
<td>β-Catenin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AJUBA</td>
<td></td>
<td>α-Catenin</td>
</tr>
<tr>
<td>Tight</td>
<td>Claudins</td>
<td>ZONAB</td>
<td>PKA</td>
<td>Cingulin (ZO-2, ZO-3, AF-6, and JAM)</td>
</tr>
<tr>
<td></td>
<td>JAMs</td>
<td>CDK4</td>
<td>Heterotrimeric G proteins</td>
<td>PAR-3 (JAMs, PAR-6, atypical PKC)</td>
</tr>
<tr>
<td></td>
<td>Occludin</td>
<td>Symplekin</td>
<td>PKC</td>
<td>PAR-6 (PAR-3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>huaASH1</td>
<td>Rho-family GTPases</td>
<td>PATJ (Pals-1, ZO-3, claudin)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ZO-2</td>
<td></td>
<td>PAL-1 (CBB1, PATJ)</td>
</tr>
<tr>
<td>Desmosomes</td>
<td>Desmoglein</td>
<td>Plakoglobin</td>
<td>PKC</td>
<td>Desmoplakin</td>
</tr>
<tr>
<td>Focal</td>
<td>Desmocollin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-Integrin</td>
<td>Paxillin</td>
<td>SRC family</td>
<td>α-Actinin</td>
</tr>
<tr>
<td></td>
<td>β-Integrin</td>
<td>Calreticulin</td>
<td>ILK</td>
<td>Vinculin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>FHL-2</td>
<td></td>
<td>Talin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hic-5</td>
<td></td>
<td>Filamin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PINCH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CDK6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zyxin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Transmembrane proteins are those that interact with homotypic or heterotypic proteins of adjacent cell or extracellular matrix. NACos are proteins that can localize to the nucleus and adhesion complexes. * Some of the specific proteins that interact with the plaque molecules are listed in parentheses. ARVCF, armadillo repeat gene deleted in VCFS (Velo-cardio-facial syndrome) thought to contribute to the morphoregulatory function of the cadherin-catenin complex (294); NECTIN, a Ca^{2+}-independent immunoglobulin-like cell-cell adhesion molecule at AJs associated with E-cadherin through their respective peripheral membrane proteins, afadin, and catenins (432); AF7/AFADIN, a peripheral membrane protein that connects nectin to the actin cytoskeleton (432); AJUBA, a cytosolic protein found at sites of cell adhesion and a member of the zyxin family of LIM proteins (290); CDK1, a human ortholog of Drosophila Crumbs, a transmembrane protein that plays an important role in epithelial cell polarity (384); zyxin and paxillin, the prototypes of two related subfamilies of LIM domain proteins that are localized primarily at focal adhesion plaques (468); CDK4 and CDK6, cyclin-dependent kinases that function as nuclear regulators of the cell cycle (19); PINCH, a five LIM domain and an integrin-link kinase (ILK) binding protein involved in the regulation of integrin-mediated cell adhesion (463); Hic-5, a paxillin-related focal contact protein that upregulates transcription of c-fos gene and is a coactivator of steroid receptor-activated transcription (494); calreticulin, α-integrin-interacting protein with nuclear function, originally identified as a chaperone in the endoplasmic reticulum (110); FHL-2, an integrin-interacting LIM-domain protein which modulates the transcriptional activity of nuclear β-catenin (473); FAK, focal adhesion kinase triggers signaling cascades important for the regulation of cell proliferation and survival (216); filamin and talin, actin binding proteins which form mechanical links to the cytoskeleton and are involved in the organization of actin networks (403); plakoglobin, known also as γ-catenin is a major component of the submembrane plaque of desmosomes where it binds to desmoglein; like its close homolog β-catenin, it can bind also to E-cadherin and can interact with LEF/TCF transcription factors (278); desmoplakin, a member of the plakin family at desmosomes that connects cytoskeletal elements to each other and to the junctional complex, plays a crucial role in orchestrating cellular development and maintaining tissue integrity (278).

It can be argued that, although there is ample evidence that TJs restrict diffusion through the paracellular permeation route, they might act as our lips, which enable our mouth to hold water, yet are not sewn together. Data supporting its role as an anchoring structure are somewhat scarce. The possibility exists that homologous interactions between transmembrane proteins located in neighboring cells (e.g., claudin/claudin) would stabilize them in this position but might not constitute a strength element holding cells together.

A. Structure

Proteins of the TJ have been divided according to several criteria, depending on whether they cross the membrane and pop up on the extracellular side, associate specifically with the cytoplasmic side of the TJ, have PDZ segments that bind them to other protein species forming stable scaffolds, anchor this structure to the cytoskeleton, belong to the TJ only transiently, for instance, during junction assembly and sealing, or because they are proteins like the NACos (see sect. vF) that shuttle between the TJ and the nucleus where they act as transcription factors (20, 63, 76, 182, 301, 451).

B. Transmembrane Proteins

Occludin and Claudins belong to the tetraspanin superfamly (256), which has four membrane-spanning domains, two extracellular loops, and the NH2 and COOH termini in the cytoplasm (159, 162). The extracellular loops of occludin are characteristically rich in glycine and tyrosine residues (300). The COOH-terminal domain binds a number of TJ plaque proteins including ZO-1 (142, 163, 226), ZO-2 (225, 477), ZO-3 (208), and cingulin (105). The NH2-terminus and the first extracellular domain modulate transepithelial migration of neutrophils (110, 219). Occlus...
din contributes to the structure and sealing of the TJ (158, 265, 304, 309, 458, 480). Although the roles of occludins in TJ assembly and functions are clearly demonstrated, null mutant mice expressed well-developed TJs (391–393).

Claudins are the major transmembrane proteins of tight junctions (159, 164). The mammalian claudin family comprises 25 members (182, 218, 451) that are the main constituents of the characteristic strands of TJs observed in freeze-fracture replicas. Claudin by itself is able to form these strands and recruit endogenous ZO-1 when expressed in fibroblasts (164, 319, 320). The COOH terminus is intracellular and has a domain that binds the PDZ proteins ZO-1, ZO-2, ZO-3 (225), PATJ (383), and MUPP-1 (205) (see below). Different claudins copolymerize along the same TJ strand and bind certain types of claudins of the neighboring cell (165). The type of claudin expressed is responsible for some specific functions of the TJ in a very strict spatial-temporal fashion (93, 94, 255, 457). Thus epithelial cells of the proximal tubule and MDCK have a characteristic low TER phenotype due to the expression of claudin-2 (160, 250, 375).

Junction adhesion molecule (JAM) is a protein of epithelial and endothelial cells with a single transmembrane domain. This protein is important for neutrophil migration through the endothelial cell layer (295). Its intracellular domain binds AF-6 (132), ASIP/Par 3 (133, 227), ZO-1 (28, 132), and cingulin (28).

C. Membrane-Associated Proteins

The cytoplasmic domain of TJ membrane proteins binds to a complex cluster of intracellular proteins like ZO-1, ZO-2, ZO-3, and Pals, which belong to the membrane-associated guanylate kinase homolog family (MAGUK) (141, 187, 239, 384, 483). Stevenson et al. (425) identified the first known TJ protein, ZO-1 (zonula occludens) (see also Ref. 5), that is a prominent associated peptide, judging from the number of associations and changes in phosphorylation it goes through. ZO proteins contain a series of domains, such as PDZ, which enable these peptides to interact with other ZO, occludins, claudins, and with the actin cytoskeleton (141–143, 226, 477, 478). MAGI (MAGUK inverted protein) colocalizes with ZO-1 (124). MAGI-1b localizes to the basolateral membrane and forms complexes with β-catenin and E-cadherin during junction formation (222) and in pallidolumysian atrophy (191). Notice that although β-catenin and E-cadherin are the key components of adherens junctions, they influence the TJ (see below). Tumorigenic proteins of adenovirus and papillomavirus target for degradation, or sequester MAGI-1 (179). MAGI-1 appears to be a tumor suppressor (179) that interacts at the TJ with the signaling molecule GEF, a guanine nucleotide exchange factor participating in the Rho signaling pathway (313). MAGI-2 and -3 interact with PTEN, an inositol trisphosphate phosphatase (488, 489), and MAGI-2 binds megalin (multi-protein receptor mediating endocytosis in kidney proximal tubules; Ref. 345). PARs are partitioning-defective proteins involved in embryonic polarity. PAR-3 localizes at the TJ through association to the COOH terminus of JAM (227) and forms a complex with PAR-6, a single PDZ-possessing molecule with a CRIB domain (232), and atypical PKCs λ and ζ (PAR-3/ASIP, atypical PKC isotype-specific interacting protein) (228). Par-6 inhibits TJ reassembly after junctional disruption induced by Ca2+ depletion (169). MUPP1 (multi-PDZ domain protein 1) functions as a cross-linker between claudin-based TJ strands and JAM oligomers in TJs (205) and retains MAGUK protein Pals1 through its MAGUK recruitment domain (MRE) (384).

AF-6 is transiently expressed at tight (491) and adherens junctions (292), suggesting a role in the formation of junctions (182). It is also an ALL-1 fusion partner at chromosome 6 associated with acute leukemia (363). PATJ is another junction-associated protein, which contains 10 PDZ domains that bind it to ZO-3 and that can also be found at the apical plasma membrane (383, 384). Cingulin is a molecule with globular domains and a central α-helical rod region necessary for dimerization (88). Through several domains spread along the molecule, specially the ZIM (ZO-1 interacting motif), cingulin cross-links with ZO-2, ZO-3, AF-6, and JAM to the F-actin and myosin cytoskeleton (28, 105, 114, 115). ZONAB is a transcription factor that regulates ErbB-2 and paracellular permeability (19, 22). Other TJ-associated proteins are symplekin, which is related to the polyadenylation of mRNA (431), the transcription factors HuASH1 and AP-1 (40, 325), MAGI-3, an atypical PKC (13, 179, 228, 427), JEAP, which appears to anchor other TJ-associated protein species (328), TH6 (247), as well as Plt (243). Another group of TJ-associated proteins is involved in vesicle traffic: Rab13 (298, 409, 497), Rab3b (426), and Sec6/8 complex (198). Finally, TJs also contain proteins associated with G proteins like G14α, G16α, G12α, G5α (119, 125, 389, 390).

D. Lipids

TJs appear on freeze-fracture replicas as continuous anastomosing strands. These strands are thought to be composed of either protein (420) or lipids (237, 358). The “protein model” depicts the strands as polymers of protein molecules in the plane of the plasma membrane that join, through their external domains, to the corresponding polymer of the neighboring cell. The preponderant participation of proteins is strongly supported by their content of transmembrane proteins such as occludin (162) and claudins (159). Transfected claudins even promote formation of strands resembling those of epithelial TJ in fibroblasts (164, 262). The “lipid model” in turn depicts the strands as cylindrical micelles with the polar groups of the lipids directed...
toward the axis, and the hydrophobic tails immersed in the lipid matrix of the plasma membrane of both neighboring cells (237). It is in keeping with the fact that the lipid-soluble probe dipicrylamine can be transferred with the aid of an applied voltage from the cell membrane of a previously loaded cell to the membrane of a neighboring one (453). It is also supported by the recovery of large bleached areas with lipids diffusing through the TJ (192) and by the cytochemical localization of phospholipids with gold complexed phospholipase A2 (240). However, several studies have failed to support or discard this model (126, 127, 326, 401, 460, 461). Lipidic messengers, like diacylglycerol, play a role in TJ formation (20, 21). Lipid phosphatase PTEN is also associated with the TJ and participates in tumor suppression (488, 489).

The possibility exists that strands would not be made exclusively of proteins or lipids, or that lipids would participate indirectly as a source of a second messenger that may in turn regulate TJs. Recent evidence supports both possibilities. Thus changing the total composition of phospholipids, sphingolipids, cholesterol and the content of fatty acids does not alter either TER or the structure of the strands, but enrichment with linoleic acid increases the paracellular flux of dextran without detectable modifications of either TER or TJs (50). Cholesterol depletion elicited by treating MDCK cells with methyl 

E. Diversity Arising From Processing TJ Proteins as Well as by the Expression of Several Isoforms

ZO-1 has three splicing variables (17, 187). ZO-1-α+ has an 80-amino acid domain, called the α-motif, inserted in the COOH-terminal half (17, 476). This variant is associated with the development of TJs and is abundant in epithelia (17, 263, 455). ZO-α− lacks the α-motif, is abundant in epithelia that express very dynamic TJs, like Ser
toli cells, endothelia (17), and even in cells like podocytes that do not express TJs (263).

Occludin is produced by a single gene (human chromosome band 5q13.1, Ref. 391) and is modified during posttranslation. Two alternative splicing isoforms are known: occludin 1B, a longer variant that has a 56-amino acids insertion in the NH₂ terminus (324), and occludin T4M(−), a short variant that lacks the fourth transmembrane domain and causes the COOH terminal to be exposed to the extracellular space (177).

The ZO-2 gene employs two alternative promoters that give rise to two ZO-2 isoforms differing at their amino-terminal portion by 23 amino acids. Although both isoforms are present in normal tissues, the longer one is absent in most pancreatic cancers (82–84).

F. Proteins That Can Localize to the Nucleus and Adhesion Complexes

Some junction-associated proteins contain nuclear address and nuclear export signals (86, 187, 224) that enable them to shuttle back and forth between the TJs as well as from other types of adhesion complexes, to the nucleus (Table 1; Fig. 3). Upon arriving at the nucleus, proteins that localize to the nucleus and adhesion complexes (NACos) activate transcription, modify mRNA processing and trafficking, activate c-jun kinase, alter the G₁/S phase transition of cell cycling, modulate histone methyltransferase, proliferation, cell density, oxidative stress, as well as interact with other molecules within the nucleus and submembrane scaffolds determine cell fate during embryonic development and are involved in tumorigenesis (23). In other words, the diverse cell junctions have specific molecular components that whenever the degree of attachment is weakened below a certain grip, they abandon the scaffold and travel to the nucleus where they switch on or off genes that have to do with proliferation.

G. Phosphorylation in TJ Assembly and Function

As mentioned above, both the assembly (20) and disassembly of the TJ (85) involve important phosphorylation steps. ZO-1 and ZO-2 become phosphorylated on Ser, Thr, and Tyr residues. Cingulin is phosphorylated on Ser residues, and occludin has several levels of phosphorylation on Ser and Thr that are required for its incorporation into the TJ (86, 394). Cingulin is phosphorylated in serine residues by a kinase insensitive to PKC activators or inhibitors (87). Likewise, the assembly of TJs depends on vinculin phosphorylation on Ser and Thr (352). ZO-1 in epithelia with low TER is more Ser-phosphorylated than in high-TER epithelia (424). Hypoxia in brain microvessels enhances phosphorylation, decreases expression, and delocalizes ZO-1 (149). However, low phosphorylation of ZO-1 has been detected in cells that lack TJs or have ZO-1 disassembled through calcium depletion (215).
Vascular endothelial growth factor increments Tyr phosphorylation and paracellular permeability (7). Epidermal growth factor induces Tyr phosphorylation of ZO-1 and relocates this protein towards the apical membrane of A431 cells (459), and tumor necrosis factor increases TJ permeability through Ser and Thr phosphorylation of occludin (89, 90). During TJ assembly ZO-1 is phosphorylated in tyrosine residues in a MAPK regulated fashion (79). Phorbol esters disassemble TJs and downregulate claudin-2. ZO-1 associates and is a substrate of ZAK, a Ser/Thr kinase (18) and of PKC (13). ZO-2 is Tyr phosphorylated when epithelial cells are transfected with v-src (433), phosphorylated in Ser and Thr residues by cAMP-dependent protein kinase (PKA) and atypical PKC when TJs are either absent or disassembled by Ca2+ removal (13). Occludin phosphorylation in Ser and Thr residues is required to recruit occludin to the TJ (394), whereas phosphorylation in Tyr is involved in disassembly and opening of TJs (118).

Although we still have no dynamic model to explain the interplay of Ca2+ levels, small GTP-binding proteins, phosphorylation, and TJ-associated molecules, there is ample evidence that this cascade involves the cytoskeleton that is linked to E-cadherin through p130, α-, β-, and γ-catenins,vinculin, α-actinin, fodrin, and spectrin (496). In fact, actin also combines with ZO-1, ZO-2, and ZO-3, occludin, cingulin, and other TJ molecules. The cytoskeleton is likely to constitute a structural framework as well as an informative network conveying signals from the adherens junctions to the TJ and back. Drugs interfering with microfilaments and microtubules block junction formation and polarization during a Ca2+ switch, and reverse these processes in fully polarized cells with already established TJs (496).

H. Biogenesis

Biogenesis of the TJs is closely related to development. In compaction, blastocyst formation, gastrulation, and neurulation, epithelia separate compartments of distinct composition. The formation of primordial epithelia results from maternal (e.g., in Xenopus) or embryonic (e.g., in mice) expression programs (151, 153). The first stage of TJs formation starts 1–2 h after compaction, with the assembly of plaque proteins ZO-1α+ and rab13 at the eight-cell stage (150, 409). This process depends on E-cadherin, protein synthesis, and assembly of microtubules. In a second stage, cingulin plaque protein is incorporated at the 16-cell stage and stabilized in a process dependent on E-cadherin-mediated adhesion (152, 230). E-cadherin seems to be necessary not only for spatial organization of the TJs, but also for maintaining their integrity, possibly by anchoring to the cytoskeleton (151, 153). Occludin and ZO-1α+ are assembled in the last stage (32 cells) (408, 410). In mouse and human, the TJ mRNAs of claudin-1, JAM, occludin TM4+ and TM4, and ZO-1α are initially inherited from maternal transcription and followed by embryonic transcription from the two-cell stage onward, and remain present throughout preimplantation development (154, 155, 176). Only ZO-1α+, ZO-2, and desmocolin-2, a component of desmosomes, are transcribed later on from the embryonic genome, during 16- to 32-cell stage in human and mouse (176, 408).

Development of TJs in Xenopus embryos is quite different from that of mouse, human, or bovine, due to the pressure imposed on Xenopus to develop rapidly swimming tadpoles, which are able to escape predators. The Xenopus egg has a very impermeable membrane, due to removal of transport proteins like the Na+-K+-ATPase through endocytosis (147, 151, 398). Xenopus embryos develop a polarized distribution of cadherins XB/U after the first cleavage, in the two-cell stage, coincidentally with the nascent blastocoel (6, 238). Sealed TJs develop at this stage, although in basal position and without constituting a fence for basolateral proteins (147, 310). TJs are initially assembled in the membrane at the animal pole, by the end of zygote cytokinesis. Cingulin is the first TJ protein to be incorporated, followed by ZO-1, then occludin. Interestingly, formation of the new apical membrane and the generation of cell polarity in Xenopus precede initiation of TJ formation, and TJ proteins assemble at the apical-basolateral boundary in the complete absence of intracellular contacts, indicating that the mechanism of TJ biogenesis relies on intrinsic autonomous cues (147).

V. SEPTATE JUNCTIONS

The first description of SJs in Hydra was made by Wood (482). While TJs are characteristic of chordates, nonchordates have instead SJs (269). SJs can be found in the blood-brain and blood-testis barriers of adult arthropods and even in the Ranvier nodes of vertebrates. Green and Bergquist (194) found that sponge junctions are difficult to locate due to their transient nature but have extremely regular intercellular spacing even in the absence of any septa. In cross-sectional views of the different types of SJ, there is little variation, as virtually all septa span a 15- to 18-nm intercellular space. Coelenterata have clear SJs. Mollusca and Arthropoda possess the well-known mollusk-arthropod pleated septate junctions (420). Tunicata do not have SJs, but rather a variation of the vertebrate TJ (175, 285). Some insects have TJs in the central nervous systems and rectal pads (266–268, 271). Furthermore, Toshimori et al. (448) described a type of TJ
in the cyst envelope of the silkworm testis that does not form extensive anastomosing arrays. These structures might not be true TJs (48, 193, 331). However, Lane and Chandler (270) clearly demonstrated the existence of what appear to be true TJs in the central nervous system of arachnids.

VI. ADHERENS JUNCTIONS

A. Structure and Function

The adherens junction is a large multiprotein cluster that plays crucial roles in tissue organization and patterning. It mediates homotypic cell-cell adhesion and the transduction of extracellular signals. E-cadherin, a classical Ca\(^{2+}\)-dependent homophilic cell adhesion molecule (434), is characterized by five extracellular structural repeats (EC1 to EC5) and a highly conserved cytoplasmic domain that associates with several cytoplasmic proteins, most prominently the catenins (344), which associate in turn to actin cytoskeleton (203, 251, 470, 495). E-cadherin is a well-known polarity-inducing protein that is able to polarize Na\(^+\)-K\(^+\)-ATPase in fibroblasts (307). In polarized MDCK cells, E-cadherin is localized to the lateral membrane (34). Newly synthesized E-cadherin is preferentially sorted to the lateral membrane (273) and binds strongly to the \(\beta\)-catenin (212), a protein that is necessary for efficient delivery of the complex to the plasma membrane (80). Basolateral delivery of E-cadherin relays upon a double leucine motif present in the intracellular domain (314). Ca\(^{2+}\) interacts with the extracellular part of E-cadherin (100, 186), more specifically with a motif of three amino acids (364). Ca\(^{2+}\) binds to the junctions between extracellular repeats of E-cadherin, changing its conformation (333, 407), and allowing it to aggregate with other E-cadherin molecules on the same cell, an arrangement that favors binding to the E-cadherin of neighboring cells (495). When adherent junctions are blocked with specific antibodies against the extracellular domain of E-cadherin, TJs do not form and, if already established, they reopen (103, 199, 200, 201). When the amino acid triplet His-Ala-Val (HAV) from the extracellular domain of E-cadherin is blocked with specific peptides containing an HAV sequence, the functions of the E-cadherin/catenin complex are interfered (330). However, HAV cannot act by itself, as flanking sequences are important in the binding selectivity of HAV peptides to E-cadherin (290, 372). Peptides from the bulge and groove regions of the EC1 domain of E-cadherin can inhibit cadherin-cadherin interactions, resulting in the opening of the cellular junctions (416).

The initial contact is restricted to small regions called “puncta” (3, 462). Vasioukhin et al. (462) demonstrated that Ca\(^{2+}\) stimulates the formation of very dynamic filopodia that penetrate and embed into neighboring cells. The initial AJ is extended in a process similar to a “zipper” closure.

This chain of reactions is interrupted by the failure in \(\alpha\)-catenin (470) and \(\beta\)-catenin, a critical component of the Wnt signaling cascade (305, 349). When wnt extracellular signals instruct cells to proliferate, dephosphorylated \(\beta\)-catenin is targeted to the nucleus and associates with the transcription factor LEF/TCF (37). In nonproliferating cells, phosphorylated \(\beta\)-catenin is removed from cell contacts and, once in the cytoplasmic pool, is bound to the axin and adenomatous polyposis coli protein complex (APC), where it is ubiquitinated and targeted to the proteasome for degradation (1, 190). Hence, the regulation of adhesion seems to be exerted, at least in part, by the titration of \(\beta\)-catenin from the cell contacts by APC. APC also binds to microtubules and participates in the formation of parallel tubulin bundles (316) involved in asymmetric cell division (353, 492).

Epithelial to mesenchymal transition (EMT) is the loss of cell-cell interactions and acquisition of a migrating phenotype of cells that changes the adhesive epithelial phenotype to a fibroblastic-like migrating one (35, 36, 38, 423). It requires loosening of the E-cadherin/catenin-dependent strong cell-cell adhesion of epithelial tissues (202, 220, 435), as well as silencing the expression of E-cadherin and TJs proteins (53, 223). Cano et al. (53) and Pérez-Moreno et al. (354) have identified the E-cadherin transcriptional repressors Snail and the mouse E12/E47 that are members of the zinc finger family and product of the E2A gene, respectively. Both interact with the E-cadherin palindromic element E-pal, present in E-cadherin promoter, and silence its expression. In addition to its effect on the E-cadherin, Snail binds directly to the E-boxes of the promoters of the claudin/occludin genes, resulting in complete repression of their promoter activity (223). In epithelial cells EMT can be triggered by the binding of the hepatocyte growth factor/scattering factor (HGF/SF) to its receptor, c-met, through the activation of the ras and MAPK signaling pathway (350). HGF/SF induces the ubiquitination of E-cadherin by Hakai, which is a member of the ubiquitination machinery, that induces endocytosis and dynamic recycling or degradation of E-cadherin, and the subsequent perturbation of cell-cell adhesions (157).

B. Relationships between Adherens and Occluding Junctions

Both types of junctions maintain a fundamental, but a highly complex relationship. TJs depend on the E-cadherin/E-cadherin interaction throughout the life of the epithelium, as TJs in mature monolayers can be opened by interfering with E-cadherin. E-cadherin does not localize in the TJ but triggers the cascade of chemical events...
resulting in TJ formation from afar (20). Nevertheless, Troxell et al. (450) found typical junctional strands and some TJs markers, such as ZO-1 and occludin, in cells whose expression of endogenous E-cadherin had been severely reduced. It is likely that E-cadherin would be also needed to hold the two neighboring cells together, so TJs can be formed. Heterotypic TJs can be established between cells from different organs of the same animal, and even from different animal species (185). This is illustrated in Figure 4, where a monolayer of cocultured MDCK (dog, unstained) and LLC-PK1 cells (pig, red) that exhibits homophilic (MDCK/MDCK and LLC-PK1/LLC-PK1) as well as heterophilic TJs (MDCK/LLC-PK1) revealed by the continuous distribution of occludin and ZO-1 molecules (green), and by the development of a TER (not shown). E-cadherin instead is only observed at homologous MDCK/MDCK borders, as indicated by DECMA-1 antibody, a result that agrees with the observation that the cell-cell contact provided by E-cadherin is a highly specific one (495). The antibody used has no specificity for E-cadherin in LLC-PK1 cells but binds to E-cadherin of MDCK cells in homotypic borders. Taken together, these results indicate that E-cadherin triggers and supports TJs at the junction between different types of epithelia cells, like the ones commonly occurring in the nephron and the gastrointestinal tract (103).

The life of higher organisms would be impossible without epithelial barriers that separate the internal from the environmental milieu. Therefore, it is conceivable that the most fundamental and evolutionary conserved role of the TJs is to act as a diffusion barrier and that finer regulation of TJ permeability, as well as their participation in the variety of functions mentioned in section I, would constitute a later development (for a recent review, see Refs. 23, 301). In this respect, it is interesting that Furuse et al. (165) deleted the COOH-terminal cytoplasmic domain of claudins and observed that these lose their ability to associate with ZO-1, ZO-2, and ZO-3. Nevertheless, these TJs still exhibit the characteristic meshwork of fibrils in freeze-fracture replicas.

VII. POLARITY

The life of a cell depends on thousands of different chemical reactions controlled by a myriad of enzyme species. Life would be impossible if these molecules had access to each other. Whenever a tissue is homogenized, temperature should be lowered below 4°C, and an abundant batteries of inhibitors should be added beforehand. Furthermore, each protein species should be targeted from the site of synthesis to the place in which it works (the Golgi apparatus, the cytoskeleton, peroxisomes, etc.), and degraded if found out of place (for a recent review, see Ref. 59). Therefore, all proteins should be assigned a specific place in the cell. In a wide sense, polarity is the consequence of targeting and selectively retaining different protein species to different sides of the cell, but the term is often restricted to the asymmetric expression of cellular features, such as microvilli, flagella, receptors, ion channels, pumps, and co- and countertransporters on a given pole of the cell, that constitute the structural basis of the asymmetric behavior of epithelia referred to in section I.

Until a couple of decades ago, it was taken for granted that polarity was a peculiar property of epithelial cells and neurons. Today it is the other way around: all cells from single ones like bacteria, yeast, and spermatozoa to hepatocytes and osteoblasts have some degree of polarization. Were it not for membrane targeting and polarity, the monomers of a receptor synthesized and sent at random to the plasma membrane would have to ramble and diffuse on the plane until they meet by chance the other partners. Polarity may therefore reveal a principle of economy that speeds up the assembly of membrane structures. The fact that polarity is so old and widely distributed is reflected in the constancy of its fundamental mechanisms and by the fact that molecules and mechanisms underlying polarity, such as E-cadherin and vinculin or genes such as PAR 3 and PAR 6, can be found to play similar roles from yeasts to Caenorhabditis, Drosophila, and humans (for recent reviews, see Refs. 59, 479). However, it is important to stress that there was a

FIG. 4. The tight junction is a promiscuous structure, yet it depends on adherens junctions which are not. Epifluorescence image of a monolayer formed by a mixed population of MDCK and LLC-PK1 cells. LLC-PK1 cells were labeled beforehand with 6.3 μM CMTMR.
very long evolution before polarity became triggered by contact clues and, besides of serving the own necessities of the cell, started to participate in the assembly of multicellular organisms (472).

A. The Transporting Epithelial Phenotype

The model put forward by Koefoed-Johnsen and Ussing (252, 456) had three basic asymmetries: 1) only the outward-facing membrane (apical) has a specific permeability to Na⁺; 2) only the inner facing membrane (basolateral) has a K⁺ specific permeability; and 3) Na⁺-K⁺-ATPase is asymmetrically situated on the inner facing membrane of the cell. In the following four decades the model of Koefoed-Johnsen and Ussing has been amply confirmed and complemented with important new findings summarized below.

B. Polarity and the TJ

Clear-cut demonstrations that TJs are not responsible for the polarized distribution of membrane proteins stem from the study of receptors for immunoglobins, ferritin, and cholesterol-binding-protein and from the observations already mentioned above that upon switching confluent MDCK cells to Ca²⁺, TJs form so rapidly that a fraction of Na⁺-K⁺-ATPase is trapped in the apical (wrong) side, but is thereafter removed and the cell attains the normal polarization of this enzyme in the next couple of hours (96). However, as discussed below, TJs may be in fact responsible for the polarized transport of fluid.

A TJ itself is polarized, as some of its molecules are targeted very precisely to and from the TJ to other places such as the basolateral membrane (165, 224, 299, 394). Actually, the experimental dissection between TJs and polarity is hard to achieve, as interference with evolutionarily conserved signaling cascades starting at membrane contacts affect both TJs and polarity. Proteins, such as PAR3, PAR4, and PAR6, identified originally in the nematode Caenorhabditis elegans (246) have homologs in Drosophila and mammals (281, 337, 427, 443, 444). Furthermore, Baas et al. (16) have recently identified a human homolog of PAR4, called LKB1, whose activation triggers the development of the whole transporting epithelial phenotype even in single, noncontacting cells. ZO-1 is a member of the same protein family as Drosophila Discs-large tumor suppressor (452, 476). The situation is in fact much more complex, because different TJ-associated signal-transduction systems might influence each other (for a recent review, see Ref. 301).

Nevertheless, the TJ is important to preserve the polarization of mobile membrane molecules such as lipids (126). But even this role does not seem to be that of a simple fence. Thus transfection of occludin with its COOH terminus truncated impairs the ability of the TJ to retain a fluorescent lipid probe in the apical domain (24).

C. Na⁺-K⁺-ATPase, Cell Attachment, and the Position of the TJ

The complex mechanisms described above are the product of a long evolution and might not have been present in primeval metazoans of pre-Cambrian times, more than 600 millions years ago. The first metazoans exchanging through epithelia must have had much simpler mechanisms. One of the simplest occurring in an epithelium would be a membrane molecule with the capacity to attach to a similar one in the neighboring cell, so the pair would get stuck at the contacting border (Fig. 5). The Na⁺-K⁺-ATPase might constitute a case in point. Axelsen and Palmgren (15) propose that the third subunit of bacterial K⁺-ATPase (KdpC) may be the ancestor of the X-K-ATPase β-subunit, due to the similar assembly functions they both perform. As discussed above, in higher species the β-subunit is tightly and specifically bound to the α-subunit (42), and the association begins as soon as they are synthesized in the endoplasmic reticulum (55) so that both reach the plasma membrane forming already a hard-to-dissociate dimer, in which β helps α to trap K⁺ in a pocket during the pumping cycle (464). However, it is not expected that they be firmly bound in early organisms Caenorhabditis elegans because sequence analyses show no association domains (338). Therefore, it is conceivable that the α-subunit became a stable resident of the lateral space of transporting epithelia by virtue of being bound to a β-subunit, which in turn has the ability to dwell in this position because of a linkage it makes with another β-subunit placed in the plasma membrane of a neighboring cell (Fig. 5).

However, a Na⁺-K⁺-ATPase located in a primitive epithelium as depicted in Figure 5 would be inefficient, as roughly one-half of the pumping fluid would leak back to the outer environment. Combinations of Na⁺-K⁺-ATPase at the cell-cell border with TJs would probably select epithelia in which TJs would be pushed toward the outermost end of the intercellular space, because it would ensure a maximal yield of fluid transported vectorially towards the inside (Fig. 5).

VIII. EVOLUTION OF THE EPITHELIAL VECTORIALLY TRANSPORTING PHENOTYPE

Evidence that mollusks can absorb ions from dilute media was already presented by August Krogh (261) (see Ref. 122). Water, ions, lipids, amino acids, and sugars were found to be transported by similar mechanisms in
the intestine in *Ascaris*, earthworms, freshwater clams, insects, as well as in the mammalian intestine (4, 107, 221, 235, 365).

Evolutionists refer to homology when two organs have the same role and evolutionary origin, but reserve analogy (an anatomical term) and homoplasy (its molecular counterpart) for organs that, in spite of playing the same physiological role, result from different phylogenetic processes. Classical examples are the wings of the butterfly and those of the bat. In this respect, the relationship between different occluding junctions is still debatable. Thus they play the role of occluding junctions (148, 308, 420) and share considerable homology between some of their molecules, such as claudins, ZO-1, and dlg (12, 485). Nevertheless, these types of junctions do show considerable differences in composition as well as in their evolution (301, 444).

The next step in evolution is constituted by *Cnidaria*, whose most conspicuous representative is the *hydra*, a freshwater coelenterate first described by Abraham Tembley in 1744 (as cited by Gierer and Meinhardt, Ref. 178). It reproduces through stem cells, and although its cells are not known to have apical/basolateral polarity, the organism does show regional differentiation, attributed to gradients of substances secreted by some of its cells (178). Fei et al. (144) have found that *Hydra vulgaris* has a homolog of ZO-1, termed HZO-1, that is a MAGUK protein, a family with members such as ZO-2, dlg-A, and TamA, which are involved in a wide variety of cellular functions such as TJ formation, cell proliferation, differentiation, and synapse formation.

### A. Evolution of the Na\(^+\)-K\(^+\)-ATPase

Thanks to the increasing number of organisms whose whole genome has been sequenced, Okamura et al. (338) could correlate the molecular evolution of the 11 subfamilies of the P-type ATPases within the establishment of the kingdoms of living things. Interestingly, heavy metal transporters (type 1B) and intracellular Ca\(^{2+}\)-ATPases (type 2A) are probably the most fundamental for life,
since these two types are found in every kingdom. They appeared very early in evolution and played critical roles in ion homeostasis. On the other hand, the ancestors of animals probably found the Na\(^{+}\)-K\(^{+}\)-ATPase and H\(^{+}\)-K\(^{+}\)-ATPase (type 2C) and got rid of the proton pump (type 3A) occurring in the lineage of plants and fungi. While the divergence of substrate specificity (ion selectivity) occurred early in the evolution of P-type ATPases and has been conserved ever since (15), the divergence of the P-type ATPase isoforms occurred after vertebrates and invertebrates separated (436). Recently, the study of evolution of P-type ATPases has been amplified with new information from the database for the nematode Caenorhabditis elegans and the fly Drosophila melanogaster. Focusing on key domains of both α- and β-subunits, such as the ouabain binding site and the α/β-assemble site, a set of novel isoforms that retain an ancestral characteristic of the Na\(^{+}\)-K\(^{+}\)- and H\(^{+}\)-K\(^{+}\)-ATPase have been identified (338, 339, 437). In the phylogenetic analysis, the β-subunits of C. elegans and D. melanogaster form a unique single cluster. Interestingly, D. melanogaster possesses two distinct types of β-subunits, one type closer to the vertebrate β-subunits and the other sharing more homology with the C. elegans β-subunits. As mentioned before, the functional expression of the Na\(^{+}\)-K\(^{+}\)-ATPase requires the assembly of the α- and β-subunits. In this regard, the newly identified invertebrate subunit isoforms (Ce2C3 and Ce2C4 and Dmβ4–6) are of particular interest because they lack the characteristic domains that have been demonstrated to be critical for α/β-assembly (140, 277, 467). Therefore, Takeyasu et al. (437) and Okamura et al. (338, 339) suggested that these nonassembling subunits may exist as lonely subunits and may play not-yet-identified function(s) other than in ATP-driven ion transport. Taken together, their studies suggest that the P-type ancestor first lacked the inhibitor binding site and the assembly domain, and therefore existed as a single subunit in lower invertebrate organisms. In its evolution, this ancestral form acquired the abilities to bind ouabain and to assemble with β-subunit, becoming the immediate ancestor of the currently known Na\(^{+}\)-K\(^{+}\)- and H\(^{+}\)-K\(^{+}\)-ATPase family. Although the information on the molecular evolution of the β-subunit is comparatively scarce, it has been suggested that it derives from the third subunit of bacterial K\(^{+}\)-ATPase (KdpC). Alternatively, the β\(_{\text{a}}\) protein of sarcoplasmic reticulum could also represent a primitive form of the β-subunit family of the X\(^{+}\)-K\(^{+}\)-ATPases.

B. Evolution of Junction Proteins

When Dictyostelium cells run out of nutrients, they polarize and adhere to form a multicellular structure that supports the spore head (260). During this process, the cells at the top of the stalk tube form a constriction with rings of cells that express AJs with the β-catenin analogous aardvark associated to the actin cytoskeleton. Aardvark is also independently required for cell signaling (197) and shares significant homology with Saccharomyces cerevisiae protein Vac8, Saccharomyces pombe SPBC354, and plant Arabidopsis thaliana AB016888 sequences. This demonstrates that in spite of lacking stable junctions, protozoa appear to have molecules that coordinate cytoskeletal dynamics, the position of the mitotic spindle and cell polarity, and that may have been precursors of molecules that make AJs and TJs in metazoa (197, 353).

β-Catenin that, as discussed above, has a dual role in cell-cell adhesion and cell signaling, is present in the nonmetazoan amoeba Dictyostelium discoideum, indicating that it evolved before the origins of metazoan (197). It seems plausible that β-catenin may have been a requisite for all multicellular development (197). In this sense, Dictyostelium has β-catenin that plays signaling and adhesion roles as in vertebrate. C. elegans instead diverged, because it has two catenins: HMP-2 for adhesion and Bar-1 for signaling to the nucleus (257).

Apical junctions in Caenorhabditis elegans have tetraspan VAB-9, which is a claudin-like membrane protein, that colocalizes with E-cadherin (HMR-1), and this localization depends on HMR-1, α-catenin (HMP-1), and β-catenin (HMP-2) (415).

Hua et al. (218) analyzed the phylogenetic trees of connexins, claudins, and occludins and found no sequence or motif similarity between the different families studied, indicating that, if they did evolve from a common ancestral gene, they have diverged considerably to fulfill separate and novel functions.

It has been recently demonstrated that Sinuous, a homolog of claudin in Drosophila, not only localizes to the septate junctions, but also confers to this structure its barrier function (33, 487). Kollmar et al. (256) only find a single claudin gene in the urochordate Halocynthia roretzi and concluded that claudins emerged when TJs replaced septate junctions. However, Asano et al. (12) found that C. elegans has no less than four different forms of claudins participating in the epithelial barrier.

Cadherins form a superfamily with at least six distinct subfamilies: classical or type-I cadherins, atypical or type II cadherins, desmocollins, desmogleins, protocadherins, and Flamingo cadherins. In addition, several cadherins clearly occupy isolated positions in the cadherin superfamily (454). Nollet et al. (332) suggest a different evolutionary origin of the protocadherin and Flamingo cadherin genes versus the genes encoding desmogleins, desmocollins, classical cadherins, and atypical cadherins. In contrast to classic cadherins, which bind catenins by the cytosplasmic domains and have genes with as much as 12 introns, nonclassic cadherins do not interact with catenins and genes have many fewer introns (485). Phylogenetic analyses suggest that there are four paralogous...
subfamilies (E-, N-, P-, and R-cadherins) of vertebrate classic cadherin proteins (168) and that these genes duplicated early in evolution (362).

Amphioxus cadherin BbC localizes to adherens junctions in the ectodermal epithelia of embryos and confer homotypic adhesive properties. It has a cytoplasmic domain whose sequence is highly related to the cytoplasmic sequences of all known classic cadherins, but its extracellular domain lacks the classical five extracellular cadherin repeats and is similar to the extracellular domain of nonchordate cadherins (336).

Choanoflagellates, a group of unicellular and colonial flagellates that resemble cells found only in Metazoa, express sequences of proteins that encode cadherin repeats. Phylogenetic analyses reveal that choanoflagellate cadherins are most similar to protocadherins and to the flamingo class of cadherins, demonstrating that these proteins evolved before the origin of animals and were later co-opted for development (248).

IX. THE DAWN OF METAZOANS AND TRANSPORTING EPITHELIA

The corelationship between multicellularity and transporting epithelia is stronger than the considerable variety of adaptive structures known to exist in many organisms. But a mere accumulation of cells might not qualify as metazoan. Thus, when nutrients are exhausted or wastes accumulate, cells may temporarily shut off their living processes, or form a transient multicellular structure (e.g., *D. discoideum*) and migrate somewhere else. Likewise, organisms such as Porifera, without real tissues nor organs, have no internal milieu because the environment can circulate through the body, all cells can exchange directly with the environment, and should not concern us here. We refer instead to stable metazoans that survive thanks to an internal milieu whose stable composition depends on transporting epithelia. Although the fossil record starts some 560–600 million years ago, there is evidence that they were already existing as early as 800–1,000 million years ago (104, 388). Ideas about metazoan phylogeny are numerous and somewhat conflicting (see Refs. 14, 104, 322, 323, 404, 475). Furthermore, our search for an ancestor of metazoans with true epithelia may start with a plausible idea of how such an organism should have been, regardless of whether it is still surviving or has been long extinct. The elaboration on a priori possible organisms might at least afford a useful working hypothesis to solve the conundrum mentioned in section 1 on the origins of metazoans and transporting epithelia.

A. The “Thrifty Sponge”

It is conceivable that in a given moment a group of cells became surrounded by a primitive epithelium, i.e., one whose cells did not exhibit vectorial transport, but that attach and become an impermeable barrier (Fig. 6, *top*). Secreted molecules such as nutrients or cAMP may thereby be momentarily conserved and used as food stuff.

**Fig. 6.** Hypothetical steps towards a metazoan surrounded by a transporting epithelium. *Top panels:* “thrifty sponge.” A: in a given moment, cells are surrounded by a primitive epithelial layer of nonpolarized cells, and secrete a valuable substance (a nutrient? a signal?). The surrounding cells not only lack polarity, but do not have a selective permeability either. B: although cells profit from the valuable substance they secrete, the internal milieu is exhausted of nutrients and polluted by wastes. C: as a consequence of intoxication, or because an increase in osmolarity of the internal milieu bursts the “thrifty sponge,” the organism is flushed by the outer environment. D: a conglomerate of cells adopts a very flat structure and is surrounded by a primitive epithelium without specific permeability, yet the area-to-volume ratio is enough to ensure a satisfactory exchange with the environment. Peristaltic movements (arrow) might help stir the internal milieu. E: “mare nostrum.” A polarized cell divides. Its descendants stay attached to each other, generate an internal milieu, and because of the orientation of the mitotic spindle, all descendant cells have the same polarization. F: perpendicular orientation of the mitotic spindle provokes the development of an internal body of cells (pink). Alternatively, this development can be originated through a process resembling epithelial-mesenchymal transition.
or signals, yet the entrapped milieu would gradually spoil and force a transient breakage of the epithelium, thus allowing the external medium to gain access to the cells and flush the organism. One may even conceive a certain synchrony between periods of secretion and epithelial tightness. In this respect, Green and Bergquist (193–195) have found that in sponges, cell junctions are apparently formed only when required for a specific purpose.

B. Very Flat Organisms

Another possibility would be a very flat organism with large surface-to-volume ratio, sufficient to allow exchange with the surroundings (Fig. 6, middle). Although these putative organisms might not have had circulatory apparatuses, peristaltic movements might have helped to stir the internal milieu and favor exchange. Actually, nature seems to have resorted to large surface-to-volume body plans and ease the survival of primitive metazoans. Thus Cnidaria, just one evolutionary step above sponges, have wide and flat structures.

C. The “Mare Nostrum” Metazoans

Up to this point we have been asking how did primeval metazoans develop their first epithelium. We cannot discard the opposite situation though. Because polarization is already observed in unicellars, the possibility exists that an already polarized cell would proliferate without completing the separation of its descendants, and somehow profit from preserving an internal milieu (Fig. 6, bottom). Furthermore, proliferating epithelial cells usually have their mitotic spindle parallel to the surface of the epithelium so that proliferation expands the area of the organ (for a review, see Ref. 353). Yet when the spindle is perpendicular to the epithelium, it provokes the formation of an outgrowth similar in many respects to the body mass of a higher organism.

X. Na⁺-K⁺-ATPase AND CELL ADHESION

A. Role of the β-Subunit in Cell Attachment

Na⁺-K⁺-ATPase arrives to the lateral membrane, interacts with ankyrin, and becomes anchored to the cytoskeleton that stabilizes the enzyme in this position (206), yet we still ignore why this enzyme binds to the cytoskeleton at the lateral borders but does not bind to this structure when expressed at other cell borders. Furthermore, Na⁺-K⁺-ATPase in Drosophila epithelia does not bind to ankyrin but is nevertheless polarized (26). To develop a more plausible explanation, we took as hints that 1) when a monolayer of cultured MDCK cells is treated with EGTA, the chicken fence image of Na⁺-K⁺-ATPase (Fig. 7A) splits into two moieties and each neighboring cell retrieves its own (Fig. 7B), indicating that each cell contributes the pumps and opening the possibility that these interact across the intercellular space. 2) In keeping with such conjunction, MDCK cells express Na⁺-K⁺-ATPase at the lateral but not at the basal borders, as if only at the intercellular space the enzyme would find an attaching partner on an opposite cell. 3) When cocultured with Ma104 cells (epithelial from monkey kidney), an MDCK cell only expresses Na⁺-K⁺-ATPase in a given border provided its neighbor is another MDCK cell (Fig. 7C), suggesting that the putative Na⁺-K⁺-ATPase/Na⁺-K⁺-ATPase interaction is a specific one (Fig. 5). 4) As mentioned above, Gloor et al. (180) have shown that the β₂-subunit of glial cells acts as a cell adhesion molecule and has the corresponding structure: a short cytoplasmic tail, a single transmembrane domain, as well as long and glycosylated extracellular fragment. MDCK cells have instead the β₁-isofrom, but its structure is almost identical to that of β₂. This suggests that the β-subunit of Na⁺-K⁺-ATPase may establish a cell-cell contact as suggested in Figure 5. 5) Furthermore, this subunit is required for the formation of the septate junction of Drosophila (486). 6) As discussed below, the β-subunit of Na⁺-K⁺-ATPase does participate in cell attachment.

On this basis, we have proposed that the polarized position of Na⁺-K⁺-ATPase at the lateral borders of epithelial cells depends on the binding ability of its β-subunit. The expression of this enzyme at the plasma membrane facing the intercellular space would be the only place where the β-subunits of neighboring cells can interact (77, 78, 98). This view has recently received experimental support from the studies illustrated in Figure 7, D and E. Briefly, it is observed that MDCK cells only express their β-subunits at the point of contact with another MDCK cell, but not in contacts with CHO fibroblasts. However, when CHO cells are transsected beforehand with β-subunit from the dog (i.e., same species as MDCK cells), MDCK cells now do express their β-subunits in heterotypic MDCK/CHO contacts.

B. (P→A), a Pump (P) Adhesion (A) Mechanism

Until a few years ago, it was assumed that, while synapses exchange valuable signals, contacts between other cell types were neutral and uneventful, and the role of cell attachment was reduced to secure the firm and definitive position of cells in a tissue. Then it was realized that attachments have to be somehow regulated, as revealed in the following circumstances: 1) when cells divide in the crypt of microvilli and migrate to the apex, and 2) cells of different types mixed in a suspension sort themselves spontaneously and group into islets of specific
cell types. 3) The points where cells attach to a neighbor or to the substrate are like tips of icebergs sunk into the cytoplasm, whose molecules form scaffolds, associate with the cytoskeleton, send and receive specific molecules to the nucleus and other organelles, as well as exhibit the complex patterns of phosphorylation/dephosphorylation in specific amino acids that we mentioned above. 4) The nucleus changes the pattern of gene expression in response to the arrival of the NACos (Fig. 3, Table 1). 5) After severing the attachment with its neighbors, cells may undergo proliferation, differentiation, migration, or apoptosis, in which batteries of genes are turned on or off. 6) The differentiation of a given cell type depends on the nature of contacts with its neighbors. Nowhere is this process more astonishing than in the self-assembly of a brain, where genes, by obeying just chemical rules, produce the most complex three-dimensional ordered object known, depending on the species of attaching molecules coded, and the timing of their expression in axons and dendrites of the neurons. 7) There is a growing amount of evidence of cancers and autoimmune diseases associated with failures in molecules involved in cell contacts (76, 77, 182). On this basis we tentatively attribute the very emergence of metazoans to special combinations of attachments between specific cells as well as between specific molecules.

In keeping with the importance of the processes mentioned in the previous paragraph, cell-cell attachment seems to be strictly regulated. Yet the information presently available on this regulation is very scant. We found a mechanism termed P→A, which transduces the occupancy of the Na⁺-K⁺-ATPase by ouabain, into a signaling to molecules involved in cell-cell and cell-substrate attachment (A), that promotes these to release the grip and results in detachment of the cell (102). Thus MDCK cells treated with ouabain increase Tyr phosphorylation and content of active MAPK, redistribute molecules involved in cell attachment (occludin, ZO-1, desmoplakin, cytokeratin, β-actinin, vinculin, and actin), and detach. Genistein and UO126, inhibitors of protein tyrosine kinase and MAPK kinase, respectively, block this detachment. The content of P190Rho-GAP, a GTPase activating protein of the Rho small G protein subfamily, is increased by ouabain, suggesting that both the Rho/Rac and MAPK pathways may be involved.

The P→A mechanism may play several potential roles, in views of the following: 1) damaged epithelial cells endanger the permeability barrier between higher organisms and the environment. An injured cell would conceivably produce less ATP, the activity of the Na⁺-K⁺-ATPase would decrease, thus triggering the P→A mechanism that promotes its own detachment and replace-

---

**FIG. 7.** Patterns in the expression of Na⁺-K⁺-ATPase in epithelial cells. A: in MDCK cells cultured in monolayers, Na⁺-K⁺-ATPase is expressed at the lateral borders, forming a chicken fence pattern. B: cell detachment provoked by 2.0 mM EGTA shows that each cell carries Na⁺-K⁺-ATPase in its borders, implying that the image in Figure 7A is given by the enzyme present in the two neighboring cells. C: a monolayer prepared with a mixed population of MDCK cells and Ma104 cells, which were previously stained with the red dye CMTPR. The use of a mouse antibody specific for the β-subunit of MDCK, followed by a goat anti-mouse fluoresceinated antibody, indicates that this subunit only occupies the cellular border of an MDCK cell, provided the neighboring cell is another MDCK one (green), but not when the border contacts an Ma104 cell. D: in mixtures of MDCK with CHO cells (fibroblasts derived from Chinese hamster ovary), MDCK cells only express Na⁺-K⁺-ATPases in homotypic (MDCK/MDCK) but not in heterotypic MDCK/CHO borders (open arrows). However, when CHO cells are transfected beforehand with dog β-subunit, MDCK cells do express their β-subunits in heterotypic contacts (E, green arrows). Bar = 20 μm.
ment. 2) Because junctions help maintain the architecture of tissues by holding neighboring cells, they would need to relax the grip whenever a change in the position of the cells is required. A few situations in which such relax would be needed are as follows: 1) during the healing of a wound, cells proliferate and migrate to occupy the space left by dead cells; 2) some tissues minimize the risks derived from errors in proliferation by restricting cell division to stem cells, and to regularly shed the mature cells of the progeny. Thus in the frog skin cells originate in the innermost stratum of the multilayered epithelium (stratum germinativum), migrate towards the outside, and only establish occluding junctions in the very last stages, as the permeability barrier thus established impairs the nutrition of all cells located beyond this point (stratum corneum). Cells of this stratum detach simultaneously and molt under the control of the hypophysis. 3) During organogenesis the various cell types involved undergo a wide range of proliferation rates and distribution that enable them to adopt the typical architecture of a given tissue. These migrations and positioning would be favored by a P→A mechanism that relaxes the adhesion between neighboring cells in a specific manner. Conceivably, this relaxation may be cell specific if each cell expresses a given isoform of α-subunit with different sensitivity to ouabain (42, 234), i.e., with different ability to detach when challenged by the hormone through the P→A mechanism. 4) Adhesion may be suspected to play a role in the development of metastasis, as cells must dislodge themselves from the mass of the main tumor and home among the cells of a distant organ. 3) Actually, the response to ouabain challenge depends on the type of cell involved. Thus it may cause apoptosis in MDCK cells from dogs, but not in Ma104 from monkeys, which may even protect the former in cocultures (45, 98, 99, 346). 4) Both pumping and enzymatic activity of the Na⁺-K⁺-ATPase can be specifically inhibited by the cardiac glycoside ouabain (397, 417) that binds to an extracellular site of its α-subunit with a high affinity (Kᵢₐ < 10⁻⁸ M). However, there is growing evidence that ouabain is a hormone normally present in the blood (403). Therefore, it is conceivable that detachment and removal of a given cell type may also be promoted by endogenous ouabain-like substances (403). 5) Metastases start by a detachment of cancer cells from the mass of the main tumor. Furthermore, Ward et al. (469) have recently described the union of ouabain to a receptor different from Na⁺-K⁺-ATPase whose function is not yet elucidated, suggesting that, eventually, the P→A mechanism may be found to be triggered through a receptor other than the α-subunit of the Na⁺-K⁺-ATPase.

Obviously, once the P→A mechanism has completely detached, a cell from its neighbors and from the substrate, all exchange of information is interrupted. Yet this is an extreme situation produced by relatively high concentrations of ouabain, but there are a variety of cell-cell contacts, as well as subtler degrees of detachment. Figure 3 illustrates the case of a NACo (β-catenin) that is addressed to the nucleus by ouabain, and another that is not (ZO-1). Taken together, this information suggests that by expressing a variety of α-subunits with different ouabain sensitivities (214, 385, 438), the Na⁺-K⁺-ATPase may be controlled by the hormone ouabain, and activate a P→A mechanism that deepens in the function of a given cell.

In summary, we cannot provide an answer to the question raised in section 1 about the origin of transporting epithelia and metazoans, but after systematizing the information obtained from widely heterogeneous fields, it seems that ancestral unicellulars already possessed most of the precursor molecules to build up transporting epithelia and form a perhaps transient metazoan that evolution might subject to selective pressures. Attachment, both at the molecular and the cellular level, seems to have played a paramount role.

We thank Dr. Catalina Flores-Maldonado and Lic. Isabel Larré for helpful discussion and furnishing their unpublished results in Figure 3, B–E. We also acknowledge the efficient and pleasant help of Elizabeth del Oso.

This work was supported by the National Research Council of Mexico (CONACyT).

Address for reprint requests and other correspondence: M. Cereijido, Center For Research and Advanced Studies, Dept. of Physiology, Biophysics, and Neurosciences, Avenida Instituto Politécnico Nacional 2508, Cédula Postal 07360, México D.F., Mexico (E-mail: cereijido@fisio.cinvestav.mx).

REFERENCES

ADHESION AND POLARITY IN THE DAWN OF METAZOANS


94. Colegio OR, Van Itallie CM, Mccrea HJ, Rahner C, and Anderson JM. Claudins create selective-channels in the paracel-


98. Crambert G, Beguin P, Pestov NB, Modyanov NN, and Geer-...


223. Ikenouchi J, Matsuda M, Furuse M, and Tsukita S. Regulation of tight junctions during the epithelium-mesenchyme transition:


ADHESION AND POLARITY IN THE DAWN OF METAZOANS


343. Perez-Moreno M, Agm A, Islas S, Sanchez S, and Gonzalez-Mariscal L. Vinculin but not alpha-actinin is a target of PKC


426. Takeya K, Renaud KJ, Taormino J, Wolitzkt BA, Barnstein AM, Tamkun MM, and Fambrough DM. Differential subunit and...


443. Tsukita S, Itoh M, Nagafuchi A, Yonemura S, and Tsukita S. 

444. Uemura T. 

445. Turin L, Behe P, Plonsky I, and Dunina-Barkovskaya A. 


