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Topical Review

Isolated-Polarized Epithelial Cells as an Experimental System for Cell Physiology Studies

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Introduction

Transporting epithelial cells are endowed with polarity, that is, different regions of the cell differ structurally and functionally. The most important aspect of epithelial cell polarity is that the transport proteins expressed in the apical and basolateral membrane are different. This property is essential for epithelia to perform transepithelial net transport (vectorial transport) when both sides are bathed in solutions of the same composition. Epithelial cell polarity involves also other biochemical differences (e.g., different phospholipid composition of the two plasma membrane domains), as well as structural differences between apical and basolateral regions, particularly in the cytoskeleton and distribution of organelles (Fig 1).

Epithelial cells contact their neighbors *via* tight junctions, desmosomes, and gap junctions. Desmosomes attach the cells mechanically, by binding of cell adhesion molecules (CAMs) of the adjoining cells. Ca^{2+}-dependent CAMs are called cadherins and are integral membrane glycoproteins with a single transmembrane domain. The one expressed in epithelia is cadherin E or uvomorulin. The intracellular domain of E-cadherin interacts with the cytoskeleton in a complex fashion (*reviewed* by Buxton, 1993).

Gap junctions are channels that communicate the cytosol of adjacent cells. Each cell contributes a connexon, which is a hexamer of transmembrane proteins called connexins (a family consisting of at least 14 members. In the connexon the six connexin molecules are parallel to each other, perpendicular to the plasma membrane, leaving a space in the middle. A gap junction (cell-to-cell channel) is formed by two connexons belonging to adjacent cells, joined in series. Gap junctions allow the permeation of molecules up to about 1 kDa. Their functional regulation is isoform-dependent and may involve phosphorylation, and effects of intracellular pH, intracellular pCa and/or membrane voltage (Bennett et al., 1991). A functional epithelium requires also contacts with the basal lamina, a region at the interface with the underlying connective tissue, via hemidesmosomes and focal contacts. Both forms of contact involve integrins, transmembrane proteins that bind to motifs in molecules of the extracellular matrix. In the intracellular side, hemidesmosomes attach to intermediate filaments *via* a desmoplakin-like protein and focal contacts attach to actin filaments *via* a complex including talin, α-actinin and vinculin. In epithelial cells, integrins are involved in the initiation of cell polarization following plating of dissociated cells (Rodríguez-Boulan & Nelson, 1989; Ruoslahti et al., 1994).

The apical and basolateral membrane domains are separated by the junctional complexes, which include the zonula occludens ("tight junction") and the zonula adherens. The zonula occludens is formed by transmembrane proteins arranged in linear polymers, bound to similar proteins in the adjacent cell. This is visualized in freeze-fracture microscopy as strands arranged in parallel, with branches and anastomoses. It is thought that in the junctions the outer leaflets of the plasma membranes are fused. However, although in many epithelia the

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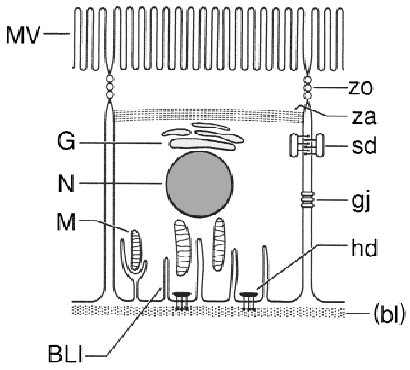

Fig. 1. Main features of epithelial cell polarity. The diagram depicts a cross section of an epithelial cell and small portions of two neighbors. The apical (luminal) surface faces up. MV = microvilli; G = Golgi apparatus; N = nucleus; M = mitochondria; BLI = basolateral-membrane infoldings; zo = zonula occludens; za = zonula adherens; sd = spot desmosome; gj = gap junction; hd = hemidesmosome; bl = basal lamina. Positions of organelles are typical of most epithelial cells. Reproduced from Reuss (1997) with permission.

junctions are quite impermeable (tight), in others they allow passage of small ions (leaky junctions). It is thought that junctional transport is passive, although it may involve permeation through pores and interactions with specific proteins, such as paracellin-1, a recently identified protein that appears to mediate the paracellular transport of Mg^{2+} in the ascending limb of the loop of Henle (Simon et al., 1999). The tight junctions have three functions (Diamond, 1977): (i) Barrier function: the junctions limit the intercellular (transjunctional) transport of polar substances. (ii) Gate function: the permeation of some small hydrophilic substances in certain junctions. (iii) Fence function: molecules that span both leaflets of the plasma membrane do not diffuse between apical and basolateral domains; in contrast, molecules contained within the inner leaflet can diffuse between the two domains (Dragsten, Blumenthal & Handler, 1981). Recent studies suggested that the protein occludin was the junctional protein accounting for cell-cell contact (Furuse et al., 1993; Gumbiner, 1993), but gene knock-out experiments revealed that tight junctions can form in the absence of occludin (Saito et al., 1998). It is likely that transmembrane proteins called claudins form part of the junction strands (Furuse et al., 1998). In liver, the strands would be formed by three proteins: occludin, claudin-1 and claudin-2. Other proteins contribute to the assembly and function of the junctions (e.g., ZO-1, *see* Anderson, Balda & Fanning, 1993; Balda & Anderson, 1993), but do not form part of the junctions themselves.

Understanding transepithelial ion transport requires the physiologist to ascertain cell function at the single-membrane level. This is a difficult endeavor on at least two grounds. First, because even the structure of the simplest ion-transporting epithelium is complex, i.e., an equivalent circuit consisting of three batteries and three resistances. This implies that the single-cell properties must be extracted from difficult-to-obtain experimental data, such as two-dimensional cable analysis, but cannot be obtained directly, except in very special instances. Second, many epithelia have two or more cell types. In these cases, the transepithelial properties are a weighed average of all cell types and the paracellular pathway(s). If the cells are electrically coupled via gap junctions, then the electrical properties assessed in single cells are also a complicated function of the entire population. If the different cell types are not communicated with one another, then their individual properties must be ascertained at the individual-cell level. Patch-clamp experiments do not have this limitation, but nevertheless are difficult in the assembled epithelium because of the mucus layer that frequently covers the apical surface, and because of the difficult access to the basal surface of the cells, attached to the basal lamina and the underlying connective tissue. Finally, the optical properties of the cells of the assembled epithelium are obscured by the presence of subepithelial layers.

For these reasons, a preparation of isolated epithelial cells retaining their functional properties, including cell polarity, may represent a major improvement over existing experimental systems for the study of the transport properties of epithelial cell membranes. This article is intended to provide an overview of the development of enzymatic methods to dissociated epithelial cells under conditions that preserve their polarized structure and function. I present arguments to validate the use of these experimental preparations, as well as examples of the experimental approaches that have been employed to study these cells from a cell physiology point of view.

Epithelia Can be Dissociated to Yield Viable Figure-Eight Cells

Polarized epithelial cells can be obtained by mechanical (Moore, Madara & MacLeod, 1994) and enzymatic methods (*see below*). Several enzymatic-dissociation procedures have been used to obtain populations of epithelial cells with a bilobated shape, hereafter, "figure-eight cells" (e.g., Supplisson, Loo & Sachs, 1991, 1993; Filipovic & Sackin, 1992; Robson & Hunter, 1994). These treatments decouple the cells from each other and from the underlying basal lamina. A subset of the dissociated cells, the figure-eight cells, remain fully polarized for many hours to days, as detailed below. In addition to figure-eight cells, these procedures yield two other populations: round cells, nonpolarized but viable, and permeabilized cells, that do not exclude trypan blue and are likely the result of mechanical or enzymatic damage (Fig. 2).

A successful cell-dissociation technique in both amphibian and mammalian native epithelia or monolayers in culture is exposure to collagenase and protease (Torres et al., 1996*a,b*). The precise concentrations and time of exposure vary depending on cell type. The percentage of polarized cells also varies among preparations and appears to be consistently higher in amphibian than in mammalian epithelia. Most investigators using these methods avoid trypsin (we found no figure-eight cells after trypsin dissociation in *Necturus* gallbladder epithelium; Torres et al., 1996*a,b*). However, Filipovic and Sackin (1992) found a low concentration of trypsin to be effective in *Necturus* renal proximal tubule in yielding a sizable population of polarized cells after dissociation, and Willumsen and Larsen (1997) were able to obtain polarized mitochondria-rich epithelial cells from toad skin after trypsin treatment. It is unclear whether these differences are ascribable to cell types, enzyme batches, or enzyme dose and time of exposure.

Two laboratories have validated this preparation at the biochemical, immunological and electrophysiological levels: Boulpaep's (Segal, Boulpaep & Maunsbach, 1996) and mine (Torres et al., 1996*a,b*, 1997; Vanoye & Reuss, 1999; Vanoye, Vergara & Reuss, 1999).

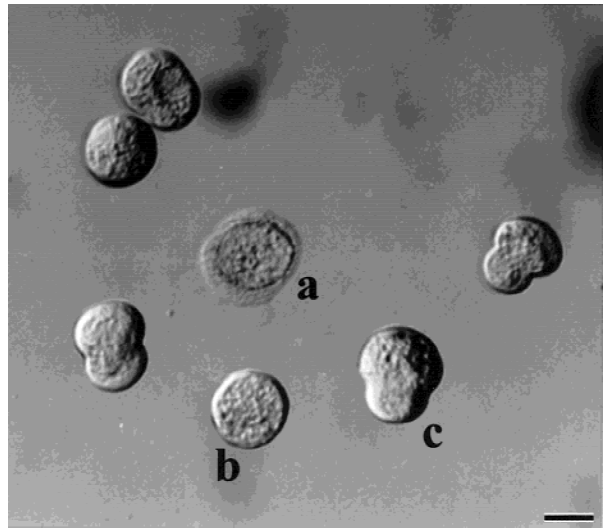

Fig. 2. Isolated cells from the gallbladder epithelium of *Necturus maculosus.* Hoffman modulation contrast. Bar: 20 (μm. Cells were isolated by exposure to collagenase and protease. Cell types found are: permeabilized, damaged cells (*a*), round cells (nonpolarized, *b*) and figure-eight cells (polarized, *c*). Reproduced from Torres et al. (1996*a*) with permission.

Figure-Eight Cells Have Typical Morphological Features

Figure-eight cells exhibit two lobes, more or less well formed, separated by a narrow region rich in actin. The small lobe corresponds to the apical-membrane domain and the large one to the basolateral-membrane domain; the latter lobe contains the nucleus. Immunofluorescence and electron-microscopy studies reveal polarization of the cytoskeleton, and polarized distribution of membrane-associated and transmembrane proteins (transport and cell-adhesion proteins, among others).

Cells isolated with the methods currently in use can last for days in vitro. Segal et al. (1996) reported that over one week there was no noticeable reduction in the percentage of polarized cells from the proximal tubule of *Ambystoma tigrinum* kidney. We have observed that under in vitro conditions, including exposure to a physiological salt solution and light, figure-eight cells from amphibian and mammalian epithelia last for many hours, sufficient for long electrophysiologic or microscopic studies.

Figure-Eight Cells Are Healthy

Several techniques can and have been used to assess the viability of the epithelial cells isolated as described above or by similar procedures. In this section, I discuss and illustrate the principal ones.

Cell Morphology

In addition to their typical shape, the figure-eight cells do not exhibit swelling, membrane blebs, membrane separation from the cytoplasm or other forms of membrane-cytoskeleton detachment.

Dye Exclusion

Trypan blue and other dyes permeate only cells whose plasma membrane is damaged. Figure-eight cells are not loaded with these dyes under control conditions that prevent endocytosis.

Cytosolic Level of Glutathione

The probe CMFDA is plasma-membrane permeable and its fluorescence is proportional to the glutathione levels (Poot et al., 1991). Therefore, a high intracellular CMFDA fluorescence denotes cell viability. In our experience, figure-eight and round cells have high CMFDA fluorescence, whereas permeabilized cells do not (Torres et al., 1996*a*).

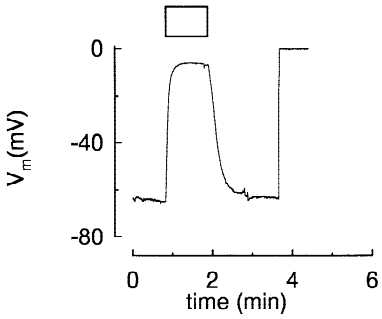

Fig. 3. An isolated figure-eight cell from *Necturus* gallbladder epithelium was attached with Cell-TakR to a dialysis membrane mounted in a microelectrode chamber, superfused with physiological salt solution and impaled with a glass microelectrode filled with 1 M KCl. When the membrane-voltage (*Vm*) record was stable, external [K^+] was transiently elevated from 2.5 to 92.5 mM for the time denoted by the bar. This caused a large, reversible depolarization. At the end, the microelectrode was withdrawn. Membrane voltage was measured in 7 cells, with similar results. Reproduced from Torres et al. (1996*a*) with permission.

Membrane Voltage

Intracellular microelectrode studies and whole-cell patch-clamp studies denote preservation of the membrane voltage in figure-eight cells (Segal et al., 1996; Torres et al., 1996*a,b*). A high membrane potential (similar to the value in the assembled epithelium) during exposure of the cells to an extracellular solution based on NaCl and $NaHCO_3$, with a low KCl concentration, reflects two facts. First, that the ionic selectivity of the plasma membrane (with the K permeability, P_K, being usually the dominant) is preserved. Second, that the differences in ion concentrations (principally K^+) between intra- and extracellular compartments are also maintained. These concentration differences depend largely on the function of the Na^+, K^+-ATPase, and therefore a preserved membrane voltage indicate that the ATPase is functional and the cytosolic ATP levels adequate. Thus, a normal membrane voltage provides very significant information about cell health. Figure 3 shows an example of determinations of membrane voltage and plasma-membrane K^+ selectivity in a figure-eight cell from *Necturus* gallbladder epithelium.

Endocytosis

The figure-eight cells exhibit temperature-dependent endocytosis. As discussed below, this is a critical point for dye-uptake studies.

Structural and Functional Criteria Demonstrate That Figure-Eight Cells Are Fully Polarized

Our experience is that cells that retain a clear figure-eight shape exhibit also the other features denoting preserved polarity (Torres et al., 1996*a,b*). These include structural and functional aspects.

Cytoskeleton Polarization

The distribution of actin in figure-eight cells is as in the native cells: preferential expression near the cell membrane domains, in particular the apical membrane and microvilli (Segal et al., 1996; Torres et al., 1997, *see also* below and Fig. 8). The belt region separating apical and basolateral domains has a high actin density and is likely an actomyosin ring.

Asymmetric Distribution of Hydrophobic Fluorescent Probes

In our studies in the gallbladder and urinary bladder of *Necturus maculosus*, as well as in certain mammalian epithelia, we exposed the isolated cells to hydrophobic fluorophores. Interestingly, the apical/basolateral membrane distribution of the fluorescence was in some cases even (di-4-ANEPPS), and in other cases very asymmetric (AFC-16), with much higher fluorescence intensity in the apical-membrane domain (Torres et al., 1996*a*). The results cannot be explained by differences in volume of phospholipid per unit area of membrane, but denote a difference in apparent probe solubility in the two domains, either because their lipid compositions are different or because putative probe-binding protein(s) differ(s) between the two domains.

Lack of Diffusion of Membrane Proteins from the Apical- to the Basolateral-Membrane Domain

A crucial feature of cell polarity is that lateral diffusion of proteins between the two membrane domains does not occur. In other words, transporters (channels, carriers and pumps), receptors, and other membrane proteins, remain segregated to one of the two membrane domains (Torres et al., 1996*a,b*). This could be explained because the membrane proteins are directly or indirectly anchored to the cytoskeleton and/or because of the existence of a barrier between the two domains (fence function of the tight junction, *see* above). We have tested whether the membrane proteins remain segregated in figure-eight cells by labeling the glycoproteins of the apical surface of the epithelium with wheat-germ aglutinin conjugated with a fluorophore (FITC). Following cell dissociation, in the figure-eight cells the label remains in the apical membrane while in the round cells the label becomes distributed in the entire cell surface (Fig. 4).

Polarized Expression of Membrane-linked Enzymes

Alkaline phosphatase is expressed exclusively on the apical membrane of *Ambystoma* renal proximal tubule cells (Segal et al., 1996).

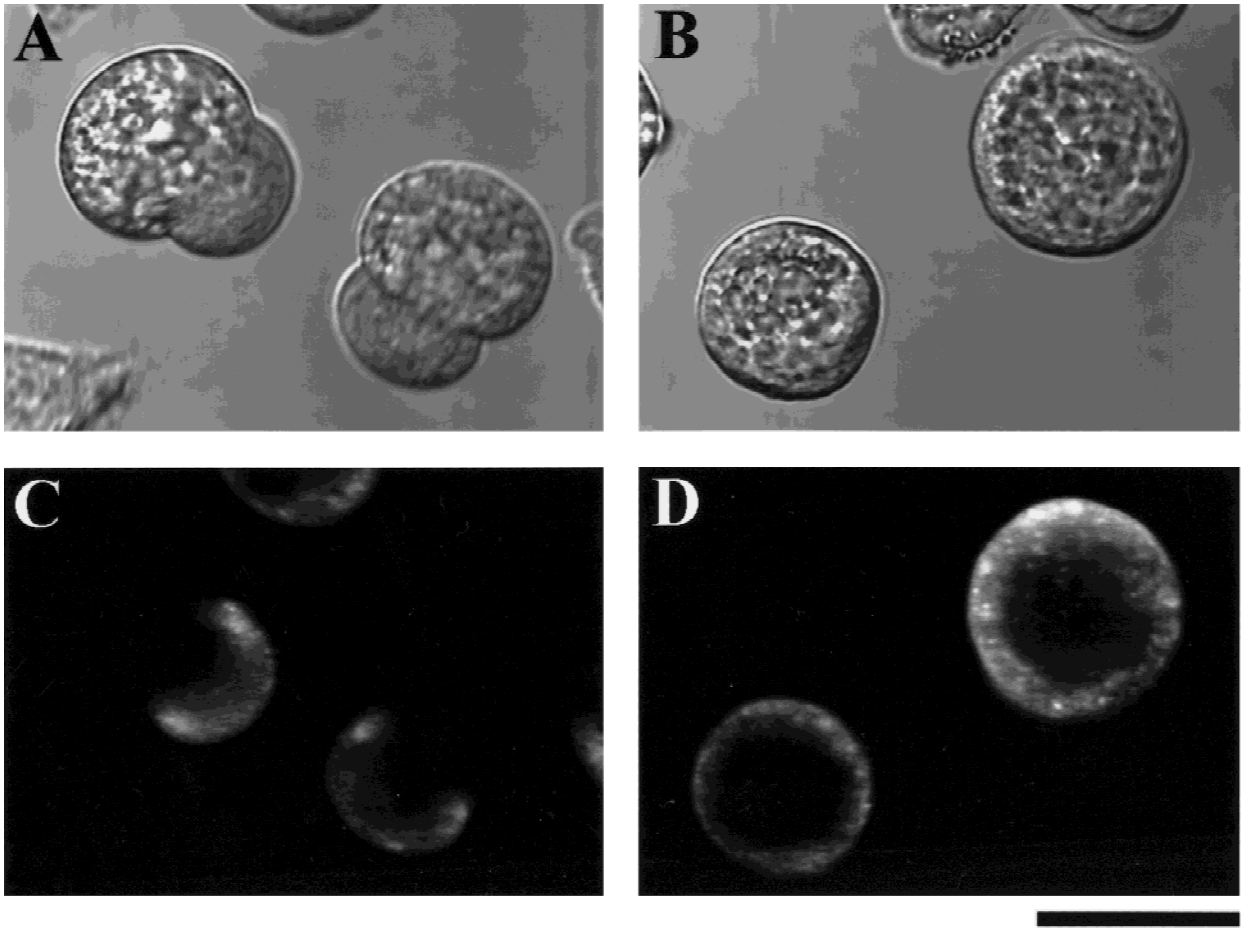

Fig. 4. *Necturus* urinary bladder epithelial cells labeled on the apical surface with WGA-FITC prior to cell isolation. (*A* and *B*) transmission images; (*C* and *D*) fluorescence images of the same cells; *A, C*: polarized; *B, D*: nonpolarized. Calibration bar: 30 μm. Reproduced from Vanoye et al. (1999).

Polarized Expression of Membrane Transport Proteins

From a functional point of view, this is the most important feature of epithelial-cell polarity. Ion-transport proteins are expressed exclusively or dominantly in one or the other membrane domain (apical or basolateral) in figure-eight cells, and this location is consonant with both the location and the function of these proteins in the intact epithelium. This has been shown by immunological methods (immunofluorescence, immunocytochemistry or immunoEM), as well as by physiological techniques. Immunological demonstrations include the following: (*a*) apical-membrane expression of the Na^+/H^+ exchanger NHE3 and the Cl^- channel CFTR (Fig. 5), and basolateral expression of the Na^+,K^+-ATPase in *Necturus* gallbladder (Torres et al., 1996*a*,*b*); (*b*) basolateral-membrane expression of the Na^+,K^+-ATPase in *Ambystoma* proximal tubule (Segal et al., 1996); (*c*) apical-membrane expression of the water pore aquaporin 1 and the $Na^+,K^+,2Cl^-$ cotransporter in apical-membrane, and of the Na^+,K^+-ATPase and the Cl^-/HCO_3^- exchanger AE2 in the basolateral membrane of rat choroid plexus cells (Wu et al., 1998). These studies demonstrate the location of the antigenic protein near a particular cell-membrane domain, but do not prove that the protein is inserted in the membrane and properly folded. Such proof has been obtained from functional studies: (*a*) Patch-clamp studies revealing different properties (single-channel conductance, open probability, ion selectivity, current-voltage relationship, regulation, and/or pharmacological sensitivity) of ion channels found in the different membrane domains (Segal et al., 1996; Torres et al., 1996*a*). (*b*) Demonstration of different ionic selectivity of the two membrane domains by regional superfusion of impaled figure-eight cells with solutions of different ion compositions (Fig. 6). (*c*) Intracellular pH recovery from an acid load (and effect of amiloride) to demonstrate function of NHE3 (Torres et al., 1996*a*). (*d*) Biphasic increase in $[Ca^{2+}]_i$ after exposure to hyposmotic solution demonstrating Ca^{2+} entry and release from intracellular stores (Torres et al., 1997).

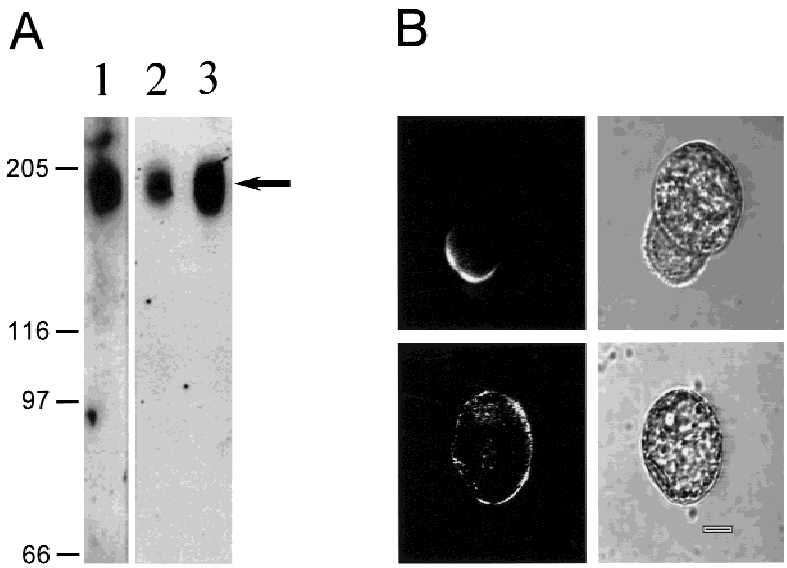

Fig. 5. Detection of a CFTR homologue in *Necturus* gallbladder epithelial cells. (*A*) CFTR immunoblot. Lane 1: 150 μg of *Necturus* gallbladder epithelium membrane proteins. Lanes 2 and 3: 12.5 and 25 (μg of T84-cell proteins, respectively. Arrow: mature (fully glycosylated) human CFTR. (*B*) Indirect immunofluorescence. In figure-eight cells, the CFTR homologue was detected at the apical membrane domain (upper left panel; right panel shows corresponding transmission image). In round cells, the CFTR homologue was also detected at the cell membrane but it did not show polarized expression (lower panels). Reproduced from Torres et al. (1996*b*) with permission.

Physiological Regulation of Transport Processes Is Preserved in Figure-Eight Cells

Functional studies in figure-eight cells with polarized expression of ion channels have demonstrated that these channels can be regulated. Examples are the modulation of basolateral-membrane K^+ channels by actin in *Ambystoma* proximal tubule cells (Segal et al., 1996), the cAMP regulation of the CFTR-mediated conductance of the apical membrane in *Necturus* gallbladder cells, and the activation of nonselective (Segal et al., 1996) or K^+ channels by membrane stretch in several isolated-cell preparations (Sackin, 1989; Filipovic & Sackin, 1992; Torres et al., 1997; Vanoye & Reuss, 1999) (Fig. 7).

Electrophysiological Studies in Isolated-Polarized Cells

Intracellular Microelectrodes; Regional Superfusion

As discussed above, microelectrode impalements followed by determination of the relative ion permeability of the cell membrane(s) provide excellent tests for cell status and retention of the properties present in the epithelial cell *in situ* (Fig. 3). This has been done in figure-eight cells from *Necturus* proximal tubule (Segal et al., 1996) and *Necturus* gallbladder (Torres et al., 1996*a*,*b*). In the latter preparation, the measurements of membrane voltage were combined with regional superfusion of the cell with solutions of altered ionic composition (Fig. 6). This allowed us to determine the ion-selective permeabilities of both membrane domains under control conditions, as well as the membrane domain responsible for the increase in Cl^- permeability elicited by cAMP (Torres et al., 1996*b*). This is the apical membrane, and the conductance is mediated by the amphibian CFTR ortholog (Torres et al., 1996*b*).

Single-Channel Studies

One of the principal uses of figure-eight cells is in the study of plasma-membrane ion channels, because both the apical- and the basolateral-membrane domains become accessible to patch pipettes. In addition, the isolated cell is a simple system to study, in contrast with a gap-junction-coupled monolayer, where in the cell-attached or whole-cell modes voltage clamp is virtually impossible. The main shortcoming is that the separation of the cell from its neighbors and/or the chemical procedure may result in changes in the expression levels or properties of membrane proteins. In our studies careful experimental validation and comparison with the native cells *in situ* have demonstrated this not to be the case, but this caveat must be kept in mind when trying this experimental approach in new systems.

Single-channel studies in figure-eight cells may be carried out with the cell-attached or the excised-patch modes. Some cells are very easy and some are more difficult to patch, but we have had success with both amphibian and mammalian figure-eight cells. We find it easier to patch figure-eight cells shortly after isolation, plated on a glass surface coated with Cell-Tak, a mixture of crustacean polyphenolic proteins that improves cell adhesion to the surface.

The most studied channels in figure-eight cells have been basolateral K^+ channels from amphibian epithelia (Fig. 7). I discuss a few examples, contrasting observations in figure-eight cells from different epithelia, dem-

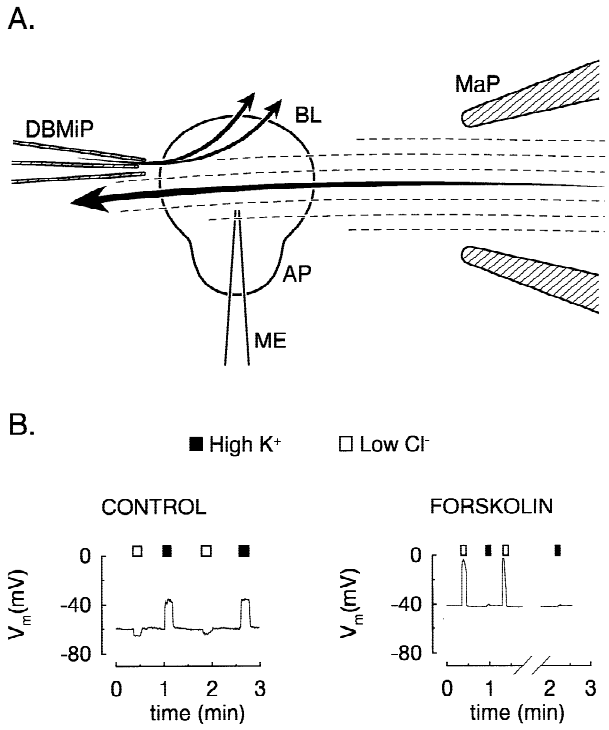

Fig. 6. (*A*) Diagram of the regional-superfusion system. Macropipette on the right (MaP) is used for gravity perfusion of the entire bath; double-barrel micropipette on the left is positioned close to the cell, to superfuse a small region (verified with hydrophilic dye). ME: microelectrode, AP = apical domain, BL = basolateral domain. (*B*) Apical-membrane superfusion under control conditions (left) and after exposure to forskolin to raise intracellular cAMP levels (right). Ionic substitutions were high K^+ (90 mM NaCl replaced with 90 mM KCl, filled bars) and low Cl^- (90 mM NaCl replaced with Na-gluconate, open bars). Under control conditions, high-K^+ solution produces a large depolarization, whereas low-Cl^- solution produces a small hyperpolarization. Following stimulation with forskolin, high-K^+ solution produces a small depolarization, whereas low-Cl^- solution produces a large depolarization. This denotes cAMP-dependent activation of apical-membrane Cl^- channels. Reproduced from Torres et al. (1996*b*) with permission.

onstrating that not all basolateral membranes express the same channels. Supplisson et al. (1991) studied *Necturus* gastric mucosa oxyntic cells, demonstrating four classes of channels, likely to result in a complex control of the membrane voltage. In *Necturus* gallbladder, the basolateral-membrane K^+ channels are different from those found in the apical membrane (Torres et al., 1996*a*). The basolateral channels have a low open probability under control conditions and are activated by membrane stretch and cell swelling by exposure to hypotonic solutions (Fig. 7). Whole-cell currents with the same properties can be demonstrated in the same cells, ruling out the possibility that the stretch-activated channels are a patch artifact (Vanoye & Reuss, 1999). In *Ambystoma* proximal tubule figure-eight cells, Segal et al. (1996) found a basolateral-membrane K^+ channel

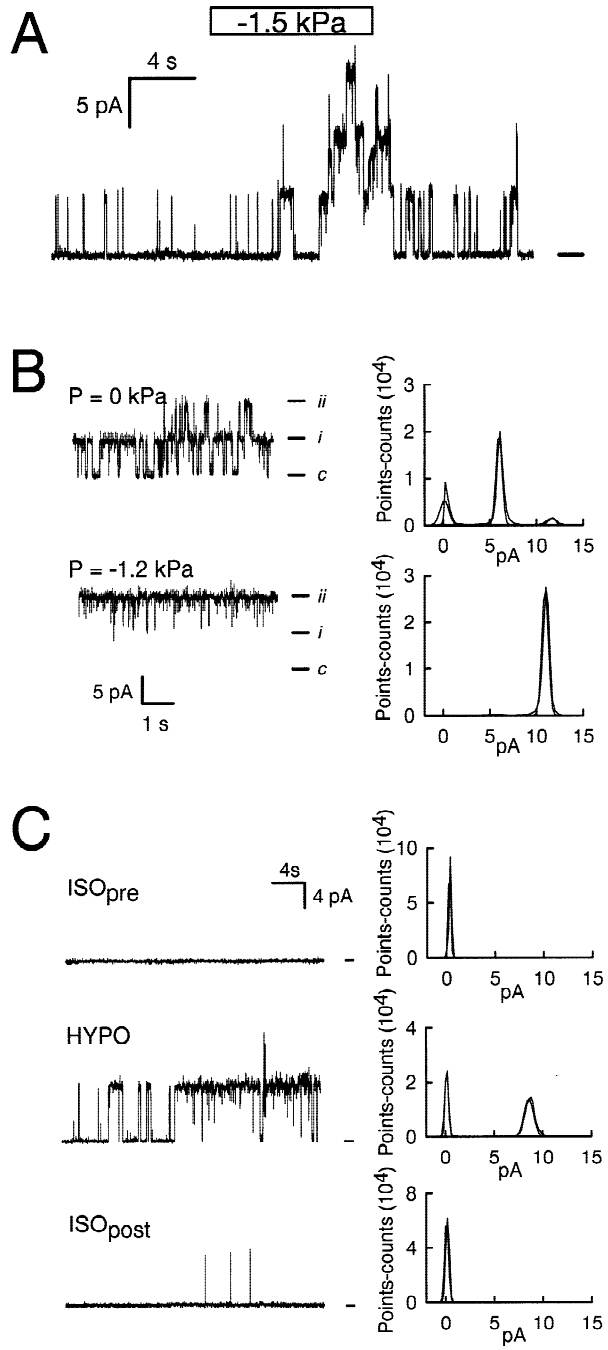

Fig. 7. Single-channel activity in the basolateral membrane domain of polarized epithelial cells isolated from *Necturus* gallbladder. (*A*) Cell-attached patch, *Vp* (pipette voltage) = 0. There is low-level K^+ channel activity under control conditions, with a large increase after rapid application of −1.5 kPa to the pipette interior (syringe connected to water manometer). Note the rapid reversibility of the effect. (*B*) Changes in steady-state channel activity by −1.2 kPa. Current histograms are depicted on the right; bin: 0.24 pA. (*C*) Effect of hyposmotic cell swelling (Δ osmolality = −28%) on K^+ channel activity. Current histograms are depicted on the right; bin: 0.20 pA. The bath solution was changed slowly; HYPO data shown were obtained near maximum swelling. Reproduced from Vanoye and Reuss (1999) with permission.

modulated by actin, as evidenced by treatment with cytochalasin D, and also a stretch-activated nonselective cation channel.

Whole-Cell Studies

In contrast with single-channel studies, whole-cell studies provide information on the function of the assembly of channels in the entire plasma membrane under conditions in which the membrane under study is untouched by the patch pipette. The use of this technique in figure-eight cells assures the investigator that both membrane domains are present; in certain round isolated epithelial cells an entire membrane domain many disappear by endocytosis (Vega-Salas, Salas & Rodríguez-Boulan, 1988).

Supplisson et al. (1993) carried out whole-cell studies of *Necturus* gastric oxynticopeptic figure-eight cells and found both K^+ and Cl^- conductances, the latter being dominant and sensitive to the Cl^--channel blocker NPPB. Although they did not test this directly, taking into account previous electrophysiologic observations by them and others, they concluded that the Cl^- channels are in the apical- and the K^+ channels in the basolateral-membrane domain. As discussed above, one approach to test this directly is to carry out regional superfusion of the cell, a technique that we have used in conjunction with microelectrode impalements as illustrated in Fig. 6 (Torres et al., 1996*b*) and could also be used with the whole-cell configuration.

A second example of the use of the whole-cell configuration of the patch-clamp technique is our analysis of K^+ and Cl^- currents in figure-eight cells from *Necturus* gallbladder epithelium. These studies were complemented with single-channel studies briefly described above and optical measurements of cell volume described below. We found that cell swelling increases basolateral P_K and decreases basolateral P_{Cl} (Torres et al., 1997). The latter effect is inconsistent with the possibility of acute cell-volume regulation by conductive effluxes of K^+ and Cl^-. Although this would support the possibility of a regulatory-volume decrease by KCl cotransport, as proposed long ago by Spring's group (Larson & Spring, 1984; Furlong & Spring, 1990), we could not detect an acute regulatory response *in situ* or in isolated cells, measuring volume by electrometric and dye-loading techniques, respectively. Acute volume regulation could occur only after activation of the CFTR-mediated apical Cl^- conductance *via* cAMP-dependent protein kinase (Torres et al., 1997). The conclusion of increased P_K and decreased P_{Cl} in swollen epithelial cells was contrary to current views (Strange, Emma & Jackson, 1996). Thus we tested its validity by measuring K^+ and Cl^- currents in *Necturus* gallbladder figure-eight cells by the whole-cell patch technique. We obtained exactly the same result (Vanoye & Reuss, 1999). On quantitative grounds, we have suggested that acute volume-regulatory decrease in other cells in culture cannot be explained by Cl^- efflux, in particular because the conductance of swelling-activated Cl^- channels is rather low at negative potentials (Altenberg et al., 1994). Thus, the physiological significance of the activation of Cl^- channels by cell swelling is not always clear.

A third example of use of the whole-cell approach is our study of figure-eight cells from the urinary-bladder epithelium of *Necturus maculosus.* We found a mildly anionic slowly-activating whole-cell current that can be elicited by cell-membrane depolarization or by decreasing the external $[Ca^{2+}]$. In low-Ca^{2+} solutions, the cells can also be loaded with small hydrophilic dyes (e.g., 5/6 CF, anionic dye of MW 376). Interestingly, 5/6 CF (valence-2) influences the current in a voltage-dependent manner, suggesting that it permeates the current-carrying pathway (Vanoye et al., 1999). We suspect that this pathway is a gap-junctional hemichannel.

Optical Studies in Isolated-Polarized Cells

Composition of the Plasma Membrane

Quantitative fluorescence with hydrophobic probes, fluorophore-conjugated lectins and antibodies (primary or secondary immunofluorescence) has been used to assess the composition of the two membrane domains in figure-eight cells.

The hydrophobic probes label the plasma membrane and the fluorescence ratio between the two domains depends on the probe used, suggesting differences in effective solubility of the probe(s) in the two domains (*see* above and Torres et al., 1996*a*). This indicates that the compositions of the membranes differ, but does not establish whether this implies phospholipids, membrane proteins, or both.

FITC-conjugated wheat-germ agglutinin labels glycoproteins. We used it to probe the domain of expression of apical-membrane glycoproteins in figure-eight and round cells by labeling them, on the apical surface only, prior to cell isolation. In figure-eight cells the label remains in the apical region, whereas in round cells it is distributed on the entire cell surface. This observation has been made in *Necturus* gallbladder and urinary bladder (Torres et al., 1996*a*, Vanoye et al., 1999), human renal-proximal-tubule cells, and several epithelial cell lines (Lopes et al., 1998; Vergara et al., 1999).

Antibodies directed against membrane-protein epitopes provide the best way to demonstrate the presence of these proteins in specific domains or the entire membrane (*see* above for a list of studies). A caveat with this method is that immunofluorescence of fixed and perme-

abilized cells indicates only the region of location of the antigen, but does not prove that the protein is inserted in the membrane. This can be assessed by separating the membrane with the biotin-streptavidin technique, or by combining the immunofluorescence study with the demonstration that the protein is transport-competent (e.g., Torres et al., 1996*a*,*b*). The advantage of the latter approach is that it demonstrates not only the membrane location of the protein, but also its proper folding.

Plasma-Membrane Permeability

Conductive ionic permeability is best assessed by the electrophysiological techniques described in the previous section. However, when ion transport is electroneutral (e.g., Na^+,K^+,$2Cl^-$ cotransport) net fluxes can be assessed by measuring changes in cell volume (*see* above and Wu et al., 1998). The experimental design must include a careful analysis of the driving forces for single substrates and transported complex, and the use of good pharmacological probes.

A second approach to the study of plasma membrane permeability in figure-eight cells is illustrated by our use of hydrophilic dyes to probe the activation of gap-junctional hemichannels, in *Necturus* urinary bladder epithelial cells (Vanoye et al., 1999) and human renal proximal-tubule (Vergara et al., 1999). Hydrophilic fluorescent dyes can enter the cytoplasm in isolated cells because of endocytosis, in which case typically the resulting fluorescence is punctuate, or because of permeation of aqueous transmembrane pathways, either selective or nonselective. Selective pores typically permeate some molecules, depending on size and/or charge, whereas nonselective pathways do not discriminate among solutes. Nonselective permeabilization of cells can be produced by detergents, several forms of physical or chemical cell damage and massive cell swelling, among other factors. Permeabilized cells do not exclude trypan blue, propidium iodide, or large-size FITC-conjugated dextran. Dyes that do not bind to intracellular components load reversibly, i.e., they can be removed by washing the cells with dye-free extracellular solution. The use of these techniques has allowed us to postulate the activation of gap-junctional hemichannels in *Necturus* gallbladder and human proximal tubule figure-eight cells. The crucial test was double loading with 5/6 carboxyfluorescein (MW 376 Da) and Texas-red dextran 3000 (MW 1,500 to 3,000 Da), at low temperature (to prevent endocytosis). Hemichannels permit cell loading with 5/6 CF, but not with the larger dextran probe, because they are not permeable to molecules larger than about 1,000 Da. Proof that the pathway is a channel was obtained by demonstrations of current-dye interaction within the pore (Vanoye et al., 1999) and block of dye uptake and current by inhibitors of gap-junctional communication. In contrast, damaged cells are loaded with both probes. In a recent study on epithelial cells from a rat mammary tumor, it was suggested that gap-junctional hemichannels may play a role in cell "isosmotic volume regulation," defined as swelling following a reduction of extracellular [Ca^{2+}] (Quist et al., 2000). This swelling is likely colloid-osmotic, ascribable to the lowering of the plasma-membrane reflection coefficient for small solutes by opening of these large channels. The study quoted provides no evidence for or against significant water flux via gap-junctional hemichannels. Our studies (e.g., Bao et al., 2000) on human proximal-tubule cells and those of John et al. (1999) on cardiomyocytes support the notion that in normal [Ca^{2+}], connexin43 hemichannels can be activated by dephosphorylation, i.e., support a pathophysiological role for hemichannels in cells undergoing ATP depletion.

Cell Volume and Composition

Cell-volume assessments are easier in figure-eight cells than in the tissue because of their optical advantages, largely the lack of other structures in series with the epithelial cells. From determinations of cell shape and cross-sectional area with a differential interference contrast microscope, Strange's group (Wu et al., 1998) demonstrated Na^+,K^+,$2Cl^-$ cotransport by rat-choroid-plexus epithelial cells. Bumetanide was used as blocker of the transporter.

An alternative method to assess relative changes in cell volume is to measure changes in the concentration of an intracellular fluorophore, ideally insensitive to changes in intracellular composition. Our group (Torres et al., 1997) used calcein.

Intracellular pH changes have been measured with BCECF (Torres et al., 1996*a*). The same kind of approach was used to measure intracellular levels of Ca^{2+} (Torres et al., 1997). Other intracellular ion concentrations can be assessed, in principle, using specific fluorescent probes.

Cell Ultrastructure

Electron microscopy has been used in figure-eight cells to study the morphological aspects of cell polarity, cell junctions (tight junctions, gap junctions), and membrane associated proteins (*see* Segal et al., 1996; Torres et al., 1996*a*). In *Necturus* proximal tubule and gallbladder figure-eight cells there is a sharp structural transition between apical and basolateral-membrane domains, evidenced in thin-section transmission EM (microvilli) and freeze fracture (tight-junction strands, density and size of intramembrane particles in the two domains) (Fig. 8).

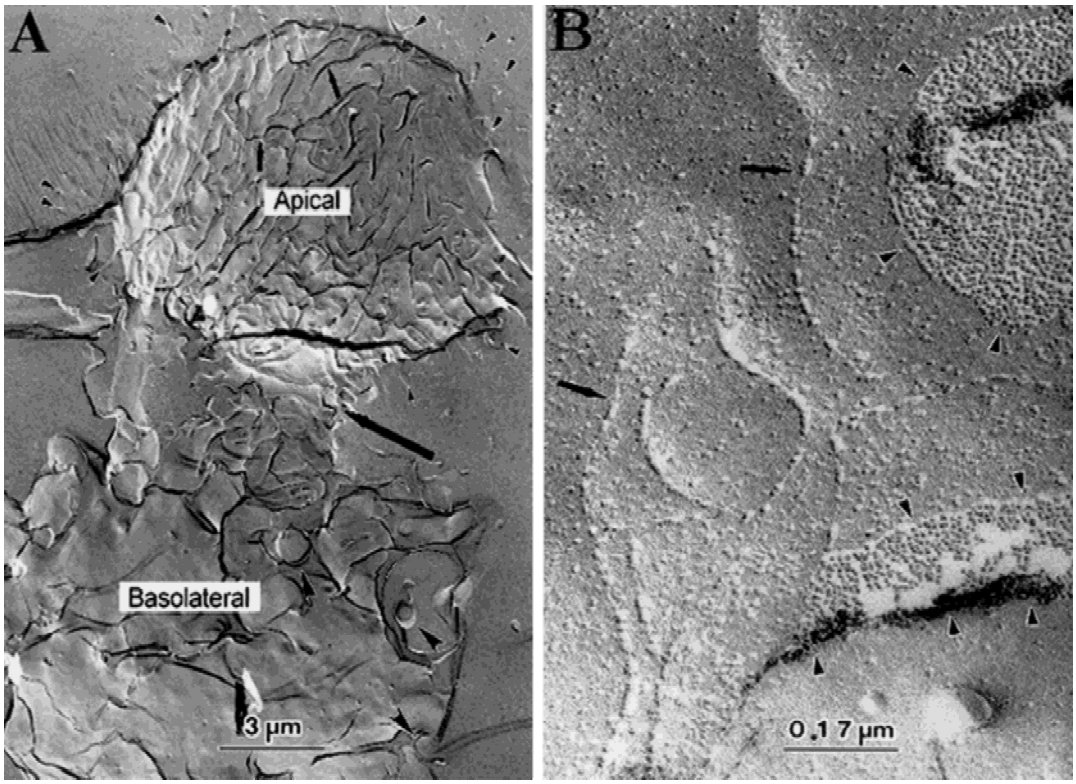

Fig. 8. (*A*) Freeze-fracture through the surface of an isolated figure-eight cell from *Necturus* gallbladder epithelium (P face). The plasma membrane of the small apical domain was identified by the presence of microvilli (arrowheads) and folds (bands that cross the surface in different directions, arrows). The plasma membrane of the larger basolateral domain lacks microvilli and folds. The round depressions and elevations observed in the fracture face of this domain are produced by vesicle fusion (depressions) and small blebs (protrusions), denoted with arrowheads. The transition between apical and basolateral domains of the figure-eight cell is an abrupt boundary (large arrow). (*B*) Freeze-fracture of the region between apical and basolateral domains. Gap junctions (arrowheads) and tight junctions (arrows) are found at the transition between the apical- and the basolateral-membrane domains. Reproduced from Torres et al. (1996*a*) with permission.

These observations are detailed in Segal et al. (1996) and Torres et al. (1996*a*). To our surprise, in *Necturus* urinary bladder figure-eight cells there are no tight-junction strands although the cells preserve their polarity, suggesting that tight-junction integrity is not necessary to maintain cell polarity (Reuss et al., *unpublished observations*).

Limitations of the Experimental Use of Figure-Eight Cells

Isolated-polarized epithelial cells have been available for a long time, but they are clearly not a widely used experimental system. There are several reasons for this. One is that all populations of isolated cells are rather heterogeneous. Even epithelia with only one cell type will yield healthy cells (both polarized and nonpolarized) and damaged cells. Hence, this preparation is clearly not the best for biochemical or molecular-biology experiments of the cell population. If a large number of cells is needed, the cells must be separated in groups by one of the many techniques available. At its current state of development, this methodology is intended for studies of individual cells using the methods described above. In many instances, it constitutes the only feasible approach. However, one must keep in mind the fact that the environment of the isolated cells is not "normal," and hence they may lose or acquire features compared with the polarized cells *in situ.* Examples of factors that can account for these differences are the lack of cell-cell contacts and the lack of cell-matrix contacts. Both can result in profound differences in cell structure and function compared to the *in situ* condition. Thus, it is indispensable to ascertain whether the property under study is present in the cell in the assembled epithelium and is not an artifact. It is possible and even likely that figure-eight cells will prove different from their precursors in the epithelium in some or many respects, but to date the findings of our group and others concerning transport properties support their use as a model for transporting epithelial cells.

Summary

Epithelial figure-eight cells can be a very useful experimental system for cell-physiological and cell-biological studies, including electrophysiology, optical functional studies in living and fixed tissue, immunolabeling, electron-microscopy, and others. Recent work from our group and others has provided a rather convincing validation of specific preparations and illustrated their use. Figure-eight cells can thus be used to assess the structural and functional features of an isolated cell that retains the properties it had *in situ*, and also to assess the mechanism(s) by which cell polarity is maintained in figure-eight cells and lost in round cells. Careful validation of the viability and functional features of each experimental validation is essential. Recent advances in molecular-biological techniques are promising in that soon it might become possible to carry out genetic studies starting with small numbers of homogeneous cell populations, e.g., figure-eight and round cells from the same preparation.

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