J. Membrane Biol. 143, 237–245 (1995)

The Journal of
Membrane
Biology

Water Pathways across a Reconstituted Epithelial Barrier Formed by Caco-2 Cells: Effects of Medium Hypertonicity

M. Parisi,[1] M. Pisam,[2] G. Calamita,[2] R. Gobin,[2] R. Toriano,[1] J. Bourguet[2]

[1]Laboratorio de Biomembranas, Departamento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires, CC 128 SUC 53 B, (1453) Buenos Aires, Argentina

[2]Service de Biologie Cellulaire, CE Saclay, CEA, France

Received 16 May 1994/Revised: 11 August 1994

Abstract. Caco-2 cells, originated in a human colonic cancer, are currently used as model systems to study transepithelial transports. To further characterize their water permeability properties, clone P1 Caco-2 cells were cultured on permeable supports. At confluence, the transepithelial net water movement (J_w), mannitol permeability (P_s), and electrical resistance (R) were simultaneously measured. The observed results were correlated with transmission and freeze-fracture electron microscopy studies and compared with those obtained, in similar experimental conditions, in a typical mammalian epithelial barrier: the rabbit rectum. When the serosal solution was made hypertonic (50 mM polyethylene glycol-PEG), the spontaneously observed secretory J_w rapidly reversed, became absorptive and then stabilized. Simultaneously, the R values dropped and P_s went up. In the case of the rabbit rectal epithelium, a similar treatment did not elicit significant changes in the water permeability during the first 20 min following the osmotic challenge while there was a significant increase in the transepithelial resistance. After exposure to serosal hypertonicity, several morphological modifications developed in the Caco-2 cells: Localized dilations in the intercellular spaces and vacuoles in the cytoplasm appeared. Nevertheless, most cells remained in contact and no evidence of cell shrinking was observed. Simultaneously, the tight-junction structure was more or less disorganized. The filament network lost its sharpness and "omega" figures appeared, bordering the intercellular spaces. In some cases the tight-junction network was completely disrupted. In the case of the rabbit rectum the structural modifications were completely different: Serosal hypertonicity rapidly induced cell shrinking and the opening of the intercellular spaces, with no noticeable change in the tight-junction structure. These results suggest that Caco-2-P1 cell membranes, contrary to the case of the basolateral membrane of rabbit rectal cells, have no water channels and that a paracellular route could play a central role in the water movements across this epithelial barrier.

Key words: Cell culture — Water permeability — Epithelial barriers — Medium hypertonicity — Freeze-fracture — Rabbit rectum

Introduction

Water movements across epithelial barriers can occur either across or between the cells, and this has been the subject of controversy between distinguished transport physiologists. Even if the recent characterization and cloning of water channels [13, 19] has clarified the field, functional studies can give additional information on the water pathways in "channel containing" and in "channel lacking" epithelial cells. Caco-2 cells grown on a permeable support provide a useful tool to study water movements across an epithelial barrier formed by a single cellular type and presenting a relatively simple topological arrangement [6, 7, 8, 12]. As in other epithelial layers, the water movements across the epithelium can be coupled to an active ionic transport or driven by an external hydrostatic or osmotic gradient [9, 15, 18]. To explore this last situation, the effects of local hypertonicity have been largely used [14, 17]. An increase in the tonicity of the medium in contact with the basolateral membrane modifies the water transfer through various

Correspondence to: M. Parisi

epithelial structures, either by interacting with transcellular and/or paracellular routes [3, 10].

We report here results with a clone (Caco-2-P1) that shows a well-differentiated enterocytic phenotype when cultured on a permeable support. The obtained results were compared with those observed in a typical mammalian epithelial barrier: the rabbit rectum. Net water fluxes (J_w) across both cell layers were recorded minute by minute, as previously reported [12]. Unidirectional 14-C-mannitol fluxes as well as transepithelial potential differences and resistances were also simultaneously measured. The effects on these parameters of serosal hypertonicity were tested and correlated with transmission and freeze-fracture electron microscopy studies. The results obtained suggest that the plasma membranes of Caco-2-P1 cells probably do not contain water channels while this is probably the case, for instance, in the rabbit rectal cells. Water movements across the Caco-2 epithelial barrier seem to proceed mainly via a paracellular route.

Materials and Methods

The Caco-2 line was initially established from a moderately well-differentiated human colon adenocarcinoma that spontaneously exhibits enterocyte-like differentiation [4]. The wild type was cloned at the Pasteur Institute and the P-1 line was cultured as previously described [12, 16]. Briefly, cells were seeded at 40,000 cells/cm^2 in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum, nonessential amino acids, and antibiotics (Eurobio-Paris). The medium was changed every two days. Cells were grown on Transwell (Costar) holders, 24 mm in diameter, having a porous (3 μm) polycarbonate bottom and under a 10% CO_2 atmosphere.

Male New Zealand rabbits were killed, after slight anesthesia, with a blow to the head. Immediately after ablation, the rectum was washed, opened along the mesenteric border and placed in a high potassium saline at low temperature (4°C). Before the experiments the mucosa and submucosa layers were dissected from the underlying tissues (always at 4°C).

Permeability Measurements

To perform permeability measurements in the Caco-2 cell layers, the Transwell holders, their bottoms covered with the confluent cell layer, were directly inserted between two Lucite chambers [12]. This defined apical (mucosal) and basolateral (serosal) compartments (15 ml each) where magnetic bars ensured a good mixing. In the case of the rabbit rectum, the dissected rectal epithelium was mounted as a diaphragm between two Lucite chambers (a nylon mesh was placed on the serosal surface). This defined apical (mucosal) and basolateral (serosal) compartments (15 ml each) where magnetic bars ensured a good mixing.

The net water flux (J_w) was recorded minute by minute, as previously described [12], in both epithelial barriers. A moderate hydrostatic pressure (5 cm of water) was applied, on the mucosal compartment. Transepithelial resistances and potential differences were measured using a Millicell electrical measurement system (ERS, Millipore). In the case of the Transwell holders, the electrical resistance of the filters (50 ± 6 Ω) was routinely subtracted. The 3 μm polycarbonate filter did not represent a significant barrier by itself to water movements. A 5 cm of water hydrostatic pressure gradient saturated our detection system. (The water flux was more than 50 $\mu l \cdot min^{-1}$).

To measure unidirectional fluxes 14-C mannitol (1 μC/ml) was added to the mucosal chamber. Samples (1 ml) were taken from the serosal bath and replaced with unlabeled medium every 5 min. Unidirectional fluxes were calculated taking into account backfluxes and sampling dilution.

The Dulbecco's modified Eagle's minimal medium (25 mEq/liter HCO_3^-, without serum, Eurobio Paris) was used in most experiments. The serosal solution was continuously bubbled with the appropriate O_2/CO_2 mixture to maintain the pH of the medium at 7.4 ± 0.1 (37°C). Hypertonic solutions were obtained by adding PEG (molecular mass 4 kDa) to the serosal buffer.

Freeze-Fracture Studies

Samples were fixed with 2% glutaraldehyde in 0.2 M cacodylate buffer for 1 hr at room temperature and then impregnated in 30% glycerol, mounted on double replica copper disks, frozen, freeze-fractured at −150°C in a Balzers 301 cryopump freeze-etch unit and replicated by Pt/C evaporation.

Transmission Electron Microscopy

Cells cultured on filters were fixed with 2% glutaraldehyde as previously described and postfixed with a 1% OsO_4 solution. Samples were dehydrated in graded series of alcohol and embedded in Epone.

Results

Permeability Properties

As previously reported [12], J_w become measurable, in Caco-2 cell layers and under a moderated hydrostatic pressure on the apical side (5 cm of water), eight to nine days after seeding. This parameter and the simultaneously measured mannitol fluxes stabilized, when using the P1 clone, after about 12 days in culture. In this situation a small and spontaneous secretory J_w was observed, as previously reported [12]. Figure 1 shows the simultaneously measured J_w, transepithelial resistance and unidirectional (mucosal to serosal) mannitol fluxes. The experimental points are the means of ten experiments (*see* Table 1). At the arrow, 50 mM PEG were added to the serosal bath, with the following results: The spontaneously observed secretory J_w rapidly reversed. The net volume flux became absorptive and then stabilized, in about 5 min, at relatively high mucosa to serosa values. Simultaneously, the transepithelial resistance dropped and mannitol permeability went up. (Table 1). All these effects were fully reversible when returning to the control conditions, except that the previously observed secretory J_w disappeared.

Figure 2 compares the results obtained, in similar experimental conditions, when an osmotic gradient was applied across the Caco-2 cell layer or the rabbit rectal epithelium. Both the J_w (Fig. 2*A*) and the transepithelial

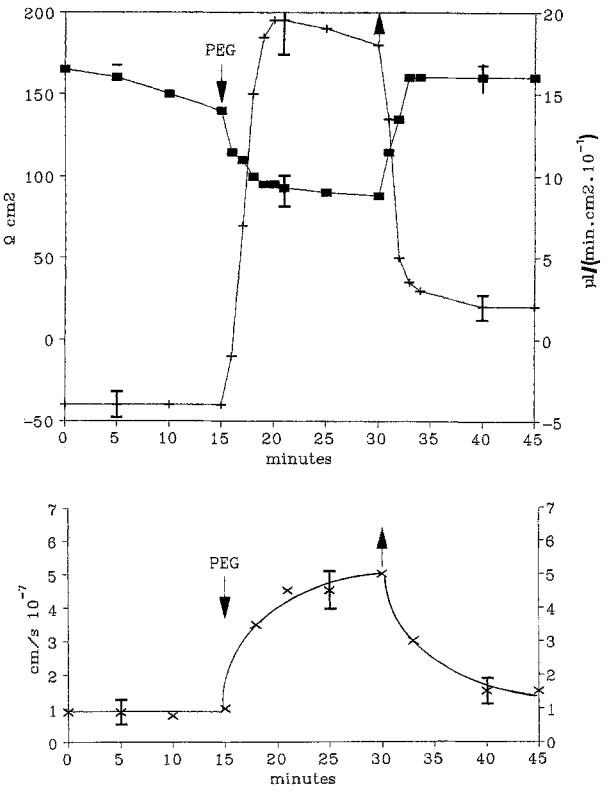

Fig. 1. Simultaneously measured net water flux (+, µl/min.cm^2 × 10 exp-1), transepithelial resistance (■, Ω · cm^2), mannitol permeability (x,P_s, (cm/sec) × 10 exp-7) as a function of time. Negative values for the net water movement indicate secretion. The experimental points are means of ten experiments (*see* Table 1). At the downward arrow 50 mM PEG were added to the serosal bath and removed at the upward arrow.

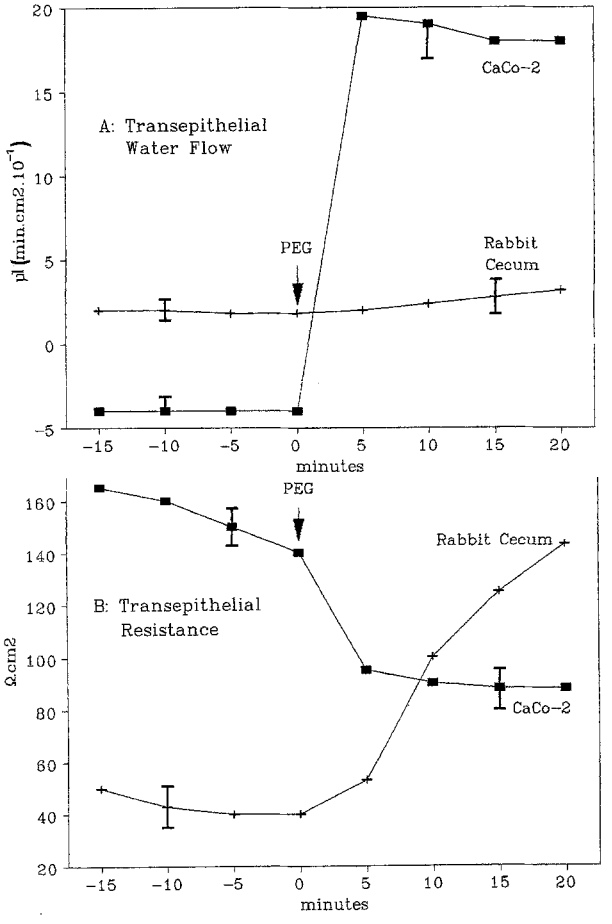

Fig. 2. Effect of serosal hypertonicity on the net water flux and the transepithelial resistance. Comparative results in the rabbit rectum and in the CaCo-2 epithelium.

Table 1. Net water fluxes (J_w, µl/(min · cm^2), mucosa to serosa positive values), transepithelial resistance (Ω · cm^2), and mannitol permeability (P_s, cm/sec) simultaneously measured in Caco-2 P1 cell layers (12 days after seeding)

	Control	Hyper	Reversal
J_w	−0.4 ± 0.2	1.9 ± 0.3	0.2 ± 0.2
Resistance	160 ± 15	93 ± 16	160 ± 18
P_s	0.9 ± 0.3	4.5 ± 0.5	1.5 ± 0.4

Control values are compared with the effects of serosal hypertonicity (hyper, 50 mOsm PEG) measured 5 min after the osmotic challenge (mean of 10 experiments). The values observed 10 min after hypertonicity removal are shown in the third column (Reversal).
Mean + SEM, n = 10.

resistance (Fig. 2*B*) were measured. It can be observed that serosal hypertonicity (40 mM PEG) only induced an important J_w in the case of the Caco-2 cell layers. Concerning the transepithelial resistance, it decreased in Caco-2 epithelium and increased in the rabbit rectal epithelium.

Ultrastructural Correlations

Figure 3 shows a general view of the epithelial barrier formed by Caco-2 cells, clone P1, at confluence. The monolayer of cells showed a well-developed brush border (Fig. 4*A* and *B*). It has been reported that Caco-2 cell lines grown on polycarbonate membranes containing 3.0 µm pores penetrate the filter and grow in the opposite surface [16]. We observed the same phenomenon with the original Caco-2 cell line (used at passage 25–32) but not with the clone used here (Caco-2-P1). Cell nuclei were not observed in the opposite surface. Only in some cases cellular processes invaded the 3.0 µm pores (Fig. 4*A*) but the epithelial layer only showed tight-junctions and microvillae at the apical border.

In control conditions (no osmotic gradient, 5 cm of water hydrostatic pressure on the apical side) the intercellular spaces appeared, both in freeze-fracture (Fig. 4*B*) and transmission electron microscopy studies (Fig. 5*A*), as tortuous and closed, with no noticeable intercellular dilations. Only a few vacuoles were observed in the

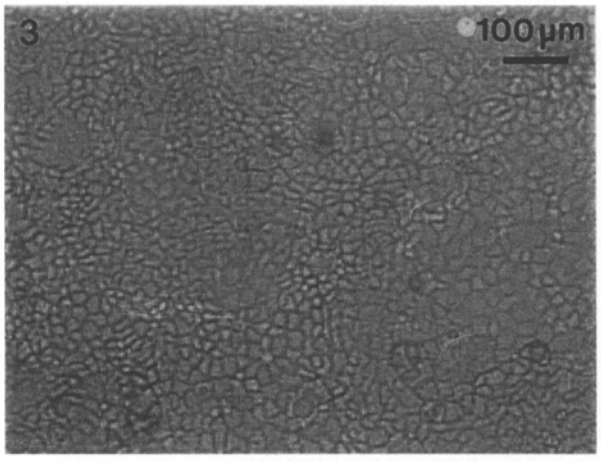

Fig. 3. General view of the epithelial barrier formed by Caco-2 cells, clone P1, at confluence.

cytoplasm. Caco-2 P1 cells formed typical tight-junction structures, as can be observed in freeze-fracture studies (Fig. 5*B*).

When the cells were fixed 5 min after imposing an osmotic gradient (50 mM PEG, serosal side, similar conditions to those described in Fig. 1), several morphological modifications developed: (i) Transmission electron microscopy studies showed localized dilations in the intercellular spaces (Fig. 5*C*) and numerous vacuoles appeared in the cytoplasm (Fig. 5*D*). Nevertheless, most cells remained in contact and no evidence of cell shrinking was observed. (ii) Simultaneously, the tight-junction structure was more or less disorganized. The filament network lost its sharpness (Fig. 6*A*) and "omega" figures, bordering the intercellular spaces (Fig. 6*B*) appeared. In some cases the tight-junction network was completely disrupted (Fig. 6*C*).

Figure 7*A* shows a cross-section on the rabbit rectal epithelium in control conditions (*compare* with Fig. 4*A*). Five minutes after imposing the osmotic gradient, the cells shrank and the intercellular spaces were wide open (Fig. 7*C*). Figure 7*B* shows the typical multistrand tight junctions observed in this tissue. No noticeable change in this structure was detected when the serosal tonicity was increased.

Table 2 gives the total thickness of the epithelial layers, measured in control conditions and five minutes after the establishment of the osmotic gradient, as well as the percentage of the total epithelial cross-section occupied by intercellular spaces and intracellular vacuoles. A significant swelling of the CaCo-2 cell layer was observed in hyperosmotic conditions.

Discussion

Intestinal epithelial barriers reabsorb and secrete substantial quantities of water. These water movements are due to a complicated interaction between osmotic, hydrostatic and salt transport dependent volume fluxes. The existence in these barriers of multiple cellular types and complex supracellular structures (crypts and villae), as well as subepithelial components (muscular, vascular and conjunctive tissues) make it difficult to analyze the mechanisms underlying the water movements. Cell lines grown on a permeable support provide a useful tool to study this problem in a single cellular type and under a simple topological arrangement, and they have been widely used to study electrolyte movements across epithelial barriers [4, 5, 12, 13, 23]. In a previous paper [12] a first study on the water handling in Caco-2 cells, cultured on a permeable support, was done. We are now analyzing the effects of external osmotic gradients and the morphostructural correlations related to the osmotically induced water movements. When an osmotic gradient generates a net water flow across an epithelial barrier, two alternative situations can be considered: (1) The water movement occurs across the cells or (2) The water movement proceeds across the intercellular spaces. In our experimental conditions serosal hypertonicity was used to generate the osmotic gradient.

Morphofunctional Correlations

Previous studies have shown that in the frog urinary bladder, a tight epithelium, serosal hypertonicity induces a rapid shrinking of the epithelial cells and the opening of the intercellular spaces [3, 14]. The same result was now observed by us when the effects of serosal hypertonicity on the epithelium of the rabbit rectum were tested (Fig. 7*C*). In these tissues the cell shrinking was not accompanied, at least during the first minutes after the osmotic challenge, by a significant increase in the transepithelial net water movement (Fig. 2*A*). The situation was completely different in the case of the Caco2-P1 cell layer. First, an important J_w appeared that arrived to a maximum in 5 min. This response to serosal hypertonicity (development of an important J_w) was previously observed in the rat jejunum [1] and in the rat cecum [2], two reputed leaky epithelia.

Simultaneously with the increase in the J_w, a drop in the transepithelial resistance and an increase in mannitol permeability were registered in the Caco-2 epithelium. Structurally, no shrinking of the epithelial cells was observed and, on the contrary, the net water movement was accompanied by an increase in the epithelial thickness and the appearance of vacuoles in the cytoplasm and localized dilations in the intercellular spaces. Finally, freeze-fracture studies showed that the tight-junction structure was altered by the osmotic challenge.

Taken together, our experimental results suggest the following conclusions: (i) The basolateral membrane of Caco2-P1 cells has a low water permeability and, be-

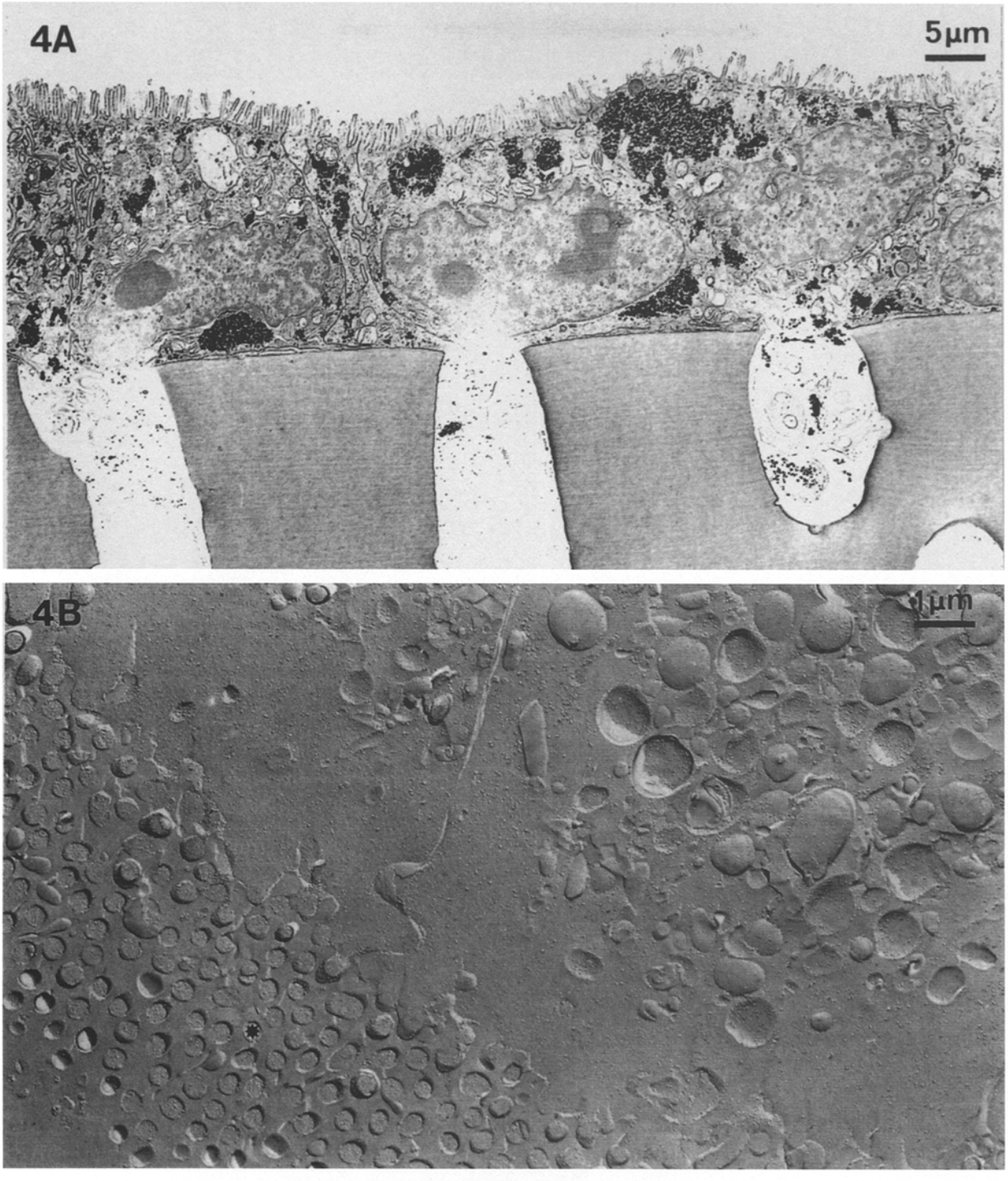

Fig. 4. (A) Caco-2 cell layer on the Nucleopore filter. The monolayer shows a well-developed brush border. Only in some cases cellular processes invaded the 3.0 µm pores but the epithelial layer only showed tight-junctions and microvillae at the apical border. (B) Freeze-fracture study showing two adjacent Caco-2-P1 cells at confluence. The interspace is closed and classical microvillae (*) at the apical border can be observed.

cause of this situation, no cell shrinking was observed when serosal hypertonicity was imposed (alternatively, but less likely, it would be possible that the epithelial cells did not change their volume because they increased their solute content). (ii) The osmotic gradient generated an important water flux across the intercellular spaces. This J_w induced structural modifications in the tight-junction structure and localized dilations in the intercel-

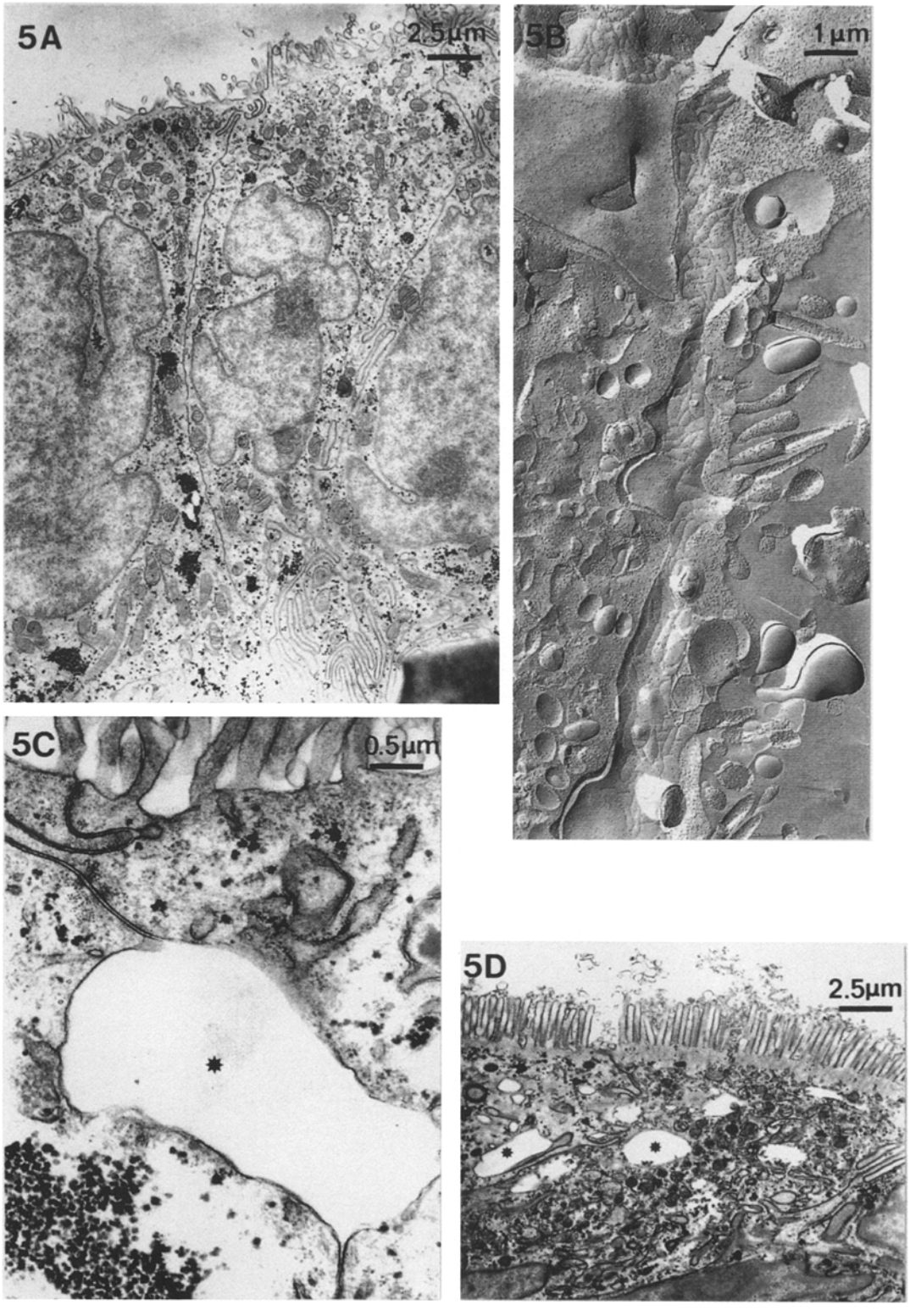

Fig. 5. (*A*) Caco-2-P1 cells at confluence in control conditions (no osmotic gradient, 5 cm of water hydrostatic pressure on the apical side). The intercellular spaces appeared as tortuous and closed, with no noticeable intercellular dilations. No apparent vacuoles were observed in the cytoplasm. (*B*) Caco-2 P1 cells formed typical tight-junction structures, as it can be observed in this freeze-fracture study. (*C*) Localized dilations in the intercellular spaces (*) were observed when the cells were fixed 5 min after imposing an osmotic gradient (50 mM PEG, serosal side, similar conditions to those described in Fig. 1). (*D*) In similar conditions to those described in *C* vacuoles appeared in the cytoplasm (*).

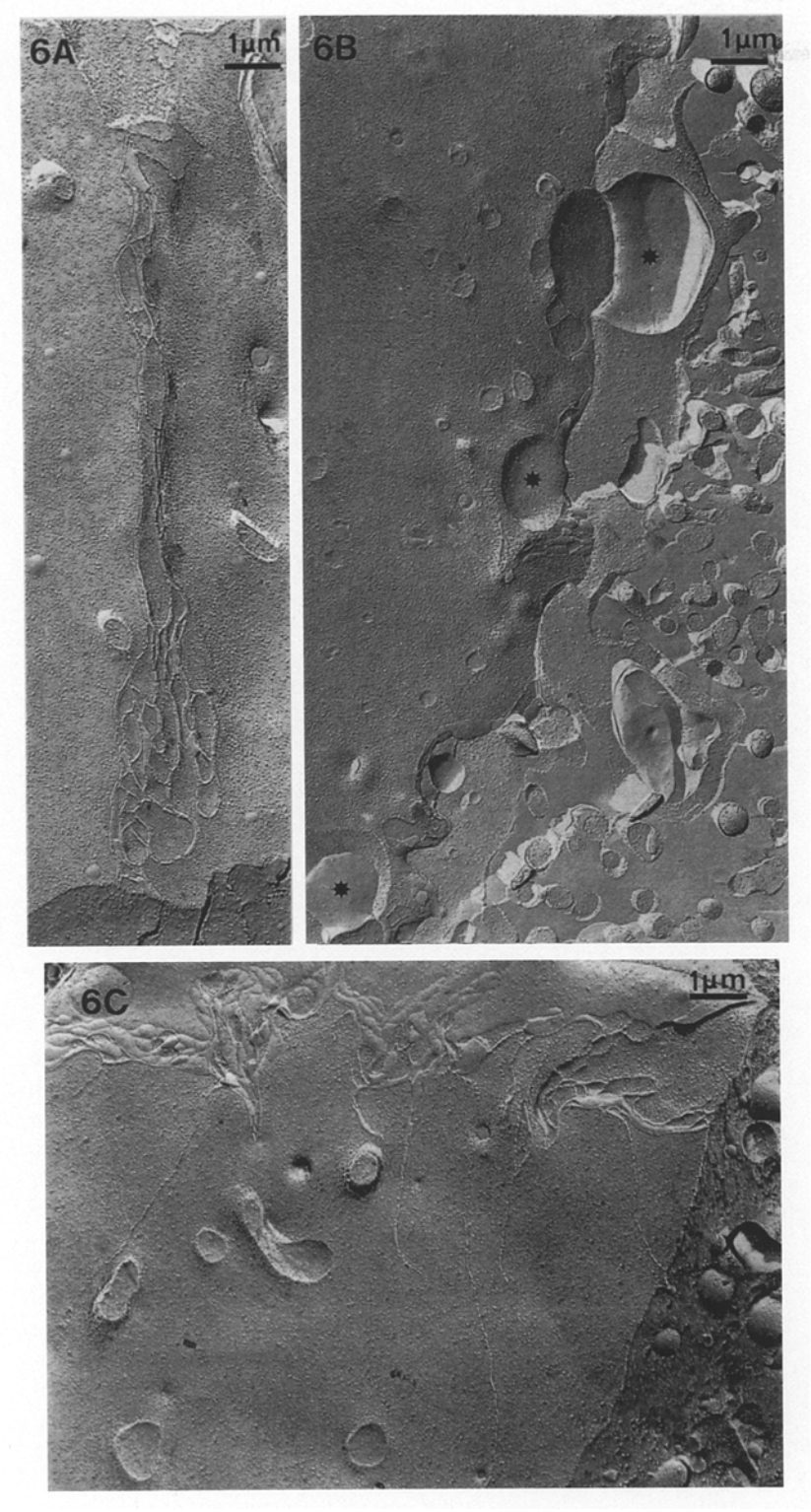

Fig. 6. (A) Five minutes after imposing the osmotic gradient the tight-junction structure was more or less disorganized. The filament network lost its sharpness (*A*) and "omega" figures appeared, bordering the intercellular spaces (* in *B*). In some cases the tight-junction network was completely disrupted (*C*).

lular spaces. (iii) The vacuoles in the cytoplasm would be generated by fluid phase endocytosis induced by the previously mentioned localized dilation in the intercellular spaces. (iv) As a consequence of this, the total thickness of the epithelium increased.

The Transepithelial Resistance and Mannitol Permeability

The effects of the serosal hypertonicity on the transepithelial resistance and mannitol permeability can be

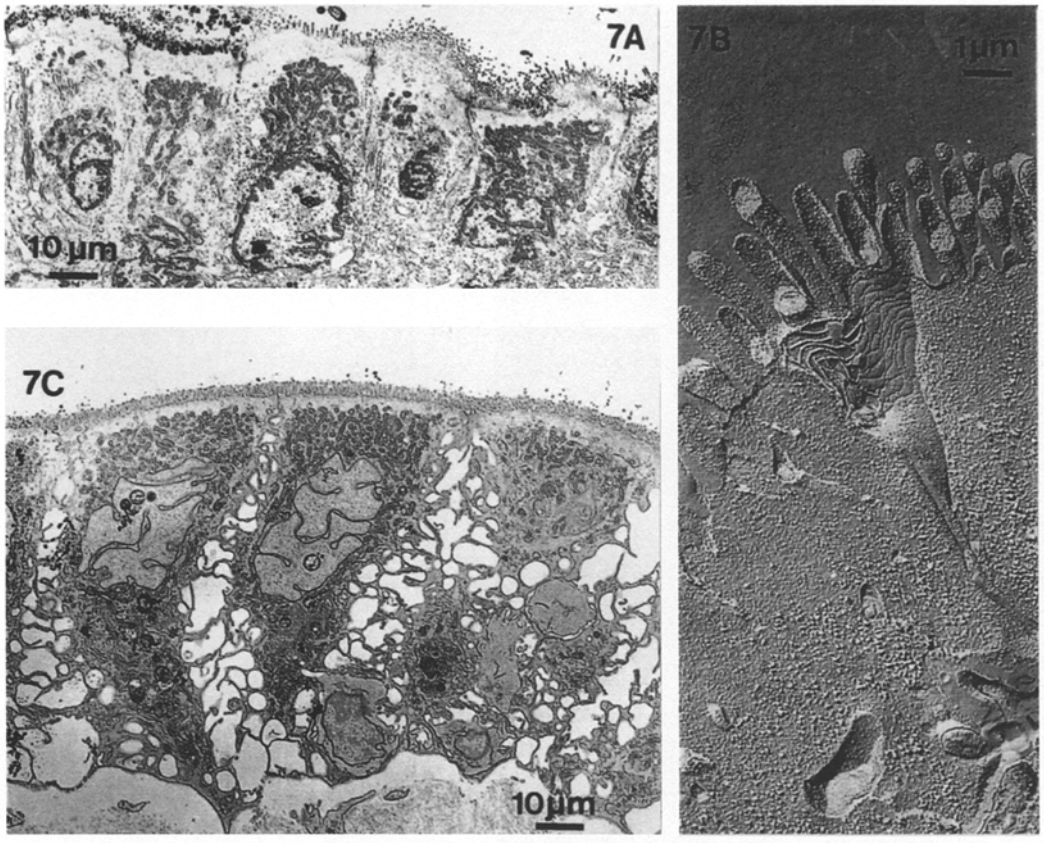

Fig. 7. (*A*) The isolated rabbit rectal epithelium in control condition (*B*). Typical multistrand tight-junction in this tissue (*C*). Five minutes after the osmotic challenge (PEG, 50 mM, serosal side) the cells shrank and the intercellular spaces were wide open.

Table 2. Thickness of the epithelium, intercellular space area (% of the total area) and vacuolar area (% of the total area), measured in control conditions and 5 min after serosal hypertonicity (hyper, PEG 50 mM) in CaCo 2 cell layers

	Thickness	Intercellular spaces	Vacuole surface
Control	28.5 ± 1.6 µm (24)		0.2 ± 0.8% (6)
Hyper	39.0 ± 3.4 µm (21)	0.4 ± 0.1%	8.0 ± 4% (6)
Diff	10.5 ± 3.8 µm		7.8 ± 0.9%
	($P < 0.01$)		($P < 0.01$)

Means ± SEM.

straightforwardly interpreted, in the Caco-2 epithelium, as due to an "opening" of the tight-junction structure. The increase in the mucose to serose mannitol movement can be due to an increase in the tight-junction permeability or to solvent-coupled mannitol fluxes. Nevertheless, in both cases a water flux across the paracellular pathway is implied. In the case of the rabbit rectal epithelium the situation is more complex and a clear dissociation between the tested parameters was observed (Fig. 2).

Water Channels in the Intestinal Epithelia

Water channels, present in those membrane cells where important osmotic fluxes are observed (red cell [13], proximal convoluted tubule [19] and collecting ducts in the kidney [5] have been recently cloned). Its presence and eventual role in intestinal barriers are not yet clearly established. Our previous [1] and present results allow us to describe two different patterns in the response to serosal hypertonicity in intestinal epithelial barriers: (1) The "water tight response," observed in the rabbit rectum and in some aspects reminiscent of the response observed in the ADH-sensitive barriers and (2) The "water leaky response," previously observed in the rat jejunum and in the rat cecum and now described in the epithelial layer formed by Caco2-P1 cells on a permeable support.

The tight water epithelia are characterized by a low permeability to water of their apical membrane, a strong dissociation between water permeability, mannitol permeability and transepithelial resistance, and a high osmotic permeability in the basolateral membrane [11]. On the other side, water leaky epithelia have a low osmotic permeability both in the apical and in the basolat-

eral membrane. A strong correlation between water permeability, mannitol permeability and transepithelial resistance is observed in this case.

Tight water epithelia are good candidates for the existence of resident water channels in the basolateral membranes. The eventual insertion of water channels in the apical border, as is the case of ADH-sensitive tissues will induce, in the presence of an osmotic gradient, a transcellular water flow. In the water leaky barriers the paracellular route would be important to move water, driven by a transepithelial osmotic gradient.

References

1. Capurro, C., Parisi, M. 1992. Water handling in the rat jejunum: effect of acidification of the medium. *Pfluegers Arch.* **421:**17–21
2. Escobar, E., Ibarra, C., Todisco, E., Pzarisi, M. 1990. Water and ion handling in the rat cecum. *Am. J. Physiol.* **259:**6786–6791
3. Fischbarg, J., Warshavsky, C.R., Lim, J.J. 1976. Pathways for hydraulically and osmotically induced water fluxes across epithelia. *Nature* **266:**71–73
4. Fogh, J., Zweibaum, A. 1983. Enterocitic like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* **47:**323–330
5. Fushimi, K., Uchida, S., Hara, Y., Hirata, Y., Marumo, F., Sasaki, S. 1993. Cloning and expression of apical membrane water channel of rat kidney collecting tubule. *Nature* **361:**549–552
6. Grasset, E.M., Bernabeu, J., Pinto, J. 1985. Epithelial properties of human clonic carcinoma cell line Caco-2: effects of secretagogues. *Am. J. Physiol.* **248:**C410–C418
7. Grasset, E.M., Pinto, A., Dussaulx, A., Zweibaum, A., Des Jeux, F.J. 1984. Epithelial properties of human colon carcinoma cell line Caco-2: electrical parameters. *Am. J. Physiol.* **247:**C260–C267
8. Hidalgo, I.J., Raub, T.J., Borchard, R.T. 1989. Characterization of the human colon carcinoma cell line Caco-2 as a model system for intestinal epithelial permeability. *Gastroenterology* **36:**736–749
9. Naftalin, R.J., Triphati, S. 1985. Passive water flows driven across the isolated rabbit ileum by osmotic, hydrostatic and electrical gradients. *J. Physiol.* **360:**27–50
10. Naftalin, R.J., Triphati, S. 1986. The role of paracellular and transcellular pathways and submucosa space in the isotonic water absorption by rabbit ileum. *J. Physiol.* **370:**409–432
11. Parisi, M., Calamita, G., Pisam, M., Gobin, R., Bourguet, J. 1994. Water pathways across the mammalian intestine: effect of hypertonicity of the medium. Membaires 94 Symposium Abstr. P37
12. Parisi, M., Escobar, E., Huet, C., Ripoche, P., Louvard, D., Bourguet, J. 1993. Water handling in CaCo-2 cells: effects of acidification of the medium. *Pfluegers Arch.* **423:**1–6
13. Preston, G.M., Carrol, T.P., Guggino, W.B., Agre, P. 1992. Appearance of water channels in Xenopus oocytes expressing red cell CHIP-28 protein. *Science* **256:**385–387
14. Ripoche, P., Bourguet, J., Parisi, M., 1973. The effect of hypertonic media on water permeability of frog urinary bladder. *J. Gen. Physiol.* **61:**110–124
15. Triphati, S., Boulpaep, E. 1988. Cell membrane water permeabilities and streaming currents in Ambystoma proximal tubule. *Am. J. Physiol.* **255:**F188–F203
16. Tucker, S.P., Melsen, L.R., Compans, R.W. 1992. Migration of polarized epithelial cells through permeable membrane substrates of defined pore size. *Eur. J. Cell Biol.* **58:**280–290
17. Van Os, C.H., Widener, G., Wright, E.M. 1979. Volume flow across gallbladder epithelium induced by small hydrostatic and osmotic gradients. *J. Membrane Biol.* **49:**1–20
18. Wright, E.M., Smulders, A.P., Tormey, J.M. 1972. The role of lateral intercellular spaces and solute polarization effects in the passive flow of water across the rabbit gallbladder. *J. Membrane Biol.* **72:**198–219
19. Zhang, R., Skach, W., Hasegawa, H., Van Hoeck, A., Verkman, A.S. 1993. Cloning, functional analysis and cell localization of a kidney proximal tubule water transporter analogous to CHIP-28. *J. Cell Biol.* **10:**359–369