Pflügers Arch – Eur J Physiol (2000) 440:609–618
Digital Object Identifier (DOI) 10.1007/s004240000308

ORIGINAL ARTICLE

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Effects of medium hypertonicity on water permeability in the mammalian rectum: ultrastructural and molecular correlates

Received: 22 July 1999 / Received after revision: 6 March 2000Accepted: 16 March 2000 / Published online: 12 May 2000

Abstract Minute-by-minute net water fluxes (J_w) were measured across the isolated rectal epithelium in rats and rabbits. Five minutes after a serosal (but not mucosal) hypertonic challenge (plus 200 mosmol/l) a significant increase in the basal J_w was recorded in both species [ΔJ_w, µl min^{-1} cm^{-2}: 0.40±0.06 (rats); 0.45±0.10 (rabbits)]. At the same time, most epithelial cells shrank markedly while the intercellular spaces were wide open (electron microscopy studies). In freeze-fracture studies multi-strand tight-junction structures (only slightly modified by serosal hypertonicity in rabbits) were observed in control conditions. No structural changes were observed after mucosal hypertonicity (both in rats and rabbits). Immunohistochemical studies showed the expression of aquaporin 3 (AQP3) at the basolateral membrane of epithelial cells in the rat. A first conclusion is that the epithelium of the mammalian rectum is a highly polarized, aquaporin-3-containing, water permeability structure. The J_w increase induced by serosal hypertonicity was sensitive to mercurial agents in both species and no changes in unidirectional [^{14}C]mannitol fluxes (P_s) or transepithelial resistance (R_T) were observed during this J_w increase. These observations suggest a transcellular route for the osmotically induced increase in water fluxes. In the rabbit rectum the initial J_w response, associated with serosal hypertonicity, was a transient one. It was followed by a second, slow and $HgCl_2$-sensitive J_w increase (a transient peak in paracellular mannitol permeability was also observed). A second conclusion is that serosal hypertonicy induces an increase in transcellular water permeability in both rat and rabbit rectum.

Key words AQP3 · AQP4 · Freeze-fracture-hypertonicity · Immunolocalization · Rabbit · Rat

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Introduction

Although the amounts of water absorbed by or secreted into the mammalian rectum are small, as compared to those in the colon, important transepithelial differences and ionic transfers are observed [8]. As in other parts of the intestine, increases in absorption or secretion induced by various physiological or pathological stimuli can be associated with diarrhea or constipation. These alterations are due to complicated interactions between osmotic, hydrostatic, and salt-dependent volume fluxes [9, 10, 21, 23]. Moreover, the presence of water channels (aquaporins) in different intestinal segments has been recently reported [17]. Nevertheless, no information is available on their presence in the mammalian rectum.

We have previously described water handling in different intestinal epithelia as either "leaky" [4] or moderately "tight" [10]. In this work we present results obtained from the isolated rectum epithelium of rats and rabbits. This tissue continues the descending colon where water must move out of the lumen against substantial opposition because of the high effective osmolality of feces [2, 22, 24, 33]. As in other epithelial layers, water movement across the rectum epithelium may be coupled to active ionic transport [30]; alternatively it can be driven by external hydrostatic [27] or osmotic [20] gradients. To explore this last situation the effects of local hypertonicity have been largely used in studies of other tissue types [3, 4, 9]. An increase in the osmolarity of the medium in contact with the basolateral membrane modifies the water transfer through various epithelial structures, by interacting with transcellular and/or paracellular routes [4, 29, 32]. Furthermore, it has been proposed recently that the capacity of the descending colon

to absorb against a high hydraulic resistance depends on its ability to generate a hypertonic absorbate [22, 24].

We have now recorded minute-by-minute, as previously reported [6], the net water fluxes (J_w) across the isolated rabbit and rat rectal epithelium. The effects on J_w of serosal hypertonicity were tested and correlated with transmission and freeze-fracture electron microscopy studies. Furthermore, unidirectional [^{14}C]mannitol fluxes as well as transepithelial potential difference (V_T) and transepithelial resistance (R_T) measurements were also made from both types of tissue. Finally, aquaporin immunolocalization studies of the rat rectum epithelium were realized to evaluate the putative presence of water channels.

Materials and methods

Animals

Male Wistar rats and New Zealand rabbits, fed with commercial chow and free access to water, were used in different experiments. The animals were killed, after light anesthesia. Immediately after, the rectum was removed, flushed with cold "standard saline solution" (described below), opened along the mesenteric border and placed in a high-potassium saline solution, at low temperature (4°C), until mounting.

Water permeability measurements

The rectal epithelium was dissected from other tissues, mounted on a nylon mesh (mucosal surface upwards) and placed as a diaphragm between two barrel Lucite hemi-chambers. This defined the apical (mucosal) and basolateral (serosal) compartments, which were continuously mixed by magnetic stirring bars. A moderate hydrostatic pressure (5 cm water), applied to the mucosal bath, pressed the epithelium against the mesh. Both chambers were initially filled with a "standard saline solution" containing (mol/l): 0.114 NaCl, 0.0045 KCl, 0.0012 $CaCl_2$, 0.0012 $MgCl_2$, 0.025 $NaHCO_3$, 0.0012 K_2HPO_4, 0.0012 KH_2PO_4, 0.025 glucose (pH 7.4 when bubbled with 5% CO_2/95% O_2). The "high-K^+ saline solution" contained (mol/l): 0.120 KCl, 0.01 $NaHCO_3$, 0.0012 $CaCl_2$, 0.0012 $MgCl_2$, 0.0012 K_2HPO_4, 0.0002 KH_2PO_4, 0.025 glucose.

The J_w was recorded minute-by-minute as previously described [6]. Briefly, the position of a liquid meniscus inside a capillary tube was photo-electrically detected. Displacements to the right or to the left were proportional to the amount of water absorbed or secreted by the epithelial layer. The mucosal chamber was limited by the nylon mesh, for this reason changes in the cell volume would not induce a meniscus displacement. The sensitivity of the system was 50 nl. Data were computed in units of µl min^{-1} cm^{-2}.

The serosal solution was continuously bubbled with the appropriate 5% CO_2/95% O_2 mixture to maintain the pH of the medium at 7.4±0.1.

Electrophysiology studies

V_T and short-circuit current (I_{sc}) were continuously recorded by an automatic voltage-clamp system (Physiological Instruments, USA) and Navycite (ME2AG4) electrodes. R_T was estimated from current deflections in response to 2-mV/s pulses every 90 s.

Unidirectional [^{14}C]mannitol fluxes

Transepithelial [^{14}C]mannitol fluxes were measured as previously reported [4]. [^{14}C]Mannitol was added to the mucosal bath (37 MBq/l or 1 mCi/l) before the start of J_w measurements. Samples (1 ml) were taken from the basolateral bath every 5 min. Mannitol permeability (P_s) was calculated, after correcting for sampling dilution and back-fluxes.

Freeze-fracture and transmission electron microscopy studies

Tissues were fixed with 2% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.4) for 20 min. For transmission electron microscopy studies the samples were post-fixed for 1 h at room temperature in a 1/1 mixture of 2% aqueous osmium tetroxide and 3% aqueous potassium ferrocyanide [16]; dehydration was in ethanol and embedding in Epon 812. Thin sections were contrasted for 2 min with lead citrate and examined at 60 kV with a Philips CM12 electron microscope. For freeze-fracture studies the samples were cryo-protected in a glycerol/Ringer solution (30% v/v) for 40 min and subsequently frozen in ^{22}Freon chilled by liquid nitrogen. Samples were then processed as usual [5] in a Balzers 301 cryopump freeze etch unit.

Immunocytochemistry and electron microscopy

The rat intestine was fixed by vascular perfusion with 4% paraformaldehyde in 0.1 mol/l cacodylate buffer, pH 7.4. Tissue blocks prepared from the rectum were post-fixed in the same fixative for 2 h, infiltrated for 30 min with 2.3 mol/l sucrose containing 2% paraformaldehyde, mounted on holders and rapidly frozen in liquid nitrogen, essentially as described previously [26]. For light microscopy analysis cryosections, approximately 0.8 µm thick, were obtained with a Reichert Ultracut S Cryoultramicrotome and were placed on gelatin-coated glass slides. After pre-incubation with PBS containing 1% BSA and 0.05 M glycine for 5 min, sections were incubated overnight at 4°C with affinity-purified anti-AQP3 [7], anti-AQP1 [35] or anti-AQP4 [31, 34] antibodies diluted in PBS with 0.1% skimmed milk. The labeling was visualized by incubation for 1 h at room temperature with HRP-conjugated secondary antibody (P448 1:100, DAKO, Glostrup, Denmark), followed by incubation with diaminobenzidine for 10 min. Sections were counter-stained with Meier stain.

Results

Effects of serosal hypertonicity on the permeability properties of the rat and rabbit rectum

Water permeability

When net J_w in the isolated epithelium of the rat or rabbit rectum was measured, a small net flux was initially recorded in the absence of any osmotic or chemical gradient (Table 1). A significant absorptive J_w developed

Table 1 Net water fluxes (J_w), transepithelial potential (V_t) and transepithelial resistance (R_T) in the rat and rabbit rectum epithelia in basal conditions

	J_W (µl min^{-1} cm^{-2})	V_T (mV)*	R_T (Ω cm^2)
Rat (n=4)	–0.25±0.10	10.2±2.1	156±5
Rabbit (n=8)	0.21±0.08	17.2±5.1	141±22

*Stabilized values

Table 2 Effects of $HgCl_2$ on the response to serosal hypertonicity, 5 min after the osmotic challenge. Increase in net water fluxes (ΔJ_w), and mannitol permeability (ΔP_S) in the rat and rabbit rectum

		ΔJ_w (µl min^{-1} cm^{-2})	$\Delta P_s \times 10^{-6}$ (cm s^{-1})
Rat	Osmotic response	0.40±0.06 (n=4)	0.26±0.10 (n=4)
	Osmotic response + $HgCl_2$	0.18±0.01 (n=4)	0.68±0.26 (n=4)
	Difference	0.22±0.06 (P<0.02)	0.42±0.28 (NS)
Rabbit	Osmotic response	0.45±0.10 (n=16)	1.45±0.7 (n=8)
	Osmotic response + $HgCl_2$	0.34±0.07 (n=8)	5.94±0.68 (n=4)
	Difference	0.11±0.12 (NS)	4.49±0.98 (P<0.001)

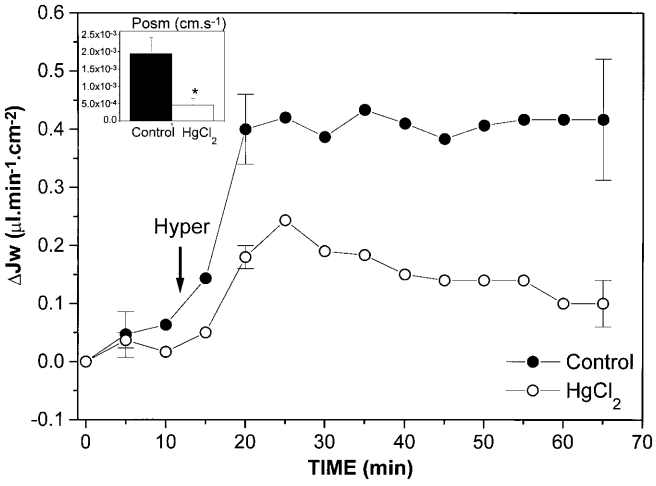

Fig. 1 Transepithelial net water flux (J_w) across the isolated epithelium of the rat rectum as a function of time in control conditions and in the presence of 0.3 mM $HgCl_2$ (both in the mucosal and serosal baths, n=4). At the *arrow* mannitol was added to the serosal bath, to a final concentration of 200 mosmol/l (total osmolarity 500 mosmol/l). The *inset* shows the effects of $HgCl_2$ on the osmotic permeability (P_{osm})

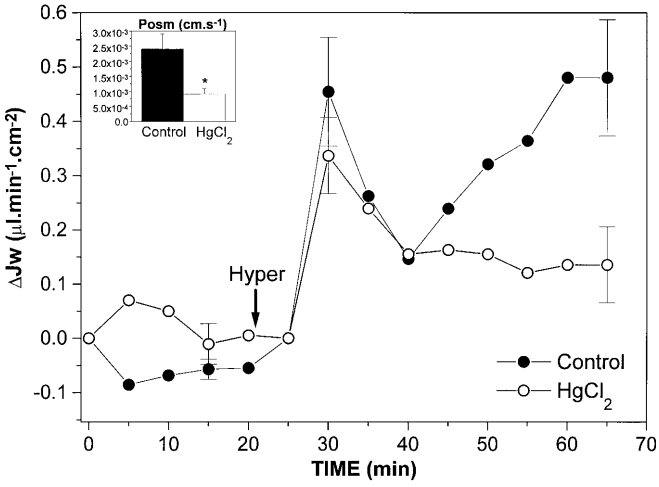

Fig. 2 Transepithelial net water flux (J_w) across the isolated epithelium of the rabbit rectum as a function of time in control conditions and in the presence of 0.3 mM $HgCl_2$ (both in the mucosal and serosal baths, n=8). At the *arrow* mannitol was added to the serosal bath, to a final concentration of 200 mosmol/l (total osmolarity 500 mosmol/l). The *inset* shows the effects of $HgCl_2$ on the osmotic permeability (P_{osm})

when the serosal bath was made hypertonic (by adding 200 mosmol/l mannitol). The observed values, 5 min after the osmotic challenge, are reported in Table 2. No significant changes in J_w were observed after mucosal hypertonicity (ΔJ_w 0.12±0.10 µl min^{-1} cm^{-2}, NS).

The time course of the response to serosal hypertonicity was further investigated in both species. While in the rat rectum the osmotic response was a sustained one (Fig. 1) in the rabbit rectum, after the initial increase, J_w values decreased to a minimum, about 15 min after the osmotic challenge. Then a slower and sustained second increase was observed (Fig. 2).

$HgCl_2$ (0.3 mM, added to both media) reduced the hydrosmotic response in both species. Nevertheless, some differences appeared: while in rats the sustained hydrosmotic response was uniformly reduced (Fig. 1), the first transient peak observed in rabbit tissue was not significantly affected while the "second phase" of the response was clearly inhibited (Fig. 2). Sixty minutes after the hypertonic challenge, the osmotic water permeability was significantly lower compared to control conditions in both species (Fig. 1 and 2, insets).

Correlation between J_w, R_T and mannitol permeability in the rat and rabbit rectum

The observed evolution of J_w after the hyperosmotic challenge in rat and rabbit rectal tissue prompted us to explore the transepithelial mannitol permeability (P_s) and electrical resistance, as paracellular permeability markers.

In the rat rectum, 5 min after the osmotic challenge, at the peak of the hydrosmotic response, no significant changes in P_s values were observed (Table 2). Even 60 min after the osmotic challenge P_s was not modified [$P_s \times 10^{-6}$, cm s^{-1}: 1.68±0.53 (5 min) versus 2.14±0.62 (60 min), n=4, NS]. P_s evolution in the presence of $HgCl_2$ (0.3 mM), added to both media, was also studied. No changes in P_s values were observed [$P_s \times 10^{-6}$, cm s^{-1}: 1.62±0.23 (basal) versus 2.29±0.23 (5 min after the osmotic challenge), n=4, NS]. In rabbits, 5 min after the osmotic challenge a rapid increase in the initial P_s values was observed (Table 2). Sixty minutes later, the observed P_s was again reduced. In the presence of $HgCl_2$ a slow increase in P_s was observed, 5 min after the osmotic challenge [$P_s \times 10^{-6}$, cm s^{-1}: 5.01±0.63 (basal) versus10.97±1.31 (5 min after the osmotic challenge), n=4, P<0.01].

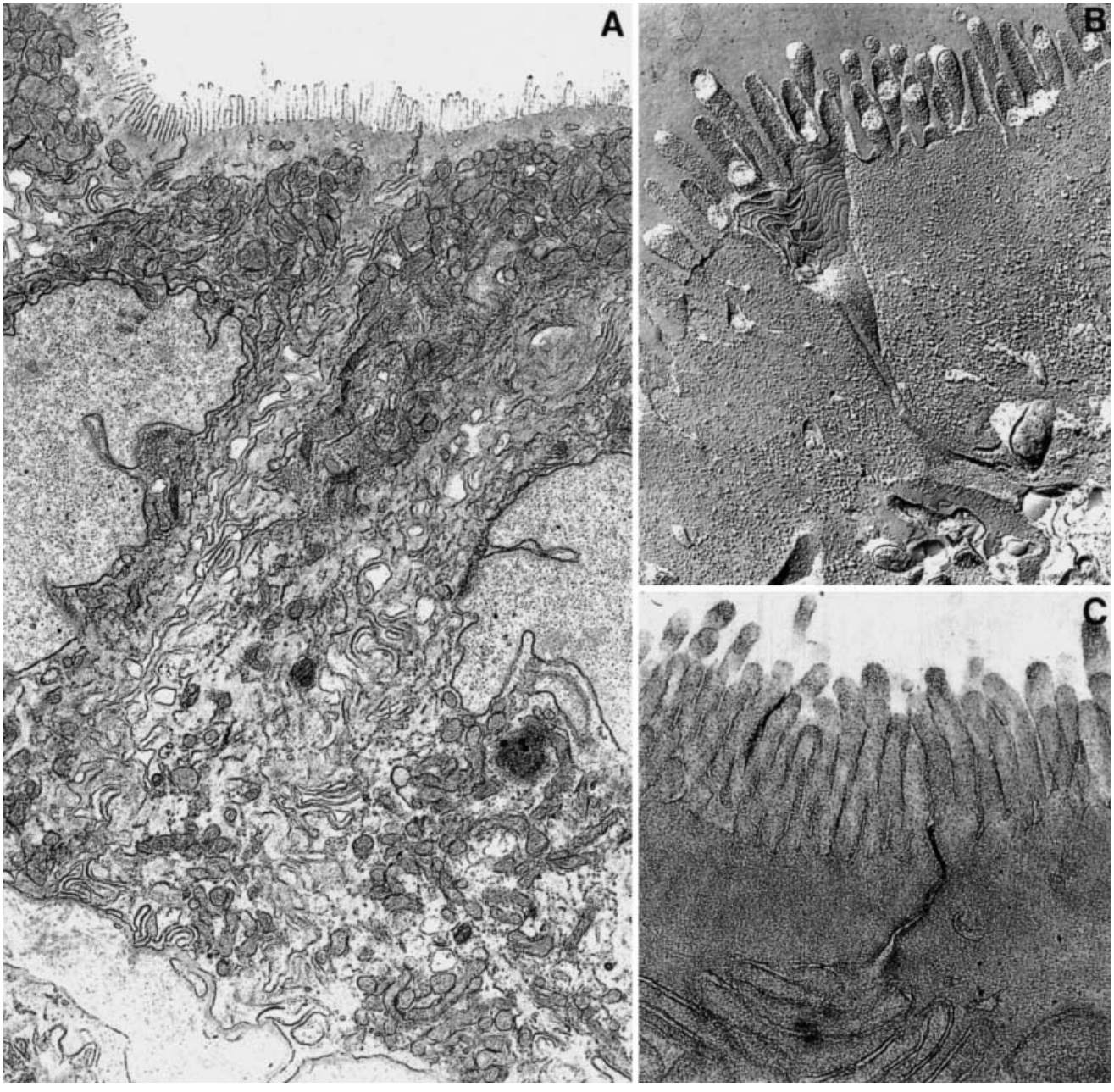

Fig. 3A–C The rabbit rectum epithelium in control conditions. **A** The intercellular spaces are slightly dilated. An organelle-free zone under the apical brush border is observed (×9000). **B** Transmission electron microscopy picture of an apical tight junction (×37,000). **C** Freeze-fracture picture at the tight-junction zone (×37,000)

Initial R_T and V_T values, shown in Table 1, did not change significantly after the osmotic challenge in either rats or rabbits.

Ultrastructural correlates

When rabbit or rat rectum epithelia were fixed in control conditions (no osmotic gradient), the principal cells presented a well developed brush-border and most intercellular spaces were closed or moderately open and quite interdigitated (Figs. 3A, 5A). Freeze-fracture and transmission studies showed that, in the rabbit rectum, quite well developed tight-junction structures were present (Fig. 3B,C). Both in transmission electron microscopy and in freeze-fracture pictures a non-structured zone (free of organelles) was evident just below the apical brush-border.

Five minutes after the increase in serosal tonicity, both in rabbits and rats, the cells had shrunk and the intercellular spaces were wide open (Figs. 4A, 5B). In the rabbit rectum, basal parts of the cells were more sensitive to the osmotic shock than the apical regions, and specially the previously described zone under the brush-border did not shrink. Figure 4B shows a freeze-fracture

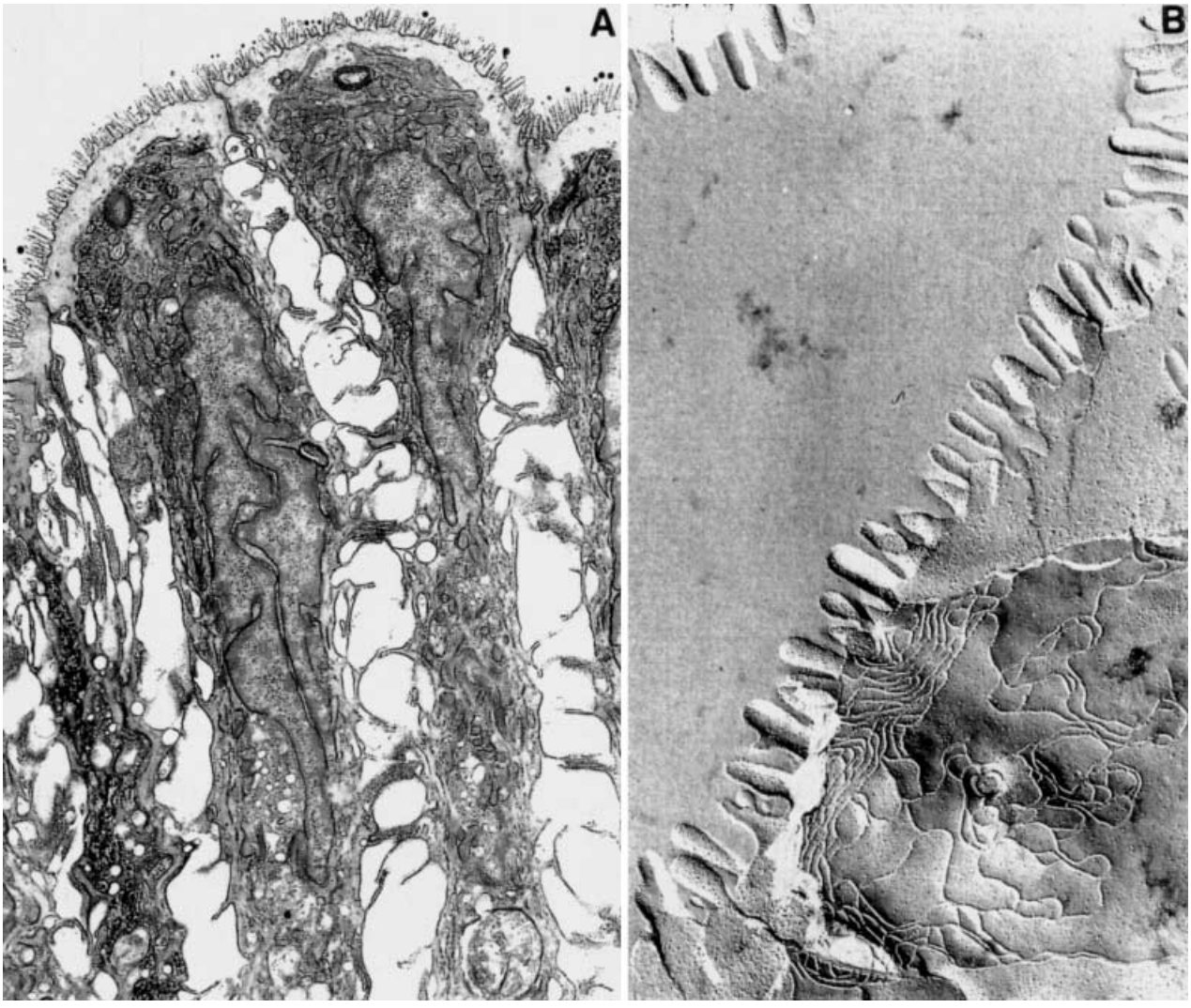

Fig. 4A,B The rabbit rectum epithelium 5 min after exposure to an osmotic gradient (plus 200 mosmol/l, serosal). **A** Electron microscopy showing the principal epithelial cells with dilated intercellular spaces (×8400). **B** Freeze-fracture showing a disrupted tight junction (×37,000)

of the rabbit rectum studied in similar experimental conditions. No important structural changes were observed. Nevertheless, the tight-junction network sometimes appeared rather disorganized (Fig. 4B). It must be remembered that a small increase in P_s was observed in this species 5 min after the osmotic challenge (Table 2).

In another experimental series the effects on the epithelial structure of an increase in mucosal tonicity, similar to that previously applied in the serosal bath, were tested. No significant structural changes were observed 5 min after the osmotic challenge, in either rabbit (Fig. 6A) or rat (not shown) tissues.

Together with the principal epithelial cells, another cell type, which was not sensitive to the osmotic shock (SH), was observed (Fig. 6B) in the rabbit rectum. These cells represent a small percentage of the epithelium and may be endocrine in nature. Frequent gap-junctions figures were present in freeze-fracture pictures at the basolateral membrane of the epithelial cells (not shown).

Immunohistochemical results

The previously described results suggest the presence of water channels (aquaporins) in the basolateral membrane of rectal epithelial cells. To identify and localize these channels, immunolocalization studies were performed using rat rectum and antibodies against different aquaporins (AQP1, AQP3 and AQP4).

Figure 7A, B shows the immunocytochemical localization of AQP3 in the rat rectum epithelium. AQP3 is abundant in the basolateral plasma membranes (arrows) of surface epithelial cells, whereas it is not present in the cells in deeper portions of the crypts (Fig. 7C). There was no specific AQP3 labeling in the apical plasma membrane (arrowheads). There was no labeling of epithelial cells in sections incubated with affinity-purified anti-AQP4 (endothelial cells in vascular structures outside the field shown were strongly labeled) (Fig. 7D).

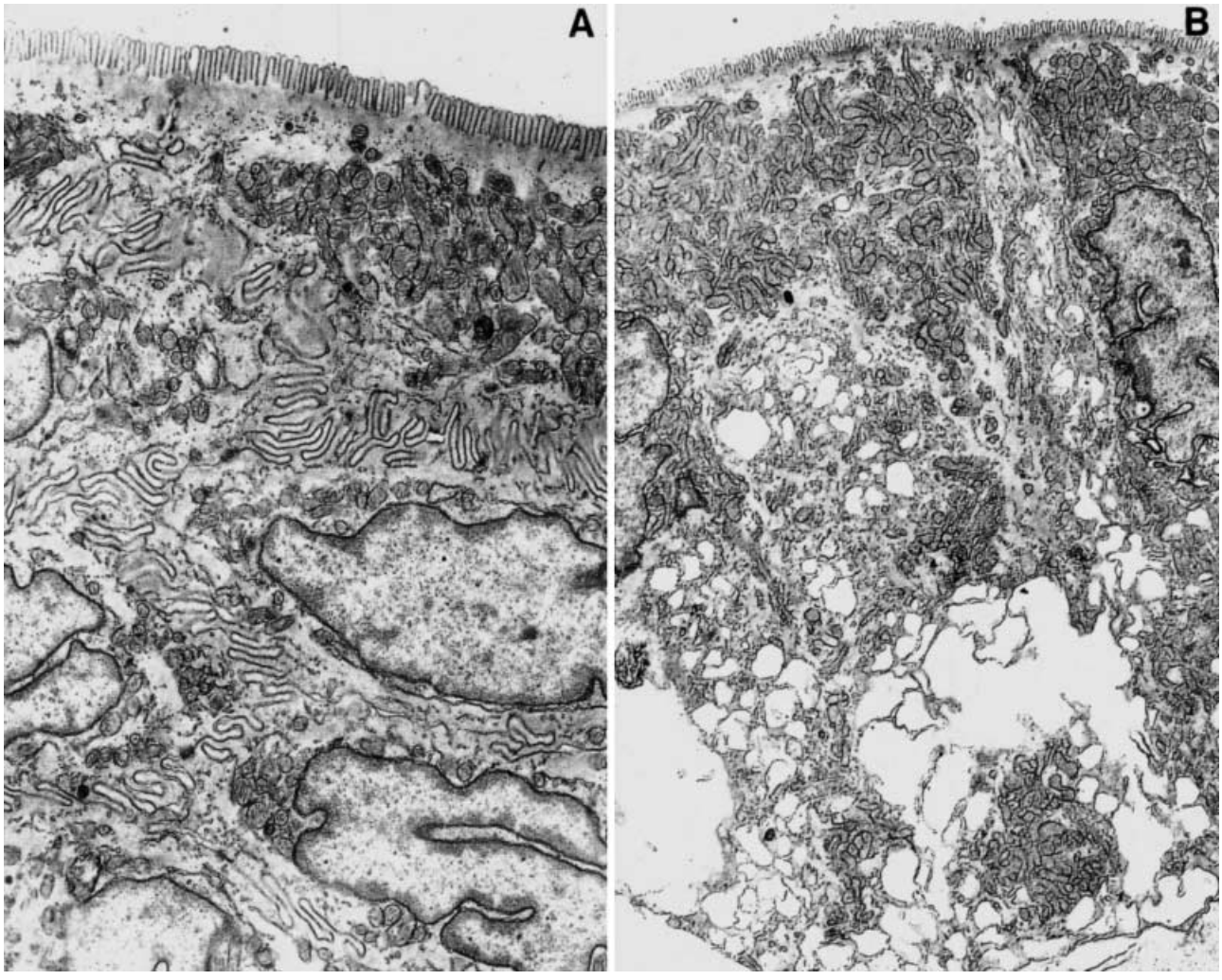

Fig. 5 **A** The rat rectum epithelium in control conditions. The intercellular spaces are slightly dilated. **B** Five minutes after exposure to an osmotic gradient (plus 200 mosmol/l, serosal)

Parallel sections were labeled with affinity-purified anti-AQP1. There was no labeling of AQP1 in the surface epithelial cells whereas endothelial cells of the vascular structures were labeled (not shown).

The antibodies used were produced in rabbits; therefore, an important non-specific signal appeared when the rabbit rectum was tested and the results were less conclusive.

Discussion

The water and solute permeability properties of the mammalian rectum play a central role in different physiological and pathological situations [1, 15, 37]. Nevertheless, even now that recent studies have given us a deeper insight into fluid absorption and feces dehydration in the large intestine [22, 24], water transfer mechanisms, particularly at the rectum level, are not completely understood. We now report that the rat and rabbit rectum have a basically similar response when the serosal tonicity is increased: a transepithelial J_w develops and is associated with an important shrinkage of epithelial cells. The apical border has a low water permeability, because no structural changes were observed 5 min after mucosal hypertonicity in either species. The strong polarity of the hydrosmotic response indicates that the J_w increase does not merely reflect the imposed osmotic gradient but a permeability change. The response is reminiscent of that previously observed in other tissues [32], suggesting that a transcellular water pathway has opened.

The cell shrinkage observed 5 min after the tonicity increase, both in rabbits and rats, can be straightforwardly interpreted as being due to the presence of water channels located in the basolateral cell membrane.

Water permeability in the rat rectum

Mannitol was used in this study in two different ways:

1. As an osmotic molecule, employed to create the tonicity gradients. Electron microscopy studies clearly

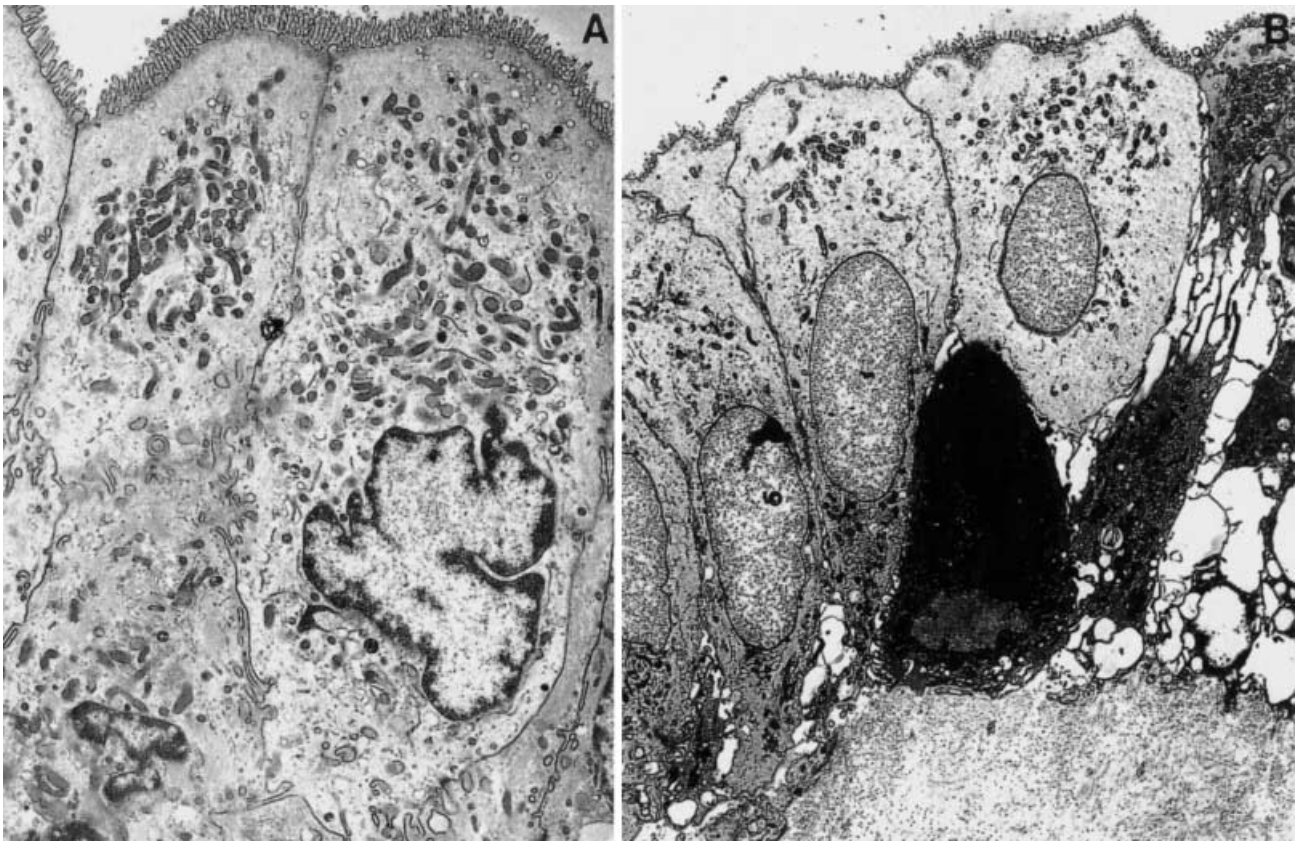

Fig. 6 **A** The rabbit rectum epithelium 5 min after an increase in the mucosal tonicity (200 mosmol/l). No significant alterations were observed (×6000). **B** Five minutes after exposure to an osmotic gradient at the serosal side (plus 200 mosmol/l). A cellular type less frequently observed and not sensitive to the osmotic gradient is shown. Not osmotically sensitive cells at the *right* (×2800)

demonstrate that its reflexion coefficient (σ) must be near to one, as previously reported for most cells [11].

2. As a marker of the paracellular route, in tracer experiments, because of its aforementioned properties [4].

After the increase in serosal tonicity no changes in P_s or R_T in the rat rectum were observed. These observations, together with J_w sensitivity to mercurial agents, strongly suggest a transcellular route for the osmotically induced increase in water fluxes. $HgCl_2$-evoked inhibition could be interpreted as the result of a blocking effect on the basolateral water channels. Nevertheless, the increase in mannitol permeability in the presence of $HgCl_2$ suggests that the "response" of the paracellular route to serosal hypertonicity is modified after a long-term preincubation (60 min) with $HgCl_2$. In this situation, a reduction in the mannitol σ coefficient would induce a simultaneous decrease in the osmotically induced J_w.

Water permeability in the rabbit rectum

The J_w initial transitory peak, observed in the rabbit rectum, could be just reflecting cell shrinkage. Nevertheless, in principle, the system used did not detect cell volume changes (see Materials and methods). Furthermore, it was not observed in rats and it was associated with a small but significant increase in P_s, suggesting the opening of the paracellular route. $HgCl_2$ did not modify this initial phase, giving additional support to the "paracellular hypothesis". Furthermore, partially disrupted tight-junctions were also observed. In contrast, the secondary J_w increase induced by serosal hypertonicity (which was reduced by $HgCl_2$) reflects a transcellular route, similar to that observed in rats.

The presence of an organelle-free zone that is apparently insensitive to osmotic changes just under the mucosal border indicates that the principal cells cannot be thought of as a homogeneous water compartment (at least in the rabbit rectum). The presence of some cells that do not shrink when exposed to a hypertonic medium (Fig. 7B) probably reflects, in this case, the absence of water channels.

The role of aquaporins in the observed permeability properties

It is now accepted that important osmotic permeabilities are associated with the presence of water channels [25,

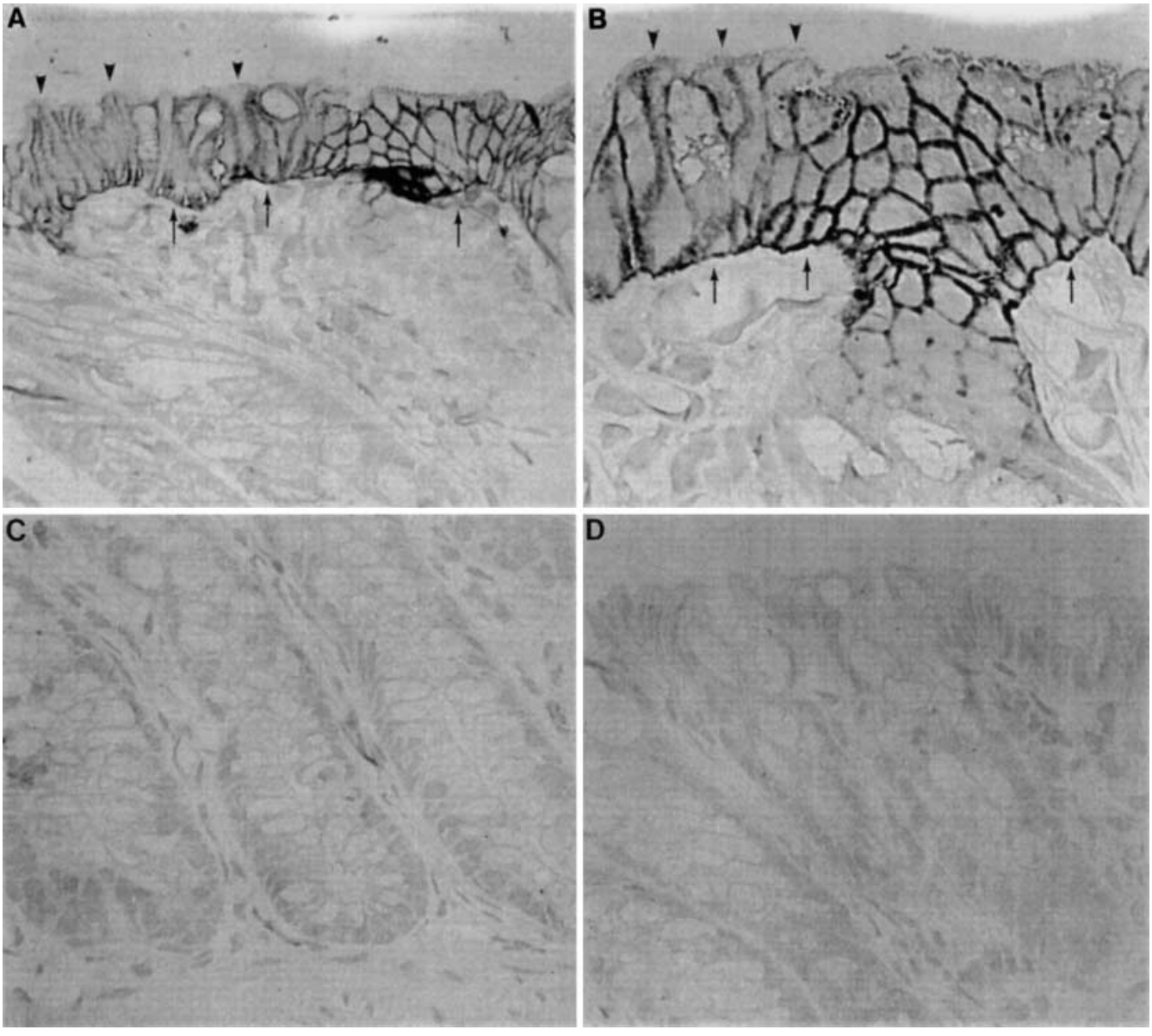

Fig. 7 **A** Immunocytochemical localization of AQP3 in the rat epithelium rectum. AQP3 is abundant in the basolateral plasma membranes (*arrows*) of surface epithelial cells whereas it is not present in cells in deeper portions of the crypts. There is no specific AQP3 labeling in the apical plasma membrane (*arrowheads*, ×480). **B** Higher magnification showing AQP3 labeling in the basolateral plasma membrane of surface epithelial cells. (×960). **C** Section of deeper portions of crypts labeled for AQP3. No labeling is observed (×480). **D** Sections incubated with affinity-purified anti-AQP4 display no labeling of the epithelial cells (endothelial cells in vascular structures outside the field shown were strongly labeled) (×960)

26, 28, 31, 36]. Expression studies have shown the presence of AQP3 and AQP4 in the basolateral membrane of colon epithelial cells in the rat [12]. In addition, in situ hybridization and Northern blot experiments indicate the presence of AQP1 and AQP3 in the intestine of humans and rats [13, 14, 17, 18]. Therefore, these are possible candidates to account for the observed high water permeability in the basolateral membrane of rabbit and rat rectal epithelial cells. Our immunohistochemical results from the study of rat rectal tissue show that AQP3, but not AQP1 or AQP4, is present in epithelial cells. AQP3 was found in abundance in basolateral plasma membranes, whereas it was not present in cells in deeper portions of the crypts. Also, there was no specific AQP3 labeling in the apical plasma membrane. The negative results for AQP1, AQP3 and AQP4 at the luminal cell border agree with our results, for both rats and rabbits, showing that epithelial cell volumes do not change after mucosal hypertonicity.

As well as AQP4 localization in the descending colon, it has been reported recently that the defecated stools of AQP4 knockout mice have a significantly greater water content compared to that of wild-type mice [17]. Our results suggest that the “aquaporin expression

pattern" is not the same in the rat distal colon and rectum, probably indicating some functional differences. However, the role of other aquaporins in the mammalian rectum must be further investigated.

The water permeability properties of the rectum determine whether hard or soft feces are formed [19], as observed in humans. It has been proposed that the feces are subjected to large fluid tensions generated by the osmotic pressure gradient across the descending colon [19]. A mechanism for removing water from the feces during the "hard feces phase" in the descending colon, in which local hypertonicity has a central role, has been reported recently [22, 24]. We propose here that an increase in the transepithelial water permeability occurs in the presence of serosal hypertonicity in the mammalian rectum. If we accept that the paracellular route cannot be the exclusive pathway for the observed water fluxes and that the basolateral membrane in which AQP3 is located is quite water permeable, a change in the permeability of the apical cell border could occur during the osmotic challenge.

It can be concluded from these results that the mammalian rectum is a "water-tight epithelium", with a highly polarized water permeability structure. The strong functional polarization observed may be attributable to the presence of AQP3 water channels in the basolateral membrane of the epithelial cells. Apical cell border permeability appeared to be very low in basal conditions. An increase in apical water permeability may be associated with the increased J_w observed after an osmotic challenge. Nevertheless, proof of this hypothesis requires more experimental evidence.

Acknowledgements We would like to thank Corinne Le Moal for the technical assistance. This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina); Universidad de Buenos Aires, Argentina; Fondo Nacional de Ciencia y Tecnica (Foncyt, Argentina) and Centre National de la Recherche Scientifique (CNRS), France.

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