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special communication

Aquaporin-2, a regulated water channel, is expressed in apical membranes of rat distal colon epithelium

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Gallardo, Pedro, L. Pablo Cid, Carlos P. Vio, and Francisco V. Sepúlveda. Aquaporin-2, a regulated water channel, is expressed in apical membranes of rat distal colon epithelium. *Am J Physiol Gastrointest Liver Physiol* 281: G856–G863, 2001.—Aquaporin-2 (AQP-2) is the vasopressin-regulated water channel expressed in the apical membrane of principal cells in the collecting duct and is involved in the urinary concentrating mechanism. In the rat distal colon, vasopressin stimulates water absorption through an unknown mechanism. With the hypothesis that AQP-2 could contribute to this vasopressin effect, we studied its presence in rat colonic epithelium. We used RT-PCR, in situ hybridization, immunoblotting, and immunocytochemistry to probe for AQP-2 expression. An AQP-2 amplicon was obtained through RT-PCR of colon epithelium RNA, and in situ hybridization revealed AQP-2 mRNA in colonic crypts and, to a lesser extent, in surface absorptive epithelial cells. AQP-2 protein was localized to the apical membrane of surface absorptive epithelial cells, where it colocalized with H^+-K^+-ATPase but not with Na^+-K^+-ATPase. AQP-2 was absent from the small intestine, stomach, and liver. Water deprivation increased the hybridization signal and the protein level (assessed by Western blot analysis) for AQP-2 in distal colon. This was accompanied by increased *p*-chloromercuriphenylsulfonic acid-sensitive water absorption. These results indicate that AQP-2 is present in the rat distal colon, where it might be involved in a water-sparing mechanism. In addition, these results support the idea that AQP-2, and probably other aquaporins, are involved in water absorption in the colon.

intestinal fluid absorption; immunofluorescence microscopy; in situ hybridization; reverse transcriptase-polymerase chain reaction; *p*-chloromercuriphenylsulfonic acid

THE SMALL AND LARGE INTESTINE are sites of abundant water transport in mammalian species. In humans, ~10 l of water are absorbed in these two segments of the gut. Indeed, the intestine served as the experimental model to establish what is today an accepted paradigm of water transport in fluid-absorbing epithelia such as the intestine and renal tubules (22). This model, in its updated version, considers the presence of an intercellular region of relative high osmotic pressure within the epithelium to which water rapidly flows across highly permeable membranes. The discovery of aquaporins (17), proteins conferring high water permeability to plasma membranes, has come to explain one key component of the paradigm.

Renal water excretion is under the influence of arginine vasopressin (AVP), which increases the osmotic water permeability of the apical membrane of collecting duct principal cells. This action is mediated by vasopressin V_2-type basolateral receptors (10). AVP regulates AQP-2 in two ways: the first is a short-term or acute effect that involves the control of intracellular traffic of AQP-2, contained in subapical cytosolic vesicles (10, 16). The second is a transcriptional effect observed in rats after 24 h of water restriction. In this condition, the relative abundance of mRNA coding for AQP-2 and the protein are increased for the acute response (5, 19, 23).

The water-conserving action of AVP seems restricted to the kidney. However, there is evidence that the colon could be a target of action for AVP. In rat distal colon, it has been shown (1, 2, 26) that AVP stimulates water absorption. However, the mechanism by which AVP stimulates colonic transepithelial water transport is unknown. Regulation of water permeability similar to that in the kidney could account for the AVP-dependent increased water absorption in colonic epithelium.

Whether water channels exist in the intestinal tract and serve a purpose in osmotically driven transepithelial water transport has been a matter of debate (12, 22). Results (24) obtained with vesicles from brush-

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border and basolateral membranes of the small intestine have suggested an absence of water channels. Recent evidence (13), however, shows that several members of the aquaporin family are expressed in epithelial cells from the gastrointestinal tract. AQP-3, AQP-4, and AQP-8 transcript or protein have been demonstrated in the epithelium lining the small or large intestine, although there are discrepancies about their distribution in different intestinal segments and cell types. AQP-3 has been reported (7, 11, 18) to be located in the basolateral membrane of enterocytes from colonic surface and small intestinal villus. AQP-4 appears localized to the basolateral membrane of colonic epithelium (7), although it has also been reported (11) to be selectively localized in basolateral membranes of deep small intestinal glands, and its knockout in transgenic mice results in a decreased osmotic water permeability (28). AQP-8 transcript has been detected (11) in the columnar epithelial cells of jejunum and colon. No aquaporin has yet been shown to be present at the apical membrane of the small or large intestine.

Here we demonstrate that water absorption in the distal colon is increased in water deprivation and inhibited by aquaporin blockade. We have also investigated whether the large intestine expresses AQP-2 and whether its expression is regulated. AQP-2 transcript is indeed present in distal colon as demonstrated by RT-PCR and in situ hybridization. AQP-2 protein can be detected by Western blot analysis and is demonstrated to be on the apical side of surface colonocytes by immunohistochemistry. The results also suggest that the level of expression of colonic AQP-2 is increased by dehydration. This aquaporin could provide an apical membrane route for regulated transepithelial water transport in the colon.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (200–250 g) were kept in plastic cages and received food and water ad libitum. Water-deprived animals had no access to water but received food ad libitum and were controlled for body weight at the start and end of the experimental period. All experiments were done according to international regulations for animal care. Blood and urine samples were taken from animals anesthetized with pentobarbital sodium (60 mg/kg ip). Net fluid and Na^+ transport from the colonic lumen was measured in vivo in anesthetized rats using 5% agarose gel cylinders inserted into the descending colon, as described previously (29). Briefly, the gel was cast into 6-mm-diameter cylinders, weighed, inserted into the distal colonic lumen, and secured by a ligature at least 1 cm distal to the gel. The gut was returned to the abdominal cavity and left for 1 h before removal. Body temperature was checked and maintained at 37°C throughout. Fluid absorption was measured from the weight difference after the 1-h period. Na^+ concentration was measured by flame photometry after overnight extraction in 0.1 M HCl, and Na^+ absorption was calculated by comparison with nonincubated gels. Fluxes are expressed per square centimeter of gel surface area.

Tissue preparation. For RNA extraction, colonic mucosa was obtained by gentle scraping. For in situ hybridization, tissues were embedded in freezing (OCT) medium and dropped into liquid nitrogen. Cryosections (7 μm thick) were kept at −80°C until use. For immunolocalization, sections were fixed in Bouin's solution for 24 h at room temperature and then embedded in paraffin.

RT-PCR and cloning. Extraction of total RNA and reverse transcription were performed as previously described for intestinal tissue (3). For PCR, the following sense and antisense primers were designed from rat AQP-2 cDNA sequence (8): sense, 5′-TCCACAACAACGCCACAGC-3′, encoding amino acids 121–127; and antisense, 5′-GCACTTCACGTTCCTCCCA-3′, encoding amino acids 246–248. The PCR profile was 35 cycles of the following: 30 s at 94°C, 45 s at 60°C, and 1 min at 72°C, followed by an extension of 7 min at 72°C. The PCR products were subcloned and sequenced. The 400-bp cDNA fragment of AQP-2 derived from colon was used to synthesize riboprobes for in situ hybridization.

In situ hybridization. Digoxigenin-labeled antisense and sense riboprobes were generated from the DNA fragment described above by using in vitro transcription with T7 and T3 RNA polymerase, respectively. The probes were used at a concentration of 10 ng/μl, as described previously (21). Tissue sections were observed and photographed on a Nikon Optiphot microscope.

Immunolocalization. Immunohistochemistry with the peroxidase-antiperoxidase method was carried out as described previously (27). For immunofluorescence, rat kidneys and colon were perfused with physiological saline and then removed. Kidney and colon sections were frozen in liquid nitrogen and stored until use. Cryostat sections (7 μm) were blocked for 30 min with 2% BSA-0.5% Triton X-100 in PBS. Purified antisera against AQP-2 (kindly provided by Dr. M. Knepper and later purchased from Alomone Laboratories) was diluted 1:300, colonic H^+-K^+-ATPase (a kind gift from Dr. T. DuBose) to 1:150, and monoclonal antibody against the α_1-subunit of Na^+-K^+-ATPase (Upstate Biotechnology) to 1:300 in PBS. The sections were incubated with the antisera overnight at 4°C. Goat antibodies against rabbit IgG coupled to Cy2 or Cy3 and goat monoclonal antibodies against mouse IgG coupled to Cy3 (Jackson ImmunoResearch) were used as secondary antibodies. In dual-labeling studies, an intermediate blocking step with normal goat serum (diluted 1:20 in PBS) was performed. Confocal laser scanning microscopy was performed on a LSM Zeiss system, and the software of the instrument was used to merge images.

Immunoblotting. This was done with crude membrane fractions prepared from the tissues indicated. Renal medulla and cortex, dissected from kidney sections, and whole liver samples were homogenized in buffer containing 250 mM sucrose and 10 mM triethanolamine. Colonocytes, isolated as described previously (4), and small intestinal epithelium, obtained by gently scraping the mucosa with a glass slide, were homogenized with the same buffer. Homogenates were centrifuged at 2,000 *g* for 10 min at 4°C. The supernatants were spun down at 100,000 *g* for 1 h at 4°C, and pellets were resuspended. The protein concentration was determined by the Bradford assay. SDS-PAGE was performed using Laemmli buffers on 12% polyacrylamide minigels. Immunoblotting was performed with enhanced chemiluminescence to reveal antigen-antibody reaction. Quantification was carried out by densitometric scanning of the film, and data were expressed as the percentage of control results.

Statistics. Data are expressed as means ± SD. Differences between means were assessed by unpaired *t*-test, and $P < 0.05$ was considered to be significant.

RESULTS

The effect of water deprivation on fluid absorption in the distal colon was studied in rats kept for 96 h without access to water but with free access to food. Figure 1*C* shows significantly increased plasma and urine osmolality in rats with water restriction. Consistent with the onset of a water-sparing mechanism, the ratio of urine to plasma osmolality was also increased in thirsted rats. These changes were accompanied by changes in the rate of fluid absorption from the colonic lumen measured in vivo. There was a statistically significant increase in water absorption in thirsted rats compared with animals with free access to water (Fig. 1*A*). Water absorption under both conditions was significantly reduced to similar levels in the presence of *p*-chloromercuriphenylsulfonic acid (PCMBS). The effect of the mercurial agent did not affect the rate of Na^+ absorption measured simultaneously (Fig. 1*B*).

The presence of AQP-2 in colonic tissue was explored by RT-PCR. Figure 2*A* shows the gene for rat AQP-2 in schematic form. The cDNA is also shown to display the position of the primers used, which were designed to encompass three different exons. Figure 2*B* shows that a single amplicon of ~0.4 kb was obtained from kidney as expected (8). No amplification product could be detected with RNA from the spleen or small intestine or in the negative controls. An amplicon of the same size as that from the kidney was, however, detected with RNA from distal colonic mucosa. Sequencing this amplicon revealed a complete identity with the published sequence (8) for rat kidney AQP-2. This finding is consistent with the presence of AQP-2 transcript in rat colon.

AQP-2 expression in colon was also verified by Western blot analysis. Immunoblotting of membranes from kidney and colon of control and thirsted rats is shown in Fig. 2*C*. The analysis revealed a band of ~29 kDa in all cases plus an additional band of >40 kDa, perhaps corresponding to glycosylated and nonglycosylated forms of the protein.

To study the tissue location of the transcript for AQP-2 in colon, we performed in situ hybridization. Figure 3*A* shows the localization of AQP-2 mRNA in distal colon from a control rat. Considerable staining could be observed only in the apical cytoplasm of epithelial cells from colonic crypts. The intensity of the signal decreased from the base of the crypt toward the mucosal surface. No hybridization was observed in sections of tissue from the small intestine (Fig. 3*A*, *inset*), liver (Fig. 3*C*), or stomach (Fig. 3*D*). Figure 3*B* shows that thirsting increased the AQP-2 hybridization signal in colonic epithelium, with the staining spreading more toward the surface epithelium. Figure 3*B*, *inset*, depicts control in situ hybridization with a sense probe in a thirsted rat colonic crypt, showing no reaction.

A considerable increase in the protein level was observed in colon from dehydrated rats by immunoblotting (Fig. 2*C*). In three separate experiments, the increase in AQP-2 with thirsting, evaluated by densitometric scanning of the lower molecular weight bands, was 1.7 ± 0.2-fold. The expected augmentation in protein level was also seen in kidney tissue, which increased 2.3 ± 0.3-fold. AQP-2 was not detected in the small intestine or liver of thirsted rats (Fig. 2*C*). The location of AQP-2 protein in colonic epithelium was investigated by immunohistochemistry with a polyclonal antibody. As shown in Fig. 4, immunolabeling for AQP-2 was present in the apical membrane of surface columnar epithelial cells. Reaction was absent from goblet cells and crypt colonocytes. No labeling was detected after preabsorbing with the peptide used in preparing the antibody or in the small intestine (results not shown). Immunhistochemistry for AQP-2 in thirsted rats also revealed an increased reaction in the apical membrane of surface colonocytes compared with control (Fig. 4*B*).

To locate AQP-2 with better resolution within the epithelium, double-labeling immunofluorescence with

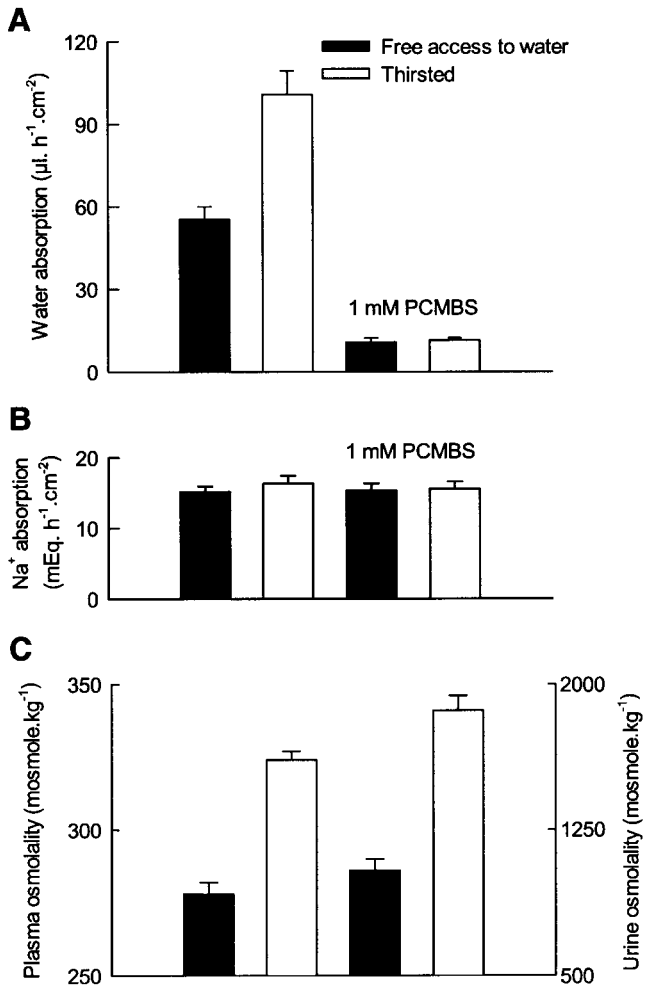


Fig. 1. Effect of water restriction on water and Na^+ absorption from the colon and on plasma and urine osmolality. *A*: water absorption measured in vivo in animals with free access to water or after 96 h thirsting. The absorption was measured from 5% agarose gels made with 150 mM NaCl with or without 1 mM *p*-chloromercuriphenylsulfonic acid (PCMBS). *B*: Na^+ absorption measured in the same intracolonic gels as in *A*. *C*: plasma (bars at *left*) and urine osmolalities (bars at *right*) of control and thirsted animals.

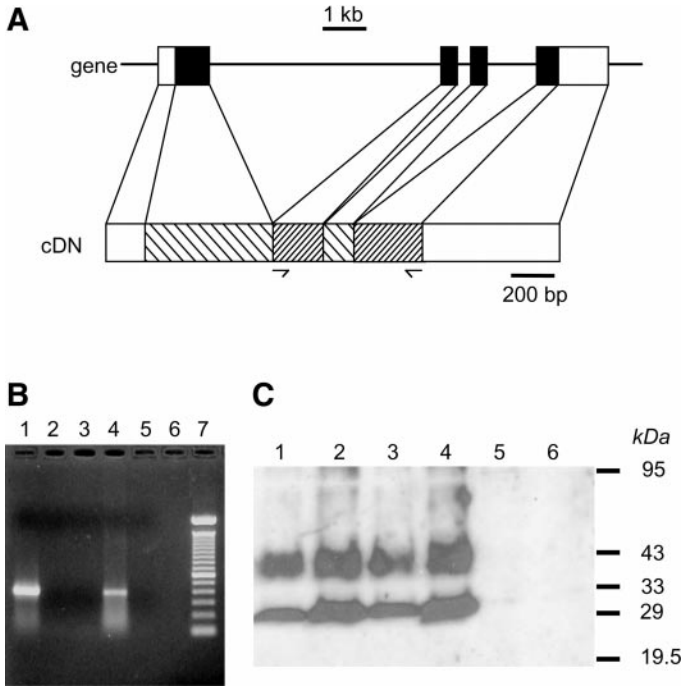


Fig. 2. Detection of aquaporin-2 (AQP-2) mRNA by RT-PCR in rat distal colon. *A*: localization of sense and antisense primers in rat AQP-2 cDNA; the sense primer is at the start of exon 2 and the antisense in exon 4. *B*: RT-PCR for AQP-2 in kidney (*lane 1*), spleen (*lane 2*), small intestine (*lane 3*), and distal colon (*lane 4*). *Lanes 5* and *6* correspond to amplification without DNA template and to a reaction without RT, respectively. *Lane 7*, molecular size markers (100-bp DNA ladder). According to the position of the primers in the cDNA, the amplicon had the expected size of 0.4 kb. *C*: immunoblot for AQP-2 of proteins from rat kidney (*lanes 1* and *2*) and distal colon (*lanes 3* and *4*). *Lanes 1* and *3* correspond to control and *lanes 2* and *4* to thirsted rats. *Lanes 5* and *6*, small intestine and liver from thirsted rats. Fifteen micrograms of protein were loaded in each lane. Blots were probed with anti-AQP-2 antiserum diluted 1:3,000.

membrane proteins localized to the apical or basolateral membrane was performed. Na^+- K^+-ATPase and H^+-K^+-ATPase were chosen, as they are known to be localized in the rat colon to the basolateral and apical membranes, respectively. In initial experiments, immunofluorescent staining with a H^+-K^+-ATPase antibody gave a distribution of label consistent with the known localization of this pump to the brush-border membrane of surface enterocytes in the distal colon (Fig. 5*J*). Simultaneous staining with a Na^+-K^+-ATPase antibody gave the expected localization to the basolateral membranes in both surface and crypt enterocytes (Fig. 5*I*). Expression of H^+- K^+-ATPase and Na^+-K^+-ATPase did not overlap (Fig. 5*K*). Figure 5, *A–D*, shows an analogous experiment but performed with an anti-AQP-2 antibody replacing the H^+-K^+-ATPase antibody. In Fig. 5, *A* and *D*, the distribution of AQP-2 and bright-field images are shown. AQP-2 is seen predominantly in what appears to be surface enterocytes and in some crypt mouths cut transversally. The label was predominantly at the apical surface of the cells, although some background staining was also present over the epithelial cells. In contrast, Na^+-K^+-ATPase was confined to basolateral membranes of the epithelium as seen in Fig. 5*B*. To test the apical membrane distribution of the aquaporin further, double labeling with H^+-K^+-ATPase antibody was carried out. Figure 5, *E–H*, shows that both AQP-2 and H^+-K^+-ATPase distribute mainly on the apical aspect of surface colonic epithelium. Superposition (Fig. 5*G*) shows that the distribution of these two proteins had significant overlap, as revealed from the emergence of yellow labeling.

DISCUSSION

The evidence presented here strongly suggests that AQP-2, the vasopressin-regulated water channel, is expressed in rat distal colon, an organ involved in water absorption and fecal dehydration. One possible way to control water absorption and thus water excretion is the mechanism that operates in the distal nephron, where AQP-2 water channels increase the osmotic water permeability of the apical plasma membrane of principal and inner medullary collecting duct cells. At the organism level, the consequence of this action is the reduction in renal water loss and the excretion of hyperosmotic urine. In fact, rats submitted to water restriction in this study showed increased plasma and urine osmolalities, and the ratio of urine to plasma osmolality was also increased compared with control rats. It is known that water restriction stimulates AVP secretion through an increase in plasma osmolality (19). In the rats used in this study, the increased urine osmolality and the ratio of urine to

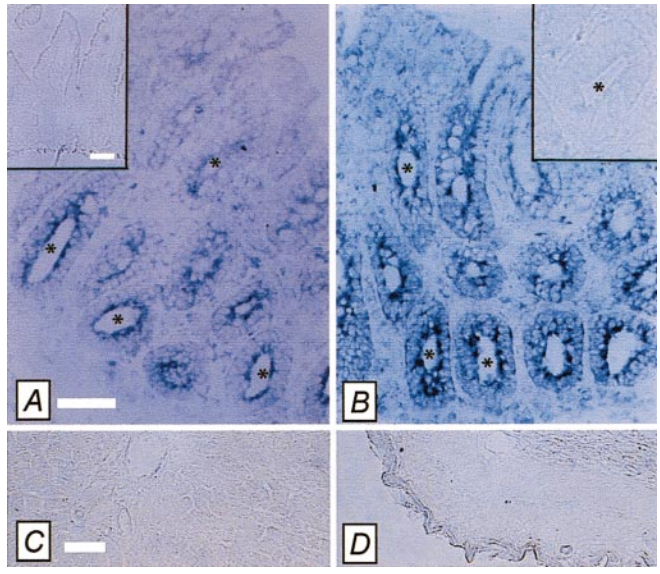


Fig. 3. Cellular identification of AQP-2 mRNA in distal colon from control and thirsted rats. *A* and *B*: in situ hybridization with digoxigenin-labeled antisense probe in distal colon from control (*A*) and thirsted rats (*B*). *Presence of the signal in apical cytoplasm of crypt epithelial cells; less intense labeling can be seen in the mucosal surface. No signal was obtained with the antisense probe in small intestine (*A*, *inset*), liver (*C*), and stomach (*D*) of thirsted rats or with the sense probe in distal colon (*B*, *inset*). Note the increased hybridization signal in colon from thirsted rats compared with control. Scale bars = 100 μm in *A* and *C* and also apply to *B*, *D*, and *B*, *inset*; scale bar = 25 μm in *A*, *inset*.

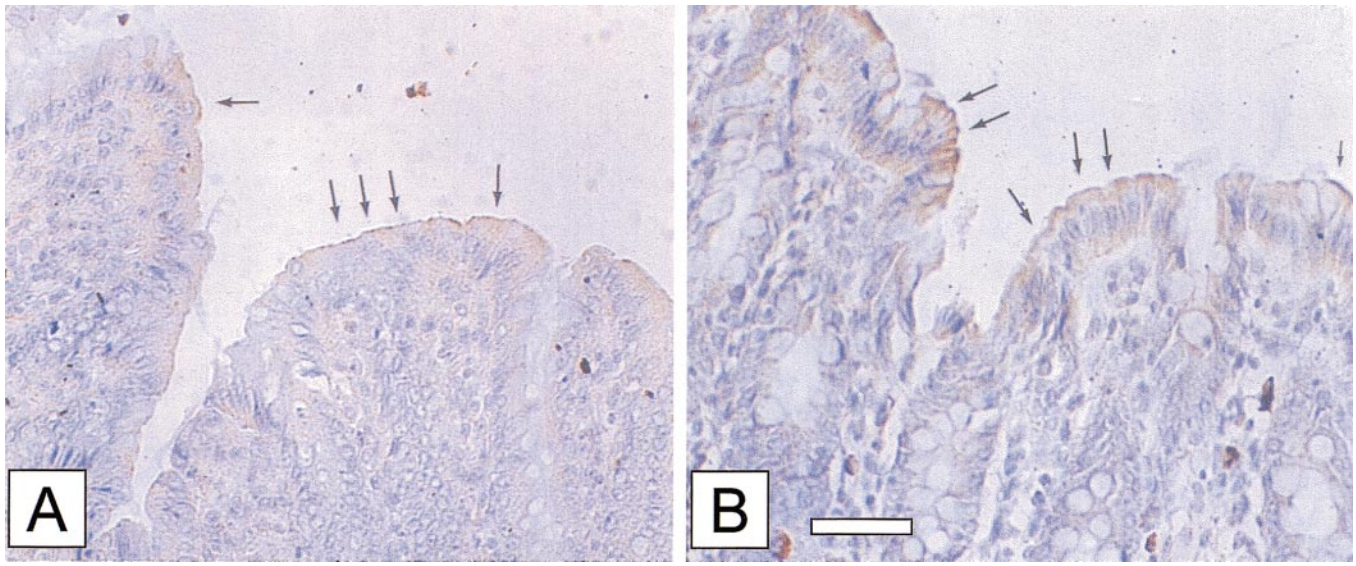


Fig. 4. Cellular identification of AQP-2 in distal colon from control and thirsted rats. Immunohistochemistry of AQP-2 in control (*A*) and thirsted rats (*B*) by the peroxidase-antiperoxidase method. Labeling is present in the apical membrane (arrows) of superficial columnar absorptive cells. Note the increased immunolabeling signal in colon from thirsted rats compared with control. Scale bar = 100 μm in *B* and also applies to *A*.

plasma osmolality are consistent with elevated levels of AVP.

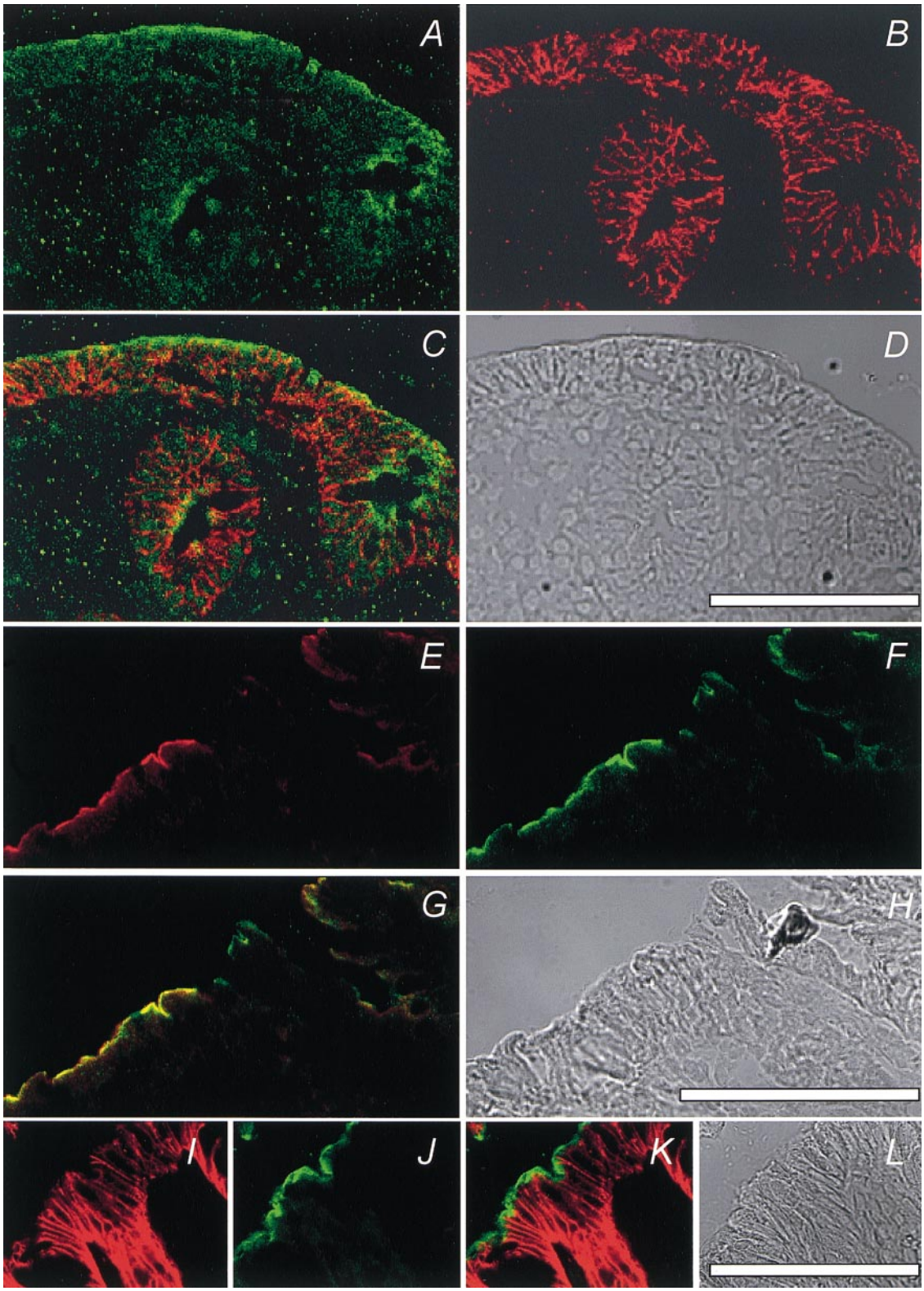
In the present study, we demonstrate that dehydration elicits an increase in water absorption in distal colon. Both basal fluid absorption and that elicited by dehydration can be abolished by the mercurial agent PCMBS, suggesting a participation of aquaporin water channels in this process. The effect of PCMBS occurs without affecting the simultaneously measured rate of Na^+ absorption, ruling out a general nonspecific effect. It is also interesting to notice that after dehydration the tonicity of the absorbate should turn from hypertonic to nearly isotonic. This might be due to the increased expression of AQP-2 in the apical membrane and suggests that significant transcellular water transport occurs. The inhibition of water absorption by PCMBS in control rats argues for a significant aquaporin-mediated basal water permeability. The in situ hybridization, immunoblotting, and immunohistochemical data also point to a constitutive expression of AQP-2, but other aquaporins could also account for this basal water permeability.

There is evidence suggesting that the epithelium of rat colon could be a target for vasopressin. In everted sacs of rat distal colon, AVP enhanced salt and water absorption (1, 2, 26), but the mechanism underlying this effect is unknown. We speculated that the mechanism of water transport regulation in the colon could be similar to that present in the kidney. We have, therefore, explored whether AQP-2 is expressed in colonic epithelium and regulated by the hydration state. Through RT-PCR using specific primers, we found evidence for the presence of the AQP-2 message in rat distal colon epithelium. Sequence analysis of the amplicon obtained from colon revealed a complete identity with the cDNA for rat kidney AQP-2 (8). The sense primer used corresponds to a sequence at the start of exon 2, and the antisense primer is in exon 4. Furthermore, the latter is part of the sequence that encodes amino acids in the cytosolic COOH-terminal tail, which is the most variable part among different members of the aquaporin family (20). Thus the fact that the amplicon encompasses three exons is consistent with the presence of the transcript rather than with the amplification of genomic DNA.

The message for AQP-2 in colon is present mainly in crypt epithelial colonocytes and less abundantly in

Fig. 5. Localization of AQP-2 in rat distal colon by immunocytochemistry and laser scanning microscopy. *A–D*: single cross section of distal colon labeled with anti-AQP-2 antibody and with anti-α_1-subunit of Na^+-K^+-ATPase. *A*: AQP-2 immunofluorescence is restricted to the apical membrane of surface mucosa as indicated by green. *B*: immunolocalization of α1-subunit Na^+-K^+-ATPase in colonic epithelium; the fluorescence is restricted to basolateral membrane of crypt and surface epithelial cells as shown by red. *C*: merging of *A* and *B* showing localization of AQP-2 and Na^+-K^+-ATPase in different membrane domains. *D*: bright-field image of the colonic section. *E–H*: localization of AQP-2 and H^+-K^+-ATPase in rat distal colon. *E*: AQP-2 immunofluorescence is restricted to the apical membrane of surface mucosa as indicated by red. *F*: immunolocalization of H^+-K^+-ATPase in colonic epithelium; fluorescence is shown by green. *G*: merging of *A* and *B* showing colocalization of AQP-2 and H^+-K^+-ATPase to the same membrane domain. *H*: bright-field image of the colonic section. *I–L*: localization of Na^+-K^+-ATPase and H^+-K^+-ATPase in rat distal colon. Distal colon section is labeled with anti-colonic H^+-K^+-ATPase antibody (*J*) or with anti-α_1-subunit of Na^+-K^+-ATPase (*I*). *K*: merging of *I* and *J* showing localization of Na^+-K^+-ATPase and H^+-K^+-ATPase in different membrane domains. *L*: bright-field image of the colonic section. Scale bar = 100 μm.

surface colonocytes. Immunostaining for AQP-2, on the other hand, is consistent with the presence of AQP-2 protein in the apical membrane of columnar absorptive cells of the mucosal surface and its absence from goblet and crypt cells. The cellular localization of AQP-2 to the apical membranes of surface colonocytes is confirmed by its codistribution with H^+-K^+-ATPase and its separateness from Na^+-K^+-



ATPase. The different distribution of AQP-2 message and protein along the crypt-villus axis is not uncommon in these cells that differentiate as they migrate from the site of cell division in the lower reaches of the crypts to the villus or surface epithelial location of mature enterocytes. Similar differing locations for protein and their messengers have been reported (9, 18) in intestinal epithelium, e.g., for AQP-3 and the SGLT1 cotransporter.

Dehydration in rats caused an increase in AQP-2 mRNA and protein of colonic epithelium that paralleled equivalent increases in renal tissue reported previously (10). It is accepted that AVP stimulation of AQP-2 gene transcription is involved in the renal effect. Another physiological signal independent of AVP might be involved in this effect, as suggested in studies (14) showing water deprivation-induced increase in renal AQP-2 mRNA under V_2-receptor blockade. The same mechanisms could be responsible for the increased AQP-2 signal found in distal colon from dehydrated rats. This would enable the colon to increase water absorption and fecal dehydration (15, 29). The finding of AQP-2 in the distal colon is novel and raises the possibility that this organ may be a site of control of water absorption in the gastrointestinal tract and thus could act in concert with the kidney to conserve water during states of thirsting.

At the cellular level, several similarities exist between principal cells of the distal nephron and columnar absorptive cells in the distal colon. First, they express the three subunits of the epithelial Na^+ channel in the apical membrane (6) and Na^+-K^+-ATPase in the basolateral membrane (25). The expression of these proteins is under the influence of aldosterone, which, as in the distal nephron, increases Na^+ electrogenic transport. Second, AQP-3 and AQP-4, which are present in the basolateral membrane of principal cells (10), are also present in the same membrane domain of absorptive cells of the distal colon (7, 18). The presence of apical membrane AQP-2 in the distal colon would complete the analogy between the two cell types. Recently (11), AQP-8 mRNA has been localized in superficial colonic cells; however, the subcellular localization of this aquaporin remains to be established. At present, no other member of the aquaporin family has been localized to the apical membrane to account for the constitutive water absorption that occurs in the colon. Further study is necessary for better understanding of the physiological role of AQP-2 in colonic water absorption and its regulation.

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