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Show me the pathway! Regulation of paracellular permeability by Na^+-glucose cotransport

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Abstract

The physiological impact of Na^+-nutrient cotransport-dependent regulation of intestinal tight junction permeability has been controversial. Nonetheless, increased permeability of small intestinal mucosae and enterocyte tight junctions as a consequence of Na^+-nutrient cotransport has been documented by a significant number of in vivo and in vitro studies. Some details of the intracellular signaling events that regulate this process have been described recently. The aims of this article are to: (i) review studies of tight junction regulation and paracellular nutrient absorption in mammalian intestine, (ii) identify potential applications of tight junction regulation, and (iii) summarize recent progress in defining molecular mechanisms that lead to altered tight junction permeability. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Tight junction; Na^+-glucose cotransport; Na^+–H^+ exchange; Myosin light chain

Contents

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1. Diversities of tight junction permeability reflect physiology

Tight junctions are essential to the organization of epithelia and endothelia. A primary function of these cells is to form a barrier that separates two distinct environments. Although cell membranes participate in this function, tight junctions are necessary to seal discontinuities at cell–cell interfaces. The permeability of tight junctions is not fixed, but varies remarkably according to the tissue examined. Reported extremes for epithelia include the 'leaky' proximal renal tubule, with a reported paracellular electrical resistance of 270 $\Omega \cdot cm^2$ [1], and the 'tight' rabbit urinary bladder epithelium, with paracellular resistance of $\geq 31{,}000\ \Omega \cdot cm^2$ [2]. The physiological reasons for these differences clearly relate to function; the proximal renal tubule is responsible for absorption of essential components from the glomerular filtrate while the bladder must prevent absorption of filtered substances and maintain urinary ion composition.

The essential role of the tight junction in renal paracellular absorption is confirmed by the recent identification of paracellin-1 [3], a member of the claudin gene family [4]. The presence of paracellin-1 is required for Mg^{2+} reabsorption in the thick ascending limb of the loop of Henle [3] (see review by Lapierre in this issue). While the precise mechanism by which Mg^{2+} wasting occurs in the absence of paracellin-1 is unknown, current evidence suggests that paracellin-1 may function as an ion-specific pore within the tight junction. Paracellin-1 and other claudins are localized to the tight junction strands, a network of anastamosing structures seen by freeze-fracture examination. The existence of proteins that form tight junction pores was predicted over 20 years ago by mathematical models linking the number of strands to tight junction resistance [5]. However, the apparent pore function of the claudins does represent a considerable divergence from the more recent focus on tight junction proteins that mediate barrier function [6]. If the proposed pore function of paracellin-1 is correct, it follows that paracellin-1 deficiency would result in an inability to recover Mg^{2+} from the renal tubules by paracellular absorption and subsequent urinary Mg^{2+} wasting. Thus, it appears likely that the claudins may be a family of tight junction pore proteins. The identity of tight junction components responsible for paracellular sealing remains unknown.

Similar to the renal tubule, the small intestine must support both transcellular and paracellular absorption of nutrients and water across the epithelial barrier. In fact, many of the protein transporters responsible for transcellular nutrient transport are expressed in both renal and small intestinal mucosa. However, the small intestine is also charged with maintaining a protective barrier separating a potentially toxic milieu, the lumen, from the interstitium. Thus, the intestinal barrier must be precisely tuned to permit water and nutrient absorption while preventing passage of toxic substances.

2. Dynamic regulation of paracellular permeability occurs in vitro

Early studies presumed that tight junction permeability was fixed. This assumption was modified after Duffey et al. reported that the transmucosal resistance of *Necturus* gallbladder epithelium increased following treatment with cyclic AMP analogues [7]. Both the charge selectivity and ultrastructure of tight junctions changed in parallel with the cyclic AMP-induced changes in resistance. These data suggested that the changes in resistance were due to modified tight junction permeability. Further studies showed that the number of tight junction strands increased along with transmucosal resistance in *Necturus* gallbladder epithelium exposed to Ca^{2+} ionophore [8]. Subsequently, it was shown that phorbol esters could increase tight junction permeability in a cultured porcine renal epithelial cell line [9,10]. Thus, it became clear that tight junction permeability could be regulated by a diverse array of intracellular signaling molecules. However, a physiological model of tight junction regulation had not yet been identified.

In 1987, Pappenheimer, Madara, and Reiss demonstrated coordinated regulation of tight junction structure and permeability in rodent small intestine [11–13]. This increase in tight junction permeability was induced by transcellular Na^+-dependent glucose transport (Fig. 1). The authors proposed a new theory of intestinal solute absorption based on this

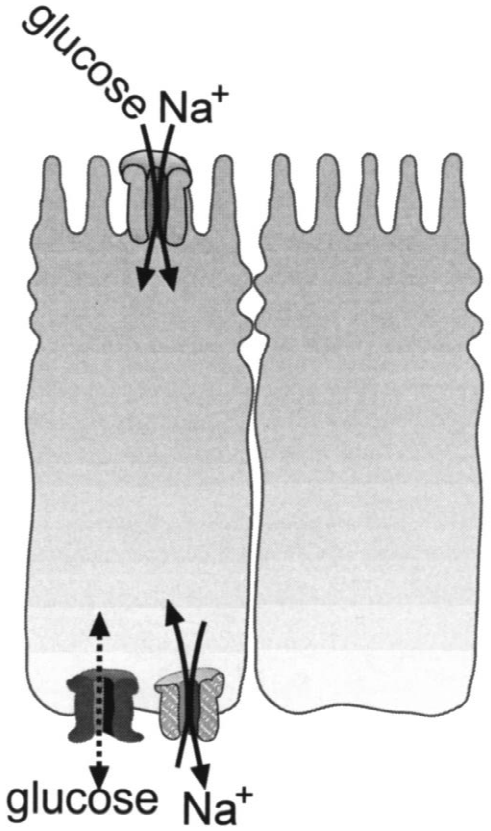

Fig. 1. Transcellular absorption of glucose. Glucose is taken up from the intestinal lumen by the brush border Na^+-glucose cotransporter via SGLT1. Glucose exits the cell via the basolateral glucose exchanger GLUT2. Na^+ traverses the basolateral membrane via ion transport channels, primarily the Na^+–K^+ ATPase.

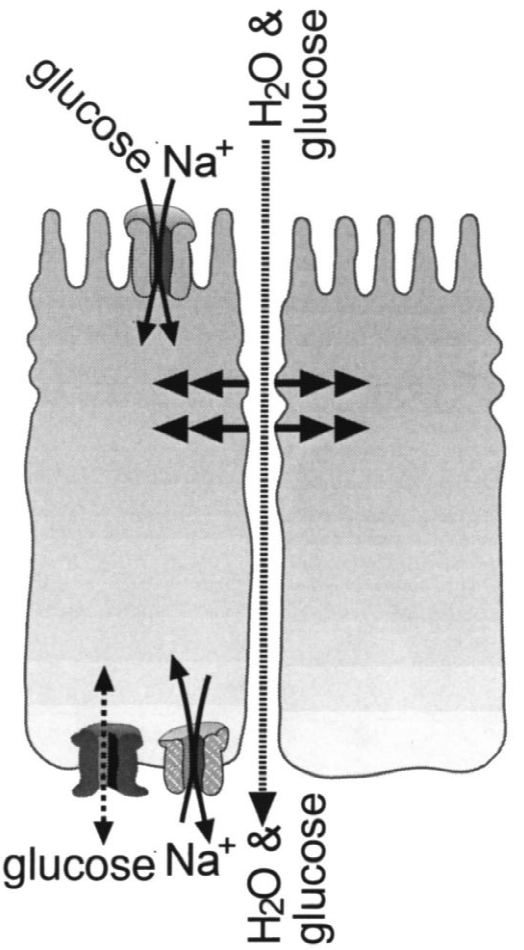

Fig. 2. Coordination of transcellular and paracellular glucose absorption. As glucose is absorbed transcellularly (Fig. 1), the paracellular pathway opens and becomes more permeable to small nutrients (double-headed arrows). The basolateral deposition of glucose and Na^+ results in an osmotic gradient that draws water down its concentration gradient. Small nutrients, e.g. glucose, are carried across the paracellular pathway with the water. The transport of these nutrients is facilitated by opening of the paracellular pathway following transcellular Na^+-glucose cotransport.

physiological tight junction regulation (Fig. 2). They showed that activation of SGLT1, the intestinal Na^+-glucose cotransporter, by luminal glucose resulted in a 91% increase in water absorption, and 193%, 6%, and 100% increases in absorption of creatinine, polyethylene glycol 4000, and inulin, respectively [13]. Parallel electrophysiological analyses showed that addition of glucose or SGLT1-transported glucose analogues resulted in two- to three-fold decreases in transmucosal impedance [12]. This was accompanied by simultaneous increases in capacitance and conductance [12]. The latter indicate increases in membrane surface area and width of intercellular junctions, respectively [12]. Similar effects on impedance, capacitance, and conductance were noted when other Na^+-coupled amino acid transporters were activated [12]. These data suggest that Na^+ cotransport of several luminal nutrients can trigger increases in tight junction permeability and paracellular absorption.

The effects of Na^+-nutrient cotransport were confirmed by subsequent studies showing that regu-

lation of transmucosal resistance did not occur in the absence of extracellular Na^+, despite the presence of glucose [14]. Glucose-induced decreases in transmucosal resistance were also prevented by the SGLT1 inhibitor phloridzin [14]. Decreases in transmucosal resistance were induced by alanine, even in the presence of phloridzin, thus providing further confirmation of the role of Na^+-dependent nutrient transport in regulating mucosal permeability [14]. A separate in situ study used isolated perfused rat intestinal segments to demonstrate the requirement for luminal Na^+-glucose cotransport [15]. Hyperglycemia, induced by intravenous infusion of glucose, did not increase paracellular permeability as assessed by L-glucose absorption [15]. Increases in paracellular permeability induced by luminal glucose (D-glucose) were not prevented by enteric nerve blockade nor were changes in permeability detected within adjacent segments that were not exposed to luminal glucose [15]. Thus, neuroregulation is not necessary for Na^+-glucose cotransport-dependent regulation of intestinal permeability [15].

3. In vivo evidence for regulation of paracellular absorption by Na^+-glucose cotransport

Some studies have shown that paracellular permeability is altered by surgical manipulation of the bowel [16]. This has been suggested as an artifactual explanation for the regulated permeability observed in studies that used isolated intestinal segments. However, the original reports of Na^+-glucose cotransport-dependent regulation of small intestinal permeability included studies in unanesthatized rats [13]. Pappenheimer and Reiss provided evidence that the phenomenon documented in vitro also occurred in vivo. In this portion of the study, unanesthetized rats were starved for 24 h and then given 50–100 mg of creatinine by gastric intubation [13]. The rats were then fed water or water with 20% glucose ad libitum. Since creatinine is only absorbed by the paracellular pathway, is confined to the extracellular space, is not metabolized, and is freely-filtered at the glomerulus, measurements of urinary creatinine recovery can serve as a surrogate for measurements of intestinal creatinine absorption (Fig. 3). Pappenheimer and Reiss showed that rats with access to glucose secreted 53%±2.4% of the exogenous creatinine load within 15 h, while rats fed only water secreted 37%±2.3% of creatinine [13]. Thus, based on this study, it appears that the data obtained from the in vitro studies of isolated mucosa are also representative of in vivo events.

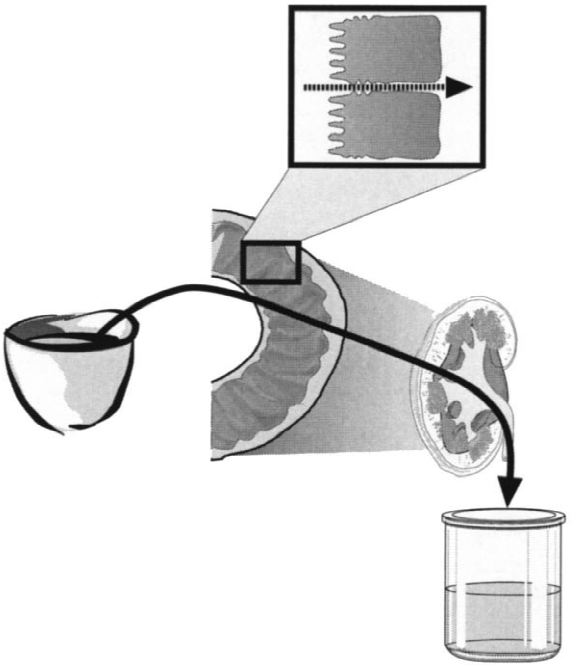

Fig. 3. Use of oral tracers as probes of paracellular permeability in vivo. Biologically inert tracers, such as creatinine, can be ingested orally. These are absorbed in the gastrointestinal tract by the paracellular pathway and ultimately enter the circulation. They are then cleared by glomerular filtration and can be recovered in urine. For tracers that are neither actively absorbed nor actively secreted in the kidney, urinary recovery can be used as an indirect measure of intestinal absorption.

We recently reported the results of a similar study assessing nutrient-induced augmentation of paracellular absorption in humans [17]. Essential criteria for the experimental approach included: (i) complete glucose absorption [18], (ii) the absence of surgical manipulation and anesthesia [16], and (iii) assay of a significant intestinal length [18]. We also sought to use molecular probes of appropriate size to allow detection of changes in paracellular absorption. Both the radius (3.2 Å) and molecular weight (113.12 g) of creatinine are similar to those of glucose (3.7 Å,

180.16 g) and other small nutrients. We therefore concluded that creatinine would be an appropriate probe to measure changes in intestinal paracellular permeability induced by luminal glucose in humans. Human subjects ingested more than 9 g of creatinine during the study [17]. Thus, absorption of even a fraction of the ingested creatinine would be sufficient to render endogenous creatinine insignificant. When ingested with glucose, urinary creatinine was recovered at an average rate of 14.2 ± 0.47 mg/min. In contrast, urinary creatinine was recovered at an average rate of 10.3 ± 1.1 mg/min when ingested with mannitol ($P < 0.01$). As shown in Fig. 4, total urinary creatinine recovery ranged from 51% to 61% of creatinine ingested with glucose (mean $54\% \pm 2\%$). When ingested with mannitol, total urinary creatinine recovery ranged from 25% to 46% of creatinine ingested with glucose (mean $38\% \pm 4\%$, $P < 0.01$). As was true of the group comparisons, creatinine recovery was greater when ingested with glucose than with mannitol for each individual subject. Thus, human intestinal paracellular absorption is increased by the presence of luminal glucose, consistent with in vivo regulation of intestinal permeability by Na^+-glucose cotransport [17].

4. Physiological significance of Na^+-nutrient cotransport-dependent regulation of intestinal paracellular permeability

It was originally proposed that the physiological role of increased tight junction permeability was to increase mass transport of nutrients by the paracellular pathway [13]. This hypothesis was based on a variety of experimental observations. First, in vivo human data show that, at up to 500 mM, intestinal glucose absorption is nearly proportional to the luminal concentration [19–21]. These observations are inconsistent with the reported K_m of 0.11 mM for the cloned intestinal Na^+-glucose cotransporter SGLT1 [22,23]. Additional studies confirmed this discrepancy for humans and showed that rates of glucose ingestion, and absorption, by mice, rabbits, and rats also exceeded the maximal rates of active transmucosal transport by as much as five-fold

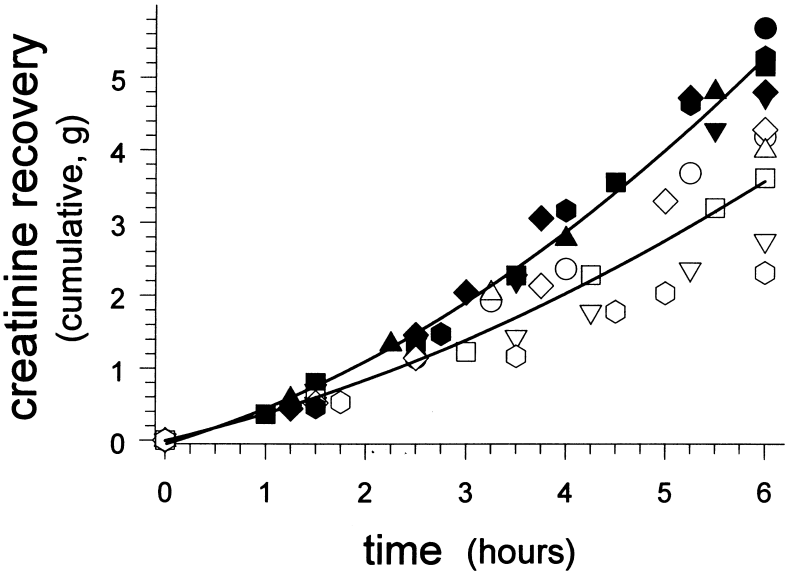

Fig. 4. Urinary creatinine recovery is increased by Na^+-glucose cotransport. Cumulative urinary creatinine recovery is plotted as a function of time. Creatinine was ingested at the beginning of the study and at 5 additional 0.75 h intervals. Urine was collected until 6 h after the initial ingestion. Filled symbols represent creatinine recovery after ingestion in a glucose-containing test solution, while open symbols represent creatinine recovery after ingestion in a mannitol-containing test solution. Unique symbol shapes (filled or open) represent each study subject. Reprinted with permission from Ref. [17].

[24,25]. Thus, Pappenheimer, Madara, and Reiss [11–13] concluded that a second mechanism of glucose absorption must be present and that this second paracellular mechanism of absorption augments transcellular nutrient absorption (Fig. 5).

Second, as described above, the paracellular flux of small nutrient-sized molecules is increased during active Na^+-nutrient cotransport. This is, in part, due to increased paracellular flow of water across tight junctions of increased permeability [13]. The increased water flow and increased permeability result in increased paracellular flow of nutrients by the mechanism termed *solvent drag* [13]. The end result of this process is paracellular amplification of transcellular nutrient absorption. This parallel mechanism of nutrient absorption may explain the fact that the glucose reabsorption in the renal tubule is easily saturated, while absorption in the small intestine appears to exceed transport maxima [25].

Thus, in the proposed model, Na^+-nutrient cotransport leads to activation of an intracellular signaling cascade that causes increased tight junction permeability. Simultaneously, transcellular transport results in the accumulation of Na^+ and nutrients within the subjunctional basolateral space. These osmotically active solutes draw water across the tight junction, resulting in increased water absorption. This water flux also carries nutrients across more permeable tight junctions. This model has been rejected by some on the basis of experimental data and theoretical considerations. Experimental data that do not support the contribution of solvent drag to nutrient absorption come from a variety of sources. Some of these studies used methods that did not effect significant paracellular fluid absorption [18,26–32]. Since the proposed mechanism of solvent drag requires paracellular water absorption, these studies cannot be considered valid refutations of the solvent drag hypothesis [18,26,27]. For example, elegant studies of human intestinal transport performed using multi-lumen catheters and functionally isolated perfused loops have failed to demonstrate increases in paracellular absorption after stimulation of Na^+-glucose cotransport [30,31]. One such study concluded that paracellular absorption of D-glucose in humans is trivial [31]. However, as analyzed in detail elsewhere [18], paracellular water absorption was limited and insufficient to demonstrate paracellular nutrient absorption. Indeed, a follow-up study documented a 129% increase in paracellular absorption of the inert marker L-xylose when Na^+-glucose cotransport was activated [30]. As would be predicted, this study also demonstrated that increased paracellular absorption required water absorption [30]. Similarly, when rats were fed tracers with dry chow, paracellular absorption of glucose was not documented [32]. Again, the likely explanation for the discordant results is the absence of paracellular water absorption [26]. This concern is emphasized by a recent a study using dogs with surgically created Thiry-Vella loops [28]. Fractional water absorption was only 5%±3% and 7%±1% for solutions with 1 mM or 50 mM D-glucose, respectively. Nonetheless, paracellular D-glucose absorption increased from 9%±4% to 24%±10% of the total D-glucose absorbed [28]. While this difference did not reach statistical significance [28], it is certainly worthy of note, as it reflects a 35-fold increase in passive D-glucose absorption (from 0.28 μmol/min to 10 μmol/min) despite similar rates of water absorption (0.235 ml/min vs. 0.329 ml/min). Thus, in contrast to the conclusions reached by the authors [28], this study may have demonstrated increases in paracellular D-glucose absorption that are triggered by transcellular D-glucose absorption.

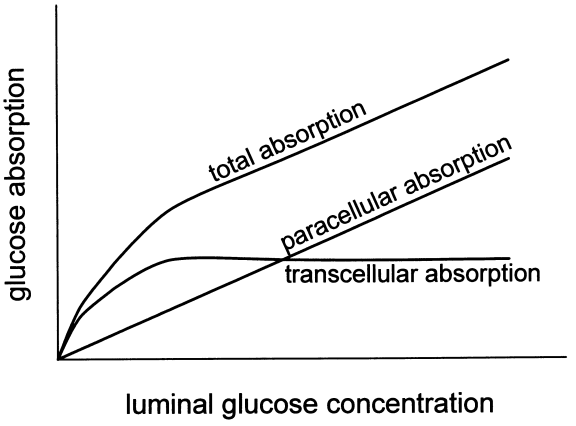

Fig. 5. Augmentation of transcellular glucose absorption by paracellular glucose absorption. Transcellular Na^+-glucose cotransport becomes maximal at low glucose concentrations. In contrast, paracellular glucose absorption increases in direct proportion to the glucose concentration. Thus, total glucose absorption continues to increase well after transcellular absorption achieves V_{max}. Drawn after Refs. [13,39].

Theoretical objections to the proposed model of paracellular solvent drag-mediated nutrient absorption are based largely on the observation that direct measurements of small intestinal luminal glucose concentrations in rats, rabbits, and dogs did not exceed 48 mM [33]. However, these measurements do not consider effects of the intestinal unstirred layer [34–38]. The depth of the unstirred layer has been variably estimated from 35 μm to 600 μm [34,36]. Thus, diffusion through the unstirred layer may be a significant limiting factor in nutrient absorption [34]. Moreover, solute concentrations within the unstirred layer and the intervillus space may greatly exceed those in the lumen [36]. Thus, glucose concentrations within the unstirred layer may significantly exceed reported luminal concentrations of 48 mM. An additional factor not considered is that the brush border, which interfaces with the unstirred layer, is rich in disaccharidases and other digestive enzymes [39]. Thus, it is reasonable to postulate that the action of these enzymes might result in high, up to 500 mM, glucose concentrations at the luminal surface of the mucosa within the unstirred layer and at the entrance to the paracellular pathway.

5. Practical exploitation of Na^+-nutrient cotransport-induced augmentation of paracellular permeability

The central components of the solvent drag hypothesis are: (i) increased permeability of the tight junctions and paracellular pathway to nutrient-sized molecules following apical Na^+-nutrient cotransport, and (ii) increased paracellular water, and nutrient, transport [11–13]. It is well established that intestinal glucose absorption effectively drives water absorption. This is the scientific basis underlying the formulation of various oral rehydration solutions [19,40–42]. Even partial inhibition of apical SGLT1-mediated Na^+-glucose cotransport with phloridzin significantly reduces mucosal water absorption [41]. Although some studies suggest some water absorption may be transcellular and, in fact, mediated by SGLT1 [43,44], the observation remains that tight junctions of leaky epithelia, such as the small intestine, are permeable to water and that the paracellular pathway is a site of significant water absorption [13,41,42,45,46].

In addition to water transport, other substances may traverse the tight junction and paracellular pathway. For example octapeptides composed of D-amino acids appear to be efficiently absorbed from the intestine, despite the absence of specific transcellular carriers [47,48]. Paracellular transport can also be exploited to deliver biologically active peptides and non-peptide drugs [49,50]. For example, absorption of the octapeptide somatostatin analog octreotide was increased 2.2-fold when 20 mM glucose was added to the jejunal delivery solution [50]. Fructose, which is absorbed via the Na^+-independent apical GLUT5 transporter, did not alter jejunal octreotide absorption [50]. A specific role for SGLT1-mediated Na^+-glucose cotransport was confirmed by the observation that addition of the SGLT1 inhibitor phloridzin to the glucose-containing solution decreased octreotide absorption to the level of non-glucose-containing solutions, while addition of phloridzin in the absence of glucose had no effect [50]. Thus, Na^+-glucose cotransport stimulated paracellular transport can be used to enhance intestinal absorption of pharmacologically active peptides. Although these data do not provide analysis of effective pore radii or reflection coefficients and, therefore, cannot verify increased paracellular permeability as a consequence of Na^+-glucose cotransport, they do support the solvent drag model of paracellular nutrient absorption [11–13].

Evidence that Na^+-glucose cotransport increases paracellular absorption of larger proteins has been generated using a rat model of intestinal anaphylaxis [51]. In this model *Trichinella spiralis* protein or ovalbumin was delivered into the duodenum of previously immunized animals and intestinal fluid volume was measured 30 min later as an indicator of net secretion [51]. Inclusion of 40 mM glucose (D-glucose) or the Na^+-cotransported nonmetabolizable β-methyl glucoside analogue with the duodenal antigen challenge resulted in a two-fold increase in fluid secretion [51]. Since glucose increases water absorption, it is possible that these values significantly underestimate the effect of luminal glucose on secretion and antigen absorption. In contrast to D-glucose and a transported analogue, the nontransported L-glucose isomer did not increase secretion.

Furthermore, the addition of phloridzin to the glucose-antigen mixture reduced the effect of glucose to near control levels, while phloridzin had no effect when added in the absence of glucose [51]. Thus, while effective pore radii cannot be determined from these data, they are highly significant since they document increased biological response to paracellularly absorbed luminal proteins during Na^+-glucose cotransport. The rapid time course of these studies effectively excludes the possibility that changes in transcellular antigen transport or processing rates are responsible for the observed effects. It remains to be seen whether this strategy can enhance primary mucosal immunization.

6. Cytoskeletal mechanisms of tight junction regulation

Ultrastructural examination of intestinal mucosae provided additional clues to the mechanistic basis for Na^+-nutrient cotransport-dependent changes in tight junction permeability [11,14,52]. These studies showed two primary changes in the tight junction region of enterocytes with Na^+-glucose cotransport-induced increases in tight junction permeability [11,14,52]. First, dilatations were seen within the tight junction [11]. These dilatations could be seen by transmission electron microscopy as lucent areas within the tight junction and by freeze-fracture electron microscopy as disruptions of the tight junction strands [11]. ZO-1 remained localized to the tight junction and could be identified in association with the dilatations [53]. Thus, these localized disruptions of tight junction structure were considered to represent the anatomic correlate of increased tight junction permeability [11]. Tracer studies showed that a small heme-conjugated undecapeptide accumulated within the tight junction dilatations in glucose-treated mucosa, but never in control (non-glucose-treated) mucosa [52]. Thus, the morphological analyses support the electrophysiological data showing that the tight junction is the site at which Na^+-nutrient cotransport alters permeability.

The second morphological change identified within the tight junction region of glucose-treated mucosa is condensation of the perijunctional cytoskeleton [11,14]. This ring of actin and myosin II encircles apical pole of columnar epithelial cells at the level of the junctional complex. Thus, condensation of the perijunctional cytoskeleton is consistent with contraction and suggests a mechanistic linkage between the cytoskeleton and tight junction permeability.

Studies using inhibitors of actin polymerization have also suggested a functional association between increased tight junction permeability and disassembly of the perijunctional actomyosin ring [54,55]. This has been investigated in greater detail more recently [56–58]. Freeze-fracture electron microscopy has shown that cytochalasin D-treatment induces a marked disruption of the network of tight junction strands, with both decreased numbers of strands and decreased numbers of anastomoses between strands [55,57]. These changes coincide with disruption of the perijunctional actomyosin ring, from a thin belt of microfilaments running parallel to the plasma membrane into irregular dense aggregates of condensed microfilaments, particularly at tricellular junctions [55]. This was interpreted as evidence of cytoskeletal contraction [55]. A subsequent study showed that while depletion of intracellular ATP with 2,4-dinitrophenol did not in and of itself alter tight junction permeability, it did prevent the effects of cytochalasin D on both tight junction permeability and the perijunctional actomyosin ring [59]. This suggests that ATP-dependent perijunctional actomyosin ring contraction may be the mechanism by which cytochalasin D alters epithelial tight junction permeability [59].

The effects of cytochalasin D on tight junction permeability are highly dependent on concentration [58]. Paradoxically, increasing concentrations of cytochalasin D induced progressively decreasing reductions in transepithelial resistance [58]. That is, while 2 μg/ml cytochalasin D induced a 58% drop in transepithelial resistance over 60 min, 20 μg/ml cytochalasin D induced only a 22% drop in transepithelial resistance over the same interval [58]. Fluorescent staining of actin in these cells showed that 2 μg/ml cytochalasin D induced gross breaks and disruptions of the perijunctional actomyosin ring, while exposure to 20 μg/ml cytochalasin D resulted in only focal discontinuities of the perijunctional actomyosin ring [58]. One interpretation of these results could be that low concentrations of cytochalasin D induce localized breaks in the peri-

junctional actomyosin ring and allow subsequent localized contraction of assembled microfilaments and the appearance of gross disruptions. In contrast, higher cytochalasin D concentrations induce a global disruption of the perijunctional microfilament network. Thus, higher concentrations of cytochalasin D may paralyze the perijunctional actomyosin ring, leaving an array of functionally disconnected perijunctional microfilaments with a relatively normal morphology by fluorescence microscopy, while lower concentrations produce only occasional breaks leading to individual foci of cytoskeletal contraction and macroscopic discontinuities.

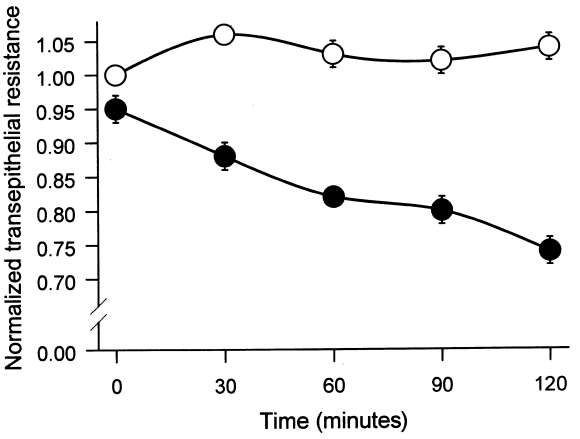

Fig. 6. Na^+-glucose cotransport-dependent tight junction regulation in cultured cell monolayers. SGLT1-transfected Caco-2 monolayers were cultured overnight with 0.5 mM phloridzin. Na^+-glucose cotransport was activated by transfer to HBSS with 25 mM glucose (filled circles) and resulted in progressive decreases in transepithelial resistance. In contrast, transfer to HBSS with 5 mM glucose and 2 mM phloridzin (closed circles) had no effect on transepithelial resistance.

7. The role of myosin light chain phosphorylation in regulation of tight junction permeability

We have recently established an in vitro model of physiological Na^+-glucose cotransport-dependent tight junction regulation [60]. Since available polarized intestinal epithelial cell lines that form monolayers with significant tight junction assembly express only low levels of the Na^+-glucose cotransporter SGLT1, we used a transfection approach. Caco-2 cells were selected for these studies since they are a well-characterized model cell line that develops an absorptive enterocyte phenotype [61,62]. The BBe subclone of Caco-2, which was selected for expression of a well-developed brush border [63], was used. We developed a clone of Caco-2 BBe that was stably transfected with SGLT1 [64]. Kinetic analyses showed that SGLT1 expressed in these cells had a K_m of 0.31 mM, a K_{Na} 43 meq/l, and a Hill coefficient of 1.96 [64]. Each of these values is comparable to that of native intestinal SGLT1. In differentiated monolayers, SGLT1 protein was apically polarized and was able to initiate vectorial Na^+ and glucose transport comparable to that observed in natural epithelia [64].

Using this transfected cell model we have shown that monolayers of these cells exhibit reversible regulation of transepithelial resistance following activation or inhibition of SGLT1 [60]. For example, activation of SGLT1 induces an approximately 30% decrease in transepithelial resistance (Fig. 6). Assays using modified Üssing chambers showed that the decrease in transepithelial resistance after SGLT1 activation is accompanied by increases in transepithelial permeability to mannitol, but not to inulin [60]. These data suggest that the SGLT1-dependent increase in permeability is due to regulation of the tight junction and paracellular pathway. Furthermore, since mannitol but not inulin flux was affected, the change is size selective, consistent with increased permeability to small nutrient-sized molecules but not to larger substances [60].

As described above, ultrastructurally evident condensation of the perijunctional actomyosin ring occurs in native mucosa after activation of SGLT1 [11]. We considered that this might represent actomyosin contraction. Thus, we evaluated a biochemical marker of actomyosin contraction, phosphorylation of the myosin II regulatory light chain. Myosin light chain phosphorylation at serine-19 by myosin light chain kinase or, possibly, by other kinases, activates actomyosin contraction. Consistent with the hypothesis that contraction of the perijunctional actomyosin ring effects tight junction regulation, we showed that activation of SGLT1 leads to increased ^{32}P incorporation into myosin light chain

[60] and that this ^{32}P incorporation occurs at ser-19 [65]. We have recently extended these observations using 2-dimensional urea-glycerol SDS–PAGE electrophoresis [65,66]. These gels resolve myosin light chain by phosphorylation state and show a clear shift from non-phosphorylated to mono-phosphorylated myosin light chain with activation of SGLT1 (Fig. 7). In order to determine whether these increases in myosin light chain phosphorylation were mechanistically linked to changes in tight junction permeability we evaluated the effects of the related myosin light chain kinase inhibitors ML-7 and ML-9 [60]. In monolayers of SGLT1-expressing Caco-2 cells inhibition of myosin light chain kinase decreased myosin light chain phosphorylation and decreased tight junction permeability while SGLT1 was active [60]. Similarly, in isolated native mucosa inhibition of myosin light chain kinase prevented the effects of SGLT1 activation [60]. Thus, we concluded that myosin light chain phosphorylation is a critical intermediate in SGLT1-dependent regulation of intestinal epithelial tight junctions.

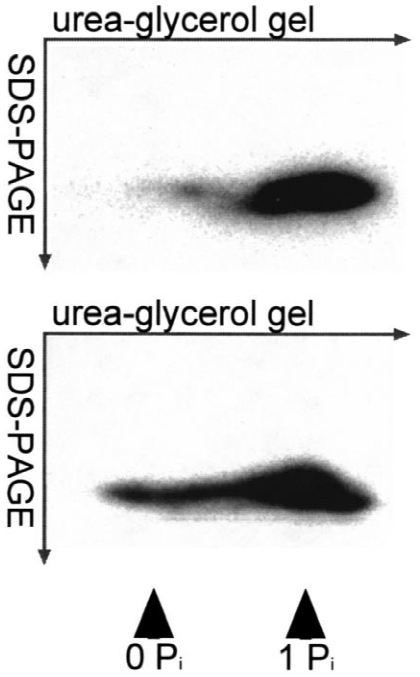

Fig. 7. Intestinal epithelial myosin light chain phosphorylation increases following activation of Na^+-glucose cotransport. Myosin light chain phosphorylation was assessed in monolayers with active or inhibited Na^+-glucose cotransport. Cell lysates were separated by two-dimensional urea glycerol PAGE – SDS–PAGE, transferred, and immunoblotted with anti-myosin light chain as described [65]. Spots corresponding to monophosphorylated myosin light chain (1 Pi) and nonphosphorylated myosin light chain (0 Pi) were detected in lysates from monolayers with inhibited Na^+-glucose cotransport (bottom panel), but only monophosphorylated myosin light chain was detected in lysates from monolayers with active Na^+-glucose cotransport (top panel).

A role for myosin light chain phosphorylation in tight junction regulation has been suggested in other intestinal epithelial models. For example, in intestinal epithelial cells, myosin light chain phosphorylation occurs after colonization with enteropathogenic *E. coli* [67]. Reorganization of the apical cytoskeleton and increased tight junction permeability occur in parallel with this myosin light chain phosphorylation and all three are prevented by inhibitors of myosin light chain kinase [67]. Similarly, the loss of barrier function induced by enterohemorrhagic *E. coli* infection is partially prevented by myosin light chain kinase inhibitors [68]. While these observations represent important examples of the regulation of epithelial tight junctions, they differ significantly from physiological SGLT1-dependent tight junction regulation in that the latter is more rapid, reversible, and tightly regulated [60]. Furthermore, the barrier defects induced by bacterial infection are massive and generalized, while SGLT1-dependent increases in tight junction permeability are limited to small nutrient sized molecules [60].

The role of myosin light chain phosphorylation in tight junction regulation has been characterized further in an elegant study using a transfected Madin-Darby canine kidney (MDCK) cell line model. These cells were transfected with a truncated myosin light chain kinase gene construct [69] that lacks the inhibitory domain necessary for calmodulin dependence [70,71]. Thus, the truncated myosin light chain kinase expressed in the transfected cells is constitutively active. When the transfected cells were grown as monolayers they developed transepithelial resistance that was less than 10% of that developed in monolayers of control cells [69]. These studies may be the strongest data available to demonstrate a direct effect of myosin light chain phosphorylation on epithelial tight junction permeability. However, since the truncated myosin light chain kinase was

expressed continuously, this model does not allow further dissection of the effects of myosin light chain phosphorylation. For example, it is not possible to use this model to differentiate effects of myosin light chain phosphorylation on tight junction assembly from effects of myosin light chain phosphorylation on permeability of the assembled tight junction.

We have recently developed a tetracycline-dependent regulated expression system in the Caco-2 intestinal epithelial cell line. We have used this model to express a truncated myosin light chain kinase construct similar to that used in the studies of MDCK cells. Removal of doxycycline from the culture media induces a marked increase in myosin light chain kinase activity and myosin light chain phosphorylation within 18 h [72]. Coincident with these increases in myosin light chain phosphorylation we observe a 20–30% decrease in transepithelial resistance and a comparable increase in transepithelial mannitol flux [72]. These data confirm that myosin light chain phosphorylation can directly regulate the permeability of tight junctions in assembled monolayers. Further evidence for the specific role of myosin light chain phosphorylation in these events comes from the observation that myosin light chain kinase inhibitors rapidly increase the transepithelial resistance of monolayers expressing the truncated myosin light chain kinase [72].

We have identified the intestinal epithelial myosin light chain kinase as a 215 kD phosphoprotein [65]. Based on Mr and immunoreactivity this intestinal epithelial myosin light chain kinase appears to be related to myosin light chain kinase cloned from human endothelial cells [73]. We have shown that the intestinal epithelial myosin light chain kinase is responsible for more than 90% of Caco-2 cell myosin light chain kinase activity [65]. Phosphorylation of the endothelial myosin light chain kinase increases after PKA activation and results in decreased kinase activity [73]. We have observed similar increases in myosin light chain kinase phosphorylation and subsequent decreases in myosin light chain phosphorylation after PKC activation in Caco-2 intestinal epithelial cell monolayers [65]. The decreases in myosin light chain phosphorylation are associated with decreases in tight junction permeability [65]. Thus, it appears that at least one mechanism by which PKC regulates intestinal epithelial tight junctions may involve the inhibition of myosin light chain kinase [65].

Phosphorylation of myosin light chain has been implicated in the regulation of endothelial paracellular permeability following stimulation with thrombin, histamine, or cyclic AMP [74,75]. These agonists promote actomyosin contraction and endothelial cell retraction [74]. This leads to the development of spaces between endothelial cells and increased paracellular permeability to macromolecules. Detailed analyses have shown that endothelial myosin light chain phosphorylation occurs shortly after an increase in intracellular Ca^{2+}, but prior to increases in cytoskeletal tension [75]. This temporal sequence supports the hypothesis that elevation of intracellular Ca^{2+} leads to activation of myosin light chain kinase which, in turn, results in myosin light chain phosphorylation and subsequent actomyosin contraction.

The in vivo trigger for endothelial contraction, inflammation, is typically a prolonged process whose pathogenesis is facilitated by macromolecular intercellular gaps. In contrast, the development of macromolecular gaps in the intestine would severely compromise the mucosal barrier. Thus, the observation that Na^{+}-glucose cotransport increases permeability to small nutrient-sized molecules while that of larger molecules remains unchanged is consistent with the physiological context. Furthermore, the in vivo stimulus for tight junction regulation in intestinal epithelium, the presence of luminal nutrients, is an event that repeats itself relatively frequently over short temporal intervals. Thus, while intermediate events in the physiologic regulation of intestinal epithelial permeability may be similar to those for endothelial contraction, both the initial stimuli and ultimate effects are distinctly different.

8. Molecular analysis of tight junction structure

While the functional association between the tight junction and the perijunctional actomyosin ring has been known for over twenty years [54], the molecular interactions necessary for that association are largely unknown. Ultrastructural studies have shown some microfilaments within the perijunctional actomyosin ring are intimately associated with the tight junction, although the majority are associated with

the adherens junction, [76]. As discussed above, activation of myosin light chain kinase and resulting contraction of the perijunctional actomyosin ring increases the permeability of epithelial and endothelial tight junctions [60,65,69,75,77]. It is also clear that the perijunctional actomyosin ring can be regulated by other signaling pathways. For example, rho kinase can phosphorylate myosin light chain directly and can also effect increased myosin light chain phosphorylation by inhibiting myosin light chain phosphatase [78,79]. Manipulation of rho activity, either through the use of bacterial toxins (see review by Hopkins et al. in this issue) or mutant rho protein expression, alters tight junction permeability [80,81]. Thus, manipulation of myosin II and perijunctional actomyosin ring contraction by a broad array of agents results in altered tight junction permeability. Nonetheless, the molecular mechanisms by which cytoskeletal-tight junction interactions are developed and maintained are largely unknown.

One candidate for linking the perijunctional cytoskeleton to the tight junction is ZO-1. ZO-1 is a ~220 kD peripheral membrane protein whose localization is restricted to the cytoplasmic face of the tight junction [82–84]. The amino half of ZO-1 contains an SH3 domain and a region homologous to guanylate kinases, although actual guanylate kinase activity has not been demonstrated [85–87]. Three PDZ domains are also located within the amino half of ZO-1 [88]. The PDZ domains, which appear to be specialized regions for protein–protein interactions, may be the sites within this region that mediate binding between ZO-1 and occludin and between ZO-1 and ZO-2 [89,90]. Separate studies have shown that the amino terminal portion ZO-1 associates with catenins in non-epithelial cells [91]. A chimera of the transmembrane portion of connexin32 and the cytoplasmic tail of occludin (containing the ZO-1 binding domain) was accurately delivered to the tight junction [92]. One caveat to the studies with this particular chimera is the observation that ZO-1 associates with connexin43 in cardiac myocytes [93], raising the possibility that the localization of the chimera may not be entirely dependent on ZO-1 binding to the cytoplasmic tail of occludin. Nonetheless, the data suggest that the interaction between ZO-1 and the cytoplasmic tail of occludin is sufficient for targeting occludin to the tight junction [92]. These data suggest that ZO-1 forms a subjunctional scaffold similar to that described for other MAGUK (membrane-associated guanylate kinase) proteins [86,92]. The carboxy terminal portion of ZO-1 contains a proline-rich domain that has been shown to bind to actin in cell free assays and to accumulate in actin structures when expressed in cells [89,91]. ZO-1 is also found in large fodrin-containing cytoskeletal aggregates after ATP depletion of MDCK cells [94]. Thus, ZO-1 can interact with the actin cytoskeleton, either directly through specific interactions with actin or indirectly via actin-binding proteins.

Given that ZO-1 can interact with both the perijunctional actomyosin ring and occludin and that this interaction may be necessary for delivery of occludin to the tight junction, it is tempting to speculate that ZO-1 is a signal transducer. Consistent with this interaction, some have found that disruption of the perijunctional actomyosin ring results in loss of occludin from tight junctions [95]. Thus, actomyosin contraction could alter ZO-1 and trigger modifications of occludin function. However, the role of occludin in forming the tight junction barrier remains unknown. One study has shown that a peptide corresponding to an extracellular domain of occludin disrupts the tight junction barrier [96]. This suggests that occludin may possess tight junction sealing properties. In contrast, tight junction strands have been observed after removal of occludin [95]. A network of tight junction strands is formed in fibroblasts after transfection of claudin, but not occludin [97]. In these transfected fibroblasts, claudin expression results in the recruitment of occludin to the network of tight junction strands [97], suggesting a physical interaction between the two. Finally, treatment of MDCK cell monolayers with the claudin-3 and claudin-4 binding enterotoxin of *Clostridium perfringens* results in removal of these claudins from the tight junction, dissolution of tight junction strands, and loss of barrier function [98]. Thus, the claudin family of proteins may simultaneously regulate barrier function and, as suggested by paracellin-1 deficiency, permeability to specific molecules [3]. While indirect evidence suggests that a multiprotein complex linking actin to ZO-1, ZO-1 to occludin, and occludin to claudin may

mediate cytoskeletal effects on the tight junction, no specific data on the underlying molecular interactions are available and other potentially critical molecular interactions continue to be identified. For example, it has recently been reported that the tight junction protein cingulin interacts directly with myosin heavy chain [99]. As more information regarding tight junction structure and the functional roles of tight junction components becomes available, it may be possible to define the mechanisms of cytoskeletal tight junction regulation in greater detail.

9. Proximal signals linking Na^+-glucose cotransport to myosin light chain phosphorylation

We have used our cultured cell model of Na^+-glucose cotransport-dependent tight junction regulation to characterize early events in the signaling pathway linking these events [100]. Cell swelling is a well-recognized consequence of SGLT1-mediated Na^+-glucose cotransport [101]. This swelling triggers a regulatory volume decrease response that normalizes cell volume within minutes [101]. We reasoned that signaling pathways activated by Na^+-glucose cotransport might trigger both regulatory volume decrease and tight junction regulation. In one model of regulatory volume decrease after hypotonic swelling of enterocytes, cytoplasmic alkalinization is necessary for the volume decrease to occur [102]. This alkalinization appears to require activation of Na^+–H^+ exchange, since both alkalinization and regulatory volume decrease can be prevented by Na^+–H^+ exchange inhibitors [102]. Thus, we explored the role of Na^+–H^+ exchange in Na^+-glucose cotransport-dependent tight junction regulation.

We found that inhibition of Na^+–H^+ exchange caused marked increases in transepithelial resistance [100]. We used RT-PCR to demonstrate that three intestinal Na^+–H^+ exchanger isoforms, NHE1, NHE2, and NHE3, were expressed in our Caco-2 cell model. Pharmacological evaluation of several Na^+–H^+ exchange inhibitors showed that the brush border NHE3 isoform, but not NHE1 or NHE2, was critical for the effects of these inhibitors on transepithelial resistance. NHE3 inhibition also caused decreased phosphorylation of myosin light chain, suggesting that activation of NHE3 is an intermediate between Na^+-glucose cotransport and increased myosin light chain phosphorylation. To better define this putative signaling pathway we verified that NHE3 activation occurs after initiation of Na^+-glucose cotransport and results in cytoplasmic alkalinization [103]. Moreover, we demonstrated that cytoplasmic alkalinization with NH_4Cl could bypass the effects of NHE3 inhibition [100]. Thus, we concluded that NHE3 activation is a critical component of the signaling pathway for Na^+-glucose cotransport-dependent tight junction regulation. While the mechanisms by which Na^+-glucose cotransport triggers further Na^+ absorption, via NHE3, are unknown, initiation of Na^+-glucose cotransport may cause the enterocyte to shift into an active absorptive state. Since the tight junction regulation that follows Na^+-glucose cotransport is thought to also enhance absorption, overlap in these signaling pathways seems plausible.

10. A unified model of Na^+-nutrient cotransport-dependent tight junction regulation

Taken as a whole, the studies described in this article provide strong evidence that the permeability of intestinal tight junctions can be regulated by activation of Na^+-glucose cotransport. This regulatory pathway appears to trigger increased paracellular flow of water and small nutrients in vivo and in isolated mammalian mucosa. Further evidence suggests that increased tight junction permeability occurring after Na^+-glucose cotransport may also allow increased transport of drugs and antigens.

Recent studies have characterized an in vitro cell culture model of Na^+-glucose cotransport-dependent tight junction regulation. These studies have identified activation of the brush border Na^+–H^+ exchanger NHE3 as an early event in Na^+-glucose cotransport-dependent tight junction regulation. These studies have also characterized activation of myosin light chain kinase, myosin light chain phosphorylation, and, ultimately, contraction of the perijunctional actomyosin ring as essential to tight junction regulation. Thus, by linking these critical events, an ordered signaling pathway between Na^+-glucose cotransport and tight junction regulation can

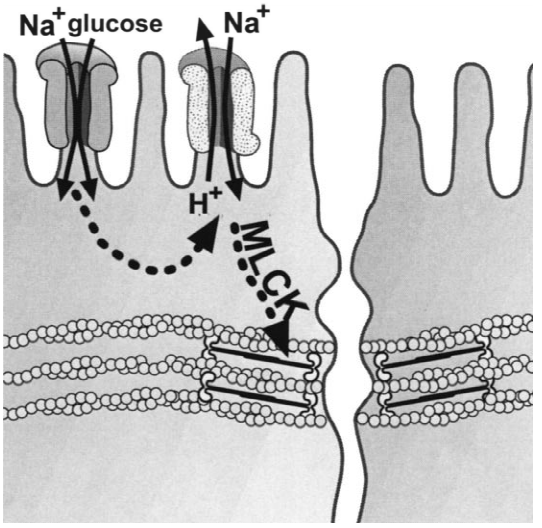

Fig. 8. Proposed signaling pathway for Na^+-glucose cotransport-dependent tight junction regulation. The initiation of Na^+-glucose cotransport leads to activation of the brush border Na^+–H^+ exchanger NHE3. This leads to increased myosin light chain kinase activity, myosin light chain phosphorylation, and contraction of the perijunctional actomyosin ring. Actomyosin contraction triggers structural tight junction modifications that result in increased permeability.

be proposed (Fig. 8). No data are currently available to define the structural events that must occur at the tight junction as a consequence of perijunctional actomyosin ring contraction. However, given the recent identification of claudins and the potential associations between claudins, occludin, ZO-1, and actomyosin filaments, it is likely that such information will be forthcoming.

As the signaling pathway and structural events that cause tight junction regulation are defined, specific strategies for manipulating intestinal permeability will become available. Such strategies may be useful in treatment of diseases with altered of mucosal permeability as well as in targeted delivery of therapeutic agents.

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