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Small Synthetic Peptides Homologous to Segments of the First External Loop of Occludin Impair Tight Junction Resealing

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Abstract. This study shows that resealing of opened tight junctions (TJs) is impaired by interaction with oligopeptides homologous to the external domain of chick occludin. The experiments were carried out with confluent A6 cell monolayers grown on collagen supports under stable transepithelial electrical resistance (TER). The monolayers were bathed on the apical side with a 75 mM KCl solution and on the basolateral side by NaCl-Ringer's solution. TJ opening was induced by basolateral Ca^{2+} removal and was characterized by a marked drop of TER. The reintroduction of Ca^{2+} triggered junction resealing as indicated by an elevation of TER to control values. Custom-made peptides SNYYGSGLSY (corresponding to the residues 100 to 109) and SNYYGSGLS (residues 100 to 108), homologous to segments of the first external loop of chick occludin molecule, impaired junction resealing when the peptides were included in the apical bathing fluid (concentrations in the range of 0.5 to 1.5 mg/ml). Peptide removal from the apical solution usually triggered a slow recovery of TER, indicating a slow recovery of the TJ seal. Changes in localization of ZO-1, a cytoplasmic protein that underlies the membrane at the TJs, were evaluated immunocytochemically following Ca^{2+} removal and reintroduction. The presence or absence of the oligopeptides showed no influence on the pattern of change of ZO-1 localization. These observations support the hypothesis that the TJ seal results from the interaction of specific homologous segments of occludin on the surface of adjacent cells. Additionally, our results show that small peptides homologous to segments of the occludin first external loop can be used as specific reagents to manipulate the permeability of tight junctions.

Key words: Tight junction — Occludin — ZO-1 — Transepithelial resistance

Introduction

The tight junction (TJ), the most apical component of the mammalian junctional complex in epithelial cells, forms a diffusion barrier that regulates the flux of hydrophilic molecules through the paracellular pathway (Grumbiner, 1987). Structurally, the TJ forms a continuous network of parallel, interconnected intramembrane strands, presumably rows of transmembrane proteins with associated cytoplasmic components (Staehelin, 1973; Lane et al., 1992; Anderson & Van Itallie, 1995; Denker & Nigam, 1998). Although the function of TJ has been well characterized little is known about its molecular composition (Kachar & Reese, 1982; Pinto da Silva & Kachar, 1982; Gumbiner, 1987; Simons 1990) and mechanism of formation (Gumbiner, 1987; Kachar & Pinto da Silva, 1981; Jaeger et al., 1997; Denker & Nigam, 1998). However, important TJ-associated proteins, including ZO-1 (Stevenson et al., 1986), cingulin (Citi et al., 1988), ZO-2 (Gumbiner et al., 1991) and 7H6 (Zhong et al., 1993) have been identified. A transmembrane protein, occludin, has been identified (Furuse et al., 1993) and appears to have characteristics of a TJ strand component (Furuse et al., 1994; Balda et al., 1996; McCarthy et al., 1996; Hirase et al., 1997; Van Itallie and Anderson, 1997). In addition, two other transmembrane proteins, claudin-1 and claudin-2 that are targeted and incorporated in the TJ strands were identified (Furuse et al., 1998). These find-

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ings suggest that multiple integral membrane proteins with four putative transmembrane domains, occludin and claudins, constitute TJ strands.

Occludin, a ~65 kDa protein, is presumed to be one of the transmembrane components of the TJ of both epithelial and endothelial cells (Saitou et al., 1998). The amino acid sequence of occludin has been determined and the analyses of the sequence predict four putative membrane-spanning segments, two 44-amino acid extracellular loops and two intracellular domains (Furuse et al., 1993). However, the nature of the interactions between occludin molecules to form the intramembrane tight junction strands and to seal the intercellular space remains to be determined.

The transmembrane topology and the amino acid sequence of the occludin are highly conserved (90%) across different species (Ando-Akatsuka et al., 1996). The least conserved region of the amino acid sequence is the first extracellular loop (53–78%); it lacks charged residues and has a high percentage of tyrosine and glycine. This unusual composition has led to the speculation that this segment of the occludin might be involved in sealing the intercellular space (Ando-Akatsuka et al., 1996).

Van Itallie and Anderson (1997) observed that the first putative extracellular loop of occludin is exposed on the cell surface. Moreover, using a suspended cell assay, they reported that a synthetic peptide containing the amino acid sequence of this loop inhibited cell adhesion.

We have now used a different competitive binding inhibition assay to test whether the sealing of the paracellular pathway by TJs in an epithelial monolayer depends on a homologous binding between occludin molecules. Small synthetic peptides (8 to 10 residues) homologous to segments of the first and second extracellular loops of chick occludin were assayed. We tested the ability of these peptides to inhibit the resealing of TJs immediately following the junctional disruption by a transient removal of basolateral Ca^{2+} (fast Ca^{2+} switch assay).

Materials and Methods

Cell Culture

A6 cells (CCL 102) obtained from American Type Culture Collection (Rockville, MD) were grown at room temperature in CL2-Amphibian medium (NIH-Media Section, Bethesda, MD), 10% fetal bovine serum (Sigma Chemical, St. Louis, MO) and 2 mM glutamine Pen-Strepto mM (Sigma). Cells at confluence were harvested with 0.25% trypsin solution (Sigma). The cell suspensions were plated, at a density adequate to reach confluence in several hours, on 6-well plates with Transwell cell culture inserts (Transwell COL, collagen-treated filters containing a mixture of collagen types I and III −4.7 cm^2 growth area and 0.4 μm pore size; Costar, Cambridge, MA). Confluent monolayers reached maximum TER averaging 1406.5 ± 106.9 Ω cm^2 ($n = 19$) around day 14. Monolayers 7 to 17 days old were used in the experiments.

Electrical Measurements

Plastic rings of 20 mm diameter were glued with ethylcyanoacrylate adhesive (Pronto CA8, 3M or Super Bonder, Loctite) to the opposite side of the support filters where the cells were attached. The monolayer fragment framed by the plastic ring was excised and immersed in Ringer solution. Then, it was mounted in a modified Ussing's chamber (Castro, Sesso & Lacaz-Vieira, 1993) exposing an area of 0.5 cm^2. Hemichambers with a recessed rim filled with high viscosity silicone grease (Dow Corning High Vacuum Grease) prevented tissue edge damage (Lacaz-Vieira, 1986). Each chamber compartment was perfused with a continuous flow of solution (up to 25 ml/min) driven by gravity. To optimize the perfusion the incoming fluid was directed towards the surface of the monolayer. Each compartment was drained through a spillway open to the atmosphere, so that the pressure inside each compartment was kept constant at the atmospheric level. Rapid solution changes were obtained without interruption of voltage-clamping by switching the inlet tubings at their connections with the chamber.

A conventional voltage-clamp (DVC-1000, World Precision Instruments) with continuous feedback was used. Saturated calomel half-cells with 3 M KCl agar bridges were used to monitor the electrical potential difference across the monolayer. Current was passed through Ag-AgCl electrodes and 3 M KCl agar bridges to give a uniform current density across the monolayer. The monolayers were kept short-circuited throughout the experiment. The clamping current was continuously recorded with a strip-chart recorder and digitized through an analog-to-digital converter at a frequency of 10 Hz (Digidata 1200 and Axotape 2.0, Axon Instruments) and recorded in a computer for further processing. A digital Gaussin Filter (Colquhoun & Sigworth, 1983) was used to remove high frequency noise of the baseline of all records used in the figures. This digital filter forms output values y_i from input values x_i by performing the arithmetic mean of three consecutive current values, so that

$$y_i = \left(\sum_{i-1}^{i+1} x_i \right) / 3.$$

Solutions and Drugs

The solutions used to bathe the basolateral side of the monolayers were (in mM): NaCl-Ringer: NaCl 115, $KHCO_3$ 2.5, $CaCl_2$ 1.0; Ca^{2+}-free NaCl-Ringer: NaCl 115, $KHCO_3$ 2.5. These Ringer solutions had pH 8.2 after aeration. The apical bathing fluid was a simple salt solution of KCl 75 mM, non-buffered, or buffered with 10 mM HEPES, pH adjusted with KOH.

Fast Ca^{2+} Switch Assay

The TJs were opened by removal of basolateral Ca^{2+}. Resealing of the TJs was induced by the reintroduction of Ca^{2+} in the basolateral fluid. The effect of the peptides on the TJs was tested by introducing the peptides into the apical bathing fluid before Ca^{2+} removal. We expected that Ca^{2+} interaction with the open TJs would affect their resealing.

Peptides

Synthesized peptides, homologous to segments of the external loops of chick occludin (Furuse et al., 1993), were obtained from the Protein Chemistry Laboratory of the Medical School of the University of Pennsylvania [supported by core grants of the Diabetes and Cancer Centers (DK-19525 and CA-16520)], and from Princeton Biomolecules, Columbus, OH. The peptides tested were: SNYYGSGLSY = [NH_2]-Ser-Asn-Tyr-Tyr-Gly-Ser-Gly-Leu-Ser-Tyr-[COOH] corresponds to the sequence of residues 100 to 109 of the first external loop of the chick occludin molecule.

YYGSGLSY = [NH_2]-Tyr-Tyr-Gly-Ser-Gly-Leu-Ser-Tyr-[COOH] corresponds to the sequence of residues 102 to 109 of the first external loop of the chick occludin molecule.

SNYYGSGLS = [NH_2]-Ser-Asn-Tyr-Tyr-Gly-Ser-Gly-Leu-Ser-[COOH] corresponds to the sequence of residues 100 to 108 of the first external loop of the chick occludin molecule.

STYLNQYIYN = [NH_2]-Ser-Thr-Tyr-Leu-Asn-Gln-Tyr-Ile-Tyr-Asn-[COOH] corresponds to the sequence of residues 210 to 219 of the second external loop of the chick occludin molecule.

The selection for size and specific amino acid sequences was made according to the following criteria: (i) conserved nature of the sequence across species; (ii) Abundance of Tyr and Gly. Because of the abundance of Tyr and Gly in the first extracellular loop and the highly conserved nature of this domain it has been suggested that this extracellular domain may be involved in the cell-cell interaction (Ando-Akatsuka et al., 1996); (iii) Selection of a region without cysteine residues to prevent inter- and intramolecular disulfide bonds. Presence of cysteines in the sequence would have required us to covalently add a protection group (such as acetamidomethyl) to prevent disulfide bond formation (Wong & Gumbiner, 1997); and (iv) We wanted to keep the peptide sequence short anticipating a potential future use for transiently opening tight junctions for drug delivery purposes.

Statistics

The results are presented as the mean ± SE. Comparisons were carried out using a Student's paired *t*-test (Neter & Wasserman, 1974).

ZO-1 Immunolocalization

ZO-1 and occludin have been shown to co-localize at the cell borders during a Ca^{++} switch assay (Contreras et al., 1997). For this reason we chose to immunolocalize only ZO-1 for monitoring TJ proteins during the fast Ca^{++} switch assay.

Plastic rings of 20 mm diameter were glued with ethylcyanoacrylate adhesive (Pronto CA8, 3M or Super Bonder, Loctite) to the opposite side of the support filters where the cells were attached. The monolayer fragment framed by the plastic ring was excised and fixed with 1% paraformaldehyde in phosphate buffer saline (PBS), pH 7.4, for 10 min at room temperature. After fixation, the cells were permeabilized with 0.5% Triton X-100 in PBS. The affinity-purified rabbit antibody against ZO-1 (Zymed Lab, San Francisco, CA, 1 mg/ml in PBS, with 0.05% NaN_3, pH 7.4) was applied and then visualized with fluorescein-conjugated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL). Both primary and secondary antibody incubations (at 1:100 dilution) were done for 45 min at room temperature. After labeling, filters were sandwiched between a glass slide and a coverslip in mounting media. The observations and photographic recording were performed under a Zeiss Jenalumar fluorescence microscope equipped with a 100× objective.

Labeling with antibodies was done in A6 cell monolayers submitted to a fast Ca^{2+}-switch assay with the peptide SNYYGSGLSY (0.5 mg/ml) in the apical bathing solution. Samples were fixed before Ca^{2+} removal, after Ca^{2+} removal, and after Ca^{2+} reintroduction.

Abbreviations and Conventions

TER: transepithelial electrical resistance, in Ω cm^2. TER was calculated from the deflections of the clamping current induced by ±5 mV shifts of the clamping potential (300 msec duration at 15 sec intervals). TER = $\Delta V/\Delta I$, where ΔV and ΔI are the changes in the electrical potential difference across the cell monolayer and clamping current, respectively.

G: transmembrane electrical conductance, in Siemens cm^{-2}, where G = 1/TER.

I: Clamping current, in μA cm^{-2}. Positive current corresponds to the transport of positive charges across the monolayer from the apical to the basolateral solution.

V: Electrical potential difference across the monolayer, in mV, i.e., between the potential of the apical solution and that of the basolateral solution.

Results

TJs of Control Monolayers Fully Recover After a Fast Ca^{2+} Switch

The electrophysiological behavior of a control monolayer submitted to a fast Ca^{2+}-switch assay that consisted in the withdrawal and reintroduction of basolateral Ca^{2+}, is shown in Fig. 1 and Table 1. Usually, after a lag period of 30 sec to 3 min after Ca^{2+} withdrawal from the basolateral solution a marked drop of TER takes place, reflecting the opening of the TJs. This process is almost immediately blocked by the reintroduction of Ca^{2+} into the basolateral bathing fluid, causing the resealing of the TJs. The resealing is characterized by a progressive rise of TER, which may take several minutes to complete. At the end of this process TER reaches values not significantly different from those observed before Ca^{2+} removal. The length of the recovery period somehow depends on the degree of TER reduction, which in turn is proportional to the time the monolayer is exposed to a Ca^{2+}-free basolateral fluid. To standardize the degree of TJ opening, so that the experiments were comparable, the process of TJ opening was terminated as soon as TER reached values of the order of 100 Ω cm^2.

Occludin Peptides in the Apical Solution Impair Junction Resealing

Before Ca^{2+} withdrawal from the basolateral medium, the flow of KCl solution through the apical compartment was stopped and the apical solution was replaced by a KCl solution (75 mM) (unbuffered or buffered with HEPES 10 mM at pH 7.0) containing the peptide to be

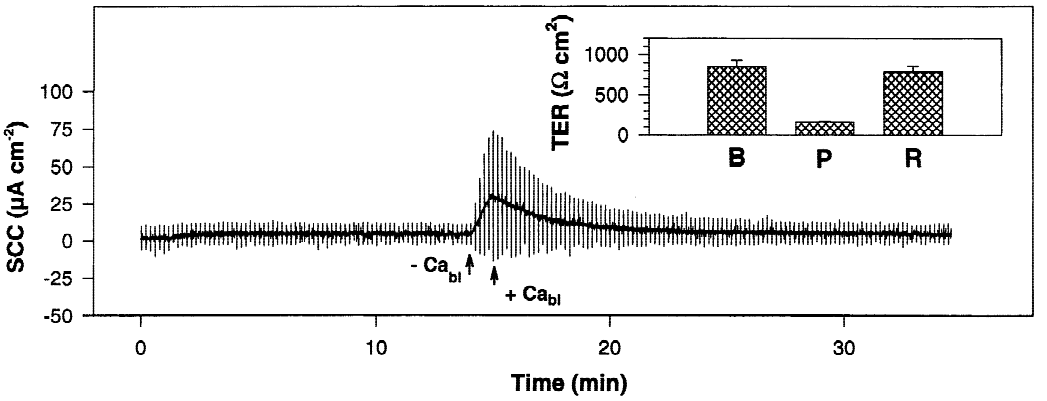

Fig. 1. Representative experiment of a fast Ca^{2+}-switch assay in a control (nontreated) monolayer, showing the effect of basolateral Ca^{2+} withdrawal ($-Ca^{2+}$) on TER and its reversibility induced by Ca^{2+} reintroduction ($+Ca^{2+}$). The monolayer was bathed by KCl 75 mM on the apical side and by NaCl-Ringer's solution on the basolateral side. The vertical bars are deflections of the short-circuit current (SCC) caused by pulses of ±5 mV in the clamping potential and are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

Table 1. Transepithelial electrical resistance, TER (Ω cm^2) response to a fast Ca^{++} switch assay in untreated monolayers (control) and in monolayers exposed in the apical solution to synthetic peptides homologous to segments of the first external loop of occludin

	Before Ca^{++} removal	Peak just before Ca^{++} reintroduction	Steady state after Ca^{++} reintroduction	Number of experiments	Statistics col 2 × col 4 paired "*t*" test
Control	843.8 ± 84.6	159.6 ± 12.0	782.1 ± 72.2	31	$P > 0.05$
SNYYGSGLSY non-buffered	500.1 ± 108.3	96.3 ± 5.6	263.2 ± 87.0	6	$P < 0.05$
SNYYGSGLSY buffered	1272.4 ± 196.8	146.9 ± 7.8	921.6 ± 180.9	11	$P < 0.05$
YYGSGLSY non-buffered	499.7 ± 57.9	125.3 ± 3.2	164.7 ± 24.0	4	$P < 0.01$
SNYYGSGLS buffered	1219.7 ± 135.1	153.7 ± 10.8	667.6 ± 139.4	10	$P < 0.01$

tested at concentrations normally in the range of 0.5 to 1.5 mg/ml. The peptide solution in the apical compartment was stirred continuously by a micropropeller driven by an electric motor. A few minutes after the peptide solution had been introduced into the apical compartment a fast Ca^{2+} switch assay was performed: Ca^{2+} was removed from the basolateral solution causing, as in the control group, a decline of TER, which was stopped, when TER reached values close to 100 Ω cm^2 by reintroduction of Ca^{2+} into the basolateral solution.

Peptide SNYYGSGLSY

Unbuffered Apical Solution

Peptide addition to the unbuffered KCl solution lowers the pH to levels between pH4 and 5, depending on the peptide concentration. The peptide in the apical solution had no effect *per se* on TER in 6 cases. In 3 other cases the peptide caused a slow decline of TER that started soon after its contact with the apical surface. This decline of TER was halted by peptide removal from the apical medium.

In the cases in which no effect *per se* was detected, the apical peptide had no effect on tissue response to basolateral Ca^{2+} withdrawal, as in the control group. In contrast, the peptide in the apical fluid substantially inhibited the recovery of TER induced by reintroduction of basolateral Ca^{2+} (Fig. 2a). In some cases, peptide removal triggered a slow recovery of TER, while in others no recovery was observed, at least in short-term experiments.

To test whether the observed effects were caused by the peptide itself or by the lower apical pH, two other experimental protocols were carried out.

Buffered Apical Solution

The apical solution of KCl 75 mM was buffered with HEPES 10 mM to pH 7.0. In a group of 11 monolayers, addition of the peptide at the concentrations mentioned above caused only minor pH falls of the order of 0.05 to 0.1 pH units and no effect *per se* on TER was ever

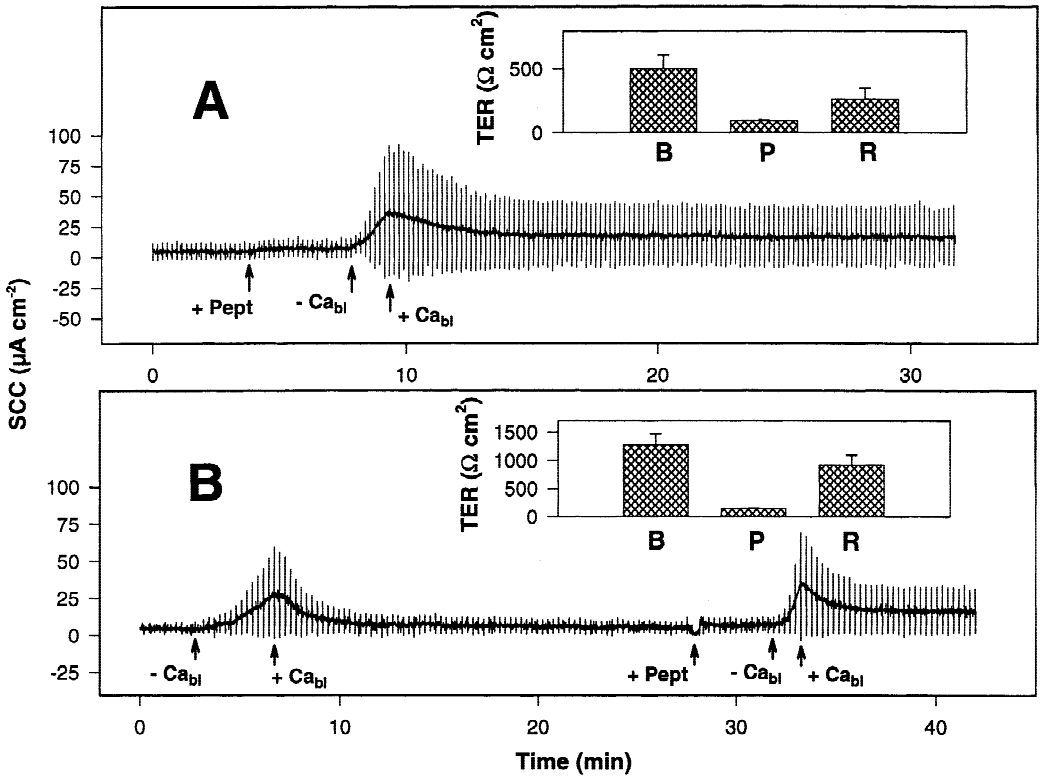

Fig. 2. Representative experiments of a fast Ca^{2+}-switch assay in peptide (SNYYGSGLSY) treated monolayers, showing the effect of basolateral Ca^{2+} withdrawal ($-Ca^{2+}$) on TER and impaired recovery in response to Ca^{2+} reintroduction ($+Ca^{2+}$). The monolayer was bathed by NaCl-Ringer's solution on the basolateral side. The vertical bars are deflections of the short-circuit current (SCC) caused by pulses of ±5 mV in the clamping potential and are proportional to the overall tissue electrical conductance (G), where G = 1/TER. (*A*) The apical solution was KCl 75 mM, and the peptide was added to this solution at a concentration of 0.8 mg/ml (+Pept). (*B*) The apical solution was KCl 75 mM, HEPES 10 mM, pH 7.0, and the peptide was added to the apical solution at a concentration of 0.4 mg/ml (+Pept). The first run is a control Ca^{2+} switch assay performed in the absence of the peptide.

observed. The results (Fig. 2*b*) were similar to those observed with the unbuffered apical solution, indicating that inhibition of TER recovery was indeed a result of the interaction of the peptide with the open TJs.

In a different group of 9 monolayers the effect of apical acidification per se was tested. The apical solution (KCl 75 mM) buffered with 10 mM MES was acidified to pH 4.0. A significant effect *per se* of this solution was observed on TER, in some cases, characterized by a small and reversible increase of TER that took place as soon as the apical medium was acidified. A decrease of TER in response to apical acidification was never observed. The response to the fast Ca^{2+} switch at low apical pH is similar to that in control condition (apical pH around 7), except for a slower recovery which can be quantified by an initial rate of TER recovery (d(TER)/dt) measured during the first minute after Ca^{2+} is reintroduced into the basolateral compartment. For control monolayers, d(TER)/dt was 5.11 ± 0.65 Ω cm^2 sec^{-1}, while for monolayers bathed with apical low pH solution, d(TER)/dt was 1.34 ± 0.19 Ω cm^2 sec^{-1} ($n = 9$) ($P < 0.01$, paired *t*-test). These results show that apical solution acidification to pH 4 does not lead to TJ opening but somehow slows the recovery process.

Peptide YYGSGLSY

Unbuffered Apical Solution

In 8 monolayers, the presence of the peptide YYGSGLSY in the apical solution had no effect *per se* in 5 cases (where the concentration of the peptide ranged from 0.5 to 0.8 mg/ml). In the other 3 cases (where the peptide concentrations were 0.5, 0.8 and 2.0 mg/ml), adding peptide to the apical compartment soon caused a slow decline of TER. Peptide removal halted the TER decline.

The experimental procedure was similar to that described for the previous groups and the results were also similar to those obtained with the peptide SNYYGSGLSY. Figure 3 shows a representative experiment of a monolayer bathed on the apical side with a solution containing the peptide YYGSGLSY and submit-

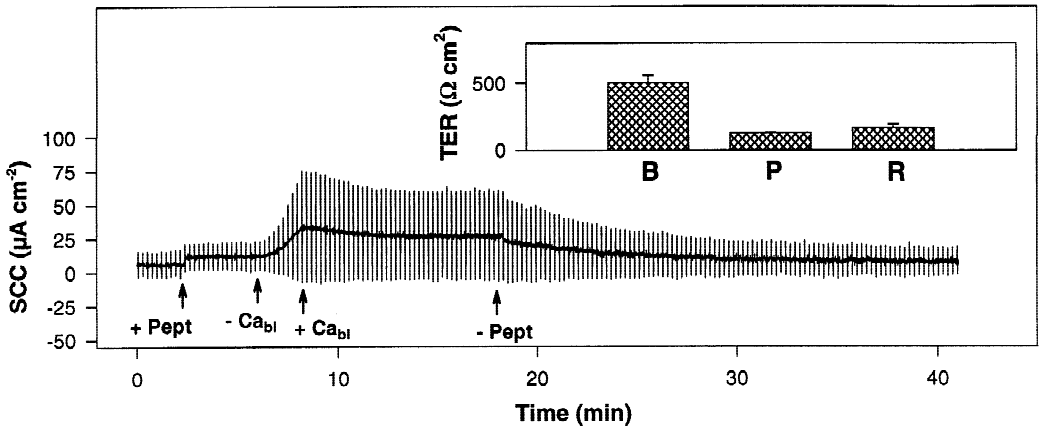

Fig. 3. Representative experiment of a fast Ca^{2+}-switch assay in a peptide (YYGSGLSY) treated monolayer, showing the effect of basolateral Ca^{2+} withdrawal ($-Ca^{2+}$) on TER and impaired recovery in response to Ca^{2+} reintroduction ($+Ca^{2+}$) and almost full recovery with peptide removal (−Pept). The monolayer was bathed by KCl 75 mM on the apical side and by NaCl-Ringer's solution on the basolateral side. The peptide was added to the apical solution at a concentration of 0.8 mg/ml (+Pept). The vertical bars are deflections of the short-circuit current (SCC) caused by pulses of ±5 mV in the clamping potential and are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

ted to a fast Ca^{2+} switch. This figure also exemplifies a case where TER recovery induced by peptide removal was clearly seen.

Peptide SNYYGSGLS

Buffered Apical Solution

The apical solution of KCl 75 mM was buffered with HEPES 10 mM to pH 7.0. In a group of 10 monolayers, peptide addition to the apical fluid caused only minor pH falls of the order of 0.05 to 0.1 pH units and no effect *per se* on TER. The results were similar to those observed with the two above mentioned peptides, in that the presence of the peptide substantially inhibited TER recovery induced by the reintroduction of Ca^{2+} into the basolateral solution (Fig. 4).

Peptide STYLNQYIYN

Experiments were carried out with the peptide STYLNQYIYN. Due to its insolubility in water, the peptide was previoiusly dissolved in dimethilsulfoxide (DMSO). The concentration of DMSO in the final solution of unbuffered KCl 75 mM solution was 10 μl/ml.

Two monolayers were tested using the peptide at 1.0 mg/ml in the apical solution. No effect was observed either *per se* or on the response to basolateral Ca^{2+} withdrawal and reintroduction. The behavior of the preparation was similar to that of the control group, with full recovery of TER upon return to Ca^{2+} in the basolateral solution.

DMSO itself was tested at 10 μl/ml; no effect was observed *per se* or on the Ca^{2+} response.

ZO-1 Immunolocalization

Despite the effects of occludin peptides on the recovery phase of the fast Ca^{2+} switch, the pattern of changes of ZO-1 immunolocalization was not altered by the presence of peptides of occludin in the apical solution. Figure 5*a* shows a monolayer before the Ca^{2+} withdrawal and before exposure to the peptide. The ZO-1 labeling appears as continuous lines on the cell borders. Figure 5*b* shows a monolayer after the withdrawal of Ca^{2+}. This monolayer shows focal points where cell-cell contact is lost and some cells retracted producing an opening in the monolayer. These cells still exhibit the belt of ZO-1 at the cell borders. In Fig. 5*c* and *d* the Ca^{2+} was reintroduced and the cells that had lost cell-cell contact became fusiform and exhibit a pattern of punctated ZO-1 labeling as they move to close the opening in the monolayer. Figure 5*e* and *f* show monolayers treated with the peptide SNYYGSGLSY, 20 min after Ca^{2+} reintroduction. These monolayers show the same pattern of resealing of the openings. The changes observed after the reposition of Ca^{2+} in both the presence or absence of the peptide suggests a regenerative process that heals the disrupted areas previously produced by the removal of Ca^{2+}.

Discussion

Tight junctions create a regulated paracellular barrier to the movement of molecules between both epithelial and endothelial cells. Recent progress has been made in

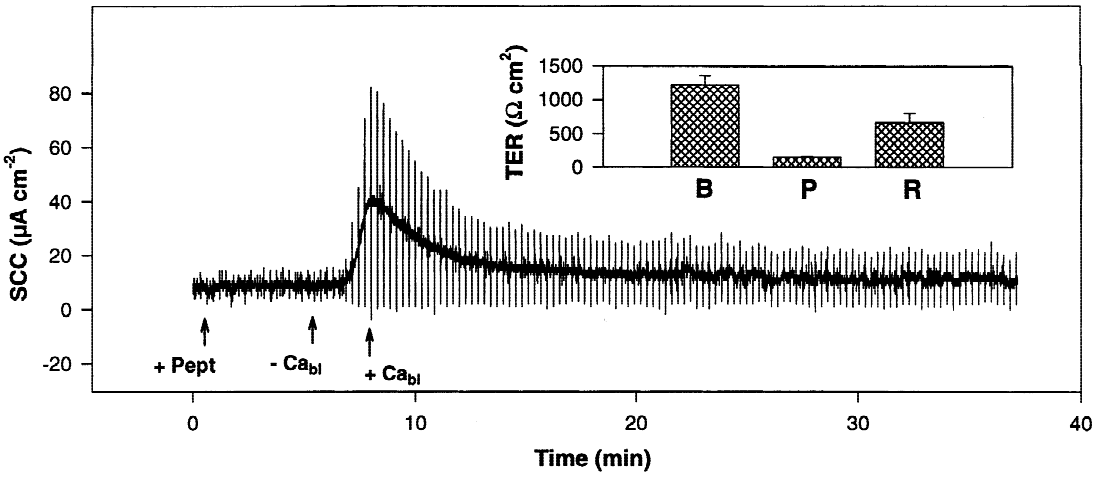

Fig. 4. Representative experiment of a fast Ca^{2+}-switch assay in a peptide (SNYYGSGLS) treated monolayer, showing the effect of basolateral Ca^{2+} withdrawal ($-Ca^{2+}$) on TER and impaired recovery in response to Ca^{2+} reintroduction ($+Ca^{2+}$). The monolayer was bathed by KCl 75 mM, HEPES 10 mM, pH 7.0 on the apical side and by NaCl-Ringer's solution on the basolateral side. The peptide was added to the apical solution at a concentration of 0.5 mg/ml (+Pept). The vertical bars are deflections of the short-circuit current (SCC) caused by pulses of ±5 mV in the clamping potential and are proportional to the overall tissue electrical conductance (G), where G = 1/TER.

identifying the proteins that create this barrier. Occludin, an integral membrane protein localized at the points of membrane-membrane interaction of the TJ (Furuse et al., 1993), where it binds to the proteins ZO-1 and ZO-2 (Anderson & Van Itallie, 1995) appears to be a component of the TJ strands (Furuse et al., 1994; Balda et al., 1996; McCarthy et al., 1996; Hirase et al., 1997; Van Itallie and Anderson, 1997). However, it has been shown recently that occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions (Saitou et al., 1998). Two other recently identified transmembrane proteins, claudin-1 and claudin-2, which do not have sequence similarity to occludin were shown to be targeted and incorporated in the TJ strands (Furuse et al., 1988). Although the precise relationship of the claudins to TJ structure and function remains undetermined, it appears that several integral membrane proteins with four putative transmembrane domains, occludin and claudins, can constitute TJ strands (Furuse et al., 1998).

The present study characterizes the behavior of A6 cell monolayers in response to a fast Ca^{2+} switch assay. It is shown that cells maintain stable TJs in the absence of apical Ca^{2+}. However, when the basolateral Ca^{2+} is removed the TJs are disrupted. This effect is reversed by the reintroduction of Ca^{2+} into the basolateral fluid which immediately triggers the recovery of the TJ. This recovery is complete after several minutes. In addition, synthetic peptides, 8 to 10 residues, homologous to segments 100 to 109, 102 to 109 and 100 to 108, of the first external loop of chick occludin molecule, when present in the apical bathing solution, impair junction resealing in response to Ca^{2+} reintroduction. This effect seems to be specific to peptides homologous to segments of the first loop of occludin, since a peptide homologous to residues 210 to 219 of the second external loop had no effect. However, peptides homologous to other segments of the second loop need to be tested in order to extend our present data, since in this study only one peptide (very insoluble) was tested. The inhibition of Ca^{2+}-induced TER recovery by the presence of first loop occludin peptides in the apical solution indicates that open TJs expose the external loops of occludin to the apical bathing fluid enabling them to interact with peptide molecules present in this fluid. This effect, apparently a competitive binding inhibition, is a strong evidence for the existence of homophilic adhesion between occludin molecules in adjacent cells, which is responsible for the sealing of the paracellular pathway (gate function).

In general the conclusions from experiments with buffered or unbuffered apical solution are similar since the sole acidification of the apical solution to pH 4 does not *per se* cause a decrease of TER nor block recovery in response to Ca^{2+} reintroduction. The observation that inhibition of TER recovery induced by the presence of first loop occludin peptides is not accompanied by marked changes of the pattern of changes of ZO-1 immunolocalization, suggests that the effects of these peptides on TER recovery are due to a competitive inhibition of the occludin-binding function. However, we cannot exclude a possible binding to other junction proteins such as claudin-1 and -2 (Furuse et al., 1998).

Recently (Wong & Gumbiner, 1997) assayed synthetic peptides (OCC1 and OCC2), corresponding, respectively, to the entire first and second extracellular

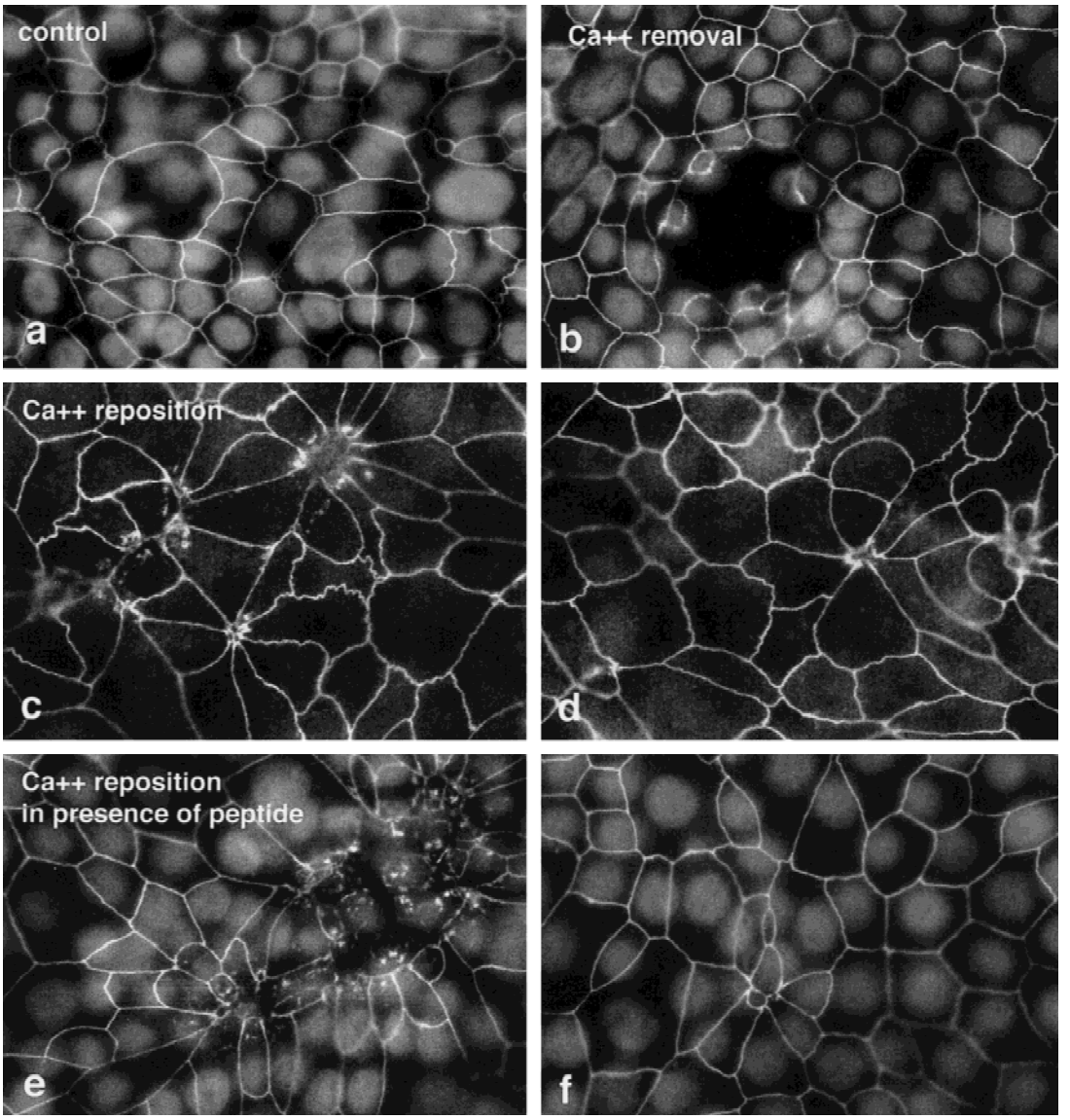

Fig. 5. Immunofluorescence localization of ZO-1 in A6 cells following disruption and resealing of tight junctions in the presence and absence of occludin peptides. (*a*) Before Ca^{2+} withdrawal (control monolayer) the cells in the monolayer are perfectly confluent and ZO-1 appears as continuous lines on the cell borders. (*b*) After Ca^{2+} withdrawal, at focal points some cells lose cell-cell contact, and their polygonal shape, and retract producing discontinuities or holes in the monolayer that after several minutes can reach the size of a few cell diameters (*see* large black area in the center of the figure). The cells bordering the opened hole in the monolayer still exhibit ZO-1 at their edges. (*c* and *d*) Monolayers 20 min after Ca^{2+} reposition showing a regenerative process which is characterized by the presence of fusiform cells that seem to stretch in order to heal (*c*) and close (*d*) the holes in the monolayer. (*e* and *f*) Monolayers 20 min after Ca^{2+} reposition in the presence of the peptide SNYYGSGLSY where the holes in the monolayers are partially (*e*) or totally (*f*) closed.

domains of occludin, for their ability to alter tight junctions in the A6 epithelial cell line. TER and paracellular tracer flux measurements indicated that only the second extracellular domain peptide (OCC2) reversibly disrupted the transepithelial permeability barrier. The experiments of Wong and Gumbiner (1997), with a time course of many hours, focused on the effect of occludin peptides on the formation of TJs in newly formed monolayers that were developing TER as well as steady-state monolayers that had completely formed maximal TER. In our experiments, with a time course of minutes, the focus was only on the effects of peptides on fully developed monolayers in which the TJs were transiently disrupted by removal of basolateral Ca^{++}. The first loop peptide apparently does not contribute to the formation of TJs (Wong & Gumbiner, 1997); however, our experi-

ments suggest that they play a significant role in the recovery of preformed TJs transiently disrupted by basolateral Ca^{++} removal.

A similarity exists, however, between our findings and those of Wong and Gumbiner (1997) regarding the peripheral TJ protein ZO-1, since in both cases it was unaltered by the treatment with the peptides.

Our results are compatible with the notion that occludin is responsible for the sealing of the TJs. Direct evidence that occludin confers cell adhesiveness in the absence of Ca^{2+}, thus ruling out cadherin-cadherin contacts, was obtained by Van Itallie and Anderson (1997) by expressing human occludin in NRK and Rat-1 fibroblasts which lack endogenous occludin and TJs. In addition, they observed that a synthetic peptide containing the amino acid sequence of the entire first extracellular loop of occludin inhibited cell adhesion induced by occludin expression. Their observation, that the first external loop of occludin is directly involved in cell-cell adhesion, is in good agreement with our results. Both results emphasize the importance of interactions between the extracellular domain of occludin in the formation and sealing of the TJs.

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