A New Principle for Tight Junction Modulation Based on Occludin Peptides

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ABSTRACT

The aim of this study was to investigate whether peptides from the extracellular loops of the tight junction protein occludin could be used as a new principle for tight junction modulation. Peptides of 4 to 47 amino acids in length and covering the two extracellular loops of the tight junction protein occludin were synthesized, and their effect on the tight junction permeability in Caco-2 cells was investigated using [^{14}C]mannitol as a paracellular marker. Lipopeptide derivatives of one of the active occludin peptides (OPs), synthesized by adding a lipoamino acid containing 14 carbon atoms (C_{14}-) to the N terminus of the peptide, were also investigated. Peptides corresponding to the N terminus of the first extracellular loop of occludin increased the permeability of the tight junctions without causing short-term toxicity. However, the peptides had an effect only when added to the basolateral side of the cells, which could be partly explained by degradation by apical peptidases and aggregate formation. By contrast, the lipopeptide C_{14}-OP$_{90-103}$, which protects the peptide from degradation and aggregation, displayed a rapid apical effect. The L- and D-diastereomers of C_{14}-OP$_{90-103}$ had distinctly different effects. The D-isomer, which releases intact OP$_{90-103}$ from the lipoamino acid, displayed a rapid and transient increase in tight junction permeability. The L-isomer, which releases OP$_{90-103}$ more rapidly, gave a more sustained increase in tight junction permeability. In conclusion, C_{14}-OP$_{90-103}$ represents a prototype of a new class of tight junction modulators that act on the extracellular domains of tight junction proteins.

Tight junctions form a paracellular barrier that limits the diffusion of solutes through the intercellular space and defines the boundary between apical and basolateral plasma membrane domains in epithelial cell layers such as the intestinal epithelium (Gumbiner, 1987; Tsukita et al., 2001). New insights into the molecular architecture of the tight junctions and their regulation theoretically offer the opportunity to up- or down-regulate this diffusion barrier with pharmacological agents, to either limit (e.g., in pathological conditions that create hyperpermeability (Gassler et al., 2001; Kucharzik et al., 2001) or enhance the diffusion of solutes (i.e., increase the absorption of membrane-impermeable drug molecules). In the latter case, the therapeutic advantages could be significant, because poor drug absorption is a major reason for failure of oral drug candidates in clinical drug development (Kennedy, 1997; Lipinski, 2000).

Consequently, numerous pharmacological agents have been investigated for their tight junction-modifying properties; as a rule, they have failed for toxicological reasons (van Hoogdalem et al., 1990; Swenson et al., 1994; Yamamoto et al., 1996). Previous tight junction modulators have generally mediated their effect by general and fairly complex intracellular pathways (such as the inositol triphosphate and diacylglycerol pathways). Activation of these pathways modulates the phosphorylation pattern of cytoskeletal proteins associated with tight junctions and eventually results in an increased permeability to solutes such as drug molecules (Lindmark et al., 1998; Hayashi et al., 1999; Hirase et al., 2001). From a therapeutic perspective, once these mechanisms have been activated, they seem difficult to control because irreversible openings in the tight junctions are obtained, which is unacceptable for toxicological reasons. In addition, many commonly used tight junction modulators are more or less surface-active, and this property further enhances the toxicity by solubilization of membrane components (Nakanishi et al., 1992; Anderberg et al., 1993).

A preferred strategy for modulating the diffusion barrier of the tight junctions would be to directly target the modulator to the extracellular proteins of the tight junctions. We speculated that such a direct intervention would reduce the short-term toxicity of the modulator. Moreover, if a peptide

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ABBREVIATIONS: OP, occludin peptide; HPLC, high-performance liquid chromatography; TOF, time of flight; MALDI, matrix-assisted laser desorption ionization; HBSS, Hanks' balanced salt solution; TER, transepithelial electrical resistance; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline.

were used, it would eventually be degraded at the level of the brush-border enzymes, which would speed up the reversibility of the process. Our hypothesis was supported by literature data indicating that some bacterial toxins have tight junction proteins as primary or downstream targets. For instance, the C terminus of *Clostridium perfringens* enterotoxin binds to the tight junction proteins claudin 3 and 4, which results in an enhanced paracellular permeability (Sonoda et al., 1999). Furthermore, the *Vibrio cholerae* enterotoxin zonula occludens toxin increases paracellular permeability by activating protein kinase C, which leads to rearrangement of the actin filaments and subsequent loosening of the tight junctions (Fasano et al., 1991, 1995). Interestingly, an endogenous analog of zonula occludens toxin was recently identified, a finding that suggests that in response to bacterial infection, the intestinal epithelium regulates its paracellular permeability by specific mechanisms (Asmar et al., 2002).

There are at least two extracellular tight junction protein targets that are available for pharmacological intervention, occludin and the claudin family (Furuse et al., 1993, 1998). The claudins have been strongly connected to the barrier function of the tight junctions (Tsukita and Furuse, 2000; Furuse et al., 2001; Tsukita et al., 2001) and may therefore seem the obvious choice. However, because of this fact and because some claudins are targets of bacterial toxins, we speculated that intervention with the claudins may result in dramatic and perhaps irreversible and toxic effects. By contrast, alteration of occludin expression levels has resulted in more modest increases in epithelial permeability, and absence of occludin from tight junctions in some tissues in vivo does not interfere with the function of the paracellular barrier (Balda et al., 1996; Saitou et al., 2000). Therefore, occludin seemed to be a safer alternative for pharmacological modulation of the tight junction barrier. This hypothesis was supported by the fact that a homologous large peptide, corresponding to the second extracellular loop of occludin, has a modest effect on the paracellular barrier function (Wong and Gumbiner, 1997). The findings that peptides corresponding to the extracellular loops of occludin seem to interact specifically with occludin, together with the fact the occludin has been shown to play a key role in the dynamic regulation of tight junction permeability (Hirase et al., 2001), made us believe that pharmacological modulation via the extracellular domains of occludin could be a promising strategy.

We therefore synthesized a series of peptides homologous to various sections of the two extracellular loops of occludin. We then used the hydrophilic paracellular marker mannitol as a model drug to study the effects of the occludin peptides (OPs) on the paracellular permeability in intestinal epithelial Caco-2 cell monolayers. Once active, nontoxic peptides were found, their efficiency was optimized using a synthetic lipoamino acid conjugate as a prodrug. Finally, the kinetics of the effect were controlled by modulation of the release of the tight junction-modulating peptide from the lipopeptide.

Materials and Methods

Peptides. Peptides corresponding to the amino acid sequence of the two extracellular loops of human occludin were synthesized, as described below. OP_{90-135} corresponds to the first extracellular loop and consists of amino acids 90 to 135, as identified by the primary amino acid sequence of human occludin (Ando-Akatsuka et al.,

1996), whereas $OP_{196-243}$ corresponds to the second extracellular loop and accordingly consists of amino acids 196 to 243. Shorter peptides (6–30 amino acids) corresponding to different sections of the extracellular loops of human occludin were also synthesized. Lipophilic amino acids composed of a linear α-side chain of six (hexyl) and twelve (dodecyl) hydrocarbons (Toth et al., 1999), denoted C_8- and C_{14}-, respectively, were added to the N terminus of OP_{90-103}, resulting in C_8-OP_{90-103} and C_{14}-OP_{90-103}. A schematic illustration of the OPs is given in Fig. 1.

Peptide Synthesis. Peptide synthesis was accomplished by a stepwise solid-phase procedure on 4-methylbenzhydrylamine-functionalized copoly (styrene-1% divinylbenzene) resin on a 0.25 to 0.75 mmol scale (Toth et al., 1999). The crude peptides were purified by reverse-phase high-performance liquid chromatography (HPLC) on a TSK-GEL C_{18} preparative column (5 μm) and lyophilized to a final purity of >95%. The purified peptides were stored in a desiccator at 4°C. Mass spectra were recorded on a Fisons vacuum gauge-time of flight (TOF) spectrometer (Thermo Finnigan, San Jose, CA) using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (MS), a VG Analytical ZAB-SE instrument (Micromass Plc., Manchester, UK) using fast atom bombardment ionization, or a MassLab Navigator quadrupole mass spectrometer (MS) using electrospray ionization (Thermo Finnigan, San Jose, CA).

Cell Culture. Unless otherwise stated, culture media and supplements were purchased from Invitrogen (Täby, Sweden). Caco-2 cells originating from a human colorectal carcinoma were obtained from American Type Culture Collection (Manassas, VA) and cultivated as described previously in detail (Artursson, 1990; Tavelin et al., 2002). In brief, Caco-2 cells were seeded at a density of 440,000 cells/cm² on uncoated permeable polycarbonate filter supports (0.45-μm pore size, 12-mm diameter; Transwell Costar, Badhoevedorp, The Netherlands). Cells at passage numbers 95 through 105 were used. The Caco-2 cell monolayers were used for transport experiments between 21 and 28 days after seeding. The Caco-2 cell culture was regularly screened during the course of this study and found to be negative for mycoplasma contamination.

Measurement of Paracellular Flux. The lyophilized peptide was dissolved in Hanks' balanced salt solution (HBSS) containing 25 mM HEPES, pH 7.4, to give a final concentration of 5 to 3000 μM. Poorly soluble peptides were dissolved in dimethyl sulfoxide (<1%) before adding HBSS. The addition of <1% dimethyl sulfoxide to HBSS did not affect the integrity of the monolayers, as determined by [14C]mannitol permeability (data not shown). Transport studies using the hydrophilic paracellular marker molecule [14C]mannitol (PerkinElmer Life Sciences, Boston, MA) at a concentration of 20,000 Bq/ml were performed in Caco-2 monolayers cultivated on permeable supports. All experiments were carried out at 37°C in HBSS, pH 7.4, on both the apical and the basolateral sides.

All solutions were preheated to 37°C and the experiments were performed at 37 ± 0.5°C on a custom-built heating plate (Detrona, Linköping, Sweden). The cell monolayers were washed with preheated HBSS and equilibrated in the same buffer for 20 min at 37°C before the transport experiments. The HBSS was removed and, unless otherwise stated, the filters were then transferred to wells containing 1.2 ml of fresh, preheated HBSS containing peptide. At the beginning of the experiment, 0.4 ml of preheated HBSS-containing peptide spiked with [14C]mannitol was added to the apical chamber. Samples were immediately withdrawn from the apical solutions to measure the initial donor concentration of [14C]mannitol. At regular time intervals, typically every 60 min, 600-μl samples were removed from the basolateral solution and replaced with 600 μl of fresh HBSS containing the peptide. All samples were analyzed using a liquid scintillation counter (1900CA TriCarb; PerkinElmer Life Sciences). The mannitol permeability in untreated Caco-2 cell monolayers was constant during this period (data not shown).

Electrophysiological Measurements. The transepithelial electrical resistance (TER) of Caco-2 monolayers grown on permeable filter supports was measured at 37°C with an Endohm tissue resis-

tance measurement chamber connected to an EVOM resistance meter (World Precision Instruments, New Haven, CT). Caco-2 cell monolayers were incubated in HBSS preheated to 37°C for 20 min. The Caco-2 cell monolayers were then transferred to preheated HBSS containing peptide on either the apical side or the apical and basolateral sides. At regular time intervals, the monolayers were transferred to the Endohm tissue resistance measurement chamber for TER measurements. The experiments were generally carried out over 360 min. The resistance of the filters without cells ($4–5 \ \Omega \cdot cm^2$) was subtracted from each TER value.

Cytotoxicity and Membrane Damage Assays. The effect of the peptides on membrane integrity was assessed by measuring the amount of lactate dehydrogenase (LDH) leakage. Caco-2 cell monolayers were incubated with HBSS containing peptide from both the apical and the basolateral sides for 360 min. Lactate dehydrogenase leakage into the apical and basolateral solutions was determined using an LDH release kit (Roche, Mannheim, Germany) according to

the manufacturer's instructions. Total cellular LDH was determined by solubilizing the cell monolayers with 1% Triton X-100 in HBSS. Cell lysates were centrifuged, and a sample of the supernatant was withdrawn and analyzed as described above. The effect of the peptides on cell viability was also assessed by measuring the intracellular dehydrogenase activity of Caco-2 cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium method (Mosmann, 1983), as described previously (Anderberg et al., 1992). Caco-2 cells were seeded at $100,000 \ cells/cm^2$ in 96-well plates in the presence of different concentrations of OP in HBSS and incubated at 37°C for 6 h before carrying out the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay.

Immunofluorescence. Caco-2 cells grown on permeable supports were washed with phosphate-buffered saline (PBS) and fixed with 3% paraformaldehyde in PBS, pH 7.4, for 10 min at room temperature. The cells were then permeabilized using 0.1% Triton X-100 in PBS for 10 min. The distribution of tight junction protein

LOOP 1

Effect

OP$_{90-135}$	H-drgygtsllggsvgypyggsgfgsygsgygygygygygyggytdpr-NH$_2$ (90 ... 135)	++
OP$_{90-113}$	drgygtsllggsvgypyggsgfgs (90 ... 113)	+++
OP$_{106-135}$	yggsgfgsygsgygygygygygygygggytdpr (106 ... 135)	-
OP$_{109-122}$	sgfgsygsgygy (109 ... 122)	-
OP$_{90-103}$	drgygtsllggsvg (90 ... 103)	++
OP$_{102-113}$	vgypyggsgfgs (102 ... 113)	-
OP$_{98-103}$	lggsvg (98 ... 103)	-
OP$_{90-99}$	drgygtsll (90 ... 99)	-
Lip-OP$_{90-103}$	H$_2$N-CH-CONH- drgygtsllggsvg (90 ... 103) R R =C$_{12}$H$_{25}$ or C$_6$H$_{13}$	++[1]

LOOP 2

OP$_{196-243}$	H-gvnptaqssgslygsqiyalcnqfytpaatglyvdqylyhycvvdpqe-NH$_2$ (196 ... 243)	-

Fig. 1. Schematic illustration of the investigated OPs. The numbers refer to the amino acid sequence of human occludin. The sequence letters refer to amino acids. The column on the right shows the effects of the peptides as tight junction modulators. −, no effect; + and ++, moderate effect; +++, strong effect. ++[1], after apical exposure of C$_{14}$-OP$_{90-103}$.

occludin was studied by indirect immunofluorescence using a rabbit polyclonal occludin antibody (1:100; Zymed Laboratories Inc., South San Francisco, CA). The cells were incubated with the primary antibodies for 1 h at room temperature, washed with PBS, and incubated with a secondary fluorescein isothiocyanate-labeled antibody against rabbit immunoglobulin (1:100 in PBS; Amersham Biosciences) for 30 min at room temperature. The samples were washed with PBS, mounted in fluorescent mounting medium (DAKO, Glostrup, Denmark), sealed, and examined under a confocal laser-scanning microscope (Leica TCS 4D; Leica LT, Heidelberg, Germany).

The distribution of occludin in Caco-2 monolayers was studied in a blinded fashion. Cells surrounded by a dense bright perijunctional ring of occludin were considered normal, whereas cells displaying a clear broadening of the occludin ring, resulting in a more diffuse staining of at least one cell border, were considered altered. Approximately 200 cells obtained from four different samples were counted in each group.

Western Blot. Caco-2 cells grown on three permeable supports (12-mm diameter) per sample were exposed to occludin peptides or lipoamino acid-peptide conjugates for 360 and 60 min, respectively. The cells were homogenized, and total protein was extracted as described in detail elsewhere (Tavelin et al., 2002). The proteins were subsequently subjected to SDS-polyacrylamide gel electrophoresis, and occludin was thereafter immunodetected using a polyclonal occludin antibody (rabbit anti-occludin; Zymed) and the enhanced chemiluminescence system (Amersham Biosciences).

Peptide Stability Measurements. The stability of the peptides (20 μg/ml) during incubation with Caco-2 cell monolayers was examined for up to 360 min. At 60, 120, 180, 240, and 360 min, 20-μl samples were removed from the apical and/or basolateral solutions. The samples were analyzed by HPLC (PerkinElmer GmbH, Überlingen, Germany) with a 238TP54 column (4.6 × 150 mm; Grace Vydac, Columbia, MD). The mobile phase was 0.1% trifluoroacetic acid/acetonitrile 80:20 and 60:40 for the analysis of OP_{90-103} and C_{14}-OP_{90-103}, respectively. The flow rate was 1.0 ml/min/ and the eluate was monitored at 214 nm.

Calculations. The apparent permeability coefficient (P_{app}, in centimeters per second) was determined according to the equation $P_{app} = (K \times V_r)/A$ (Artursson and Karlsson, 1991), where K is the steady-state change in concentration in the receiver chamber (C_t/C_0) versus time (seconds), C_t is the concentration in the receiver compartment at the end of each time interval, C_0 is the initial concentration in the donor chamber at each time interval (moles per milliliter), V_r is the volume in the receiver chamber (milliliters), and A is the surface area of the filter membrane (square centimeters).

Statistics. The experiments were repeated at least twice using different batches of peptides and four monolayers each time, unless otherwise stated. The results are expressed as mean values ± S.D. Significance was tested using the two-tailed Student's t test for comparison of two mean values or one-way analysis of variance followed by Dunnett's post test for comparison of multiple mean values.

The minimum effective concentration (C_{min}) was defined as the OP concentration at which a significant, more than 2-fold increase in mannitol permeability was observed compared with control monolayers. The concentration of peptide with half the maximum effect (EC_{50}) was determined by fitting the sigmoidal dose-response curve to the data: $P_{app} = (P_{app(min)} + [P_{app(max)} - P_{app(min)}])/(1 + 10^{[(\log EC_{50} - \log \text{conc.}) - \gamma]})$, $P_{app(min)}$ is the P_{app} in control monolayers, $P_{app(max)}$ is the maximum P_{app} of cell monolayers incubated with peptide, log conc. is the logarithm of peptide concentration, and γ is the slope factor.

Results

Effect of Native Occludin Peptides. First, the two peptides corresponding to the two complete extracellular loops of occludin (Fig. 1) were investigated for their effects on tight junction permeability. In the presence of OP_{90-135} (32 μM), the permeability to the paracellular marker mannitol of Caco-2 cell monolayers increased in a time-dependent manner. After 120 min, a 3-fold increase compared with the control monolayers was observed ($p < 0.001$), whereas no increase in permeability could be seen in Caco-2 cell monolayers after incubation with $OP_{196-243}$ (Fig. 2A). Based on these results, we hypothesized that peptides corresponding to the first loop are more likely to modulate tight junction permeability, and consequently we set out to identify shorter active peptides from this loop.

A peptide that corresponds to the N-terminal half of the first extracellular loop, OP_{90-113} (20 μM), increased the mannitol permeability of Caco-2 cell monolayers in a time-dependent manner. The increase in permeability compared with control monolayers was 11-fold after 240 min (data not shown) and 45-fold after 360 min (Fig. 2B) ($p < 0.001$). OP_{90-113} did not cause short-term toxicity at concentrations as high as 50 μM, as measured by LDH release (data not shown). $OP_{106-135}$ (50 μM), a peptide that corresponds to the C-terminal fragment of the first extracellular loop, increased the permeability to mannitol by only ~2-fold after 360 min of incubation ($p > 0.05$). Based on these results, we concluded that tight junction-modulating peptides should preferably correspond to the N terminus of the first extracellular loop. Next, we studied the effects of two shorter peptides: OP_{90-103}, which corresponds to the N terminus of OP_{90-113}, and $OP_{102-113}$, which corresponds to the C terminus (Fig. 1). With OP_{90-103} (100 μM), a 5-fold increase in the permeability to mannitol was seen after 240 min (data not shown) and a 22-fold increase was seen after 360 min ($p < 0.001$) (Fig. 2C). The other truncated peptide, $OP_{102-113}$, was inactive at 250 μM (Fig. 2C). Further truncation of OP_{90-103} resulted in peptides (OP_{98-103} and OP_{90-99}) (Fig. 1) that were inactive (data not shown). No significant short-term toxicity was observed after exposure to OP_{90-103} at concentrations as high as 3 mM, as judged by measurements of the intracellular dehydrogenase activity (Fig. 2D).

Concentration-Dependent Effect. Both OP_{90-113} and OP_{90-103} increased the Caco-2 cell monolayer permeability to mannitol in a dose-dependent manner (Fig. 3). The C_{min} and EC_{50} of OP_{90-113} were 5 and 22 μM after 360 min, respectively. The effect reached a plateau at approximately 50 μM, at which the permeability was around 200-fold higher than that of the control monolayers. After 360 min of incubation, the C_{min} and EC_{50} of OP_{90-103} were ~50 μM and ~175 μM, respectively, and EC_{50} reached a plateau at ~500 μM, when the permeability of mannitol was about 80-fold higher than that of the control cells ($p < 0.05$).

Apical and Basolateral Effects of OP_{90-113} and OP_{90-103}. In agreement with previous studies on tight junction-modulating peptides, the OPs were added to both the apical and the basolateral sides of the cell monolayers in all of the above experiments (Wong and Gumbiner, 1997). Because pharmacological agents that modulate tight junctions would be administered to the luminal side, the apical effect is a prerequisite. However, when OP_{90-103} (200 μM) was added only to the apical side of the Caco-2 cell monolayers, no significant effect on tight junction permeability was observed ($p > 0.05$) (Fig. 4A), whereas a significant (11-fold) increase in permeability was observed when the peptide was added only to the basolateral side ($p < 0.05$). This effect was much lower than the 68-fold increase in permeability obtained after exposure of both the apical and the basolateral side. A similar

pattern was observed for the more potent OP_{90-113} (data not shown). We therefore concluded that basolateral exposure is required for a significant effect of OP_{90-103} in Caco-2 cell monolayers and that there is a synergistic effect of the apical and basolateral exposure of OP_{90-103}.

Degradation and Aggregation of Occludin Peptides. One possible explanation for the lack of apical effect of OP_{90-113} and OP_{90-103} was that the peptides were rapidly degraded during the course of the experiments. We therefore examined the stability of OP_{90-103} incubated with Caco-2 cell monolayers. When 8 μM OP_{90-103} was added to the basolateral side, no significant degradation of the peptide was observed (Fig. 4B). However, after apical addition, 0.51 ± 0.01 nmol/h (corresponding to 16%/h) was degraded. The degradation rate was reduced to 0.14 ± 0.06 nmol/h (4.4%/h) by adding peptidase inhibitors. A similar degradation rate was observed at as much as 500 μM of OP_{90-103}, indicating that OP_{90-103} is significantly degraded in the active concentration range used in this study (data not shown). However, no apical effect was observed for OP_{90-103} in combination with the peptidase inhibitors. We therefore began looking for additional explanations for the lack of apical effect.

MALDI-TOF-MS experiments tuned to detect possible multimer aggregation of peptides revealed that several OPs aggregated. For example, one of the inactive peptides, $OP_{109-122}$, formed a pattern of multimers ranging from dimers to hexamers (Fig. 4C). This OP is part of the glycine- and serine-rich repetitive amino acid sequence (Fig. 1), and we speculate that it may be part of a homologous binding site of native occludin. OP_{90-103} also formed aggregates, but to a lower extent than $OP_{109-122}$, resulting in a small peak corresponding to a dimer of OP_{90-103} (data not shown). Consequently, this aggregation may also have reduced the number of OP_{90-103} molecules available for interaction with the tight junctions from the apical side because of the increased size of the aggregates. The effect of OP_{90-103} from the basolateral side may therefore be explained by better accessibility of the peptides aggregated, at least in part, to the tight junctions from the basolateral side (Sonoda et al., 1999; Sinaga et al., 2002). We conclude that the lack of apical effect of OP_{90-103} could be caused by degradation by apical peptidases as well as aggregation followed by reduced access to the tight junctions from the apical side.

Effect of Lipoamino Acid Conjugates. Conjugation of lipoamino acids to peptides has previously been shown to inhibit peptide degradation in Caco-2 cells (Yamamoto, 1998; Toth et al., 1999). We therefore conjugated a lipoamino acid $[H_2N-CH(C_{12}H_{25})-COOH]$ to the N terminus of OP_{90-103} to inhibit the peptidase-mediated degradation of the peptide. In addition, we hypothesized that the lipoamino acid moiety may insert into the cell membrane and thus concentrate OP_{90-103} at the cell surface. Interestingly, in contrast to
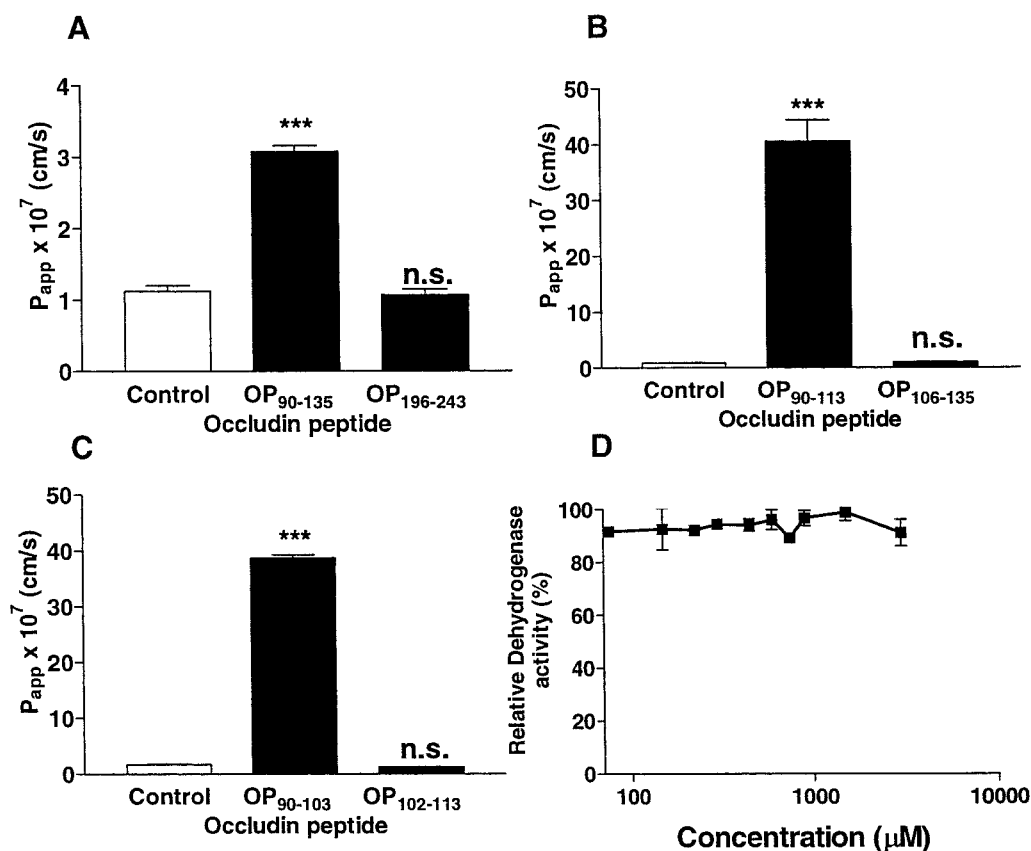
Fig. 2. Effect on Caco-2 cell monolayers exposed to OPs. A, after exposure to OP_{90-135} for 120 min, the permeability to mannitol in Caco-2 cell monolayers increased 3-fold compared with control monolayers. The permeability to mannitol did not change after exposure to $OP_{196-243}$. B, after exposure to OP_{90-113} for 6 h, the permeability to mannitol increased 45-fold compared with control monolayers. The permeability to mannitol increased by only ~2-fold after exposure to $OP_{106-135}$. C, after exposure to OP_{90-103}, the permeability to mannitol increased 22-fold, whereas the permeability to mannitol was unchanged after exposure to $OP_{102-113}$. D, no significant short-term toxicity could be seen after exposure to OP_{90-103} at concentrations as high as 3 mM, as judged from measurements of intracellular dehydrogenase activity in the Caco-2 cells. Values are given as mean values $\pm$ S.D. ($n = 4$). ***, $p < 0.001$ compared with control. n.s., not significant.
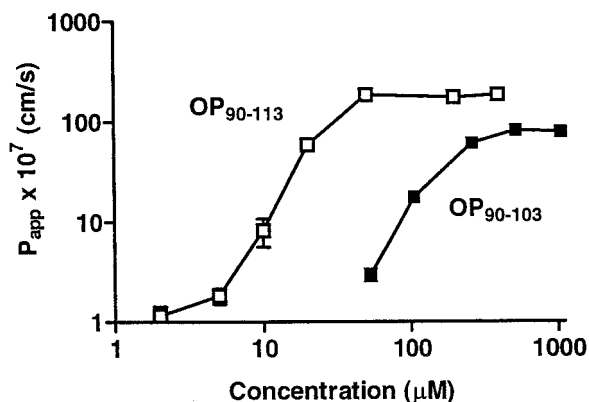
OP_{90-103}, the lipoamino acid conjugate of OP_{90-103} (C_{14}-OP_{90-103}) did not form significant amounts of homodimers, as judged by ES-MS experiments (Fig. 5A). However, C_{14}-OP_{90-103} formed aggregates with both OP_{90-103} and OP_{90-135}, suggesting that C_{14}-OP_{90-103} can bind to the corresponding extracellular domains of occludin (data not shown).

Compared with the control cells, C_{14}-OP_{90-103} (75 μM) added to both the apical and the basolateral side increased the tight junction permeability by 35-fold (Fig. 5B). The lipoamino acid alone was inactive (data not shown). Although the effect on the permeability to mannitol was similar for OP_{90-103} and C_{14}-OP_{90-103}, the effects on the TER, a measure of ion flux (mainly reflecting the ion flux across the tight junctions) of the Caco-2 cell monolayers, were quite different. It was seen that OP_{90-103} decreases TER in a time-dependent manner (Fig. 5C). On the other hand, C_{14}-OP_{90-103} caused an initial increase in TER, followed by a gradual decrease (Fig. 5C). However, TER never reached values lower than those of the control monolayers within the time frame used in this experiment. The derivative C_{14}-OP_{90-103} did not cause short-term toxicity to Caco-2 cells at concentrations as high as 1 mM, as judged by measurements of the intracellular dehydrogenase activity (Fig. 5D). At higher concentrations, a gradual decrease in dehydrogenase activity was observed.
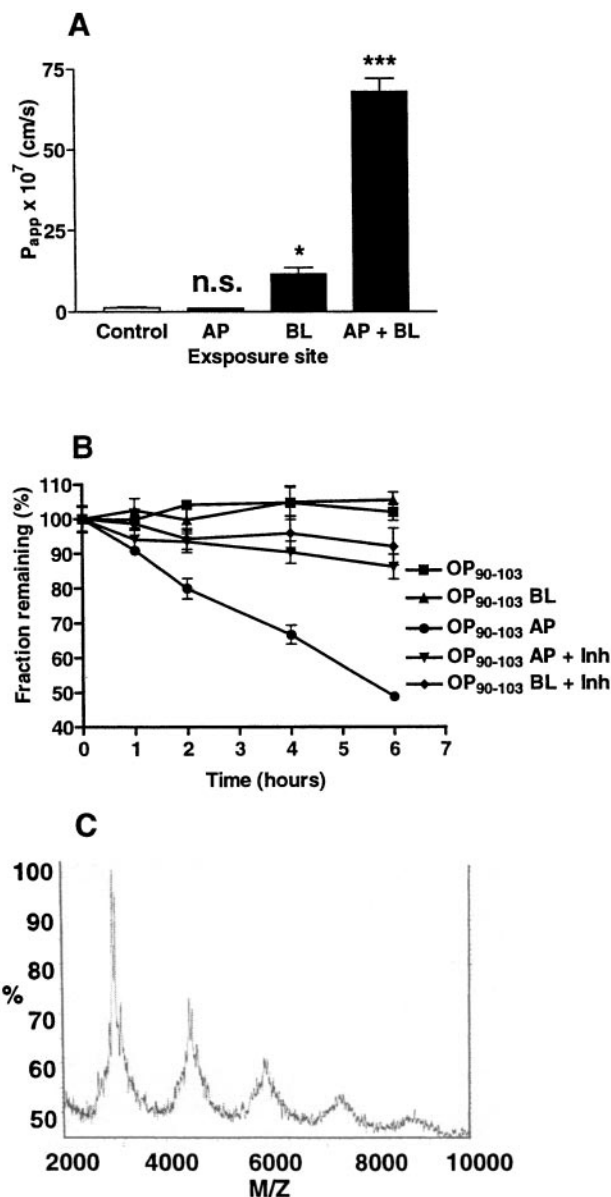
In contrast to OP_{90-103}, the lipophilic derivative C_{14}-OP_{90-103} was active from the apical side in a dose-dependent manner. For example, an apical dose of 500 μM C_{14}-OP_{90-103} resulted in a 3.5-fold increase in the permeability to mannitol compared with control monolayers. To study the effect of the chain length of the conjugated OP_{90-103}, another lipophilic derivative of OP_{90-103}, C_8-OP_{90-103}, was synthesized. The apical effect of C_8-OP_{90-103} was negligible in the concentration interval studied (data not shown). We tentatively conclude that this was an effect of the β-hydrocarbon chain of the C_8 lipoamino acid, which is too short to insert into the apical cell membrane.

Difference in Effect between D-Isomers and L-Isomers of C_{14}-OP_{90-103}. C_{14}-OP_{90-103} exists as a racemic mixture of the two diastereomers, D-C_{14}-OP_{90-103} and L-C_{14}-OP_{90-103}, that were separated by HPLC and subsequently characterized as separate isomers. First, we examined the stability of D- and L-C_{14}-OP_{90-103} added to the apical side of the Caco-2 cell monolayers (Fig. 6). The L-isomer released OP_{90-103} at a 15-fold faster rate (5.01 $\pm$ 0.11 nmol/h) than the

D-isomer (0.33 $\pm$ 0.60 nmol/h). Only intact OP_{90-103} was released, indicating that the lipoamino acid protected the released OP_{90-103} from further degradation. Thus, after 1-h incubation of 6.40 nmol L-C_{14}-OP_{90-103} on the apical side of Caco-2 cell monolayers, 4.20 $\pm$ 0.33 nmol (n = 4) OP_{90-103} was found in the apical medium, corresponding to a recovery of approximately 84% of OP_{90-103}. Furthermore, no degradation of the released OP_{90-103} was observed over a 6-h incubation (data not shown), which is in agreement with previous

Fig. 4. A, apical exposure of OP_{90-103} did not increase the permeability to mannitol in Caco-2 cell monolayers, whereas the permeability increased 11-fold after basolateral exposure. Exposure to both apical and basolateral solution resulted in a 68-fold increase in the permeability to mannitol. B, degradation of OP_{90-103} after incubation with Caco-2 cells. After 6 h, approximately 50% of the OP_{90-103} was degraded on the apical side, whereas no degradation was observed in the basolaterally exposed OP_{90-103}. The apical degradation of OP_{90-103} was inhibited by a peptidase inhibitor cocktail [0.29 mM bestatin, 1 mM diprotin A, and 1 mM captopril (Pauletti et al., 1997)]. Values are given as mean values $\pm$ S.D. (n = 4). C, MALDI-TOF-MS spectrum showing formation of dimers and multimers of an inactive peptide ($OP_{109-122}$). *, $p < 0.05$; ***, $p < 0.001$ compared with control. n.s., not significant.

Fig. 3. Dose-dependent increase in tight junction permeability to mannitol after exposure of OP_{90-113} and OP_{90-103}. Sigmoidal dose response curves were obtained with both peptides, but OP_{90-113} was approximately 10 times more potent than OP_{90-103}. Values are given as mean values $\pm$ S.D. (n = 4).

studies indicating that the lipoamino acid conjugation inhibits enzymatic degradation of peptides (Toth et al., 1999).

Surprisingly, in our preliminary study, only the L-isomer, which released OP_{90-103} more rapidly than the D-isomer, seemed to increase the permeability to mannitol (Fig. 7A). However, in a more detailed time study, both isomers increased the permeability by ~15-fold compared with controls after only 20 min. The permeability was further increased to ~40-fold compared with the control monolayers after 40-min incubation for the L-isomer, whereas the effect of the D-isomer diminished with time (Fig. 7B). The increase in permeability could be efficiently inhibited by peptidase inhibitors [0.29 mM bestatin, 1 mM diprotin A, and 1 mM captopril (Pauletti et al., 1997)], indicating that release of OP_{90-103} from $C_{14}-OP_{90-103}$ is required for effect (Fig. 7C). The effect of the two isomers on TER was in close agreement with that observed for the two isomers on mannitol permeability, with a rapid decrease in TER for both isomers after 10 min. Whereas exposure of the L-isomer resulted in a further decrease that leveled off after ~10 min, when the TER was reduced to 30% of control values, the effect of the D-isomer on TER was reversible and returned to values comparable with control values within 20 min (Fig. 7D). In contrast to the results obtained for the racemic mixture of the isomers presented in Fig. 5, no elevation of TER was observed for the separate isomers when added only to the apical side. Thus, the paradoxical increase in TER observed in Fig. 5 most likely arises from the basolateral exposure. Although this result is difficult to explain, one possibility is that the lipopeptide, when added to the basolateral side, either forms clusters within the basolateral space that specifically restrict the diffusion of ions (but not solutes, such as mannitol) or interacts with the basolateral cell membrane components of the tight junctions accessible only from the basolateral side in a more specific way, with the same result.

The functional studies were supported by immunofluorescence studies. Thus occludin was distributed as distinct continuous bands along the cell borders in untreated Caco-2 cell monolayers (Figs. 8, A and D). In Caco-2 cell monolayers exposed to 200 μM L-or D-$C_{14}-OP_{90-103}$ from the apical side, a broader and more diffuse occludin staining was observed in a fraction of the cell borders already after 10 min (Figs. 8, B and C). In agreement with the permeability studies, the altered staining pattern of occludin after 60-min incubation with the D-isomer was reversible and indistinguishable from that of the untreated control cells (Fig. 8E). In contrast, the staining pattern of occludin in Caco-2 cells exposed to the L-isomer remained broad banded and diffuse (Fig. 8F). Interestingly, no redistribution of the rhodamine-phalloidin stained perijunctional actin ring or appearance of stress fibers (Hirase et al., 2001) was observed, indicating that the effect was relatively specific (data not shown). Blinded examination of the occludin-stained samples indicated a redistribution of occludin from the dense and bright perijunctional ring in the untreated control cells to a clear broadening of the occludin ring, resulting in a more diffuse staining at least one cell border after exposure to the isomers. Thus, after 10 min of incubation with the L- and D-isomers, respectively, 13% and 15% of the cells had and altered occludin distribution; after 60 min of incubation, the number of affected cells had increased to 29% after exposure to the L-isomer, whereas the number of affected strands after exposure to the D-isomer had returned to the values of the control cells.

Although changes in the distribution of occludin were apparent after exposure to the L- and D-isomers, no change in the total amount of occludin was detected by Western blot
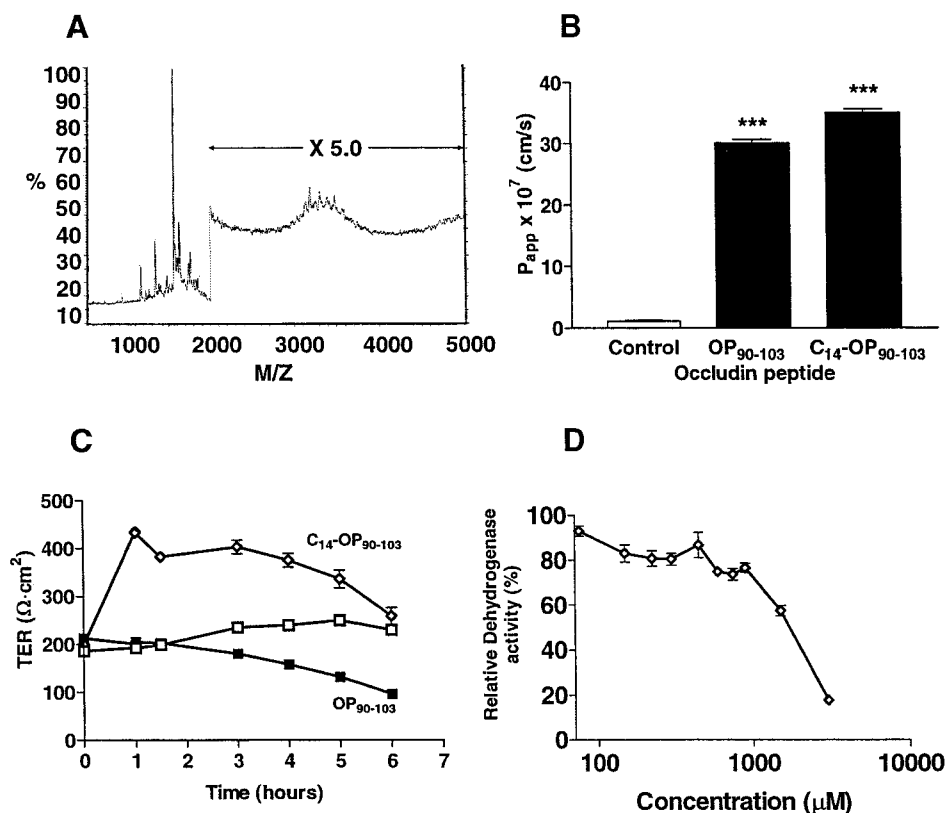
Fig. 5. A, no significant formation of dimers or multimers could be seen for $C_{14}-OP_{90-103}$ (m/z values >2000 are amplified five times). B, OP_{90-103} and $C_{14}-OP_{90-103}$ increased the permeability to mannitol approximately 30- and 35-fold, respectively. C, the change in TER of the Caco-2 cell monolayers in the presence of either 200 μM OP_{90-103} $C_{14}-OP_{90-103}$ added to both the apical and the basolateral side differed, with a gradual decrease in TER for OP_{90-103}, whereas an initial increase in TER, followed by a gradual decrease to levels comparable with control monolayers, was observed for $C_{14}-OP_{90-103}$. D, no short-term toxicity of $C_{14}-OP_{90-103}$ was observed at concentrations as high as 1 mM, as seen by dehydrogenase activity in Caco-2 cells. At higher concentrations, a gradual increase in toxicity was seen. Values are given as mean values ± S.D. ($n = 4$) (B–D). ***, $p < 0.001$ compared with control. n.s., not significant.

after 60 min of incubation (Fig. 8G). Similarly, no difference in occludin expression could be detected after 360 min of incubation with the native peptides OP_{90135} and $OP_{196-243}$ (Fig. 8G).

Discussion

Our finding that the peptides corresponding to the whole, or parts, of the first extracellular loop of occludin increased the tight junction permeability to mannitol in Caco-2 cells, whereas the peptide corresponding to the whole second loop ($OP_{196-243}$) was inactive, is only partly in agreement with published data. For instance, in Wong and Gumbiner (1997), it was reported that the second loop peptide increased tight

Fig. 6. Degradation of C_{14}-OP_{90-103} after apical exposure to Caco-2 cell monolayers. L-C_{14}-OP_{90-103} (■) was degraded more rapidly than the D-isomer (●). No degradation of the L- (□) and D-isomers (○) in HBSS was observed after 6-h incubation. Values are given as mean values ± S.D. ($n = 4$).

junction permeability but that the first loop peptide was inactive. On the other hand, another study reports that peptides corresponding to the first loop of occludin inhibit tight junction resealing after the Ca^{2+} switch (Lacaz-Vieira et al., 1999). The main difference between the two cited studies and results presented here is that the previous studies were carried out using peptides corresponding to chicken occludin and cells originating from *Xenopus laevis* toad kidney epithelia, whereas the peptides in our study correspond to the extracellular loops of human occludin and the cells were of human origin (Caco-2). It is therefore possible that the different results may be related to species differences, especially given that the first extracellular loop of occludin is only 53% conserved between the chicken and human (Ando-Akatsuka et al., 1996). Further support for our finding that OP_{90-135} was active was found in studies showing that the first extracellular loop of occludin is accessible for immunostaining in nonpermeabilized Caco-2 cells and that a peptide corresponding to the first extracellular loop of human occludin inhibits occludin-mediated cell aggregation (Van Itallie and Anderson, 1997).

One fundamental question that arises is how the present and previous (Wong and Gumbiner, 1997; Lacaz-Vieira et al., 1999) results can be reconciled with the knowledge that an occludin-null mouse showed no deficit in intestinal barrier function (Saitou et al., 2000). In this context, we believe that it is important to distinguish between the events occurring during morphogenesis [where the lack of expression of one protein can perhaps be compensated by the expression of other(s)] and the situation described in the present manuscript (where a differentiated and functional protein complex is targeted directly). If occludin is absent during the development of tight junctions (as in the case of the occludin-null
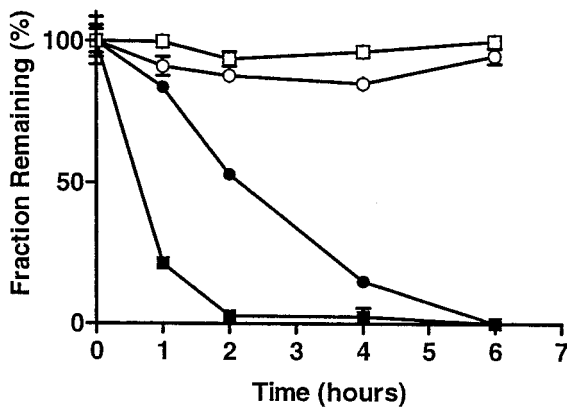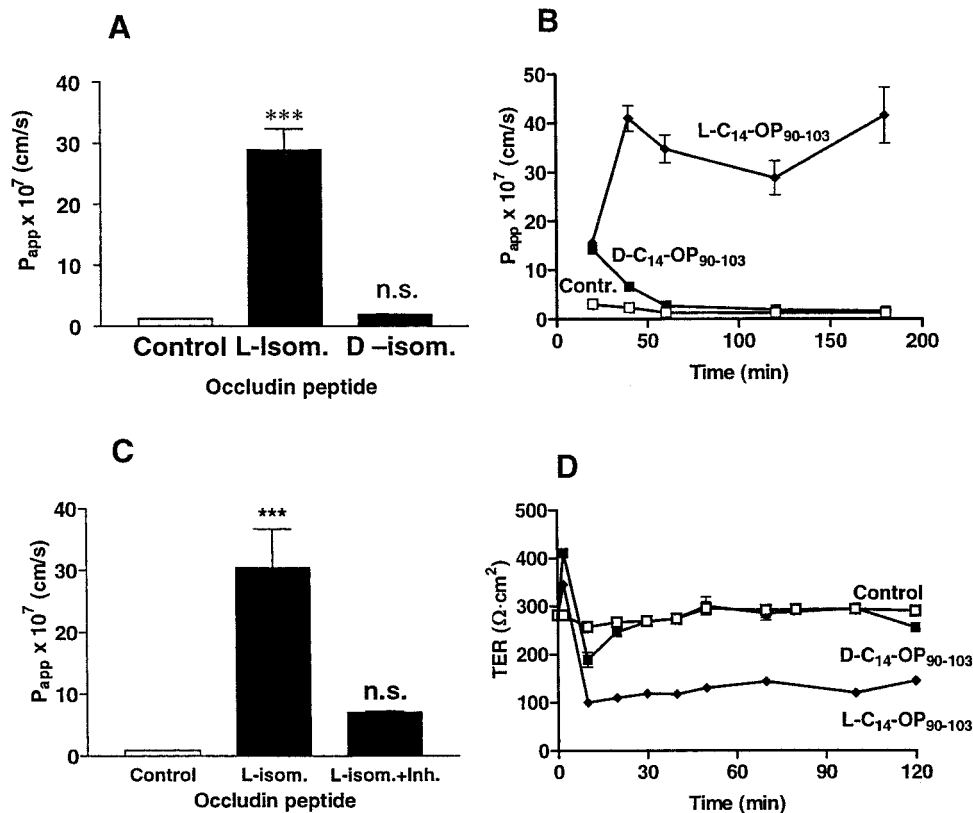
Fig. 7. Apical effects of the L- and D-isomer of C_{14}-OP_{90-103}. A, after 360 min, only L-C_{14}-OP_{90-103} showed significant (30-fold) activity, whereas no activity could be seen for D-C_{14}-OP_{90-103}. B, in time-dependent studies, both L-C_{14}-OP_{90-103} and D-C_{14}-OP_{90-103} (200 μM) induced a rapid, 15-fold increase in mannitol permeability. The activity was further increased to approximately 40-fold after 30 min for the L-isomer, whereas the effect reversed to baseline levels with time for the D-isomer. C, inhibition of L-C_{14}-OP_{90-103} activity by peptidase inhibitors [0.29 mM bestatin, 1 mM diprotin A, and 1 mM captopril (Pauletti et al., 1997)], showing that release of OP_{90-103} from C_{14}-OP_{90-103} is required for effect. D, the effects on TER of the two isomers were in close agreement with those observed for mannitol permeability, with a rapid initial decrease in TER for both isomers, followed by a return to control levels for the D-isomer, whereas the L-isomer maintained a reduced TER for at least 2 h. Values are given as mean values ± S.D. ($n = 4$). ***, $p < 0.001$ compared with control. n.s., not significant.

mice) it can be hypothesized that other components of the tight junctions (e.g., the claudins) are regulated in such a way as to compensate for the lack of occludin. However, because the precise functions of occludin and the individual claudins are incompletely understood, this hypothesis remains to be tested.

Pharmacological agents are generally administered to the luminal side of epithelia covering mucosal surfaces. A prerequisite, therefore, is that they are active from the luminal (apical) side. To increase accessibility of the OPs to the tight junctions from the apical side, we synthesized a lipoamino acid derivative of the shortest active peptide, OP_{90-103}. This conjugate has several potential advantages over the free peptide: first, the conjugate of a lipoamino acid of sufficient length would insert into the cell membrane; second, it would release the active peptide through the action of brush border peptidases (i.e., at the site adjacent to the tight junctions); third, it would limit degradation of the released peptide by apical peptidases; and fourth, it would perhaps also reduce

aggregation. Consequently, we chose a lipoamino acid with a hydrocarbon chain of sufficient length to interact spontaneously with the cell membrane. Only intact released peptide was found after incubation with Caco-2 cells. The use of C_{14}-OP_{90-103} as a prodrug therefore inhibited the peptidase-mediated degradation of OP_{90-103}. A similar inhibition of degradation of the parent peptide by lipoamino acid conjugation has been observed previously, and it has been speculated that the released lipoamino acid inhibits further peptidase activity on the released peptide (Toth, 1994). Although we did not visualize the cellular distribution of the C_{14}-OP_{90-103} conjugate, indirect proof of the necessity of membrane interaction was obtained from experiments using the corresponding conjugate with a shorter lipoamino acid. The shorter C_8-OP_{90-103} thus used was inactive under all conditions.

A prerequisite to in vivo applicability of a tight junction-modulating agent is that it should have a rapid onset of action. If the effect kinetics are too slow, the peptide may be degraded or washed away from the intestinal surface before
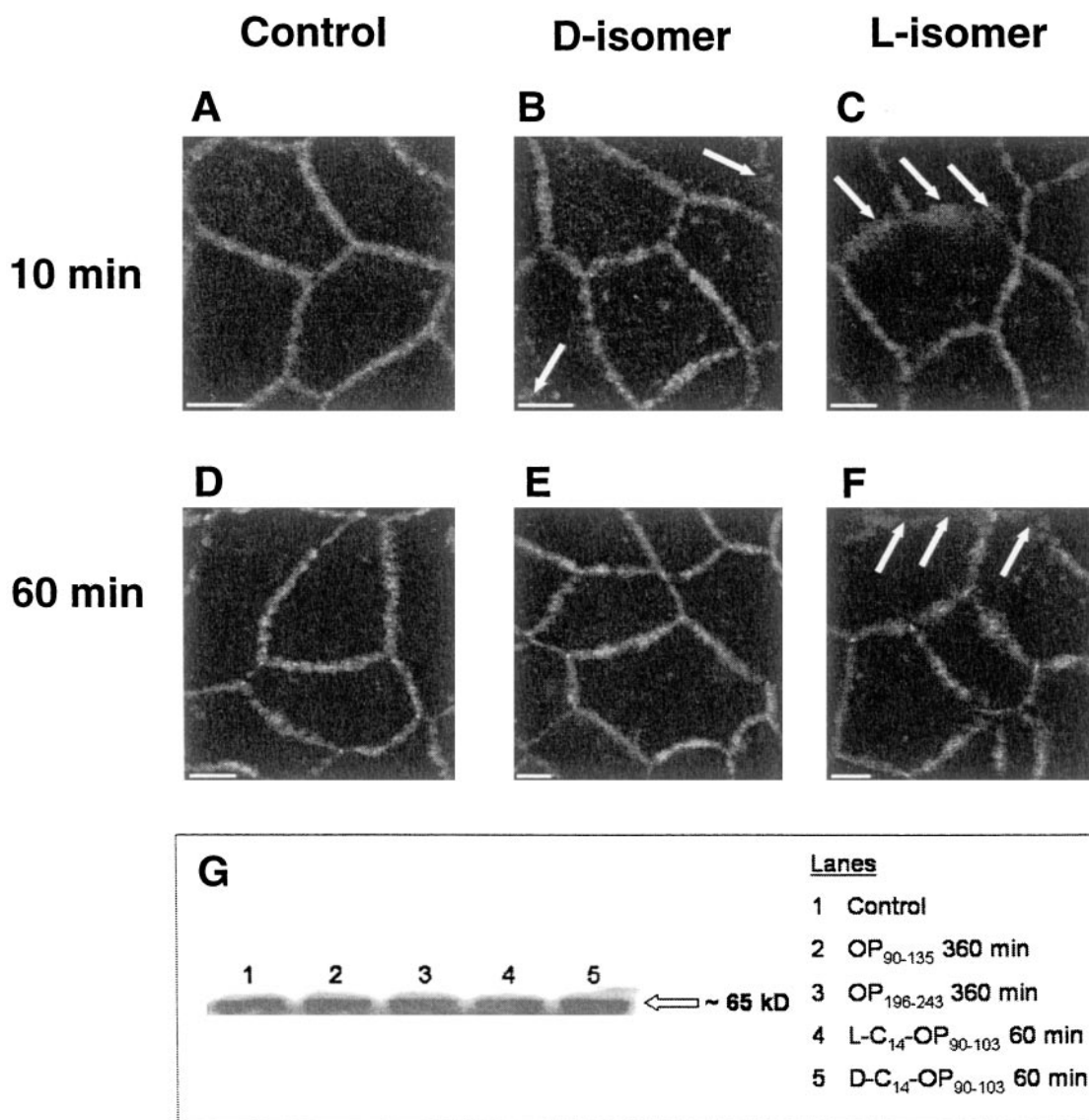
Fig. 8. Immunofluorescence detection of occludin in Caco-2 cell monolayers stained after 10 min (A–C) and after 60 min (D–F). Left, Caco-2 cell monolayers incubated with HBSS without peptides (A and D); middle, Caco-2 cell monolayers exposed to 200 μM D-C_{14}-OP_{90-103} (B and E); right, Caco-2 cell monolayers exposed to 200 μM L-C_{14}-OP_{90-103} (C and F). Areas of diffuse distributions of occludin are highlighted with arrows in the figures. Total levels of occludin in Caco-2 cells after exposure to OPs or C_{14}-OP_{90-103} were no different from the level in unexposed Caco-2 cells (G).

it becomes effective and, more importantly, the coadministered drug will have been transported further away from the site of action of the peptide. All native peptides investigated in this study required up to several hours to significantly enhance the paracellular permeability of the Caco-2 cell monolayers and up to 6 h to obtain more than 10-fold permeability enhancement, which is probably far too slow for any clinical application, such as an additive in a pharmaceutical dosage form.

In contrast to the effects of the native peptides, the effects of the L- and D-isomers of C_{14}-OP$_{90-103}$ were much more rapid but quite different. Conjugation of the C_{14} lipoamino acid to OP$_{90-103}$ also resulted in a much faster onset of action compared with the unconjugated OP$_{90-103}$. This was most probably caused by the concentration of peptide at the cell surface adjacent to the tight junctions, which was mediated by the insertion of the lipoamino acid moiety into the cell membrane. Initially, both isomers increased the tight junction permeability. However, only the L-isomer displayed a sustained effect, whereas the effect of the D-isomer gradually reversed to baseline levels. These results were supported by immunofluorescence studies of the occludin distribution. Thus, alterations in the occludin distribution correlated with the increased paracellular permeability of the tight junctions. The more diffuse staining pattern of occludin after exposure to the L- or D-isomers of C_{14}-OP$_{90-103}$ indicates that the ordered occludin structures observed at the untreated cell borders had been disturbed through the interaction with the occludin peptide. However, disturbance of the ordered occludin distribution was not necessarily expected for a fine-tuned regulation of paracellular permeability mediated by our OPs. In our minds, it is still unclear whether these changes (occurring in minority of the tight junctional strands) are actually the cause of the increase in tight junction permeability or are merely a side effect. However, we note that despite the relatively large changes in occludin distribution that appeared after exposure of the L- or D-isomers of C_{14}-OP$_{90-103}$, all cell junctions remained intact (as seen by actin staining), and no cells lost contact with their neighboring cells. Further studies of the distribution of additional tight junction proteins are required to more precisely investigate the specificity of C_{14}-OP$_{90-103}$ on the protein organization in the tight junctions.

The L-isomer readily and completely released OP$_{90-103}$, whereas the D-isomer released the peptide much more slowly. This fact led us to tentatively conclude that the release of intact peptide is a prerequisite to the sustained effect of C_{14}-OP$_{90-103}$. We found support for this hypothesis when we observed that the effect of L-C_{14}-OP$_{90-103}$ is readily inhibited by addition of peptidase inhibitors. Direct proof of the C_{14} lipoamino acid's inefficacy was obtained from experiments with the C_{14} lipoamino acid alone and in combination with the D-isomer (which also releases the lipoamino acid with time), in which the C_{14} lipoamino acid was inactive and showed a reversible effect, respectively. In addition, we also found that a combination of C_{14} lipoamino acid and OP$_{90-103}$ added to the apical side of Caco-2 cell monolayers was inactive. Obviously, to be active, the lipoamino acid and the peptide have to be presented together as a conjugate to the epithelial cells. This suggests that the lipoamino acid does not trigger the epithelial cells into action such as cytoskeletal

contraction, and that it does not subsequently expose the tight junctions to intervention by the peptides.

In conclusion, we present in this study a new concept for tight junction modulation based on peptides from the first extracellular domain of occludin. A rapid apical effect was obtained after coupling of a lipoamino acid to the N terminus of the peptide. In contrast to results of previous pharmacological approaches to tight junction modulation, the lipopeptides in the present study did not cause short-term toxicity in vitro. The kinetics of the effect of L- and D-diastereomers of C_{14}-OP$_{90-103}$ differed. The derivative D-C_{14}-OP$_{90-103}$ displayed a rapid and transient effect. This is a desirable feature in many respects, because a short duration of action limits unrestricted permeation of potentially toxic agents and also reduces the risk of harmful autoimmune responses. For sustained effect, the lipopeptide must rapidly release intact peptide by a mechanism mediated by apical peptidases, and this was the case only with the L-isomer. Together, these data support our finding that C_{14}-OP$_{90-103}$ may represent a prototype of a new class of tight junction modulators that act on the extracellular domains of tight junction proteins.

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