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Effects of *Escherichia coli* heat-stable enterotoxin and guanylin on the barrier integrity of intestinal epithelial T84 cells

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Abstract

Tight junctions contribute to the formation and establishment of intestinal epithelial barriers against microbial infections. However, a variety of enteric pathogens have developed strategies to adhere to and invade epithelial cells and disrupt epithelial integrity. The aim of this study was to ascertain if enterotoxigenic *E. coli* heat-stable enterotoxin (STa) can cause deterioration of epithelial barrier integrity. Since STa shows amino acid similarity to guanylin, we evaluated the effects of both of these molecules on T84 epithelial cells. T84 epithelial monolayers were grown on 24-well Transwell filters and barrier integrity was assayed by measurement of transepithelial electrical resistance (TER). Macromolecular permeability of the monolayers was determined by measuring the paracellular passage of FITC-labeled dextran 4000 from apical to basolateral compartments of the Transwell filter culture. Treatment of T84 monolayers with either ST or guanylin did not increase paracellular permeability to FITC-dextran. However, although guanylin, which is a physiological guanylate cyclase activator, did not change TER in polarized T84 monolayers, ST did elicit a reduction in TER within 2 h, at concentrations above 4 μM. These data suggest that STa causes not only induction of water secretion but also intestinal barrier dysfunction.

Keywords: enterotoxin; intestinal barrier; tight junction

Abbreviations list

diarrheagenic *E. coli* (DEC), Dulbecco's Modified Eagle's Medium (DMEM), enterotoxigenic *E. coli* (ETEC), heat-stable enterotoxin (STa), tight junction (TJ), transepithelial electrical resistance (TER) heat-labile enterotoxin (LT), ethylene glycol tetra acetic acid (EGTA)

1. Introduction

Escherichia coli (*E. coli*) is the predominant facultative anaerobe of human colonic flora. Generally, most of the *E. coli* strains in the intestinal lumen of healthy people are harmless. However, some *E. coli* clones are virulent and can cause a spectrum of diseases such as sepsis, urinary tract infection, and diarrheal disease. Enterotoxigenic *E. coli* (ETEC) are some of the best-known diarrheagenic *E. coli* in both livestock and humans (Croxen and Finlay, 2010; Turner et al., 2006). These organisms colonize intestinal mucosa using species-specific colonization factors. However, heat-labile enterotoxin (LT) and heat-stable enterotoxin (STa) are present in both animal and human ETEC strains. LT and STa cause an increase in intraepithelial cAMP and cGMP levels, respectively, and subsequently mediate decreased absorption of sodium and chloride ions, and increased secretion of bicarbonate and chloride ions, via activation of the cystic fibrosis transmembrane conductance regulator (Fasano, 1999). Thus, ETEC cause watery diarrhea by changing epithelial function from absorption to secretion.

Tight junctions (TJ) of intestinal epithelial cells contribute to the formation and establishment of barriers between the intestinal lumen and subepithelial tissues. However, a variety of enteric pathogens have developed strategies to disrupt epithelial integrity as well as to adhere to and invade the epithelial cells (Bonazzi and Cossart, 2011). For example, *Clostridium perfringens* and *C. difficile* use toxins to induce the deterioration of tight junctions.

A reduction in transepithelial electric resistance and the paracellular passage of FITC-labeled dextran are recognized as indices of the decreased barrier integrity of epithelial cells infected with microbes. The objectives of this study were to assess whether these indices of barrier integrity could be reduced by STa and to compare the effects of STa on these indices with the effects of guanylin. A comparison with guanylin was carried out because the amino acid sequence

of STa is functionally similar to that of the physiological modulator guanylin, which is known to activate guanylate-cyclase-C in intestinal epithelial cells and to induce water secretion from epithelia (Taxt et al., 2010).

2. Materials and methods

2.1. Epithelial cell lines

The T84 human colon cell line was routinely maintained in medium containing a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan) and Ham's F-12 (Nissui Pharmaceutical) supplemented with 2 mM L-glutamine, 0.15% $NaHCO_3$, 5% FBS (Invitrogen, Grand Island, NY), 63 mg/ml penicillin (Sigma, St. Louis, MO), and 100 mg/ml streptomycin (Sigma). The cells were seeded at high density in 25 cm^2 polystyrene tissue culture flasks and were maintained in humidified incubator with 5% CO_2 at 37 °C. To generate polarized T84 cell monolayers, approximately 4×10^4 cells were seeded onto 0.33 cm^2 polystyrene Transwell permeable support cell culture inserts (0.4 μm pore size; Costar) and were grown for 7-9 days. Transepithelial electrical resistance (TER) was calculated as $\Omega \cdot cm^2$ using the electrical resistance measured with a Millicell Electrical Resistance System (Millipore, Bedford, MA) and the surface area of the filter. Monolayers were used for experiments in which TER was increased up to >1,000 $\Omega \cdot cm^2$.

2.2. Test for epithelial barrier integrity

T84 cells were seeded on 24-well Transwell polyester cell culture inserts as described above. The growth medium in the apical and basolateral compartments of the Transwell inserts were exchanged for DMEM/F12 (1:1) containing 0.5% FBS. After the medium change, T84 cells were maintained in 5% CO_2 at 37 °C for 2 h for equilibration. Peptides or chemicals were then added into the apical compartment of the Transwell inserts and TER was measured. The background reading of a cell-free control filter was subtracted from sample readings. Ethylene glycol tetra acetic acid (EGTA; Kanto Kagaku, Tokyo, Japan) treatment (4 mM) functioned as a positive control for induction of tight junction dilation and forskolin (Wako, Osaka, Japan) was used as a substitute for LT and a positive control for adenylate cyclase activation.

Paracellular macromolecular permeability through T84 cell monolayers was determined by measuring the paracellular passage of FITC-dextran (molecular weight 4 kDa) (Sigma) from apical to basolateral compartments of the Transwell filter culture. The test substance and the FITC-dextran (2 mg/ml) were dissolved in phenol red-free DMEM/F12 (1:1) containing 0.5% FBS and the mixture (150 μl) was loaded onto the apical side of the monolayer. The FITC-dextran concentration in the basolateral compartment was determined longitudinally on the basis of fluorescence intensity analyzed using a spectrofluorophotometer (Wallac 1420 ARVOSX; PerkinElmer Life Sciences, Boston, MA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.3. Assay of cGMP

The cGMP level of T84 cells was measured using the Cyclic GMP EIA Kit (Cayman Chemical C., Ann Arbor, MI) after reaction of the cells with the test substance for 4 h.

3. Results and Discussion

T84 epithelial monolayers were treated with enterotoxin STa, and its effect on TER was compared with the effects of the endogenous peptide guanylin. Forskolin, a well-known cAMP inducer, was used as a positive control for decrease in TER (Fig. 1). STa reduced TER within 2 h at doses ranging between 4×10^{-6} and 10^{-5} M, and this reduction was similar to that of forskolin. In contrast, the guanylin, an endogenous guanylate-cyclase-C ligand, showed no effect on TER. Neither STa nor guanylin increased paracellular permeability as assessed by measurement of the paracellular passage of FITC-dextran although, the FITC-dextran did pass through the epithelia in 8 h when EGTA was added as a positive control of TJ dilation (Fig. 2 A and B). Not only STa but also guanylin increased cellular cGMP within 4 h at doses between 10^{-6} and 10^{-5} M (data not shown).

T84 epithelial monolayers were reconfirmed as a useful in vitro model for the detection of effectors of TJ. Several studies have shown that toxins often have the ability to dilate TJ and increase paracellular permeability (Guttman and Finlay, 2009; Hofman, 2003; Soong et al., 2008). Both STa and forskolin (Fig. 2C) decreased TER but did not allow the passage of dextran-FITC, suggesting that both of these substances induced a moderate increase in paracellular permeability. In contrast, the physiological substance guanylin increased cGMP to the same level as that induced by STa, but did so without affecting TER. The observed effect of STa on TER is likely to play a role in the pathogenesis of ETEC.

It was recently reported that LT could cause not only a reduction in TER but also in the passage of dextran-FITC by disrupting the TJ of T84 cells (Kreisberg et al., 2011); the lack of

dextran passage in the forskolin-treated cells would suggest that LT causes barrier permeability for large molecules in an cAMP independent way. On the other hand, STa-producing ETEC organisms did not affect the TER in that study. Since we observed a decrease in TER only when STa was added to T84 cells at doses of more than 4×10^{-6} M, it is possible that the STa-induced reduction of TER may only occur at the limited number of epithelial sites to which the ETEC organisms adhere and at which they elaborate the STa.

5. Conclusion

Although both STa and guanylin induced cellular cGMP production in T84 epithelial monolayers, only STa reduced barrier integrity. The effect of STa on epithelial tight junctions may contribute to enterotoxic activity.

Acknowledgements

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Conflict of Interest statement

We declare that we have no conflict of interest in connection with this paper.

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Figure legends

Fig. 1. Effect of forskolin, ST and guanylin on longitudinal changes in transepithelial electrical resistance (TER) through T84 monolayers. Forskolin (A), STa (B), or guanylin (C) were added to T84 monolayers and incubated for the indicated times. TER was then measured using a Millicell Electrical Resistance System (Millipore). Forskolin, an adenylate cyclase activator, was used as a substitute for the heat-labile enterotoxin of ETEC. EGTA, which loosens tight junctions, was used as a positive control.

Fig. 2. Effects of ST and guanylin on macromolecular paracellular permeability of T84 monolayers. Cell permeability was longitudinally determined by measuring the paracellular passage of FITC-labelled dextran 4000 from apical to basolateral compartments of the Transwell filter culture after addition of STa (A), guanylin (B) or forskolin (C) to the apical side. EGTA, which loosens tight junctions, was used as a positive control.

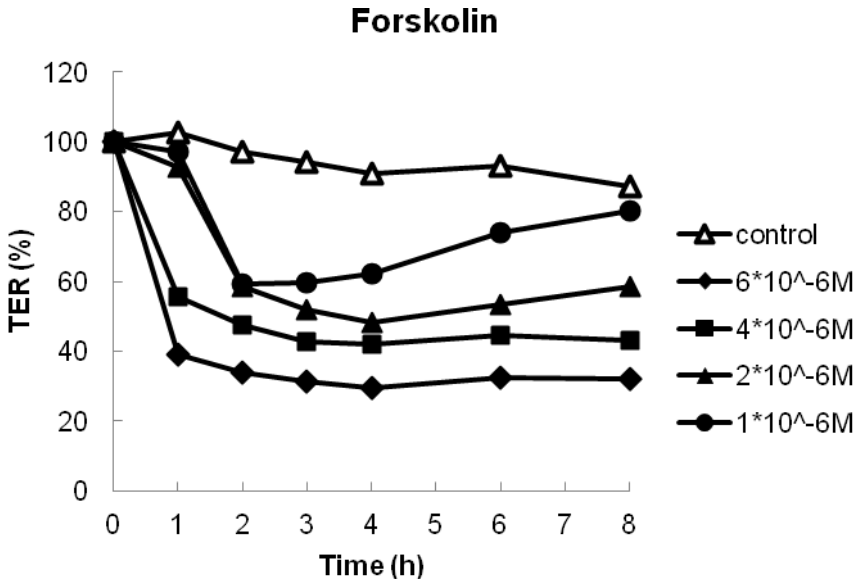
Forskolin
120
100
80
60
40
20
0
TER (%)
0 1 2 3 4 5 6 7 8
Time (h)
control
6*10^-6M
4*10^-6M
2*10^-6M
1*10^-6M

Fig. 1A

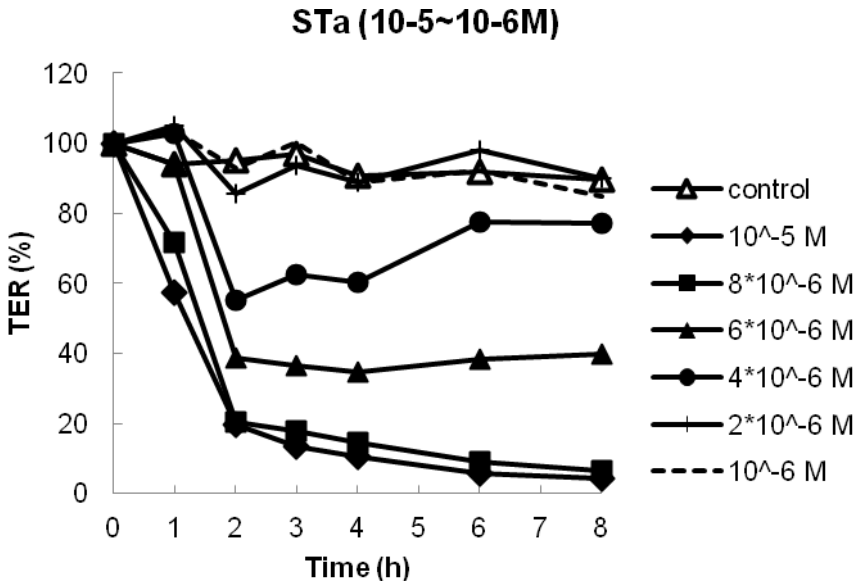
STa (10-5~10-6M)
120
100
80
60
40
20
0
TER (%)
0 1 2 3 4 5 6 7 8
Time (h)
control
10^-5 M
8*10^-6 M
6*10^-6 M
4*10^-6 M
2*10^-6 M
10^-6 M

Fig. 1B

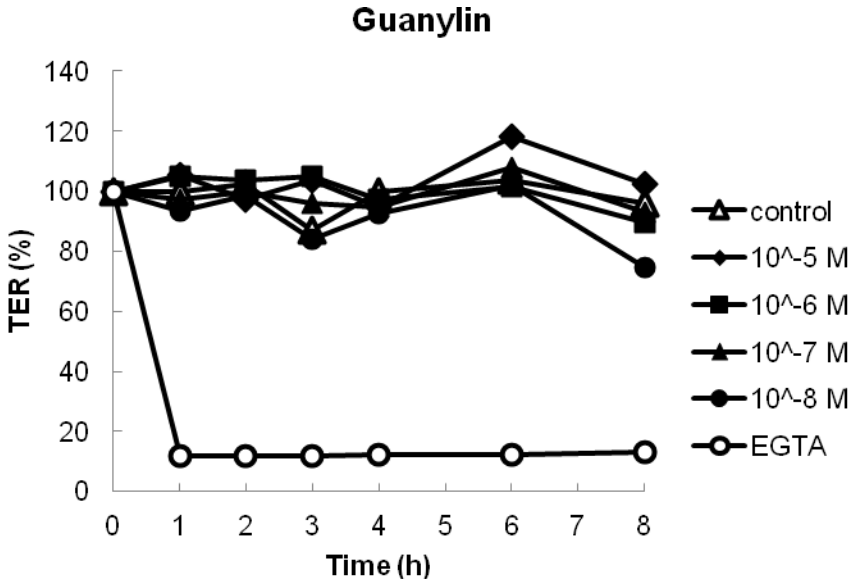
Guanylin
140
120
100
80
60
40
20
0
TER (%)
0
1
2
3
4
5
6
7
8
Time (h)
control
10^-5 M
10^-6 M
10^-7 M
10^-8 M
EGTA

Fig. 1C

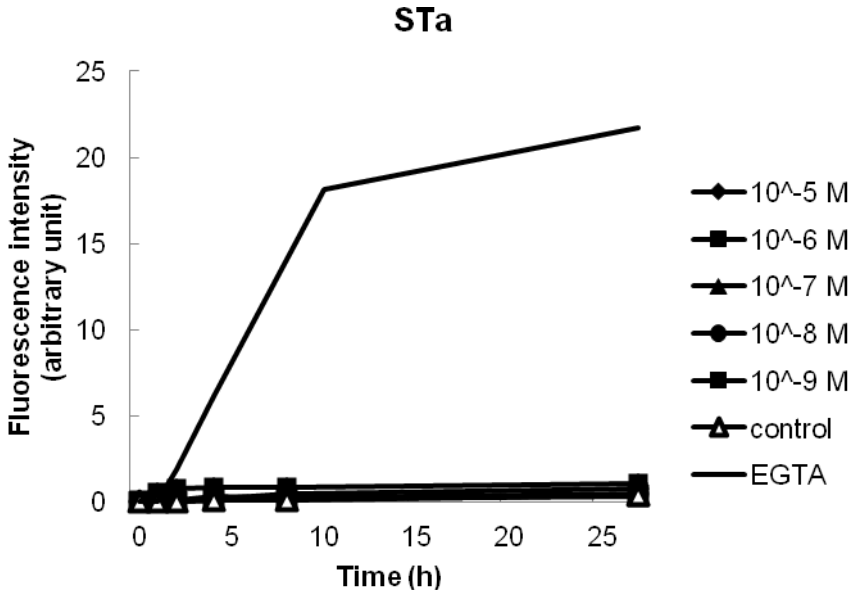
STa
Fluorescence intensity (arbitrary unit)
25
20
15
10
5
0
0
5
10
15
20
25
Time (h)
10^-5 M
10^-6 M
10^-7 M
10^-8 M
10^-9 M
control
EGTA

Fig. 2A

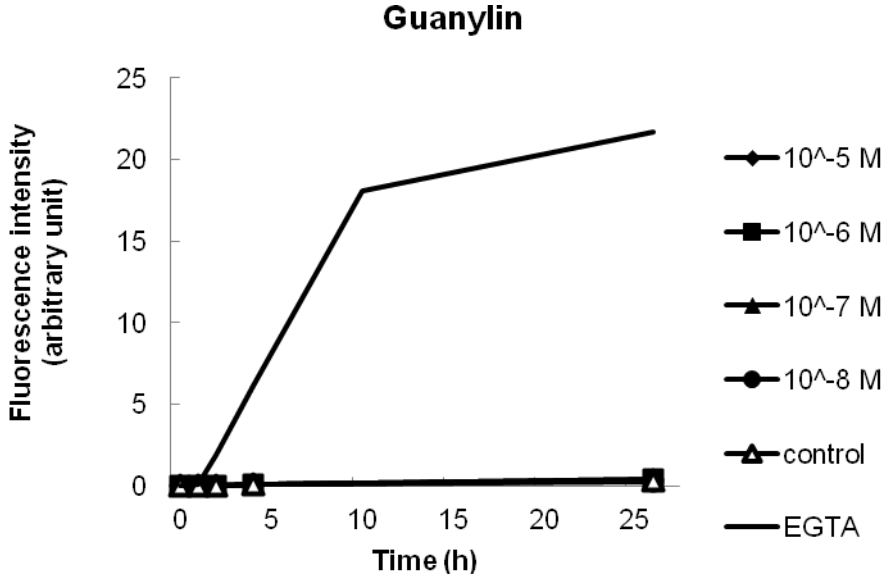
Guanylin
Fluorescence intensity (arbitrary unit)
25
20
15
10
5
0
0
5
10
15
20
25
Time (h)
10^-5 M
10^-6 M
10^-7 M
10^-8 M
control
EGTA

Fig. 2B

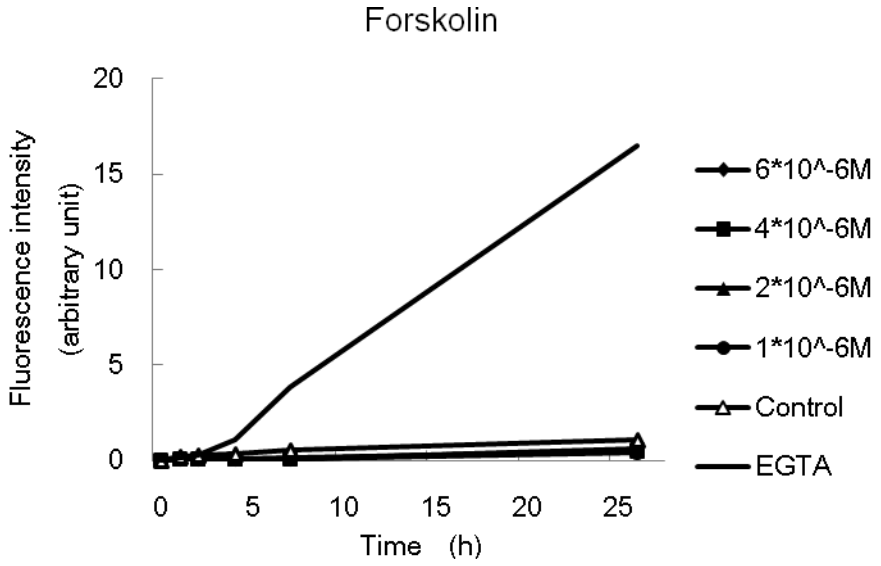
Forskolin
Fluorescence intensity (arbitrary unit)
20
15
10
5
0
0
5
10
15
20
25
Time (h)
6*10^-6M
4*10^-6M
2*10^-6M
1*10^-6M
Control
EGTA

Fig. 2C