**ABSTRACT** The present report demonstrates the effect of α toxin from *Clostridium perfringens* on electrophysiological indexes of jejunal mucosa from laying hens pretreated with inulin and N-acetyl-l-cysteine (ACC), a mucolytic agent. In a first set of experiments, the effect of α toxin with or without pretreatment with ACC on the electrophysiological parameters was determined when jejunal tissues from laying hens were mounted in Ussing chambers. The short-circuit current remained unchanged when α toxin was added mucosally in the tissues whether pretreated with ACC or not. The change in the transmural tissue conductance (∆Gt) was higher (P = 0.18) after 90 min exposure of toxin independent of pretreatment with ACC. The effect of α toxin on ∆Gt became significant (P ≤ 0.05) after 120 min of incubation. In the second set of experiments, the effect of α toxin on the jejunal tissues preincubated with inulin (0.1%) was investigated. The effect of toxin was also time dependent, and ∆Gt became significantly higher (P ≤ 0.05) after 120 min of incubation independent of preinubation with inulin. Inulin did not influence the ∆Gt during the experimental period when compared with control tissues. In conclusion, α toxin from *C. perfringens* can impair the intestinal mucosal barrier. The effect is obviously not dependent on the presence of a mucolytic agent nor can it be affected by direct addition of inulin under in vitro conditions. Whether there is an effect of inulin after long-term supplementation in feeding trials or it is due to fermentation bacterial metabolites remains an open question.

**Key words:** alpha toxin, *Clostridium perfringens*, poultry, Ussing chamber, inulin, N-acetyl-l-cysteine

**INTRODUCTION**

*Clostridium perfringens*, a low G+C gram-positive anaerobic spore-forming bacterium, produces different toxins and the expression pattern is the basis for the commonly used toxin typing scheme that assigns *C. perfringens* isolates to 1 of 5 types (Type A-E) based upon their production of α, β, epsilon, and iota toxins (McDonel, 1980). The α toxin from *C. perfringens* has a phospholipase C activity and exhibits hemolytic, necrotic, vascular permeabilizing, and platelet aggregating properties (Titball et al., 1999). It hydrolyzes the phosphatidylcholine and sphingomyelin moieties in the presence of calcium ions and promotes membrane disorganization (Titball et al., 1999). Inoculation of α toxin into the ligated intestinal loops of adult pigs failed to induce substantial lesions or fluid loss (Jestin et al., 1985). However, when administered intragastrically to neonatal piglets, α toxin caused edema of villi and neutrophilic inflammation of the small intestine (Johannsen et al., 1993a,b). Administration of semipurified ultrafiltered α toxin in ovine ileal and colonic loops induced accumulation of fluid in the lumen (Miyakawa and Uzal, 2005).

In poultry, the functional importance of α toxin in the pathogenesis of necrotic enteritis (NE) is unclear. Intraduodenal infusion or oral inoculation of α toxin in chickens induced necrosis of the small intestinal epithelium (Al-Sheikhly and Truscott, 1977). In one report, no difference in the α toxin levels was noted when in vitro α toxin levels were compared between isolates from diseased and healthy birds (Gholamiandekhordi et al., 2006). In another recent study, it was demonstrated that α toxin is not an essential virulence factor in the pathogenicity of NE (Keyburn et al., 2006). Serosal addition of α toxin to rat colon induced a biphasic increase of short circuit current (Isc), whereas mucosal addition of α toxin did not induce any change in Isc (Diener et al., 1991; Hug et al., 1996). Previously,
we found similar results when jejunal mucosal sheets from laying hens were mounted in the Ussing chambers. However, sodium-dependent glucose transport was diminished in the jejunum of birds preincubated with α toxin compared with the jejunal tissues that were not preincubated with α toxin (Rehman et al., 2006). We hypothesized that the effect of mucosally added α toxin on Isc or on the glucose transport might be due to production of mucus that could hinder the penetration of toxin across the mucosal wall. Therefore, in the present study, we treated mucosal sheets with ACC, a sulphydryl reagent that has been reported to destroy the cross-linkages of mucus when added in the mucosal compartment of Ussing chambers (Greger et al., 1991).

Inulin, a prebiotic extracted from chicory (Cichorium intybus), contains both oligosaccharide and polysaccharides components. Because of β (2→1) glycosidic bond, it is resistant to host-derived digestive enzymes and is believed to enhance the growth of health-promoting bacteria. In our previous study, we found a lower transmural tissue resistance in the jejunal sheets from broilers fed a diet supplemented with 1% inulin (Rehman et al., 2007). We were not sure whether the lower transmural tissue resistance was due to direct effect of inulin (interaction with enterocytes) or indirectly due to bacterial fermentation metabolites that have been found to be influenced by dietary inulin (Rehman et al., 2008a,b). The bacterial metabolites may cause irritation to the intestinal mucosa that could lead to impaired intestinal barrier functions (Bruggencate et al., 2005). Mucosal application of different nondigestible saccharides has been reported to increase the paracellular permeability of Caco-2 cell monolayer planted in Ussing chambers (Suzuki and Hara, 2004). Addition of inulin in the mucosal compartment of Ussing chambers has been shown to increase the transport of calcium across the intestinal mucosa of cecum and distal colon in rats by increasing paracellular permeability (Raschka and Daniel, 2005).

Therefore, the objective was to study in association with C. perfringens α toxin 1) whether there is any role of mucus, 2) whether inulin affects the transmural tissue conductivity directly, and 3) whether inulin can modulate the transmural tissue conductivity in toxin-exposed tissue.

**MATERIALS AND METHODS**

**Birds, Housing, and Diet**

Lohmann Brown laying hens 40 wk of age (n = 30) were purchased from a commercial farm (Cobb Germany Avimex GmbH, Germany) and were housed individually in cages. The birds were fed a basal diet primarily composed of wheat and corn. The diet contained 17.1% crude protein, 2.8% crude fat, 5.2% crude fiber, and 0.4% methionine and met the recommendation for laying hens (Gesellschaft für Ernährungsphysiologie, 1999). The light:dark schedule was 16L:8D. Birds had free access to feed and water.

**Tissue Preparation**

The birds were killed by stunning and bleeding. The jejunum was removed and rinsed several times with ice-cold Krebs-Henseleit (KH) buffer (4°C). The KH buffer contained 2.0 g/L of d-glucose. Sodium chloride and sodium bicarbonate were added at the time of preparation of buffer. The segments were taken from 5 cm proximal to Meckel’s diverticulum, and tissue flaps were prepared as described earlier (Rehman et al., 2006, 2007). Tissues were placed in the same cold KH buffer and gassed with carbogen (O2/CO2, ratio 95:5) until they were mounted in Ussing chambers.

**Chemicals**

All chemicals were purchased from Sigma-Aldrich, Steinheim, Germany. Alpha toxin (Phospholipase C, EC 3.1.4.3, Type 1) from C. perfringens was dissolved in KH buffer. The specific activity of toxin was 5.9 units/mg of solid. The ACC, inulin and mannitol solutions were prepared in KH buffer, whereas ouabain solution was prepared in Ringer solution. The inulin from chicory was 99% pure and contained less than 0.05% of glucose and 0.05% of fructose according to technical report of the manufacturer.

**Measurements of Electrophysiological Traits**

The Isc and transmural tissue conductance (Gt) were measured in Ussing chambers with a microprocessor system based on a voltage/current clamp device (Müslar, Microclamp, Aachen, Germany). The modified Ussing chambers were connected with a computer interface that allowed for real-time data acquisition. The epithelial sheets were mounted in the modified Ussing chambers with an exposed tissue area of 0.9 cm2. The serosal and mucosal surfaces of the tissues were bathed in 15 mL of KH buffer (pH 7.4). The bathing medium in the chambers was aerated with 95% O2 and 5% CO2 and maintained at 39°C in a water bath. The solution was continually stirred and oxygenated by bubbling into the chamber by means of a gas lift. The electrode potential and the solution resistance were determined at the beginning of every experiment and were automatically corrected before tissues were placed in the chambers. The tissues were first incubated under open circuit conditions for 30 min for equilibration and were short-circuited afterward by clamping the voltage at 0 mV. The chemicals were added when the base line was achieved. Variations in the electric parameters were measured continuously for 120 min after the addition of toxins. The effects of the chemicals on the intestinal electrophysiology were compared using ΔIsc and ΔGt.
where $\Delta I_{\text{sc}} = (I_{\text{sc}} \text{ at time } t) - (I_{\text{sc}} \text{ at time zero})$ and
where $\Delta G_{t} = (G_{t} \text{ at time } t) - (G_{t} \text{ at time zero})$.

**Experimental Design**

**Experiment 1.** This experiment was conducted to determine the effect of ACC (10 mmol/L) in combination with $\alpha$ toxin on the electrophysiological indexes of jejunal sheets mounted in the Ussing chambers. For equilibration, mannitol (10 mmol/L) was also added in the serosal compartment. The $\alpha$ toxin (100 U/L) was added on the mucosal side after 30 min incubation with ACC. Preliminary experiments revealed that addition of ACC in the mucosal compartment of Ussing chambers did not elicit any variation in the electric parameters of the jejunal tissues.

**Experiment 2.** This experiment aimed to investigate the effect of $\alpha$ toxin on the electrophysiological indexes in the jejunal mucosa preincubated with inulin (0.1%) on the mucosal side. The $\alpha$ toxin (100 U/L) was added after 30 min preincubation of tissues with inulin. No attempt was carried out to adjust the osmolality of the buffer in both compartments after the additions of inulin because it has been reported that inulin (1.0%) did not change the osmolality of the Ussing buffer when added on the mucosal side (Raschka and Daniel, 2005).

After the experiments, the vitality of tissues was checked by adding ouabain (10 mmol/L), a Na$^{+}$-K$^{+}$-AT-Pase inhibitor, in the serosal compartment resulting in a decreased $I_{\text{sc}}$ within 5 min after addition (data not shown).

**Statistical Analysis**

Statistical program SPSS (version 12; SPSS GmbH, SPSS Inc., Munich, Germany) was used for data analysis. The Kolmogorov-Smirnov test was used to test the normal distribution of the data. Results were expressed as means ± SEM. One-way ANOVA was employed to compare differences. The group differences were determined by the Duncan’s multiple range test at $P \leq 0.05$.

**RESULTS**

Addition of $\alpha$ toxin in the mucosal compartment ($t = 0$) with tissue pretreated with ACC or not showed no change in the $I_{\text{sc}}$ throughout the experimental period (data not shown). The basal values of $G_{t}$ of control group (3.16 ± 0.62) was comparable ($P = 0.68$) with toxin (3.48 ± 0.44) or toxin plus ACC pretreated tissues (3.90 ± 0.64). The influence of toxin on $\Delta G_{t}$ was time-dependent. After 90 min of exposure, $\Delta G_{t}$ tended to be higher ($P = 0.18$) in tissues treated with $\alpha$ toxin and $\alpha$ toxin plus ACC compared with control tissues that were not treated with toxin (Figure 1). However, the effect of toxin became statistically significant ($P \leq 0.05$) after 120 min of toxin exposure because $\Delta G_{t}$ was significantly higher ($P \leq 0.05$) in toxin or toxin plus ACC-treated tissues compared with control (Figure 1).

In the second experiment, there was no significant difference in the basal value of $G_{t}$ for control group (4.9 ± 0.74) or tissue treated with inulin (5.72 ± 0.59) or $\alpha$ toxin (3.83 ± 0.50) or $\alpha$ toxin plus inulin (4.06 ±
0.42). Neither inulin nor toxin affected the Isc during the course of the experiment (data not shown). Like in the first experiment, the ∆Gt, at \( t = 90 \), tended to be higher (\( P = 0.10 \)) in the toxin and toxin plus inulin treated tissues compared with tissues that were either not treated (control) or treated with inulin only (Figure 2). The transmural conductivity went on increasing and ∆Gt became higher (\( P \leq 0.05 \)) in α toxin-treated tissues at the end of experiment (Figure 2).

**DISCUSSION**

The present study reveals that mucosal application of α toxin in the Ussing chamber alone or with ACC failed to affect Isc, which is in accordance with our previous findings (Rehman et al., 2006). It has been reported that mucosal addition of α toxin in rat colon did not induce any change in Isc, but a biphasic increase of Isc was observed when toxin was added serosally (Diener et al., 1991; Hug et al., 1996). In the present study, mucosally added α toxin induced changes in the transmural conductivity that became evident after 90 min of incubation. The ∆Gt continued to increase with time of incubation and finally statistically higher ∆Gt (\( P \leq 0.05 \)) was obtained in toxin-treated tissue compared with control tissue independent of pretreatment with inulin or ACC at \( t = 120 \) min (Figures 1 and 2). The transmural tissue conductivity of stripped epithelia results from the contribution of the transcellular and paracellular pathways (Frizzell and Schultz, 1972). Therefore, the results may either be explained by respective alterations of cellular or paracellular conductance, or both. In the present study, we were unable to conclude whether one or both pathways were affected by toxin treatment.

The regulation of intestinal transport functions mainly depends on the junctional complex connecting enterocytes together (Pácha, 2000) that also determines the extent to which solutes and water are absorbed or secreted. The tight junctions selectively control the passive diffusion of ions and other small solutes through the paracellular pathway. The presence of paracellular pathways with high permeability, as in the small intestine, permits rapid transepithelial diffusion and precludes the presence of transepithelial electrical potential. The impaired intestinal barrier facilitates the transport of antigenic and toxic substances across the intestinal mucosa. In rats, it has been suggested that the phospholipase C activity of α toxin impaired the intestinal mucosal barrier and increased the permeability of the intestine through activation of phospholipase \( \Lambda_2 \) (Otamiri, 1989). Collier et al. (2003) observed that paracellular permeability was higher in tissues from chickens infected with *C. perfringens* than those infected chickens treated with tylosin, a drug that decreases the intestinal concentrations of *C. perfringens*. It can be assumed that interaction of α toxin with enterocytes results in the disruption of intercellular tight junction that could alter the intercellular permeability. The disruption of intercellular tight junction could result in higher ∆Gt. It has been demonstrated that addition of α toxin in the mucosal bathing of Ussing chambers mounted with ovine intestinal tissue resulted in a biphasic decrease of net transepithelial water flux (Miyakawa and Uzal, 2005). The higher ∆Gt in the present study and diminished sodium-dependent glucose transport...
described in a previous study (Rehman et al., 2006) in the toxin-treated jejunal flaps may partly explain reabsorption-secretion imbalance induced by α toxin.

Dietary inulin has been found to increase jejunal acetate and lactate and cecal butyrate levels in broilers (Rehman et al., 2008a,b). Dietary inulin decreased the transmural tissue resistance when jejunal tissue flaps from inulin-fed broilers were mounted in Ussing chambers (Rehman et al., 2007). In vitro addition of non-digestible saccharides, disaccharides, oligosaccharides, and inulin (1%) to rat intestinal tissues mounted in Ussing chambers increased the paracellular permeability resulting in a higher calcium conductance (Mineo et al., 2001, 2004; Raschka and Daniel, 2005). Mineo et al. (2002) determined the permeability of lucifer yellow (paracellular passage marker) and transepithelial electrical resistance of the intestinal mucosa. Mucosal addition of melibiose, difructose anhydride III, or difructose anhydride IV increased lucifer yellow permeability and decreased electrical resistance in the cecum and colon flaps of rats planted in Ussing chambers. However, this effect could not be observed in the jejunal flaps with melibiose and difructose anhydride III. This suggests that some oligosaccharides may directly interact with tight junction and increase the permeability of tissues. However, in the current study, transmural tissue conductance remained unaffected by the mucosal addition of inulin (0.1%). It can be hypothesized that the lower concentration of inulin might have been insufficient to stimulate a “sensory system,” if present, in the intestinal tissues as proposed by Suzuki and Hara (2004) in the Caco-2 cell monolayer. Another reason could be that inulin affects the transmural tissue conductance indirectly mainly in association with intestinal microflora and bacterial fermentation metabolites that may affect the intestinal mucosa resulting in higher transmural tissue conductance.

In conclusion, mucosal addition of α toxin increased the transmural tissue conductance independent of pretreatment with N-acetyl-L-cysteine or inulin. Inulin had no potential influence on the electrophysiological parameters under present experimental conditions. Further studies are needed to investigate molecular mechanisms involved in the α toxin-induced impairment of intestinal barrier.

REFERENCES


Rehman, H., J. Böhm, and J. Zentek. 2008a. Effects of differentially fermentable carbohydrates on the microbial fermentation profile of