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Studies on the Mechanism of Diarrhoea Induced by *Escherichia coli* Heat-Stable Enterotoxin (STa) in Newborn Calves

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ABSTRACT

Enterotoxigenic *Escherichia coli* (ETEC) produces a heat-stable enterotoxin (STa) that binds to and activates a putative intestinal receptor, guanylate cyclase, causing an increase in the intracellular levels of cyclic guanosine monophosphate (cGMP). Using flow cytometry and ^{125}I-STa binding assays, we studied the distribution of STa-receptors on enterocytes isolated from different segments of the newborn calf's intestinal tract. We also investigated the effect of STa on the intracellular levels of cGMP and ion transport to the intestinal lumen. More STa-receptors were found on enterocytes prepared from the ileum than on enterocytes obtained from the other segments of the intestinal tract. Guanylate cyclase activity was higher in the ileum of STa-challenged calves than in the ileum of control calves. No changes were observed in the guanylate cyclase activity of the other intestinal segments of the STa-challenged and control calves. Na^+ levels, as measured by atomic absorption spectroscopy, were significantly increased in the luminal contents of the ileum of STa-challenged calves, whereas serum Cl^- levels were significantly lower in the STa-challenged calves than in control calves. This study supports previous observations on the role of guanylate cyclase in the initiation of STa-induced secretory diarrhoea and suggests that Na^+/Cl^- coupling may be the major mechanism for the loss of ions in the diarrhoeal response that is mostly induced in the ileum of newborn calves.

Keywords: calf, *Escherichia coli*, enterocyte, flow cytometry, guanylate cyclase, heat-stable enterotoxin, ionic flux, neonate

Abbreviations: BBM, brush border membrane; BSA, bovine serum albumin; cGMP, cyclic guanosine monophosphate; ETEC, enterotoxigenic *Escherichia coli*; GTP, guanosine 5′-triphosphate; HPLC, high-performance liquid chromatography; I-STa, iodinated STa; FITC, fluorescein isothiocyanate; LT, heat-labile enterotoxin; ST, heat-stable enterotoxin; STa, methanol-soluble ST; STb, methanol-insoluble ST

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of colibacillosis in calves (House, 1978; Roussel *et al.*, 1988). The incidence of diarrhoea caused by ETEC varies widely between herds and probably is affected by geographic, managemental, seasonal, and diagnostic variables (Acres, 1985). Strains of ETEC that cause diarrhoea in young farm animals have pili that facilitate adhesion to the intestinal mucosa and produce enterotoxins that cause a net secretion of fluid and electrolytes (Acres, 1985; Dean and Isaacson, 1985; Roussel *et al.*, 1988; Jaso-Friedmann *et al.*, 1992). ETEC produces two types of enterotoxins, heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT). LT has been studied extensively and found to be similar to cholera toxin in its structure and function (Sears and Kaper, 1996). On the other hand, ST is unique in its structure and function and consists of two subtypes, STa (methanol-soluble) and STb (methanol-insoluble) (Sack, 1980; Sears and Kaper, 1996). Most ETEC isolated from calves produce mainly STa (Acres, 1985). STa has a low molecular weight (2 kDa), is poorly immunogenic, and induces a secretory response in infant mice (Chan *et al.*, 1983; Acres, 1985; Roussel *et al.*, 1988, 1993).

STa induces secretory diarrhoea by stimulating intestinal guanylate cyclase (Field *et al.*, 1978; Rao *et al.*, 1981; Epstein *et al.*, 1986). STa binds to a specific receptor, guanylate cyclase-C, present on the apical surface of enterocytes and induces intestinal secretion through an increase in the level of cyclic guanosine monophosphate (cGMP) in affected enterocytes (Giannella *et al.*, 1983; Schulz *et al.*, 1990; Cohen *et al.*, 1993). STa-receptors are found throughout the small intestine and the colon of rats and pigs (Krause *et al.*, 1994). STa-induced diarrhoea is associated with enhanced intestinal secretion of Cl^- and water (Kuhn *et al.*, 1994). Increased secretion of Cl^- from target tissues exposed to STa has been directly demonstrated in the cultured human colon carcinoma cell line T84 and in isolated rat intestinal mucosa maintained in the Ussing chamber (Rao *et al.*, 1981; Guandalini *et al.*, 1982; Argenzio *et al.*, 1984). Studies of electrolyte transport in ligated jejunal loops of weanling pigs have also demonstrated an increased net secretion of Na^+ and Cl^- in the presence of crude preparations of STa or cholera toxin (Argenzio *et al.*, 1984).

In this study, using flow cytometry, we studied the distribution of STa-receptors throughout the intestinal tract of newborn calves. In addition, we investigated the effect of STa on brush border membrane (BBM) guanylate cyclase activity in different segments of the small and large intestine of newborn calves. We also measured ion transport to the intestinal lumen in STa-challenged and control newborn calves.

MATERIALS AND METHODS

Experimental animals

Six male 1 to 4-day-old colostrum-fed Holstein calves were used. Three of the calves were challenged orally with 2 mg of STa mixed with 100 ml of 10% glucose solution. The rest of the calves received 100 ml of 10% glucose solution. The animals were

monitored for the onset of diarrhoea. After 2 h, the calves were euthanized by intravenous injection of Beuthonasia-D Special (Schering-Plough Animal Health Corp., Kenilworth, NJ, USA). Each ml of this solution contains 390 mg pentobarbital sodium and 50 mg phenytoin sodium. Each calf received 1 ml/kg body weight.

Preparation of calf enterocytes and brush border membranes

Twenty centimetres from the middle of each intestinal segment (anterior jejunum, posterior jejunum, ileum and colon) were thoroughly washed with ice-cold phosphate-buffered saline (PBS), 0.01 mol/L, pH 7.2. The segments were filled with ice-cold PBS that contained 1.5 mmol/L ethylenediaminetetraacetic acid and 0.5 mmol/L dithiothreitol and were then incubated on ice for 2 h. The segments were gently agitated every 15 min. The enterocytes were harvested by centrifugation at 1000*g* for 15 min and were resuspended in PBS containing 1% bovine serum albumin (BSA). Enterocyte counts and viability were determined after exclusion of 0.2% trypan blue-stained enterocytes by microscopic examination. Enterocyte populations with $>70\%$ viable cells were used for the flow cytometry and ^{125}I-STa binding assays.

Brush border membranes were prepared by scraping the intestinal mucosa from the different segments of the calf intestine. Scrapings were suspended in homogenate medium (50 mmol/L mannitol, 2 mmol/L Tris-HCl, pH 7.4) at a ratio of 20 ml of medium per gram of mucosal scrapings. The scrapings were homogenized for 60 s at setting 3 of an Omni-2000 homogenizer (Omni International, Waterburg, CT, USA) and centrifuged for 15 min at 200*g*. $MgCl_2$ was added to the supernatant to give a final concentration of 10 mmol/L, and the supernatant was stirred in the cold room (4°C) for 15 min and then centrifuged for 15 min at 2400*g* to pellet the precipitate. The resultant supernatant was centrifuged for 30 min at 19 000*g* to generate the crude BBM. The supernatant was poured off, and 6 ml of resuspension medium (300 mmol/L mannitol, 50 mmol/L Hepes-Tris, pH 7.0) was divided between the centrifuge tubes. The pellets were resuspended by repeated passage through a 26-gauge needle, pooled, divided into 0.25-ml aliquots, and frozen at –120°C until the day of use.

Heat-stable enterotoxin of Escherichia coli

STa was produced and purified as described by Al-Majali and colleagues (1999a).

Flow cytometry

Enterocytes isolated from different segments of calf intestine were processed for flow cytometry as described previously (Al-Majali *et al.*, 1999a). Briefly, the enterocytes were washed twice with PBS containing 0.5% BSA. They were then incubated with 50 µl of HPLC-STa at 37°C for 45 min. After washing with PBS, the cells were incubated at 4°C for 30 min with 50 µl of 1:10 diluted STa-specific rabbit antiserum prepared as

described previously (Al-Majali *et al.*, 1999a). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit-IgG (KPL, Gaithersburg, MD, USA), 50 µl diluted 1:100 in PBS, was added to the enterocyte suspensions and incubated for 30 min on ice. The enterocytes were then washed three times with PBS-BSA, resuspended in 1.0 ml of PBS, and kept on ice until flow-cytometric analysis was performed. As negative controls, similar samples (but without STa) were incubated with STa-antibody and FITC-conjugated anti-rabbit-IgG antibody and used to determine the threshold of specific staining. Flow-cytometric analysis was performed using an Epics ELITE flow cytometer (Coulter Electronics, Hileah, FL, USA). The flow cytometer was set to read 10 000 cells from each enterocyte preparation, FITC-stained cells were excited using 15 mW of 488 nm argon laser light. FITC-conjugated beads had previously been run through the cytometer and the mean fluorescence intensity set at a fixed value.

Radiolabelled STa binding assay

STa was iodinated and separated from free iodine as previously described (Al-Majali *et al.*, 1999b). Reaction mixtures containing calf enterocytes (1×10^4), PBS-BSA and ^{125}I-STa (20–640 nmol/L) were incubated in a final volume of 200 µl for 40 min at 37°C in a shaking water bath. Unbound ^{125}I-STa was removed from bound ^{125}I-STa by vacuum filtration (Millipore Corp., Bedford, MA, USA) using 1 µm, 2.5 cm GF/B glass filters (Whatman, Maidstone, UK). The total binding was measured in a reaction mixture that did not contain the unlabelled STa, whereas nonspecific binding was measured in a reaction mixture that contained the labelled STa plus a 1000-fold excess of unlabelled STa. Specific binding was calculated by subtracting nonspecific binding from the total binding. Scatchard plots for the ^{125}I-STa specific binding data were constructed by plotting the bound ^{125}I-STa against the ratio between the bound and the free ^{125}I-STa. The dissociation constant (K_d) and the maximum number of STa receptors (B_{max}) were then calculated using the Rosenthal–Scatchard equation (Scatchard, 1949; Matthews, 1993).

Guanylate cyclase assay

The guanylate cyclase activity associated with the brush border membrane of the different segments of the calf intestine was assayed as described previously (Kimura and Murad, 1974). Briefly, the guanylate cyclase was assayed in a reaction mixture containing 50–100 µg BBM, 5 µmol Tris-hydrochloride (pH 7.4), 1 µmol theophylline, 0.75 µmol phosphocreatine, 3.5 U creatine phosphokinase, 0.1 µmol GTP, 0.5 µmol $MgCl_2$, and 0–400 µg STa per ml in a total volume of 0.1 ml. The reaction was started by the addition of the GTP-$MgCl_2$ and was stopped, after 10 min incubation at 37°C, by the addition of 0.1 ml of 0.5 mol/L sodium acetate (pH 4.0). The cGMP formed was measured by EIA using a cGMP correlate kit (Assay Designs, Inc., Ann Arbor, MI, USA).

Atomic absorption spectrometry

The contents from the different intestinal segments of the two groups of calves were diluted 1:1 in water and freeze dried. Samples were ashed in a high-temperature oven before digestion in acid to dissolve metal ions. The digests were quantitatively taken up in acid to known volumes. Atomic absorption analysis was performed on these solutions, with quantification based on a standard curve for each element determined. These analyses were performed by the Atomic Absorption Laboratory, Purdue University. Serum samples from STa-challenged and control calves, before and after challenge, were collected and sent to the Clinical Pathology Laboratory, Purdue University for serum ion concentrations, which were measured using a Vitros-750 spectroscope (Johnson & Johnson Inc., Manchester, NY, USA).

Statistical analysis

Statistical analysis of the data on flow cytometry, guanylate cyclase activity and ion measurements was performed using the two-tailed Student's *t*-test for unpaired samples. The receptor stoichiometry data were analysed using one-way ANOVA followed by pairwise comparison probabilities (Bonferroni correction). Values of $p < 0.05$ were considered to be significant. All data were analysed for statistical significance using Statistica V5 software (Statsoft Inc., Tulsa, OK, USA).

RESULTS

Flow cytometry

The fluorescence level associated with enterocytes isolated from the ileum was higher than that associated with enterocytes from other segments of the calf intestine (Figure 1). Stronger fluorescence signals were detected on enterocytes from the posterior jejunum than on those from the anterior jejunum and colon. The signals on the enterocytes from the anterior jejunum and colon were similar to those of the negative control.

STa receptor stoichiometry

The binding of ^{125}I-STa to enterocytes prepared from different segments of the calf intestine was saturable and reached a plateau. The specific binding of ^{125}I-STa to enterocytes prepared from the ileum was higher than that to enterocytes prepared from the other segments of the calf intestine (Table I). Both the density and the affinity of the receptors to STa were relatively high in enterocytes isolated from the ileum. The values of the affinities and the densities of the STa-receptors on enterocytes prepared from the posterior jejunum were higher than those from the anterior jejunum and colon. These differences in the affinity and density of the STa-receptors on enterocytes isolated from the different segments of the intestine of newborn calves were statistically significant ($p < 0.05$).

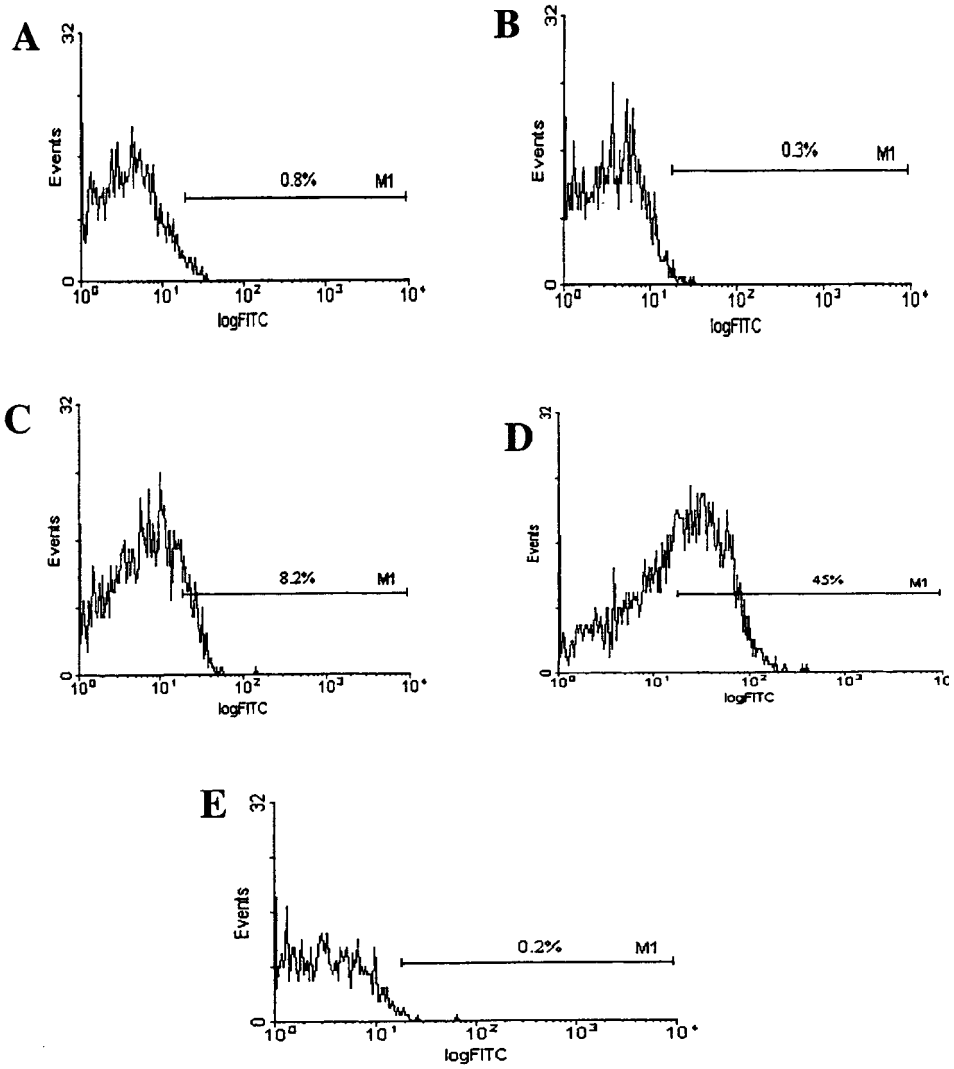

Figure 1. Representative flow cytometry histograms for STa interaction with enterocytes from different segments of newborn calf intestinal tract. (A) Negative control; (B) enterocytes from anterior jejunum; (C) enterocytes from posterior jejunum; (D) enterocytes from ileum; (E) enterocytes from colon. All the enterocytes were stained with STa, anti-STa-rabbit serum and anti-rabbit-IgG-FITC antibodies, except for the negative controls, where no STa was used

TABLE I
Binding properties of ^{125}I-STa to enterocytes prepared from different segments of the intestine of newborn calves

Intestinal segment	Specific binding (%) (mean ± SD)	Nonspecific binding (%) (mean ± SD)	Dissociation constant (K_d) (nmol/L) (mean ± SD)	STa-receptors (receptors/ enterocyte) (mean ± SD)
Ileum	87 ± 5.3[a]	13 ± 5.3[a]	10.9 ± 1.4[a]	16 171.1 ± 3367.2[a]
Anterior jejunum	32 ± 5.7	68 ± 5.7	77.0 ± 9.0	611.2 ± 93.8
Posterior jejunum	48 ± 6.2	52 ± 6.2	26.2 ± 7.8	3 715.5 ± 710.1
Colon	19 ± 3.0	81 ± 3.0	105.9 ± 20.8	248.8 ± 62.4

[a]The differences between the ileum and other intestinal segments were statistically significant ($p < 0.01$) using one-way ANOVA followed by pairwise comparison probabilities (Bonferroni correction)

Guanylate cyclase activity

Before being stimulated with STa, the guanylate cyclase activity was higher in the ileal segments from the STa-challenged calves than in the ileal segments from the control calves (Figure 2). Upon adding different amounts of STa to stimulate the guanylate cyclase and to measure the residual activity of this enzyme, ileum from STa-challenged calves showed significantly higher ($p < 0.05$) activities as the amount of STa was increased (Figure 3). The guanylate cyclase activities in the anterior jejunum, posterior jejunum and colon from the STa-challenged calves were similar to those of control calves.

Ion measurements

The sodium concentrations in the luminal content of the ileum of STa-challenged calves were significantly ($p < 0.05$) higher than those in the luminal content of the ileum from control calves (Figure 4). No differences in Na^+ concentration were noticed in the contents of other intestinal segments from either STa-challenged or control calves. In the control calves, the mean concentrations (±SD) of Ca^{2+} were 6 (±1.5), 6 (±1.6), 7 (±1.9), and 11 (±3) mg/g of the contents of the anterior jejunum, posterior jejunum, ileum, and colon, respectively. The mean concentrations (±SD) of K^+ were 12 (±1.5), 11 (±2), 10 (±2.1), and 17 (±2.3) mg/g of the contents of the anterior jejunum, posterior jejunum, ileum, and colon, respectively. No significant changes were observed in the concentrations of Ca^{2+} or K^+ in any of these intestinal segments from STa-challenged newborn calves. A significant decrease ($p < 0.05$) in the concentration of Cl^- in the serum was observed in the STa-challenged calves (Figure 5). The mean serum concentrations (±SD) of K^+, Na^+ and Ca^{2+} in newborn calves before STa

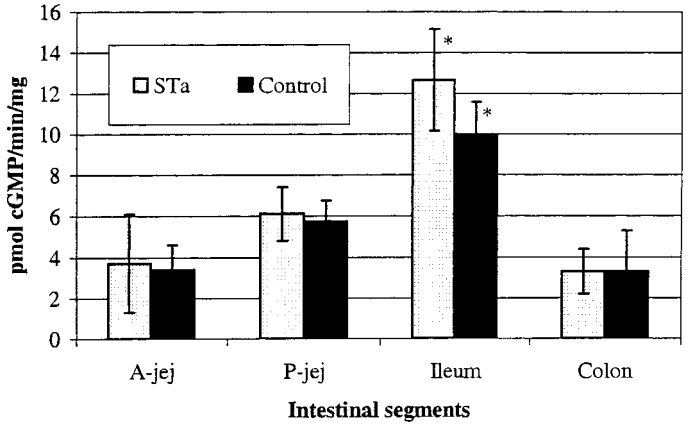

Figure 2. Basal guanylate cyclase activity in different intestinal segments of STa-challenged and control calves. A-jej, anterior jejunum; P-jej, posterior jejunum. Each point represents mean ± SD of values obtained from 3 animals. *The difference between the means is significant ($p < 0.05$)

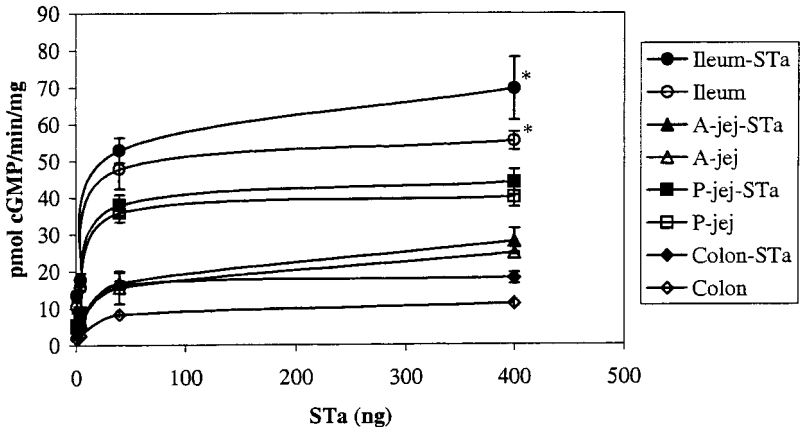

Figure 3. STa-stimulated guanylate cyclase activity in different intestinal segments of STa-challenged and control calves. A-jej, anterior jejunum; P-jej, posterior jejunum. Each point represents the mean ± SD of values from 3 animals. *The difference between the means is significant ($p < 0.05$)

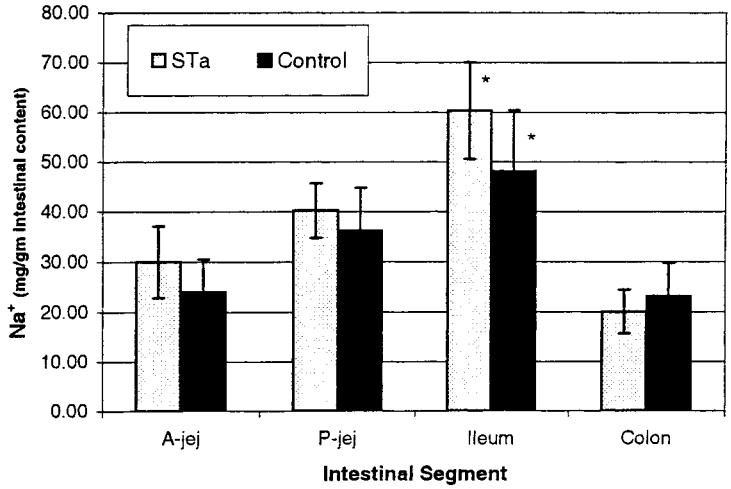

Figure 4. Concentration of Na^+, measured by atomic absorption spectrometry, in the luminal contents of different intestinal segments of STa-challenged and control calves. *The difference between the means is significant ($p < 0.05$)

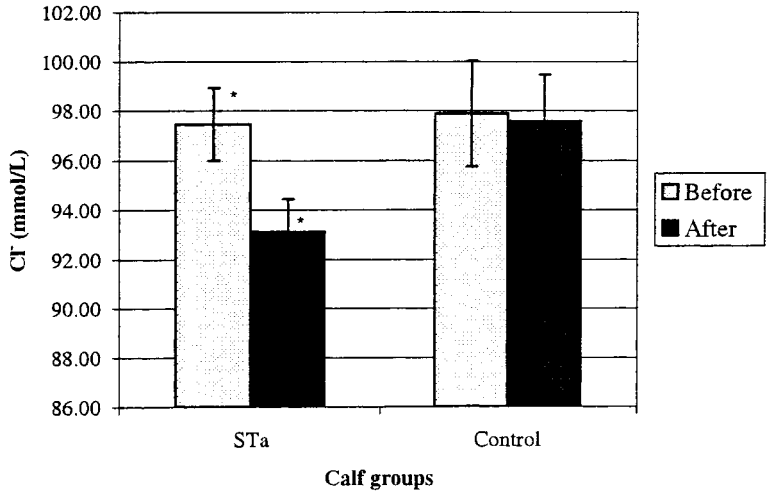

Figure 5. Levels of Cl^- in the serum of STa-challenged and control calves before and after challenge with STa. *The difference between the means is significant ($p < 0.05$)

challenge were 140 ($\pm$2) mmol/L, 5.6 ($\pm$0.4) mmol/L and 9.2 ($\pm$1) mg/dl, respectively. No significant changes occurred in the concentrations of these ions in sera from the same calves after STa-induced diarrhoea.

DISCUSSION

The diarrhoea induced by enterotoxigenic *Escherichia coli* in calves is mediated, in large part, by the secreted heat-stable enterotoxin (STa) (Acres *et al.*, 1982; Acres, 1985; Gyles, 1994; Sears and Kaper, 1996). Loss of fluid and electrolytes results from activation of guanylate cyclase enzymes located in the apical surfaces of the intestinal epithelial cells (Guerrant *et al.*, 1980; Rao *et al.*, 1981; Guandalini *et al.*, 1982; Epstein *et al.*, 1986). Only the particulate form of intestinal guanylate cyclase is stimulated by STa (Guandalini *et al.*, 1982), in contrast to cholera toxin and the *E. coli* heat-labile enterotoxin, which activate the adenylate cyclase of both intestinal and non-intestinal cells (Ahrens and Panichkriangkrai, 1985; Sears and Kaper, 1996). The mechanism of action of STa is not completely understood, but it does appear to involve increased intracellular levels of cGMP (Guerrant *et al.*, 1980; Rao *et al.*, 1981; Guandalini *et al.*, 1982; Epstein *et al.*, 1986; Volant *et al.*, 1997). In this study, we have characterized the interaction of STa with its receptor throughout the small intestine of newborn calves.

Flow cytometry suggested that a stronger interaction occurred between STa and its receptor on enterocytes prepared from the ileum than on enterocytes prepared from other segments of the intestine. The significant increase in the fluorescence intensities on the enterocytes prepared from the ileum may be attributed to an increase in either the density of STa-receptors or the affinity of these receptors for STa toxin. The ^{125}I-STa data suggested that there was a higher density of STa-receptors present on enterocytes from the ileum than on enterocytes from other intestinal segments. Unlike previous reports in humans, birds and rats (Cohen *et al.*, 1986; Nobles *et al.*, 1991; Krause *et al.*, 1994, 1995), which suggested a decrease in the numbers of STa-receptors going distally along the small intestine, this report suggests an increase in the numbers of STa-receptors as the distal end of the small intestine is approached. In addition to the change in receptor numbers, we observed that receptor affinity for STa also increased. STa-receptor affinity was higher in enterocytes prepared from the ileum compared to those from the other intestinal segments. This is the first report that describes the changes in STa-receptor density and affinity along the intestinal tract of newborn calves.

To further characterize the interaction between STa and its receptor throughout the intestinal tract of newborn calves, we measured the guanylate cyclase activity on BBMs prepared from different segments of the intestine. The guanylate cyclase activity profiles on BBMs from STa-challenged and control calves further suggest that the ileum is the major segment of the intestine that is affected by STa-induced diarrhoea.

The changes in intestinal ion transport produced by STa are of interest because the toxin is clinically important and because it is a unique probe for the effect of cGMP on transport of intestinal electrolytes (Guandalini *et al.*, 1982; Argenzio *et al.*, 1984; Cox *et al.*, 1988; Matthews *et al.*, 1993). The net movement of fluid and electrolytes across

the intestine is the difference between two unidirectional fluxes: from the intestinal lumen to the blood (insorption), and from the blood to the intestinal lumen (exsorption) (Kutchai, 1996). The effect of STa on the net movement of Na^+ may have resulted from increased exsorption, decreased insorption, or both (Bywater, 1973). It seems that it might further help in understanding the mode of action of STa if the effect of STa can be defined in terms of changes in the unidirectional fluxes of fluids and Na^+.

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