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MICROBIAL PATHOGENICITY

Chloride secretion induced by thermostable direct haemolysin of *Vibrio parahaemolyticus* depends on colonic cell maturation

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Vibrio parahaemolyticus **produces a thermostable direct haemolysin (TDH) that has been implicated in the pathogenesis of diarrhoeal disease caused by this organism. In previous work, TDH induced Cl^- secretion in human colonic epithelial cells that was dependent on the intracellular Ca^{2+} concentration, $[Ca^{2+}]_{in}$. This study investigated whether Cl^- secretion induced by TDH is influenced by the stage of maturation of intestinal epithelial cells. Two different human colonic cell lines, villus cell-like Caco-2 cells and crypt cell-like T84 cells, cultured by different methods to obtain differentiated samples, were used. When these cells were exposed to butyrate, a transcriptional regulator of differentiation genes, or co-cultured with 18Co cells, a human colonic fibroblast cell line, they showed increased trans-epithelial resistance and villus cell marker enzyme activity. In Caco-2 cells, exposure to butyrate or co-culturing with 18Co cells resulted in increased TDH binding, higher short-circuit currents (Isc) and greater $[Ca^{2+}]_{in}$. These results suggest that sensitivity to TDH is affected by the stage of cellular differentiation of cultured intestinal epithelial cells.**

Introduction

Vibrio parahaemolyticus is a widespread and important cause of gastro-enteritis, but the mechanisms underlying the diarrhoeic action of this pathogen are not completely understood. Thermostable direct haemolysin (TDH) secreted by *V. parahaemolyticus* is considered to be a major virulence factor [1, 2] and has various biological activities, including haemolytic activity, cardiotoxicity, mouse lethality and enterotoxicity [2, 3]. Several reports have shown that TDH induces intestinal secretion and is involved in the pathogenesis of diarrhoea caused by *V. parahaemolyticus* [4, 5].

Other research has indicated that TDH induces intestinal chloride secretion with an increase of Ca^{2+} as a second messenger in rabbit ileum [5]. In erythrocytes [6] and Intestine 407 cells [7], TDH increases the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$). The effect of TDH on human colonic epithelial cells follows three steps: (1) TDH binds to the epithelial cells; (2) there is an increase in $[Ca^{2+}]_{in}$, which is associated with activation of protein kinase C (PKC); (3) Cl^- secretion is stimulated, as indicated by increased short-circuit currents (Isc) [8]. Thus, TDH-induced Cl^- secretion, which depends on $[Ca^{2+}]_{in}$, is one of the reasons why *V. parahaemolyticus* infection induces diarrhoea.

Reports have shown that *V. parahaemolyticus* attaches to the rabbit intestinal brush border [9] and to Caco-2 cells [10] which are differentiated to the mature villus epithelium [11]. On the other hand, *V. parahaemolyticus* attaches to M cells but has a poor affinity for the villus cells in the human small intestine [12]. However, the target of *V. parahaemolyticus* is still not clearly defined in the human colonic epithelium. TDH added on the mucosal side did not stimulate Cl^- secretion or increase Ca^{2+} in T84 cells, a human colonic crypt-like cell line [13]. Crypt cells are relatively undifferentiated proliferating cells. However, as they migrate up the villus, intestinal cells differentiate and acquire the enzymic and morphological characteristics of mature villus epithelium [14, 15]. TDH increased Cl^- secretion when added to Caco-2 cells that were stimulated to

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differentiate by treatment with butyrate. Recently, sensitivity to Shiga toxin (STX), which also induces diarrhoea, has been associated with cell differentiation [14]. As cells mature, they express receptors to which STX can adhere and sensitivity to STX depends on the extent to which these receptors are expressed. There have been no reports as to whether intestinal cells differ in sensitivity to TDH depending on their stage of differentiation.

To investigate the effect of TDH, Caco-2 cells and T84 cells were prepared under different conditions: (1) in simple cultures; (2) by addition of butyrate to the simple cultures; (3) by co-culturing with 18Co cells; (4) by a combination of butyrate addition and co-culturing with 18Co cells. Butyrate is normally found in the intestinal lumen and induces the increased differentiation of many types of cells [15–18] including Caco-2 cells [14, 16, 17]. The 18Co cell line is a human colonic cell line that exhibits most of the known characteristics of intestinal subepithelial myofibroblasts *in situ*. It has been suggested that these cells are a source of differentiation factors that can have an effect on water or Cl^- secretion from epithelial cells [19, 20]. The stages of differentiation were assessed by examining changes in enzymic activities that are readily detected on colonic villus [14, 15, 21]. In this study, the effects of TDH on Cl^- secretion, $[Ca^{2+}]_{in}$ and TDH binding with these various differentiated human colonic cells were examined.

Materials and methods

Cells

T84, Caco-2 and 18Co cells were purchased from the American Type Culture Collection (Rockville, MD, USA). T84 cells, derived from human colon carcinoma, develop crypt cell-like properties during growth to confluence. Caco-2 cells are also derived from human colon carcinoma, but develop villus cell-like properties during growth to confluence. 18Co cells were derived originally from a colonic mucosal biopsy of a 2-month-old female infant. All cell lines were maintained in Eagles' Minimal Essential Medium supplemented with fetal bovine serum (FBS) 10% v/v, gentamicin 100 U/ml and non-essential amino acids 1%. The medium and supplements were obtained from Gibco BRL (Gaithersburg, MD, USA). Cells were grown in 75-cm^2 flasks in air with CO_2 5% at 37°C, fed every 2 days and passaged weekly. To promote differentiation, cells were cultured in different ways (Fig. 1). Stock cells, consisting of Caco-2 cells or T84 cells, were trypsinised and suspended at 2×10^5 cells/ml in medium. Cells were seeded at confluent density on to 1.0-cm^2 transwell inserts (Costar, Cambridge, MA, USA) (Fig. 1A and B). After the Caco-2 or T84 cells had been cultured for 2 days, butyrate was added to the culture medium (Fig. 1B and D). 18Co cells were inoculated on the reverse side of the filter in the transwell inserts (Fig. 1C–F). After these cells had been cultured for 2 days, Caco-2 or T84 cells were

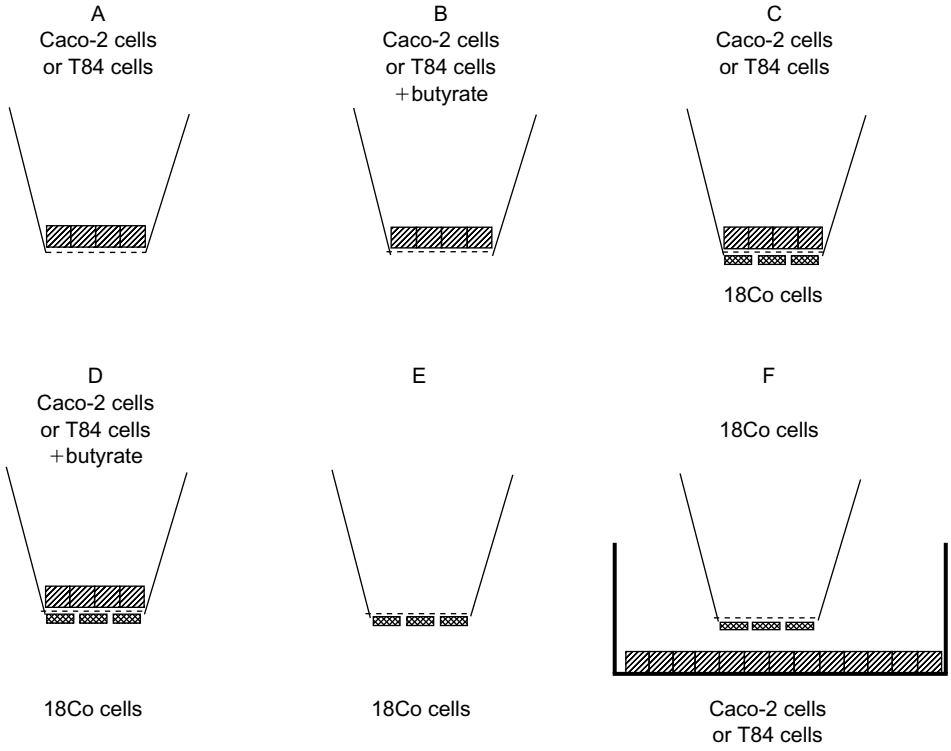

Fig. 1. Methods of cell culturing on transwell inserts. (**A**) Caco-2 cells or T84 cells in simple culture; (**B**) butyrate added to the simple culture medium after 2 days; (**C**) 18Co cells cultured on the lower side of the transwell insert filters and Caco-2 cells or T84 cells cultured on the other side of the same filter; (**D**) butyrate added to the culture medium after 2 days, as in (C); (**E**) 18Co cells cultured on the lower side of the transwell insert filters; (**F**) 18Co cells cultured on the lower side of transwell insert filters and Caco-2 cells or T84 cells cultured on the floor in a 12-well plate (see *Materials and methods*).

inoculated on the other side of the filter in the same transwell inserts (Fig. 1C and D) or on to the base of 12-well culture plates (Fig. 1F). After the Caco-2 cells and T84 cells had been cultured for a total of 6 days, transwell inserts with cultured cells were used for trans-epithelial resistance measurements and the Ussing chamber experiments. The effects of TDH on T84 cells from passages 50–80, Caco-2 cells from passages 30–55 and 18Co cells from passages 4–12 were investigated.

Trans-epithelial resistance measurements

The trans-epithelial resistance of cells cultured on the transwell inserts was measured with an electrical voltmeter (EVOM, WPI, Sarasota, FL, USA).

Isolation of TDH

Recombinant plasmid pKK223-3, which harbours the structural gene of wild-type TDH (pT101), was introduced into *Escherichia coli* JM109 by transformation. Transformants were cultivated at 37°C for 16 h with rotary shaking in Luria-Bertani Broth (Bacto-tryptone, Difco, 1%; yeast extract, Difco, 0.5%; NaCl 0.5% containing ampicillin 100 μg/ml. Recombinant toxin was purified by a previously reported method [22]. Fractions containing TDH were assayed by haemolysis on rabbit blood agar plates [23]. The purity of samples was evaluated by SDS-PAGE by the method of Laemmli [24].

Intracellular Ca^{2+} measurements

The concentration of intracellular free Ca^{2+} was evaluated by microfluorimetry, with the fluorescent dye 1-(2-(5′-carboxyoxazol-2′-yl)-6-aminobenzofuran-5-oxy)-2(2′amino-5′-methylphenoxy)ethane-N,N,N′,N′-tetraacetic acid, pentaacetoxy methyl ester (Fura-2/AM) at excitation wavelengths of 340 nm and 380 nm and at an emission wavelength of 510 nm with a specially designed chamber and an ARGUS-50/CA system (Hamamatsu Photonicus, Japan). The cells were cultured on coverslips (18 × 18 mm) for 6 days. At the end of this period, each sample was loaded with Fura-2/AM (2 μM) (Molecular Probes, Eugene, OR, USA) for 30 min and washed twice with PBS. Coverslips were inserted into the chamber and each series of measurement was completed in 30 min.

Ussing chamber experiments

To measure the trans-epithelial Isc, the bath solution contained (mM) 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.8 K_2HPO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$ and 10 glucose. The pH of this solution was 7.4 at 37°C when gassed with a mixture of O_2 95% and CO_2 5%. Transwell cell culture inserts, with the cultured cells, were mounted in an Ussing chamber (laboratory-made) and the tissue samples were continuously short-circuited with a voltage and current clamp amplifier (Model DVC1000-E; World Precision Instruments, FL, USA). After compensation for fluid resistance, trans-epithelial resistance was measured by applying a 5-mV pulse at 40–50-s intervals, and the resistance was calculated by using Ohm's law. TDH, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), glybenclamide and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (all from Sigma) were added only to the mucosal bath solution in the Ussing chamber at the concentrations indicated.

Enzyme assays

Alkaline phosphatase. After they had been cultured for 6 days in transwell inserts, the cell samples were washed twice with PBS. Then, 100 μl of *p*-nitrophenyl phosphate (1 mg/ml in 100 mM Tris-NaCl buffer, pH 9.5, containing 5 mM $MgCl_2$) were added to right side of each transwell insert and PBS to the other side. After incubation for 10 min at room temperature, the absorbance at 405 nm (A_{405}) was measured and compared with that from cells without substrate. Activity was expressed as the relative increase in A_{405} compared with the control. After measuring the alkaline phosphatase activity, cell numbers were counted after the cells were digested with trypsin.

Sucrase. After the Caco-2 cells or T84 cells had been cultured for 6 days in transwell inserts, they were washed as above, then 100 μl of 0.3 M sucrose in 0.1 M KPO_4 buffer, pH 6.0, were added to right side of each transwell insert and PBS to the other side, after which the samples were incubated at 37°C for 30 min. Liberated glucose was detected with a V-glucase kit (Nissui, Japan). The A_{520} of a glucose standard 1 mg/ml was 0.367. Activity was expressed as the A_{520} against the cell count in the sample.

Immunofluorescence staining

T84 cells or Caco-2 cells were grown on 18 × 18-mm coverslips (Matsunami, Osaka, Japan) by simple culture for 4 days, butyrate was added and 2 days after butyrate addition they were co-cultured with 18 Co cells for 6 days. Then the cells were washed and fixed with methanol : acetic acid (1 : 1) and incubated at 37°C for 3 h with TDH 10 μg/ml. The cells were washed with PBS and incubated for 1 h with rabbit polyclonal antibody to TDH [1] (1 in 1000 dilution in PBS solution). The cells were washed again and incubated for 1 h with rhodamine-conjugated anti-rabbit IgG antibody (1 in 1000 dilution in PBS) (Gibco BRL). The coverslips were removed, thoroughly washed with PBS containing bovine serum albumin 0.1% and examined by fluorescence microscopy.

Other chemicals

Guaranteed reagent grade inorganic salts were purchased from Wako Pure Chemicals (Tokyo, Japan).

Statistical evaluations

The data were evaluated by the Student's *t* test for unpaired samples, and $p < 0.05$ was considered significant.

Results

Effect of butyrate or co-culture on trans-epithelial resistance

Trans-epithelial resistance increased with the time of cell culture (Fig. 2). The resistance of 18Co cells, cultured on the reverse side of the transwell inserts and with the Caco-2 cells or T84 cells cultured on the base of the plate, did not change (Fig. 2). T84 cells had a higher trans-epithelial resistance than Caco-2 cells. Both Caco-2 cells and T84 cells which had been exposed to butyrate or co-cultured with 18Co cells had a higher trans-epithelial resistance than samples cultured without these additions. Peak resistance values occurred after culture for 5–7 days and decreased gradually thereafter (Fig. 2). Thus, for subsequent studies, cells that had been cultured for 6 days were used.

Effect of butyrate or co-culture on enzyme activity

To assess the extent of cell differentiation to villus-like cells, alkaline phosphatase and sucrase activity were measured (Fig. 3) [14, 15, 21]. Sucrase activity increased after treatment with butyrate or co-culturing with 18Co cells and activity in Caco-2 cells was slightly higher than in T84 cells (Fig. 3a). Alkaline phosphatase activity in Caco-2 cells was higher than in T84 cells. After treatment with butyrate or co-culturing, alkaline phosphatase activity in Caco-2 cells increased substantially (Fig. 3b). The greatest alkaline phosphatase activity was recorded in samples of Caco-2 cells co-cultured with 18Co cells. These results indicate that Caco-2 cells that had been exposed to butyrate or to 18Co cells differentiated more toward the villus-like cells than T84 or Caco-2 cells in simple culture. Trans-epithelial resistance of Caco-2 cells or T84 cells that were exposed to butyrate or co-cultured with 18Co cells was higher (Fig. 3c) than in samples cultured without these treatments. The number of Caco-2 cells or T84 cells/cm^2 did not vary significantly between the four different culture methods.

Effects of TDH

Fig. 4a shows the effects of TDH 10 μg/ml on trans-epithelial Isc of T84 cells cultured by the four different methods shown in Fig. 1A–D. T84 cells in simple culture did not show any increase in Isc. There was a

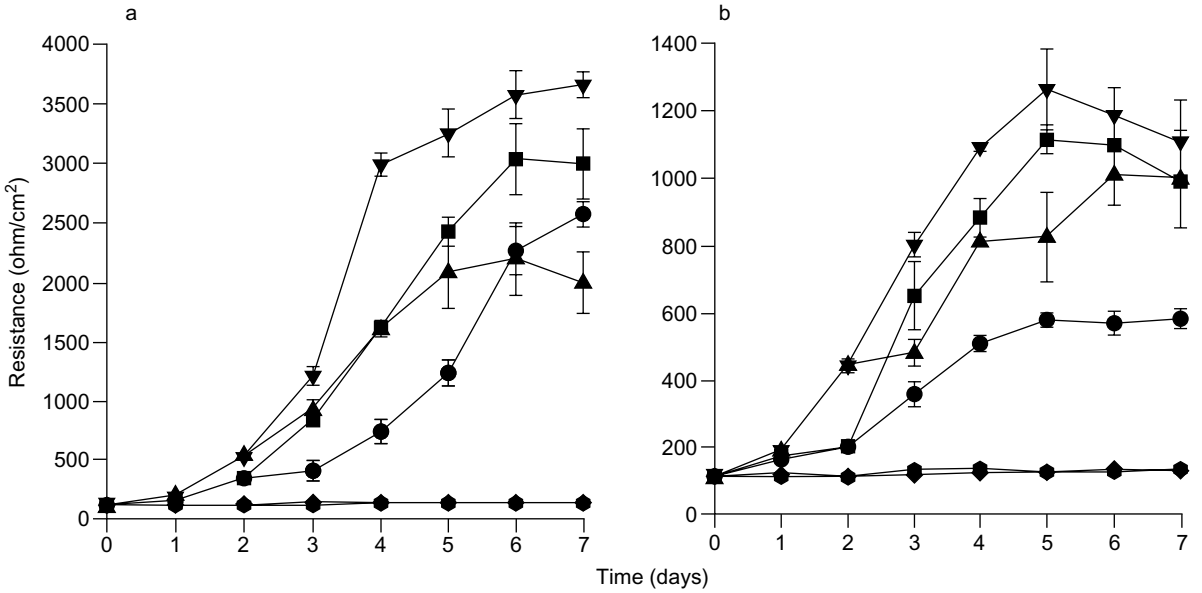

Fig. 2. Changes in trans-epithelial resistance according to number of days of culturing. (**a**) ●, T84 cells cultured by method in Fig. 1A; ■, T84 cells cultured by the method in Fig. 1B; ▲, T84 cells and 18Co cells cultured by the method in Fig. 1C; ▼, T84 cells and 18Co cells cultured by the method in Fig. 1D; ◆, 18Co cells cultured by the method in Fig. 1E; ⬣, 18Co cells cultured with T84 cells by the method in Fig. 1F. (**b**) ●, Caco-2 cells cultured by the method in Fig. 1A; ■, Caco-2 cells cultured by the method in Fig. 1B; ▲, Caco-2 cells and 18Co cells cultured by the method in Fig. 1C; ▼, Caco-2 cells and 18Co cells cultured by the method in Fig. 1D; ◆, 18Co cells cultured by the method in Fig. 1E; ⬣, 18Co cells cultured with Caco-2 cells by the method in Fig. 1F. Values are expressed as mean and SD ($n = 5$).

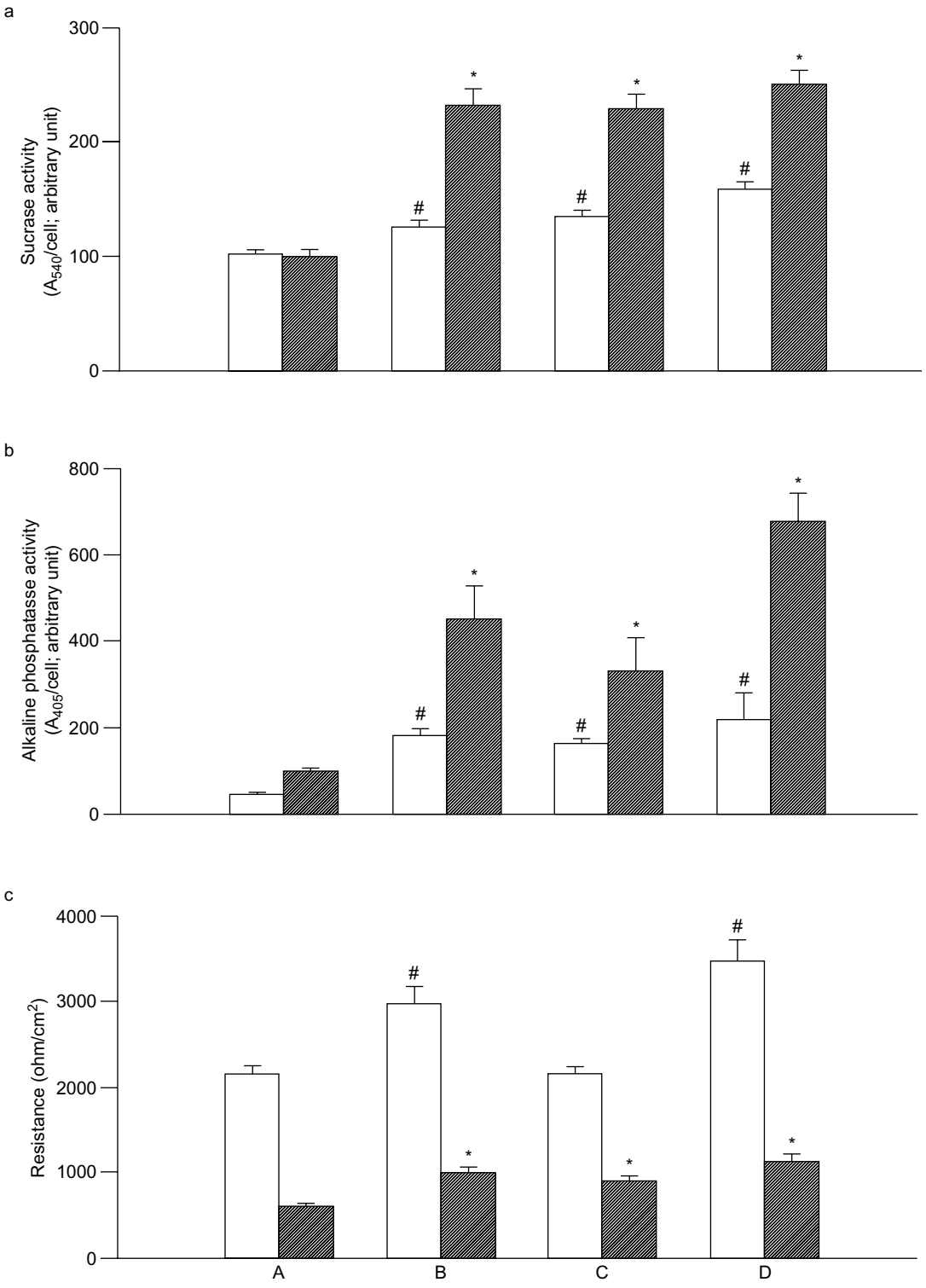

Fig. 3. Effects of exposure to butyrate or co-culturing with 18Co cells, or both, on enzyme activities and trans-epithelial resistance. □, T84 cells cultured on the upper side of the transwell inserts. ▨, Caco-2 cells cultured on the upper side of the transwell inserts. (**A**) Cells cultured by the method in Fig. 1A; (**B**) cells cultured by the method in Fig. 1B; (**C**) cells cultured by the method in Fig. 1C; (**D**) cells cultured by the method in Fig. 1D. (**a**) Sucrase activity of T84 cells or Caco-2 cells. (**b**) Alkaline phosphatase activity of T84 cells or Caco-2 cells. (**c**) Trans-epithelial resistance of T84 cells or Caco-2 cells. All cell samples were cultured for 6 days. Values are expressed as mean and SD ($n = 5$). *Significant difference $p < 0.05$ (*t* test) *versus* (A) Caco-2 cells. #Significant difference $p < 0.05$ (*t* test) *versus* (A) T84 cells.

readily detectable Isc increase after TDH was added to T84 cells that had been exposed to butyrate or co-cultured with 18Co cells. Fig. 4b shows the effects of TDH on the trans-epithelial Isc of Caco-2 cells cultured by the four different methods. With Caco-2 cells treated with butyrate or co-cultured with 18Co cells, the Isc increase was greater than with similarly treated T84 cells. Caco-2 cells in simple culture did not show

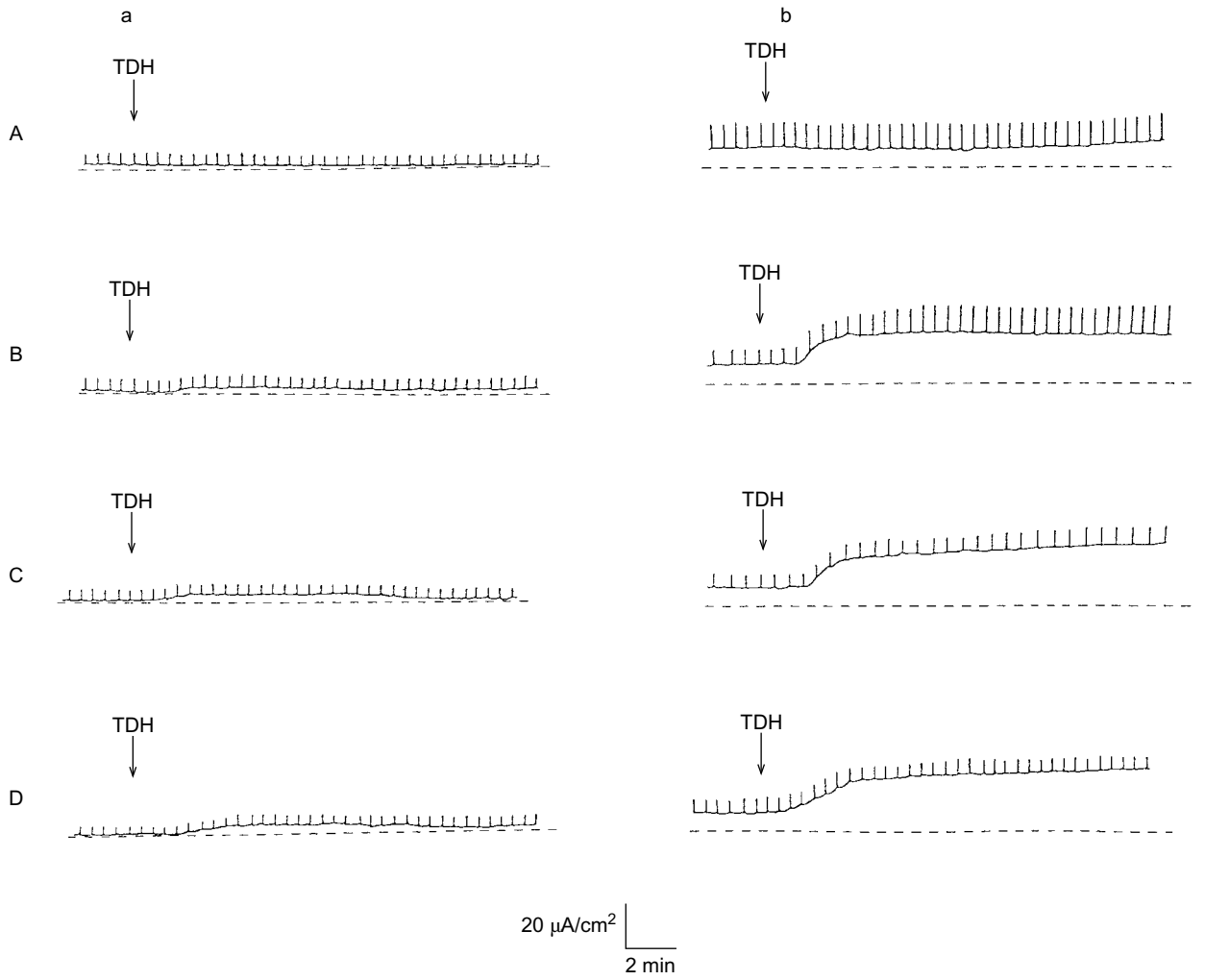

Fig. 4. Typical changes in Isc after adding TDH 10 μg/ml to the mucosal side of T84 or Caco-2 cells that had been cultured for 6 days on transwell inserts. (**A**) Cells cultured by the method in Fig. 1A; (**B**) cells cultured by the method in Fig. 1B; (**C**) cells cultured by the method in Fig. 1C; (**D**) cells cultured by the method in Fig. 1D. (**a**) T84 cells cultured on the upper side of transwell inserts; (**b**) Caco-2 cells cultured on the upper side of transwell inserts.

any increase in Isc. These results suggest that the increase in Isc that followed exposure to TDH occurred only in cells that had matured into villus-like epithelia.

Effect of Cl^- channel inhibitors on Isc increase by TDH

Fig. 5 shows the effects of Cl^- channel inhibitors on Isc of samples that had been exposed to TDH. Fig. 5a shows data for T84 cells cultured by the four methods as shown in Fig. 1A–D. There was a small increase in Isc for simply cultured T84 cells; however, for Caco-2 cells cultured without additional treatment, Cl^- channel inhibitors had very little effect. Caco-2 cells that had been exposed to butyrate or co-cultured with 18Co cells, or both, showed increased Isc (Fig. 5b). In these samples, Isc stimulated by TDH was inhibited by DIDS, an inhibitor of the Ca^{2+}-dependent Cl^- channel [25, 26] and the inhibition in cells cultured by the three different methods (Fig. 1B–D) was similar. Glybenclamide, an inhibitor of cAMP-dependent Cl^- channel [27, 28], and NPPB, an inhibitor of the Cl^-channel [25, 27], did not affect Isc (Fig. 5b).

Effect of TDH on intracellular Ca^{2+} concentration

In Caco-2 cells that had been treated with butyrate or co-cultured with 18Co cells, or both, the intracellular Ca^{2+} concentration increased (Fig. 6b). On the other hand, the $[Ca^{2+}]_{in}$ concentration changed very little in T84 cells, even after exposure to butyrate or co-culturing with 18Co cells (Fig. 6a). These results suggest that the increase in the $[Ca^{2+}]_{in}$ that followed exposure to TDH occurred only in cells that had matured into villus-like epithelia.

Binding of TDH to cell monolayers

Immunofluorescence staining of the cell monolayers with rabbit anti-TDH and rhodamine-conjugated anti-rabbit IgG indicated that TDH bound more efficiently to Caco-2 cells that had been exposed to butyrate or co-cultured with 18Co cells, or both, than to T84 cell monolayers or Caco-2 cell monolayers in simple culture (data not shown).

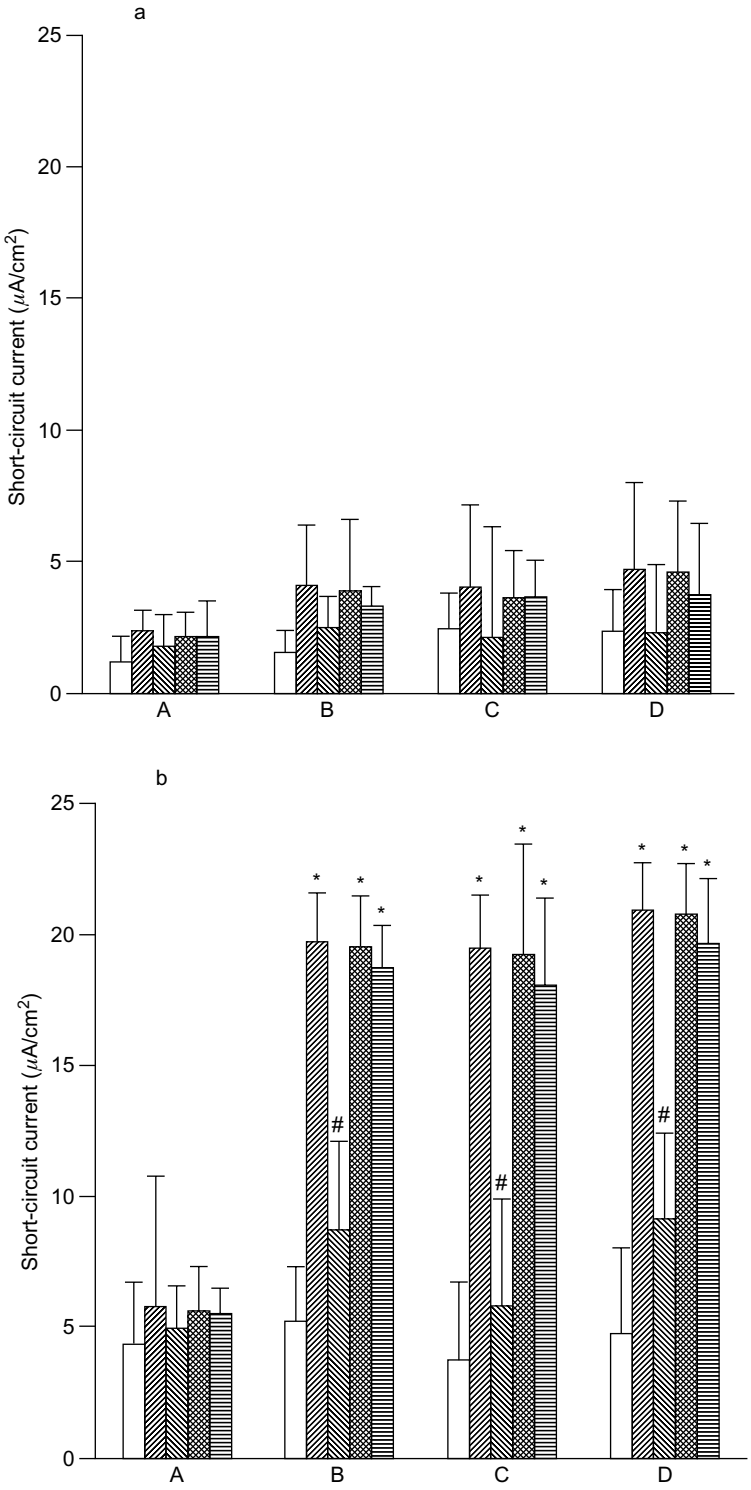

Fig. 5. Changes in Isc after adding TDH 10 μg/ml on the mucosal side of T84 or Caco-2 cells that had been cultured for 6 days. (**a**) T84 cells cultured on the upper side of Transwell inserts; (**b**) Caco-2 cells cultured on the upper side of transwell inserts. (**A**) Cells cultured by the method in Fig. 1A; (**B**) cells cultured by the method in Fig. 1B; (**C**) cells cultured by the method in Fig. 1C; (**D**) cells cultured by the method in Fig. 1D. □, baseline; ▨, TDH added on the mucosal side of the T84 cells or Caco-2 cells (Isc was measured 15 min after TDH addition). ▧, TDH added, then DIDS added on the mucosal side (Isc was measured 15 min after DIDS addition). ▩, TDH added, then glybenclamide added and Isc was measured after 15 min. ⊟, TDH added, then NPPB added and Isc measured after 15 min. *Significant difference $p < 0.05$ (*t* test) *versus* each of the baseline values (□). #Significant difference $p < 0.05$ (*t* test) *versus* each of the TDH values (▨).

Discussion

The effects of TDH on undifferentiated and well-differentiated human colonic cells prepared by different culture methods were investigated. Caco-2 cells differentiated towards villus-like cells after exposure to butyrate, by co-culturing with 18Co cells, or both, whereas T84 cells cultured by the same methods

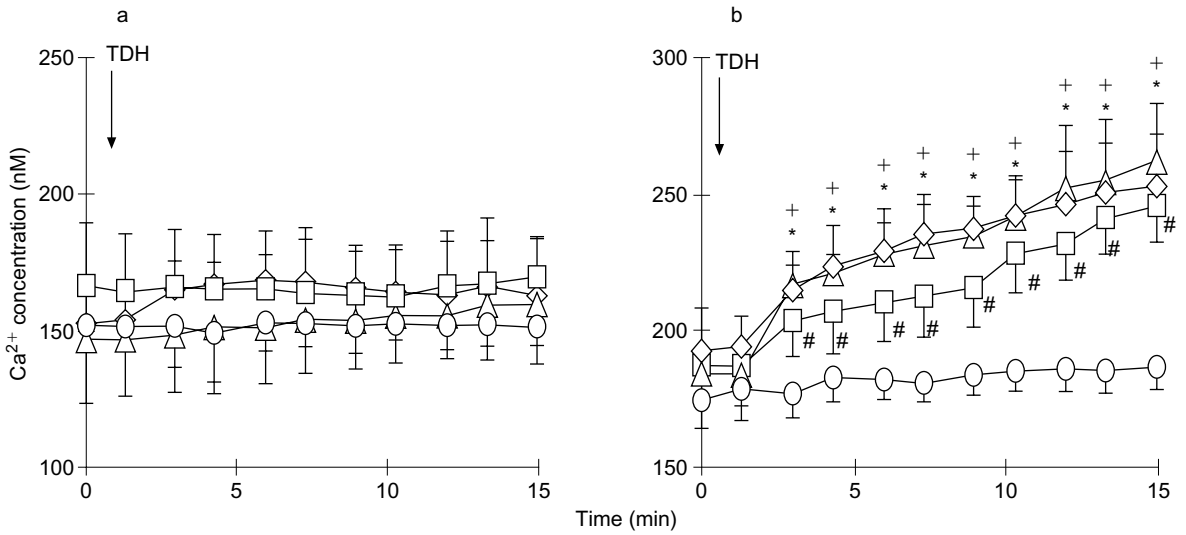

Fig. 6. Changes in the intracellular Ca^{2+} concentration after addition of TDH 10 μg/ml. TDH was added at the points indicated. (**a**) T84 cells; (**b**) Caco-2 cells. ○, T84 cells or Caco-2 cells cultured on coverslips for 6 days; □, T84 cells or Caco-2 cells were cultured on coverslips for 6 days then butyrate was added and cells were cultured for a further 2 days; #significant difference $p < 0.05$ (*t* test) *versus* ○ value at each time. Δ, T84 cells or Caco-2 cells cultured with 18Co cells for 6 days; +significant difference $p < 0.05$ (*t* test) *versus* ○ value at each time. ◇, T84 cells or Caco-2 cells cultured with 18Co cells for 6 days then butyrate was added and cells were cultured for a further 2 days; *significant difference $p < 0.05$ (*t* test) *versus* ○ at each time. The data were collected every 30 s, but the values presented are the averages over 90 s. Values are expressed as mean and SD ($n = 7$).

showed a much lower degree of differentiation towards villus-like cells. Furthermore, Caco-2 cells that were co-cultured with 18Co cells and exposed to butyrate showed higher alkaline phosphatase activities and sucrase activities than Caco-2 cells that were exposed to butyrate without co-culturing. Thus, it is possible that the differentiation induced by exposure to butyrate and co-culturing with 18Co cells are different. These data confirm previous reports that butyrate or 18Co cells enhance cell differentiation to villus-like cells [12, 14, 17, 19, 29], although the mechanisms are not completely understood. The methods used to produce five types of undifferentiated and three types of well-differentiated colonic epithelial cell monolayers are described.

To evaluate the effect of TDH on colonic epithelial cells, $[Ca^{2+}]_{in}$, TDH binding and Isc were measured – the latter to provide evidence of Cl^- secretion. After exposure to TDH, Isc and $[Ca^{2+}]_{in}$ increased only in differentiated Caco-2 cells. DIDS inhibited the increase in Isc brought about by exposure to TDH, but glybenclamide and NPPB did not. These pharmacological profiles are the same as in previous findings that showed increased Isc after exposure to TDH in butyrate-treated Caco-2 cells [8]. Consequently, it is possible that the greater Isc detected in samples exposed to TDH is due to activation of Ca^{2+}-activated Cl^- channels. T84 cells that had been exposed to TDH showed very small changes in Isc and $[Ca^{2+}]_{in}$. In this study, cells that were co-cultured with 18Co cells or exposed to butyrate were stimulated to develop villus-like cell functions as indicated by the type of enzyme activities found with villus cells [14, 15, 19, 21]. This suggests that the Cl^- secretion induced by TDH occurs in those cells that matured to become similar to epithelial villus cells.

There was greater binding of TDH to Caco-2 cells that had been stimulated to differentiate than to T84 cells or Caco-2 cells that had not been treated. This suggests that the expression of a receptor to which TDH can bind occurs during cell maturation. If the binding of TDH to cell receptors is the first step in the effect that TDH has on colonic epithelial cells, then the stimulation of Cl^- secretion and the increase in $[Ca^{2+}]_{in}$ may be regulated by binding of TDH to the cells during the process of cell differentiation.

It is generally accepted that butyrate induces cell differentiation that leads to the expression of villus-like enzymic and absorptive activity *in vivo* [18, 29, 30]. Butyrate does occur naturally in the normal human colon as a by-product of the metabolism by colonic bacterial flora of unabsorbed starch and non-starch polysaccharides, and this butyrate is used as an energy source by colonocytes [30]. Normal faecal levels of butyrate in man may be as high as 20 mM [18]. When butyrate is absent, as in the surgically excluded colon and in antibiotic therapy that destroys the intestinal mucosal flora, a syndrome known as diversion colitis ensues; this condition is ameliorated by the administration of butyrate [29]. Such evidence suggests that the butyrate is normally produced *in vivo* and affects intestinal epithelial cells.

18Co is a human colonic myofibroblast cell line. Intestinal subepithelial myofibroblasts are found at the

interface between enterocytes and the lamina propria [12, 19, 31]. Differentiation, proliferation and apoptosis of enterocytes and intestinal subepithelial myofibroblasts along the crypt–villus axis are spatially correlated in the human colon [12, 19, 32]. It has been suggested that intestinal subepithelial myofibroblasts secrete growth and differentiation factors that act on overlying epithelial cells [19, 33, 34].

Thus, it is likely that butyrate and subepithelial fibroblasts also regulate intestinal cell differentiation *in vivo*. Because the effects of TDH depend on the differentiation of colonic epithelial cells, it is plausible to suggest that manipulation of butyrate or the functions of subepithelial fibroblasts may alter the interaction of TDH and affect the symptoms caused by *V. parahaemolyticus*.

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