Calcitonin receptor-mediated CFTR activation in human intestinal epithelial cells

Hongguang Liu a, Amika Singla b, Mei Ao b, Ravinder K. Gill a, Jayashree Venkatasubramanian b, Mrinalini C. Rao b, Waddah A. Alrefai a, c, *, Pradeep K. Dudeja a, c, #

a Section of Digestive Diseases and Nutrition, Department of Medicine, University of Illinois at Chicago, Chicago, IL, USA
b Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL, USA
c Jesse Brown VA Medical Center, Chicago, IL, USA

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Abstract

High levels of calcitonin (CT) observed in medullary thyroid carcinoma and other CT-secreting tumours cause severe diarrhoea. Previous studies have suggested that CT induces active chloride secretion. However, the involvement of CT receptor (CTR) and the molecular mechanisms underlying the modulation of intestinal electrolyte secreting intestinal epithelial cells have not been investigated. Therefore, current studies were undertaken to investigate the direct effects of CT on ion transport in intestinal epithelial cells. Real time quantitative RT-PCR and Western blot analysis demonstrated the expression of CTR in intestinal epithelial T84 cells. Exposure of T84 cells to CT from the basolateral but not from apical side significantly increased short circuit current ($I_{SC}$) in a dose-dependent manner that was blocked by 1 μM of CTR antagonist, CT8–32. CT-induced $I_{SC}$ was blocked by replacing chloride in the bath solutions with equimolar gluconate and was significantly inhibited by the specific cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor, CFTR127inh. Further, biotinylation studies showed that CT increased CFTR levels on the apical membrane. The presence of either the Ca$^{2+}$/H11001 chelator, bis(2-aminophenoxy)ethane tetraacetic acid-acetoxymethyl (BAPTA-AM) ester or the protein kinase A (PKA) inhibitor, H89, significantly inhibited $I_{SC}$ induced by CT (~32–58% reduction). Response to CT was retained after permeabilization of the basolateral or the apical membranes of T84 cells with nystatin. In conclusion, the activation of CTR by CT induced chloride secretion across T84 monolayers via CFTR channel and the involvement of PKA- and Ca$^{2+}$-dependent signalling pathways. These data elucidate the molecular mechanisms underlying CT-induced diarrhoea.

Keywords: calcitonin • calcitonin receptor • chloride secretion • CFTR • diarrhoea

Introduction

Calcitonin (CT) is a 32-amino acid peptide secreted from the parafollicular cells (C-cells) of the thyroid gland and belongs to a family of peptides including CT gene-related peptide, amylin, adrenomedullin, intermedin and CT receptor (CTR)-stimulating peptide [1–4]. CT is a hypocalcaemic hormone that induces Ca$^{2+}$ deposition in bones and stimulates calcium excretion into urine [5–7]. Clinically, CT has been used for treatment of osteoporosis, Paget’s disease and hypercalcemia. CT functions by binding to its receptor, CTR, which is a class II G-protein-coupled receptor predominantly expressed in osteoclasts. CTR in osteoclasts has been previously shown to be coupled to Gαs and Gqα proteins that links the receptor to both adenylate cyclase-cAMP–protein kinase A (PKA) and Ca$^{2+}$-protein kinase C (PKC)-dependent pathways [8].

High levels of CT lead to diarrhoea. For example, diarrhoea has been reported to occur in 28–39% of patients with medullary thyroid carcinoma (MTC) associated with elevated levels of CT [9–11]. The diarrhoea in patients with MTC is usually severe, watery and lacks specific treatment and therefore, the mortality is high. Diarrhoea is also observed in other cases such as infusion of CT for hypercalcemia, CT-secreting pancreatic micro-tumours and small cell lung tumours [12–16]. Previous perfusion studies have shown that CT infusion in healthy humans and rabbits inhibited active sodium absorption and induced active chloride secretion [17, 18]. However, the detailed molecular mechanisms of CT-induced chloride secretion in intestinal epithelial cells are poorly understood.
Intestinal chloride secretion plays an important role in body fluid homeostasis and diarrhoea. Several transport processes present on the basolateral and the apical membranes of intestinal cells are involved in driving chloride secretion into the intestinal epithelial lumen. The main chloride channel expressed in small intestine and colon is the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is expressed in many epithelial tissues, where it has been found to have multiple putative functions, the major one being that of a chloride channel. Activation of CFTR as a Cl⁻ channel requires cyclic AMP, PKA and ATP [19]. Mutations in the gene encoding CFTR leading to a decrease in chloride channel function, is the primary defect in cystic fibrosis, a disease that affects approximately 30,000 patients in the United States alone [20]. On the other hand, activation of CFTR by cholera toxin leads to a massive secretory diarrhoea and life-threatening dehydration [21]. Whether the activation of CFTR by CT also stimulates chloride secretion via CFTR is not known.

Therefore, current studies were undertaken to investigate the expression of CFTR in intestinal epithelial cells and to examine the effect of CT on electrolyte secretion in colonic T84 cell line. Our data showed that CT induced chloride secretion via CFTR in a Ca²⁺- and cAMP-dependent manner. Our current studies provide novel insights into the molecular basis of CT-induced chloride secretion that may unravel potential targets for better therapy of diarrhoea associated with high levels of CT.

Materials and methods

Cell culture

Experiments were performed with the human intestinal T84 cell lines as previously described [22]. DMEM/F12 with 6% calf serum was used for T84 cells. For simultaneous measurements of [Ca²⁺] and increased short-circuit current (Isc), cells were seeded onto Snapwell membranes (Costar, Corning, NY, USA) with 0.4 μm pore diameter (culture area 0.1 cm²). Cells reached confluency after 7 to 8 days, with a resistance greater than 500 - 10³ Ω cm², and then mounted in Ussing chambers (Physiologic Instruments, San Diego, CA, USA) for electrical measurements.

Real time quantitative RT-PCR analysis

RNA was extracted from T84 cells using Qiagen RNeasy kits (Qiagen, Valencia, CA, USA). Equal amounts of RNA from T84 cells were reverse transcribed and amplified in one-step reaction for β-actin and CTR by using Brilliant SYBR Green quantitative RT-PCR (QRT-PCR) Master Mix kit (Stratagene, Santa Clara, CA, USA). Real-time QRT-PCR was performed using Mx3000P (Stratagene). Human CTR was amplified with gene-specific primers (sense primer: 5'-GCAGGAAGATGTATGCTTTGA-3' ; antisense primer: 5'-CCAGGAAGGCTTGAA-3') [23]. Human β-actin was amplified as an internal control by using gene-specific primers (sense primer: 5'-CATGTGTAGACCTCATACAC-3' ; antisense primer: 5'-GGACAGGAAGGCTTGAA-3') [24].

Immunoblotting

For immunoblotting studies, briefly, cell lysates were prepared from T84 cells using radio immunoprecipitation assay (RIPA) buffer. A total of 100 μg protein from each of the T84 cells lysates was solubilized in Laemmli sample buffer (2% SDS, 100 mM dithiothreitol, 60 mM Tris, pH 6.8, 0.01% bromophenol blue) and was separated on 8% Tris/glycine SDS-PAGE. For CFTR, 75 μg protein from T84 cell lysate was used. The blot was then probed with primary rabbit anti-C TR antibodies (1:500, Abcam, Cambridge, MA, USA) or rabbit anti-CTR antibody (1:1000) from SantaCruz (Santa Cruz, CA, USA) or rabbit anti-actin antibody (1:5000) from Sigma (Saint Louis, MO, USA) for loading control. Goat anti-rabbit antibody (1:2000, SantaCruz) was used as secondary antibody. The bands were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Piscataway, NJ, USA).

Measurement of intracellular Ca²⁺ and cAMP levels in T84 cells

Fluo calcium indicator, Fluo-4-AM (Invitrogen, Carlsbad, CA, USA) was used for measuring intracellular [Ca²⁺] changes after CT treatment according to company's suggested protocol. Briefly, transwell cultured T84 cells were incubated with Fluo-4-AM (5 μM) in cell culture incubator for 45 min. After washing with 1× phosphate-buffered saline (PBS), cells were incubated in 1× PBS for 30 min. to allow complete de-esterification of intracellular AM esters. The cells were then mounted on a Carl Zeiss LSM 510 laser (Carl Zeiss, Jena, Germany) scanning confocal microscope for live calcium imaging. Beam of 488 from a UV laser was used for excitation. CT was added to basolateral side at a concentration of 10 nM. Images were captured every 5 sec. for 5 min. Intracellular C AMP levels were determined using the Amersham Direct Biotrak EIA kit. On the day of assay, cells were harvested and assayed according to company's suggested protocol. Each assay point was performed at least in triplicate.

Measurements of short-circuit current

Agonist-induced anion secretion was measured in T84 monolayer as described [25, 26]. Briefly, cells grown on a Snapwell membrane were incubated with bicarbonate-buffered Krebs-Henseleit solution contained (in mM or mmoles/l) NaCl, 117; NaHCO₃, 25; KCl, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.2; CaCl₂, 2.5 and D-glucose, 11, pH 7.4, when bubbled with 5% CO₂, 95% O₂. Cl⁻-free solution was prepared by isosmotically replacing NaCl and KCl with sodium gluconate and potassium gluconate, respectively; CaCl₂ was replaced with 11 mM calcium gluconate to counteract the chelating effect of gluconate anion. The potential difference was clamped to 0 mV, and I sc was simultaneously measured using a voltage-clamp amplifier. Both signals were digitized and recorded. For antagonist experiments, cells were pre-incubated with the antagonist 45 min. before CT was added.

I sc in nystatin-permeabilized T84 monolayers

T84 cell monolayers were mounted in the Ussing chamber and bathed in normal Krebs-Henseleit solution while the I sc was measured as described above. Apical membrane Cl⁻ currents, defined as I ap, were measured in
cells permeabilized basolaterally with 300 µg/ml nystatin in the presence of asymmetrical buffers that imposed an apical to basolateral Cl\(^{-}\) gradient. Basolateral NaCl was replaced by equimolar sodium gluconate. Nystatin was added to the basolateral membrane 30 min. before the addition of drugs. Under these asymmetrical conditions, activation of the apical membrane Cl\(^{-}\) conductance would cause a rapid downward current deflection. Basolateral membrane K\(^{+}\) currents, defined as \(i_{\text{K}^{+}}\), were measured in cells permeabilized apically with 300 µg/ml nystatin for 30 min. in the presence of asymmetrical buffers that imposed an apical-to-basolateral K\(^{+}\) gradient. The apical NaCl was replaced by equimolar K-gluconate, whereas basolateral NaCl was substituted with equimolar sodium gluconate. In all gluconate-containing solutions, CaCl\(_2\) was increased to 11 mM to compensate for the Ca\(^{2+}\) chelating effect of the gluconate anion [27].

**Cell surface biotinylation**

Cell surface biotinylation studies were performed in T84 monolayers utilizing Sulfo-NH-SS-Biotin (1.5 mg/ml; Pierce, Rockford, IL, USA) in borate buffer (in mM: 154 NaCl, 7.2 KCl, 1.8 CaCl\(_2\), 10 H\(_3\)BO\(_3\), pH 9.0) as described previously [28]. Labelling was allowed to proceed at 4°C to prevent endocytosis and internalization of antigens for 60 min. The biotinylated antigens were immunoprecipitated utilizing streptavidin agarose beads, and the biotinylated proteins were released by boiling in Laemmli buffer containing 100 mM dithiothreitol. Proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes for immunoblotting.

**Statistical analysis**

In all stances, data shown are the mean ± S.E. of three to six independent experiments. Difference between control versus treated was analysed using t-test and \(\alpha\) = 0.05. Differences were considered significant at \(P < 0.05\).

**Results**

**Calcitonin receptor is expressed in intestinal epithelial cells**

First set of studies were undertaken to determine the expression of CTR in human intestinal epithelial cells. As shown in Figure 1A, real-time PCR analysis demonstrated the expression of CTR mRNA in human colonic T84 cell line. Western blot analysis utilizing CTR-specific antibodies also showed the presence of a ~56 kD protein band representing CTR protein (Fig. 1B). These results indicate that T84 cell line represents suitable *in vitro* cellular model for investigating the effects of CT on intestinal electrolyte transport.

**Calcitonin induces short circuit current in T84 cells**

Human colonic T84 cells are widely used as an *in vitro* model to study the secretory processes across colonic epithelial cells by the measurement of \(I_{SC}\) [29, 30]. Therefore, to examine the effect of CT on \(I_{SC}\), we utilized T84 monolayers mounted in Ussing chambers. Exposure of cells to 10 nM CT added to the basolateral compartment caused a sharp increase in \(I_{SC}\) (Fig. 2C). The addition of CT from the apical side had no effect suggesting that CTR is expressed on the basolateral membrane of T84 cells. The increase in \(I_{SC}\) began within 20 sec. of addition of CT, peaked at 5–10 min. and started to return to baseline slowly by 45–60 min. When compared to the actions of carbachol (CCH, 100 µM) (Fig. 2A) or Forskolin (FSK) (10 µM) (Fig. 2B), CT-induced \(I_{SC}\) was much longer in duration than CCH, but shorter than FSK; it was slower than CCH but more rapid than FSK to reach its peak. CT-induced \(I_{SC}\) in the T84 monolayer was concentration dependent, i.e. 10 nM produced higher effects than 1 nM, and interestingly, 100 nM had less effect than 10 nM (Fig. 2D). Pre-incubation (30 min.) of the T84 monolayer with increasing concentrations of CTR-specific antagonist, CT\(_{8–32}\) resulted in a dose-dependent blockage of CT-induced \(I_{SC}\), with 1 µM of CT\(_{8–32}\) almost completely abolishing CT- (10 nM) induced \(I_{SC}\) (3.1 ± 1.1% of control) (Fig. 2E). Inhibition only occurred when CT\(_{8–32}\) was added to the basolateral not the apical side, further indicating a functional basolateral distribution of CTR in this cell line.

**CT-induced \(I_{SC}\) in T84 cell monolayer is Cl\(^{-}\) dependent**

To test whether CT-induced \(I_{SC}\) in T84 cells results from Cl\(^{-}\) secretion, T84 monolayer was mounted in Ussing chambers and Cl\(^{-}\) was replaced with equimolar gluconate in the bathing solutions. As shown in Figure 3A, Cl\(^{-}\)-free conditions produced a significant reduction of \(I_{SC}\) (12.5 ± 6.0% of Cl\(^{-}\)-containing medium, \(P < 0.001\)) at 1 min., and was abolished (5.4 ± 1.3% of Cl\(^{-}\)-containing medium, \(P < 0.001\)) at 5 min. after basolateral application of CT. To further determine the nature of the CT-induced \(I_{SC}\), we tested the effects of inhibitors of transport pathways involved in chloride secretion. As depicted in Figure 3B and C, the addition of 100 µM bumetanide, a specific blocker of sodium, potassium, chloride co-transporter (NKCC1), to the basolateral side caused a significant decrease (12.5 ± 6.0%, compared to 80 ± 7.6% of peak \(I_{SC}\) with
PKA- and Ca$^{2+}$-dependent pathways are involved in CT-induced Cl$^{-}$ secretion

Activation of CTR in osteoclasts has been previously shown to stimulate both adenylate cyclase/cAMP/PKA and Ca$^{2+}$/PKC intracellular signalling pathways [8]. In order to determine if CT-induced chloride secretion in T84 cells involves Ca$^{2+}$-dependent pathways, we examined if there were changes in intracellular Ca$^{2+}$ in these cells in response to CT. The results depicted in Figure 4A showed that intracellular Ca$^{2+}$ signal increased almost immediately after addition of 10 nM of CT to the basolateral side, and kept elevated for more than 5 min. We also measured intracellular cAMP levels in T84 cells to determine if CT-induced chloride secretion involves the cAMP signalling mechanism. Intracellular cAMP levels increased from baseline 93 ± 12 fmol/μg protein to 9000 ± 346 fmol/μg protein after 20 min. incubation with 10 nM CT (Fig. 4B). The CT effect was blocked (9000 ± 346 fmol/μg protein versus 157 ± 19 fmol/μg protein) by co-incubation with 1000 nM of the CT antagonist CT8–32, while CT8–32 alone had no effect on intracellular cAMP level (93 ± 12 fmol/well versus 125 ± 25 fmol/well protein). FSK (10 μM), used as a positive control, similarly increased cAMP level (17105 ± 2421 fmol/μg protein).

We next examined the effect of H-89, a PKA inhibitor, and RpcAMP, a cAMP antagonist to further confirm the involvement of the PKA-cAMP pathway in CT-induced chloride secretion. As shown in Figure 4C, pre-treatment of T84 cells with the H-89 (10 μM) or RpcAMP (25 μM) for 45 min., reduced CT-mediated ISC to 45.8 ± 8.2% and 68.5 ± 3.9% of control, respectively.

In order to assess the role of Ca$^{2+}$- or PKC pathways in CT-induced chloride secretion in T84 cells, BAPTA-AM (Ca$^{2+}$ chelator) and bisindolylmaleimide (BIM) (general PKC inhibitor) [31] were used. Pre-incubation of T84 cells with 20 μM BAPTA-AM for 45 min. resulted in 42.4 ± 13.2% of CT-induced ISC, whereas BIM (5 μM) had no effect (Fig. 4C). These results indicate that cAMP and Ca$^{2+}$-dependent but not PKC-dependent pathways are involved in the effects of CT. Interestingly, pre-incubation with both 20 μM BAPTA-AM and 10 μM H-89 produced an additive effects (decreased to 25.2 ± 2.3% of control, Fig. 4C) suggesting each of the cAMP and Ca$^{2+}$-pathways has separate effects on CT-induced ISC.

CT stimulates ISC across the apical and basolateral membranes of intestinal epithelial cells

To further delineate the involvement of apical conductance and/or basolateral conductance in the T84 monolayer, the pore-forming

vehicle alone). Barium chloride (5 mM), a general inhibitor for K$^+$ channels, also resulted in significant decrease in ISC induced by CT (13.3 ± 3.3%, compared to 80 ± 7.6% of peak ISC with vehicle alone) when added from the basolateral side (Fig. 3D and F). Furthermore, the addition of 10 μM of CFTRinh172 (a specific inhibitor for CFTR), significantly reduced CT-induced ISC when added to the apical side as shown in Figure 3E and F (ISC induced by CT decreased to 11.7 ± 4.4%, compared to 80 ± 7.6% of peak ISC with vehicle alone). These data suggested that the CT-induced ISC in T84 cells is contributed by K$^+$ channels, CFTR and NKCC.
antibiotic nystatin was used to selectively permeabilize either cellular membrane [25]. The appropriate trans epithelial ion gradients were also established to measure the apical $I_{ap}$ and basolateral $I_{bl}$ currents. CT was added to basolateral side in both conditions. $I_{ap}$ was measured after nystatin permeabilization of the basolateral membrane. For studying the effects of different inhibitors, CT (10 nM) was added from the basolateral side after establishment of the baseline. Ten minutes later, inhibitors: (B) control = vehicle, (C) Bumetanide (100 μM), (D) barium chloride (5 mM) were added from the basolateral side, and (E) CFTRinh-172 (10 μM) was added from the apical compartment of the T84 monolayer. One representative tracing of each is shown here. (F) Data shown here are the values of $I_{sc}$ 10 min. after addition of different inhibitors (control = vehicle, Bumetanide, barium chloride, CFTRinh-172) (arrowhead in B, C, D and E), calculated as percentage of control. Results represent mean ± S.E.M. of three or more independent experiments in all above figures. **$P < 0.001$. 

Fig. 3 CT-induced $I_{sc}$ is chloride dependent. (A) CT (10 nM) was added from the basolateral side in regular Cl$^-$ containing Krebs-Henseleit medium and Cl$^-$ free Krebs-Henseleit medium where Cl$^-$ was replaced with equimolar gluconate. Data shown here are 1 and 5 min. after adding CT, calculated as percentage of Cl$^-$ containing Krebs-Henseleit medium. For studying the effects of different inhibitors, CT (10 nM) was added from the basolateral side after establishment of the baseline. Ten minutes later, inhibitors: (B) control = vehicle, (C) Bumetanide (100 μM), (D) barium chloride (5 mM) were added from the basolateral side, and (E) CFTRinh-172 (10 μM) was added from the apical compartment of the T84 monolayer. One representative tracing of each is shown here. (F) Data shown here are the values of $I_{sc}$ 10 min. after addition of different inhibitors (control = vehicle, Bumetanide, barium chloride, CFTRinh-172) (arrowhead in B, C, D and E), calculated as percentage of control. Results represent mean ± S.E.M. of three or more independent experiments in all above figures. **$P < 0.001$. 

antibiotic nystatin was used to selectively permeabilize either cellular membrane [25]. The appropriate transepithelial ion gradients were also established to measure the apical $I_{ap}$ and basolateral $I_{bl}$ currents. CT was added to basolateral side in both conditions. $I_{ap}$ was measured after nystatin permeabilization of the basolateral membrane. The CT-induced $I_{ap}$ was significantly blocked (28.4 ± 8.7%, Fig. 5A and C) by H89 (10 μM, 45 min. pre-incubation), while BAPTA-AM (30 μM, 45 min. pre-incubation) alone had no effect in this setting (86.1 ± 13.5%, Fig. 5C). This current was also sensitive to CFTRinh172, which represented a CFTR current (Fig. 5A). After nystatin permeabilization of the apical membrane, application of CT generated a small $I_{bl}$, which was signifi-

antly blocked by 45 min. pre-incubation of 20 μM of BAPTA-AM and 10 μM of H89; however, H89 had much less effect (21.2 ± 7.3% of control for BAPTA-AM and 50.0 ± 11.2% of control for H89, $P < 0.01$, Fig. 5B and D). This current was not found to be sensitive to NKCC inhibitor, bumetanide, but sensitive to K$^+$ channel blocker, barium chloride, which represented a K$^+$ channel current (Fig. 5B). These results suggest that CT evokes both $I_{sc}$ across the apical membrane via CFTR and $I_{sc}$ current across the basolateral membrane via K$^+$ channels. These findings also suggest that CAMP signalling component of CT-induced $I_{sc}$ involves both apical and basolateral processes, while Ca$^{2+}$ component mostly involves basolateral process induced by CT.
CT increased CFTR levels on the apical membrane

A mechanism of activation of CFTR is an increase in its trafficking from the intracellular compartments to the membrane. Aberrations in trafficking are among the causes underlying the manifestation of the disease in CF patients. To examine whether CT increases ksc via altering the membrane levels of CFTR, cell surface biotinylation studies were performed. Results showed that CT treatment (20 min.) of T84 cells significantly increased the surface level of CFTR compared to control (Fig. 6).

Discussion

Previous studies have shown that CT stimulates active Cl− secretion in adult rat ileum in vitro [32], rabbit in vivo [17, 33] and healthy humans in vivo [18]. The current studies provide more in depth understanding of the mechanisms of intestinal chloride secretion induced by CT. The major findings of current studies are: (i) CTR is expressed in human colonic epithelial cells; (ii) direct activation of CTR by CT in intestinal epithelial cells; (iii) CT-induced Cl− secretion involves both Ca2+ and cAMP-dependent pathways; (iv) CT-induced Cl− secretion occurs via CFTR chloride channel.

Earlier studies demonstrated the expression of CTR in several cell types including cells in the central nervous system, renal epithelial cells, breast, prostate cells and abundantly in mature osteoclasts [34]. CT was shown to inhibit sodium, hydrogen exchanger (NHE) activity and the Na+, K-ATPase in the kidney cell line LLC-PK1 [29]. CT was also found to inhibit proton extrusion in resorbing rat osteoclasts via PKA [30]. Although, previous studies showed that CT-induced chloride secretion was observed in human beings and rabbits, no information was available regarding the mechanisms of CT-induced chloride secretion, including CTR expression and its direct activation in intestinal epithelial cells. Our findings provided novel evidence showing the expression of CTR in colonic T84 cell line both at the mRNA and protein levels. These
findings provided a compelling evidence for the suitability of these intestinal cell lines to be used as an in vitro cellular model to investigate the molecular mechanisms underlying electrolyte transport alterations by CT.

In the T84 cell model, when CT was added to the basolateral side of T84 monolayer, an fsc was generated almost immediately, which was blocked by CTR-specific antagonist CT8–32 in a dose-dependent manner, while no current was produced when CT was added from the apical compartment. These findings indicate that this fsc was induced by CTR located on the basolateral membrane of the T84 cells, which is consistent with the fact that CT is a blood borne hormone. It is interesting that CT at 10 nM concentration induced chloride secretion more than at 100 nM concentration. As 10 and 100 nM CT-induced cAMP levels to the same extent (data not shown), we believe that this observation is likely due to a receptor de-sensitization. Indeed, diarrhoea occurs in about 28–39% of patients with MTC associated with high CT. These individual differences may reflect different levels of CTR expression in the intestinal epithelial cells or may indicate individual variations in the mechanisms involved in receptor desensitization.

Further experiments confirmed that CT-induced fsc in T84 monolayers was indeed due to increases in chloride secretion involving apical CFTR and basolateral NKCC and potassium channels (Figs 2 and 3). Further, biotinylation studies showed that CT increased CFTR levels on the apical membrane. Our findings also demonstrated that after activation of CTR by CT, two intracellular signalling pathways are triggered in T84 cells: cAMP- and Ca²⁺-dependent pathways. The earlier studies in rats, rabbits and humans, measured only the total amount of electrolyte movements across the intestinal epithelia. There are no data delineating the signalling pathways in intestinal epithelial cells. Our data showed that the direct activation of CTR by CT in T84 monolayers, significantly increased intracellular cAMP levels, which then either directly activated CFTR or stimulated basolateral membrane K⁺ channel activity. The basolateral K⁺ channels, promote potassium recycling across the basolateral membrane by extruding K⁺ entering either via the NKCC cotransporter or the Na, K-ATPase pump, and thereby increasing the electrochemical gradient for Cl⁻ entering via NKCC to exit the cell via apical membrane chloride channels (Figs 4 and 5). Our findings also demonstrated that CT caused an immediate increase in Ca²⁺ and the BAPTA data showed that the role of Ca²⁺ in CT evoked chloride secretion was through K⁺ channels on the basolateral membrane (Figs 4 and 5). Although activating distinct pathways, Ca²⁺ and cyclic nucleotides often modulate each other’s activities and the fact that CT activates both provides some intriguing possibilities for exploring the cross-talk between...
the two cascades. Responses to cyclic nucleotide-mediated agonists are sustained, whereas those to Ca\(^{2+}\)-mediated agonists are transient even though levels of intracellular Ca\(^{2+}\) can remain elevated after the secretory response has resolved [35]. This may explain the fact that CT-induced chloride secretion has a unique feature: quick response that could be due to activation of Ca\(^{2+}\)-dependent pathway like CCH and longer duration that may result from activation of cAMP-dependent pathway like FSK (Fig. 2).

Based on our findings, we propose the following cell signalling pathways of CTR mediated CFTR activation (Fig. 7). CT binds and activates CTR located at the basolateral membrane of T84 cells, which then evokes cAMP- and Ca\(^{2+}\)-dependent pathways stimulating CFTR and secreting chloride. In cAMP-dependent pathway, cAMP not only affects CFTR directly, either by activating CFTR channels or by increasing CFTR levels on the apical membrane, but also activates K\(^+\) channel on the basolateral membrane. In Ca\(^{2+}\)-dependent pathways, Ca\(^{2+}\) activates the K\(^+\) channel at the basolateral side providing the driving force for chloride influx via NKCC.

The current findings of CTR expression in intestinal epithelial cells provide a possible target for managing CT-induced severe diarrhoea in MTC patients and other clinical situations where severe diarrhoea is developed in CT-secreting pancreatic micro-tumour, and small cell lung tumours, etc.

In summary, the current study showed for the first time the expression of CTR in intestinal epithelial cells. Our data further demonstrated that CT, a hormone that regulates calcium homeostasis, directly activates CTR and induces CFTR-mediated chloride secretion in intestinal epithelial cells via cAMP- and Ca\(^{2+}\)-dependent signalling pathways. Our results define the molecular mechanisms underlying CT effects on intestinal ion transport and may enhance the possibility of developing novel therapeutic approaches for the treatment CT-induced diarrhoea.

Conflict of interest

The authors confirm that there are no conflicts of interest.

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