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Activation of apical CFTR and basolateral Ca^{2+}-activated K^+ channels by tetramethylpyrazine in Caco-2 cell line

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Abstract

We have previously demonstrated that tetramethylpyrazine (TMP) could stimulate colonic and pancreatic anion secretion. The present study investigated the signaling pathways and cellular mechanisms underlying the effect of TMP using human colonic Caco-2 cells, with permeabilized apical or basolateral membranes, in conjunction with Ussing chamber technique, intracellular cAMP and Ca^{2+} measurements as well as competitive RT-PCR for mRNA expression of cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-dependent Cl^- channels (CACC). Basolateral addition of TMP induced a short circuit current (I_{SC}) response, which could be mimicked by forskolin and 3-isobutyl-1-methylxanthine (IBMX). Adenylate cyclase inhibitor, MDL12330A, and intracellular Ca^{2+} chelator, BAPTA-AM, significantly inhibited the TMP-induced I_{SC}. In basolateral membrane-permeabilized cells, TMP, as well as forskolin and IBMX, induced an I_{SC} response, which was sensitive to MDL-12330A, H_{89}, and specific channel blocker $CFTR_{inh\text{-}172}$, but insensitive to apical application of 4-4′-didsothiocyanostilbene-2, 2′-disulfonic acid (DIDS) and basolateral pretreatment with BAPTA-AM. In apical membrane-permeabilized cells, TMP, similar to forskolin and IBMX, produced a very small current increase, which was sensitive to K^+ channel blockers, $BaCl_2$ and tetraethylammonium (TEA), but not Chromanol 293B and charybdotoxin (ChTX), alone or combined. However, in intact Caco-2 monolayers, the TMP-induced I_{SC} could be partially inhibited by ChTX. TMP (5 mM) could stimulate intracellular cAMP production. Intracellular Ca^{2+} was also increased by TMP (5 mM) in both Ca^{2+}-containing and Ca^{2+}-free bathing solutions. RT-PCR showed that the expression of CFTR in Caco-2 cells was 5.2 fold higher than that of Ca^{2+}-activated Cl^- channel (CACC). In conclusion, TMP stimulates Cl^- secretion by activating cAMP and $[Ca^{2+}]_i$ signaling pathways leading to subsequent activation of apical CFTR and basolateral K^+ channels.

Keywords: Tetramethylpyrazine; Cystic fibrosis transmembrane conductance regulator; cAMP; K^+ channel; Membrane permeabilization; Caco-2 cell line

1. Introduction

Tetramethylpyrazine (TMP), also known as ligustrazine, is a main active compound purified from *Ligustium wollichii franchat* (Sutter and Wang, 1993). It has been used clinically for treatment of cardiovascular disorders (Lin et al., 1997; Kwan et al., 1990) and acute ischemic cerebrovascular disease (Zhang et al., 1994, Cai et al., 2000) by improving microcirculation. Several signaling pathways, including intracellular cAMP and $[Ca^{2+}]_i$ have been proposed to account for TMP action on vasodilation and antiplatelet activity (Lin et al., 1993; Liu and Sylvester, 1994; Li et al., 2001). We have previously demonstrated that TMP also exerts a stimulatory effect on anion secretion by colonic and pancreatic duct epithelial cells (Zhu et al., 2004; Zhao et al., 2003). However, the signaling pathways and cellular mechanisms involved in mediating the effect of TMP in the secretory activity of the gastrointestinal tract remain unknown.

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It is well known that for secondary active Cl^- secretion in many epithelia including the colon, two sets of ion channels, Cl^- channels on the apical membrane and K^+ channels on the basolateral membrane, are required. Cl^- enters the cells across basolateral membrane by the means of electrically neutral Na^+–K^+–Cl^- cotransporter, driven by the Na^+ gradient set up by the Na^+–K^+-ATPase, and exits across apical membrane via Cl^- channels. The K^+ entering the cells via the Na^+–K^+-ATPase is recycled by basolateral K^+ channels, which firstly prevents K^+ accumulation causing cell swelling and secondly induces a hyperpolarization of the cells that subsequently provides the driving force for Cl^- exit across the apical membrane (Kunzelmann and Mall, 2002). It is well known that both apical Cl^- and basolateral K^+ channels in the colon can be regulated by intracellular cAMP or Ca^{2+}. Cystic fibrosis transmembrane conductance regulator (CFTR) is a cAMP-dependent Cl^- channel (Gregory et al., 1990; Cheng et al., 1991) predominantly expressed on the apical membrane of most epithelial cells (Sugi et al., 2001; Merlin et al., 1998; Grasset et al., 1985; Chang et al., 1991) and shown to play an important role in epithelial anion secretion, especially cAMP-dependent neurohormonal regulated secretory activities. Although CFTR has also been implicated in Ca^{2+}-dependent anion secretion (Bernard et al., 2003; Namkung et al., 2003), the detail mechanisms, especially the involvement of specific types of basolateral K^+ channels in CFTR-mediated anion secretion, remain largely unknown.

We undertook the present study to investigate the possible signaling pathways involved in mediating the stimulatory effect of TMP on anion secretion by a human colonic cell line, Caco-2 using the short-circuit current technique in conjunction with intracellular cAMP and Ca^{2+} measurements as well as competitive RT-PCR for mRNA expression of cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+}-dependent Cl^- channels (CACC). Efforts were also made to investigate the involvement of specific apical and basolateral ion channels by permeabilizing the basolateral and apical membrane, respectively. The results show that TMP may stimulate both cAMP and Ca^{2+}-dependent pathways leading to activation of both apical CFTR and basolateral Ca^{2+}-activated K^+ channel.

2. Materials and methods

Human colonic Caco-2 was purchased from American Type Culture Collection (Rockville, MD). The Caco-2 cells (passages from 45 to 60) were routinely maintained in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum with 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were fed 2–3 times a week and $2–3\times10^5$ of them were plated on to the floating permeable support, which was made of a Millipore filter with a silicone rubber ring attached on top of it for confining the cells (0.45 cm^2). For the cytosolic free Ca^{2+} concentrations measurement cells ($2–3\times10^4$) were grown on Transwell-col membranes (Costar, Cambridge, with 0.4 μm pore diameter culture area 0.1 cm^2). Cultures were incubated at 37 °C in 95% O_2–5% CO_2 for 5 days before experiments.

2.1. Short-circuit current measurement

The measurement of I_{SC} has been described previously (Zhu et al., 2004). Between the two halves of the Ussing chamber, in which the total cross sectional area was 0.45 cm^2, the monolayers of cells grown on permeable supports were clamped, which were bathed in both sides with Krebs–Henseleit solution (K–HS) (NaCl, 117 mM; KCl, 4.7 mM; $MgCl_2$, 1.2 mM; KH_2PO_4, 1.2 mM; $NaHCO_3$, 24.8 mM; $CaCl_2$, 2.5 mM; glucose, 11.1 mM) and maintained at 37 °C by a water jacket enclosing the reservoir. The K–HS was bubbled with 95% O_2 and 5% CO_2 to maintain the pH of the solution at 7.4. Drugs could be added directly to apical or basolateral side of the epithelium. Transepithelial potential difference for every monolayer was measured by the Ag/AgCl reference electrodes (World Precision Instrument, USA) connected to a preamplifier that was in turn connected to a voltage-clamp amplifier DVC-1000 (World Precision Instrument, USA). In most of the experiments, the change in I_{SC} was defined as the maximal rise in I_{SC} following agonist stimulation and it was normalized to current change per unit area of the epithelial monolayer (μA/cm^2). The total charges transported for 20 min (the area under the curve of the agonist-induced I_{SC} response) were also used to describe the agonist-induced response (μC/cm^2).

2.2. Basolateral membrane permeabilization

To evaluate the effect of TMP on the apical cAMP-dependent Cl^- channels, we used the pore-forming nystatin to selectively permeabilize the basolateral membranes. Firstly, Cl^- gradient (apical to basolateral) was achieved by replacing basolateral NaCl of K–HS with equimolar Na^+-gluconate, and elevated Ca^{2+} of K–HS from 2 mM to 4 mM. After the I_{SC} reached steady state, the basolateral membrane was permeabilized by the addition of nystatin (360 μg/ml) for 30–45 min. Under these conditions, agonist-induced I_{SC} represents the Cl^- current as Cl^- moves down its concentration gradient through the Cl^- channels in the apical plasma membrane.

2.3. Apical membrane permeabilization

To study the effect of TMP on the basolateral K^+ conductance, the apical surface of the epithelium was permeabilized by using nystatin (180 μg/ml) for 30–45 min, and a K^+-gradient applied in the apical-to-basolateral

direction which was achieved by replacing NaCl of the K–HS with equimolar potassium gluconate in the apical and with equimolar sodium gluconate in the basolateral solutions, and Ca^{2+} of K–HS was elevated from 2 mM to 4 mM. Under these conditions, agonist-induced I_{SC} represents the K^+ current as K^+ moves down its concentration gradient through the K^+ channels in the basolateral plasma membrane.

2.4. Reverse transcription PCR (RT-PCR) analysis

Total RNA (15 μg) was extracted from the Caco-2 cells. Expression of CFTR and CACC were analyzed by competitive RT-PCR. The specific oligonucleotide primers for human-CFTR were: AGC TGG ACC AGA CCA ATT TTG AGG AAA for sense and CCA CAC GAA ATG TGC CAA TGC AAG TCC for antisense corresponding to nucleotides 184–738 with expected cDNA of 554 bp; the specific oligonucleotide primers for human-CACC-2 (Agnel et al., 1999) were: GGC ACT TGG GCA TAC AAT CT for sense and ACA TTG GCT CCA AGA ACA GG for antisense corresponding to nucleotides 1715–1914 with expected cDNA of 200 bp. The conditions were: denaturation at 94 °C for 45 s; annealing at 55 °C and 58 °C, for 45 s; extension at 72 °C for 45 s; 30 and 31 cycles for human-CFTR and human-CACC, respectively. Internal marker, GAPDH was used for semi-quantitative analysis of hCFTR and hCACC-2 expressions in Caco-2 cells. The specific oligonucleotide primers for GAPDH was TCC CAT CAC CAT CTT CCA G for sense and TCC ACC ACT GAC ACG TTG for antisense corresponding to nucleotides 249–764 bp with expected cDNA of 515 bp (Usui et al., 2001). Intensity ratios were calculated for the bands of hCFTR/GAPDH and hCaCC-2/GAPDH.

2.5. Measurement of [^{3}H]-cyclic AMP production

The cAMP measurement has been described previously (Rowlands et al., 2001). After an overnight incubation (18 h) with [^{3}H]-adenine (2 μCi/ml; 1 μCi/well), the medium was aspirated and the cells washed twice with 1 ml HEPES-buffered saline (HBS mM: HEPES 25, pH 7.4, NaCl 135, KCl 3.5, $CaCl_2$ 2.5, $MgCl_2$ 1.0, glucose 3.3). Cells were challenged with test compounds (TMP, forskolin and IBMX) for 30 min at 37 °C in assay buffer (HBS containing 3 mM indomethacin to inhibit tonic prostanoid synthesis). The reaction was stopped by addition of ice-cold trichloroacetic acid and ATP, at a final concentration of 5% and 1 mM, respectively. The plates were left for at least 30 min on ice before separating the [^{3}H]-cyclic AMP from [^{3}H]-ATP by column chromatography 80. Cell samples were loaded onto Dowex AG50W-X4 (200±400 mesh) columns and [^{3}H]-ATP eluted with 3 ml distilled water. A further 10 ml distilled water was added and the eluant loaded directly onto a neutral alumina column, which was eluted with 6 ml 0.1 M imidazole buffer, pH 7.5, to give a fraction containing [^{3}H]-cyclic AMP. Scintillator (OptiPhase 'HiSafe' 3, 10 ml) was added for scintillation counting. The production of [^{3}H]-cyclic AMP from cellular [^{3}H]-ATP, i.e. adenylyl cyclase activity, was estimated as the ratio of radio-labeled cyclic AMP to total AXP (i.e. cyclic AMP, ADP and ATP), and is expressed as [cyclic AMP]/[total AXP]× 1000. All assays were performed in triplicate. Solvent controls were run as appropriate, but neither dimethyl sulfoxide nor ethanol interfered with the assay at the concentrations used.

2.6. Intracellular Ca^{2+} measurement

Effects of TMP on the intracellular Ca^{2+} concentrations in Caco-2 cells were determined by the fluorescent dye technique with fura-2 AM. Cells grown on Transwell-col membrane (Wong and Ko, 2002) were loaded with Fura-2 by incubation (45 min at 37 °C) in K–HS containing 3 μM Fura-2-acetoxymethylester and 1.6 μM pluronic F127. The membranes bearing Fura-2-loaded epithelia were then mounted in a miniature chamber attached to the stage of an inverted microscope (Olympus IX70). The cells were then viewed with a 10× magnification and the Fura-2 fluorescence ratios were recorded (MetaFlour software, Universal Imaging Corp.). The signal was digitized and recorded to computer hard disc.

2.7. Data analysis

Results were expressed as mean±standard error of mean (S.E.M). Experiments were normally repeated in different batches of culture to ensure that the data were reproducible. The number of experiments represents independent measurements on separate monolayers. Comparisons between groups of data were made by either the Student's *t*-tests (two-group comparison) or one-way ANOVA with Newman–Keuls post hoc test (three or more-group comparison). A P value of less than 0.05 was considered statistically significant.

2.8. Drugs

Injectio Ligustrazini Hydrochloridi (Tetramethylpyrazine) was purchased from the Fourth Pharmaceutical Factory of Beijing (Beijing, China). CFTR-inh172 was a gift from Prof. Ma Tong Hui (Membrane Channel Research Laboratory, Northeast Normal University, Changchun, P.R. of China). Tetraethylammonium (TEA), 3-isobutyl-1-methylxanthine (IBMX) and 4-4′-didsothiocyanostilbene-2,2′-disulfonic acid (DIDS) were obtained from Sigma Chemical Company (St. Louis, MO). Calbiochem (San Diego, CA) was the source for glybenlamide, thapsigargin, bumetanide, forskolin, H_{89}, nystatin (Mycostatin) and MDL-12330A. Trans-6-cyano-4-(*N*-ethylsulfonyl-*N*-methylamino)-3-hydroxy-2, 2-dimethyl-chromane (chromanol 293B) was

obtained from Hoechst (Frankfurt/Main, Germany). Fura-2 and pluronic F127 were from Molecular Probes (Eugene, OR).

3. Results

3.1. Dependence of TMP-evoked I_{SC} response on intracellular cAMP and Ca^{2+}

As shown in Fig. 1A, baseline values of transepithelial Potential Difference (PD), Short Circuit Current (I_{SC}) and resistance (R_t) in Caco-2 cells were 1.17±0.09 mV, 1.51±0.15 μA/cm^2 and 0.41±0.05 kΩ cm^2, respectively. The TMP-induced biphasic response, with an initial transient peak followed by a sustained plateau, was mimicked by an activator of adenylyl cyclase, forskolin, and an inhibitor of phosphodiesterase, IBMX, both of which are well known for elevating intracellular cAMP (Kopito, 1999). Pretreatment with MDL-12330A (20 μM), an inhibitor of adenylate cyclase, 10 min prior to the addition of TMP inhibited the TMP-induced I_{SC} response by 70.3%, from 3813.5±638 (n=6) to 1132.5±67.5 μC/cm^2 (n=4, P<0.001; Fig. 1B). Pretreatment with BAPTA-AM (50 μM), a membrane permeable Ca^{2+} chelator, or thapsigargin (3 μM), endoplasmic reticulum Ca^{2+} pump inhibitor, prior to TMP administration inhibited the TMP-induced I_{SC} response by 51%, from 5947±561.9 to 2917±259.6 μC/cm^2 (n=5, P<0.001; Fig. 1C) and by 45.3%, from 4996±324.7 to 2732±239.4 μC/cm^2 (n=9, P<0.001; Fig. 1C). However, the current kinetics remained unchanged (Fig. 1C).

3.2. Effects of TMP on apical Cl^- and basolateral K^+ current

TMP-induced Cl^- secretion could be achieved by directly increasing the apical membrane Cl^- conductance or by enhancing the electrochemical driving force for Cl^- secretion, which might be produced by modulation of basolateral membrane K^+ conductance. To further investigate the conductance pathways, the pore-forming antibiotic nystatin was used to selectively permeabilize either the basolateral or apical membrane, and the transepithelial ion gradients were appropriately established to measure apical Cl^- and basolateral K^+ current, respectively (see Materials and methods).

3.2.1. TMP-induced apical transepithelial Cl^- currents

Baseline values of PD and I_{SC} were changed from 1.07±0.1 to −14.7±1.51 mV and 1.27±0.18 to −45.4±5.97 μA/cm^2, respectively after permeabilizing basolateral membrane with nystatin and imposing Cl^- gradient. TMP (5 mM), similar to forskolin (10 μM) and IBMX (100 μM), stimulated an I_{SC} response in Caco-2 cells, with nystatin-permeabilized basolateral membrane, in a direction consistent with the imposed Cl^- gradient (117 mM) from apical to basolateral side (Fig. 2). The current increase was completely blocked by apical application of glibenclamide (1 mM; n=9), but not by apical addition of DIDS, a blocker of

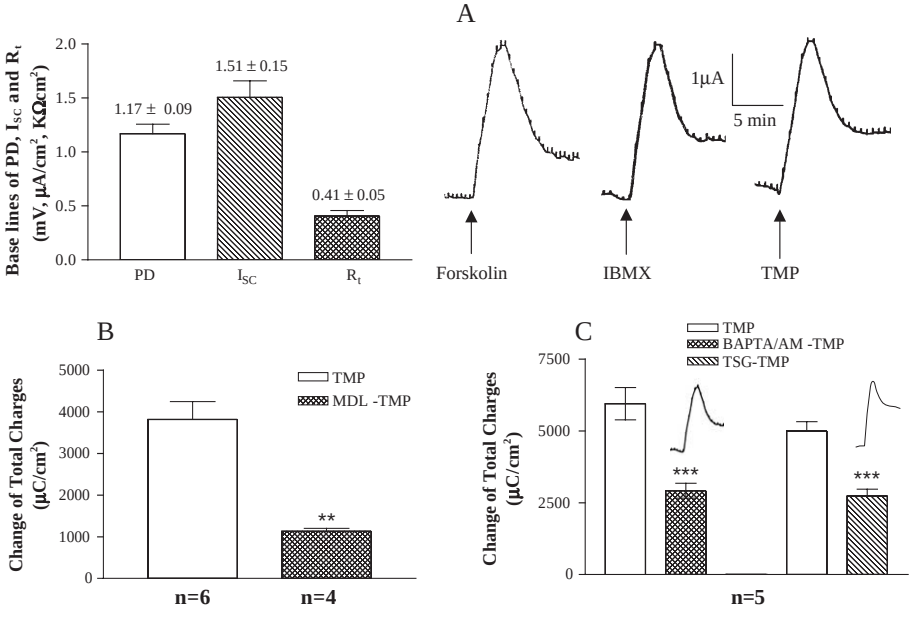

Fig. 1. Dependence of the TMP-induced I_{SC} on intracellular cAMP and Ca^{2+}. (A) Baseline values of transepithelial Potential Difference (PD), Short Circuit Current (I_{SC}) and resistance (R_t) in Caco-2 cells (left). Representative I_{SC} recordings in response to forskolin, IBMX and TMP (right). (B) Summary of the effects of the inhibitor of adenylyl cyclase, MDL 12330A (10 μM, basolateral), and the inhibitor of phosphodiesterase, IBMX (100 μM, basolateral) on TMP-induced I_{SC}. (C) Summary of the effects of the Ca^{2+} chelator, BAPTA-AM (50 μM, basolateral), and endoplasmic reticulum Ca^{2+} pump inhibitor, thapsigargin (3 μM, bilateral), on TMP-induced I_{SC}. Values are means±S.E.M.; ***P<0.001.

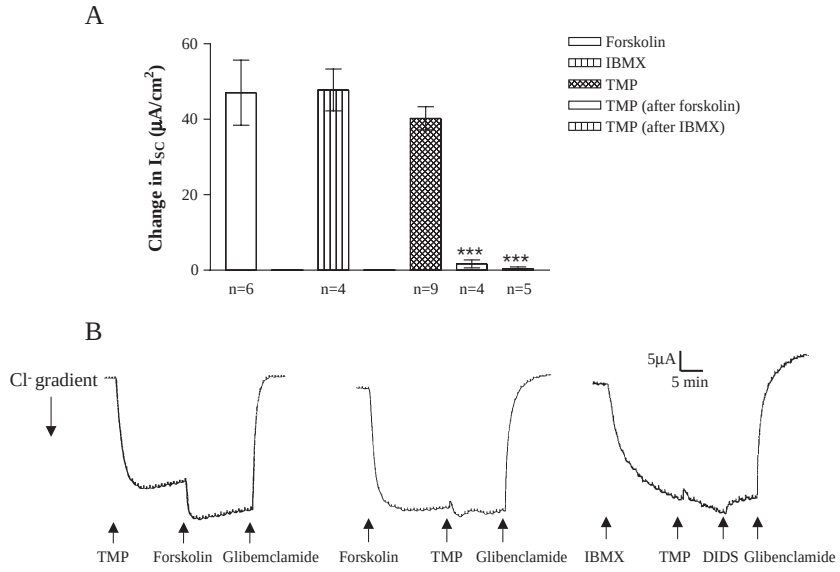

Fig. 2. Comparison of TMP-, forskolin- and IBMX-induced I_{SC} in Caco-2 cells with basolaterally permeabilized membrane. (A) Comparison of the effect of pretreatment with forskolin (10 μM) or IBMX (50 μM) on TMP (5 mM)-induced I_{SC} with Cl^- gradient shown. (B) Representative I_{SC} recordings in response to TMP (5 mM), forskolin (10 μM), IBMX (50 μM), TMP (5 mM) and apical addition of DIDS (100 μM) and glibenclamide (1 mM). Arrowheads indicate the time of chemicals' addition. TMP could not evoke additional I_{SC} increase after pretreatment with forskolin and IBMX.

CACC (Fig. 2A). Almost no further increase in the I_{SC} could be observed when TMP was added after the response to forskolin or IBMX (Fig. 2A, B), indicating that TMP may overlap with the cAMP pathway (i.e. more than 95% of TMP-induced I_{SC} was masked by forskolin- and IBMX-induced current responses). Conversely, after the TMP response, the forskolin- and IBMX-induced I_{SC} responses were also significantly reduced (data not shown). To further confirm the involvement of intracellular cAMP in TMP-induced I_{SC} response, the effects of MDL-12330A (20 μM) and an inhibitor of protein kinase A (PKA), H_{89} (50 μM) were examined. The results showed that MDL-12330A and H_{89}, when administrated 15 and 45 min prior to the addition of TMP, inhibited the TMP-induced I_{SC} response (n=9) by 69.1% and 76.6%, respectively, from 40.25±3.1 to 12.4±5.1 μA/cm^2 (n=5, P<0.001) and to 9.4±4.0 μA/cm^2 (n=5, P<0.001) (Fig. 3A). Bilateral pretreatment BAPTA-AM (50 μM/each side) for 50 min did not significantly affect the TMP-induced current response (n=4, P>0.05). A specific blocker of CFTR, $CFTR_{inh-172}$ (3 μM), inhibited the TMP-induced I_{SC} increase by 91.8%, from 49.1±6.0 to 4.9±1.9 μA/cm^2 (n=5, P<0.001; Fig. 3B).

3.2.2. TMP-induced basolateral transepithelial K^+ currents

To examine the effects of TMP on the basolateral K^+ channels of Caco-2 cells, the apical membrane was permeabilized with nystatin in the presence of an apical to basolateral K^+ gradient (117 mM). As shown in Fig. 4A, basolateral addition of TMP (5 mM) produced a small current increase with a more sustained plateau, which was resistant to basolateral application of 293B (10 μM; n=5), a blocker of cAMP-dependent K^+ channels, or ChTX (100

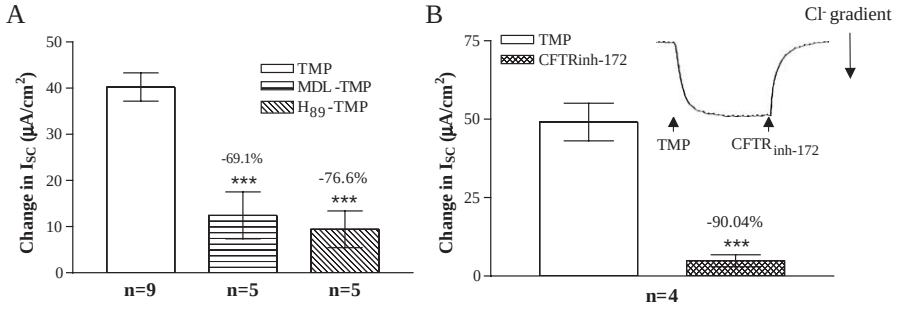

Fig. 3. Inhibition of the TMP-indced I_{SC} by pharmacological agents in Caco-2 cells with basolaterally permeabilized membrane. (A) Comparison of the effect of MDL12330A (20 μM) and H_{89} (50 μM) on TMP (5 mM)-induced I_{SC}. (B) Apical addition of $CFTR_{inh-172}$ (3 μM) blocked TMP (5 mM)-induced current increase; representative I_{SC} recordings in response to TMP (5 mM) and apical addition of $CFTR_{inh-172}$ (3 μM). Arrowheads indicate the time of chemicals' addition. Values are means±S.E.M.; ***P<0.001.

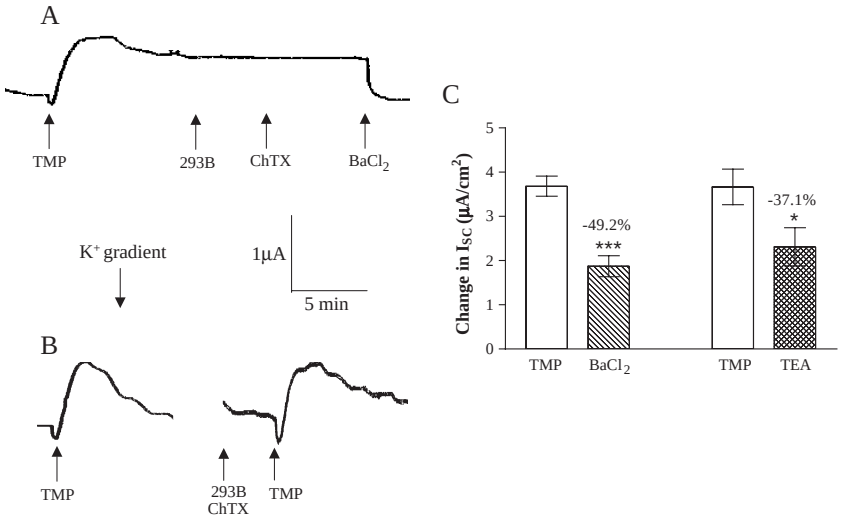

Fig. 4. Effects of K^+ channel blockers on the TMP-induced current increase in apically permeabilized Caco-2 monolayers. Representative I_{SC} recordings in response to TMP (5 mM), 293B (10 μM), ChTX (100 nM) and $BaCl_2$ (5 mM) without ouabain pretreatment (A), and TMP (5 mM) and pretreatment with 293B (10 μM) and ChTX (100 nM) with ouabain pretreatment imposed K^+ gradient is shown in arrow (B). (C) Comparison of the effect of $BaCl_2$ (5 mM) and TEA (5 mM) on TMP (5 mM)-induced I_{SC}. Values are means±S.E.M.; *P<0.05, ***P<0.001.

nM; n=4), a blocker of Ca^{2+}-dependent K^+ channels, either alone or combined (n=3). Similar I_{SC} response with the same pharmacological profile was observed with basolateral administration of IBMX (0.5 mM) and forskolin (10 μM) (data not shown). When ouabain (0.1 mM) was used to inhibit basolateral Na^+–K^+-ATPase, TMP only produced a transient current response lasting less than 3 min (n=3), which was not affected by 293B, ChTX or both (n=3, Fig. 4B). Pretreatment with BAPTA-AM (50 μM/each side, n=6) or MDL12330A (10 μM/each side, n=3) did not significantly affect the TMP-evoked I_{SC} response in apical membrane-permeabilized cells. $BaCl_2$ (5 mM) and TEA (5 mM), the non-specific blockers of K^+ channels, however, inhibited TMP-induced I_{SC} increase by 49.2%, from 3.68±0.23 to 1.87±0.24 μA/cm^2 (n=7, P<0.001) and 37.1%, from 3.67±0.40 to 2.31±0.43 μA/cm^2 (n=5, P<0.05; Fig. 4C), respectively. In intact Caco-2 epithelia, ChTX (100 nM), but not 293B (10 μM), reduced the TMP-induced current increase by about 42.1% (n=3; Fig. 5A), and basolateral addition of $BaCl_2$ (5 mM) totally blocked the TMP-induced current increase (n=6; Fig. 5B).

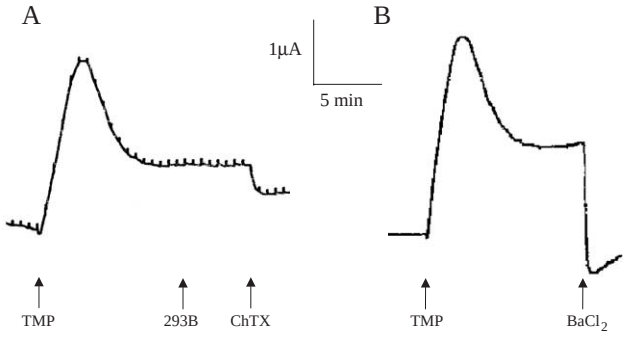

Fig. 5. Effects of K^+ channel blockers on the TMP-induced current in intact Caco-2 monolayers. Representative I_{SC} recordings in response to TMP (5 mM), 293B (10 μM) and ChTX (100 nM) (A) and to non-specific K^+ channel blocker, $BaCl_2$ (5 mM) (B).

3.3. Effects of TMP on intracellular cAMP and Ca^{2+} concentrations

Measuring conversion of [^{3}H]-ATP to [^{3}H]-cAMP showed that TMP (5 mM) and forskolin (10 μM) elevated cellular cAMP production from 1.2±0.1 to 2.7±0.7 (n=3, P<0.05) and 4.0±0.1(n=3, P<0.001), respectively, (Fig. 6A). Intracellular Ca^{2+} measurements (Fig. 6B) showed that TMP (5 mM) slightly increased $[Ca^{2+}]_i$, the Fura-2 fluorescence ratios from baseline 0.56±0.02 to 0.71±0.4, (n=7, P<0.01) in Ca^{2+}-containing solution, and from 0.51±0.02 to 0.60±0.4 (n=5, P<0.05) in Ca^{2+}-free solution.

3.4. RT-PCR analysis of CFTR and CACC expression in Caco-2 cells

The expressions of CFTR and CACC in Caco-2 cells were examined by RT-PCR. The result showed that the level of CFTR expression was much higher than that of CACC (Fig. 7A). The semi-quantitative analysis showed that the nucleotide expression ratio of CFTR and CACC-2 to GAPDH in Caco-2 cells was 1.51±0.22 and 0.29±0.04, respectively (n=4, P=0.0015; Fig. 7B).

4. Discussion

The present study investigated the possible signaling pathways and the coupled cellular mechanisms underlying TMP effect on anion secretion in human colonic cells, Caco-

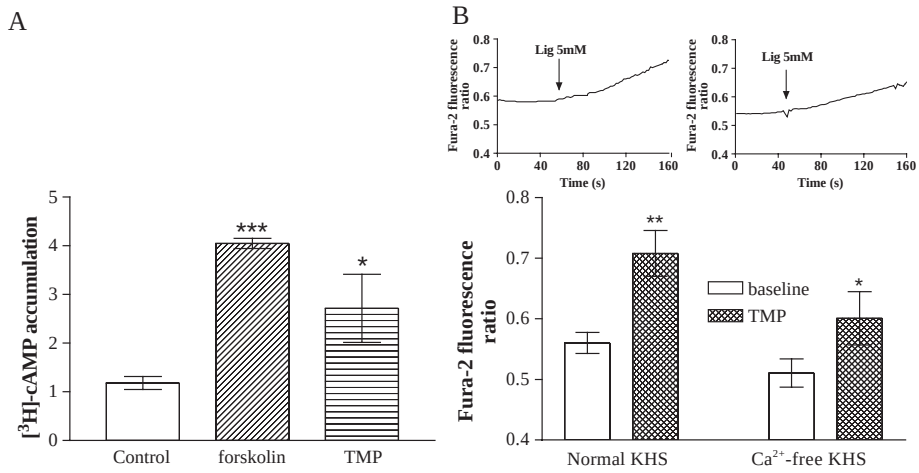

Fig. 6. Effects of TMP on intracellular cAMP and Ca^{2+} levels. (A) Comparison of cAMP production induced by forskolin (10 μM) and TMP (5 mM). (B) Comparison of the effects of TMP (5 mM) on the intracellular $[Ca^{2+}]_i$ with the illustrations of the time course in Ca^{2+}-containing and Ca^{2+}-free solution. Each column represents the means±S.E.M.; *P<0.05, **P<0.01, ***P<0.001, compared with control.

2. It appears that TMP may trigger both intracellular cAMP and Ca^{2+} increases in the cells. However, our results suggest that only the cAMP-activated Cl^- channel, CFTR, but not the Ca^{2+}-activated Cl^- channel, CACC, is responsible for the Cl^- exit from the apical membrane in Caco-2 cells. Several lines of evidence support this notion. First, the TMP-induced I_{SC} responses in intact and basolateral permeabilized Caco-2 monolayers can be mimicked by cAMP-evoking agents, forskolin and IBMX, and inhibited by inhibitors of adenylate cyclase and PKA. Second, there is small or no additive effect of TMP on forskolin or IBMX-induced I_{SC}, and vise versa, indicating that TMP as well as the cAMP-evoking agents, forskolin and IBMX, are using the same common signal pathway. Lastly, in intact or basolateral permeabilized Caco-2 monolayers the TMP-induced current increase can be inhibited by blockers known to inhibit CFTR, but not by blocker of Ca^{2+}-dependent Cl^- channel. It should be noted that $CFTR_{inh\text{-}172}$, a small molecule identified by high-throughput screening with most potent inhibitory effect on CFTR (Ma et al., 2002), has recently been demonstrated to be effective in producing

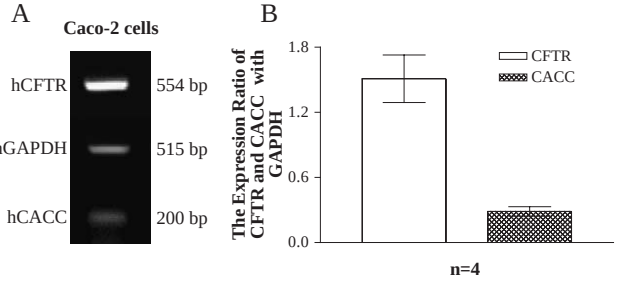

Fig. 7. RT-PCR analysis of mRNA expression of CFTR and CACC in Caco-2 cells. (A) RT-PCR results with products of CFTR, CACC and housekeeping gene, GAPDH in Caco-2 cells. (B) Semi-quantitative analysis of CFTR and CACC expressions in Caco-2 cells, which were shown in ratios of CFTR and CACC to GAPDH (internal marker). Values are means±S.E.M.

cystic fibrosis-like submucosal gland fluid secretions in normal airways (Thiagarajah et al., 2004a) and preventing toxin-induced intestinal ion and fluid secretion (Thiagarajah et al., 2004b). Taken together, these data suggest that CFTR is the apical transporting mechanism responsible for mediating the TMP-induced I_{SC} in Caco-2 cells. The high expression of CFTR and very low expression of CACC in Caco-2 cells, as demonstrated by RT-PCR, are in good agreement with a predominate role of CFTR in mediating the effect of TMP. It has also been reported that Cl^- secretion in Caco-2 cells is predominantly mediated by CFTR and that luminal Ca^{2+}-activated Cl^- channels is not functional in these cells (Davenport et al., 1996).

Although cAMP-dependent CFTR, but not Ca^{2+}-activated Cl^- channel, appears to be involved in mediating the effect of TMP, the TMP-induced I_{SC}, however, does seem to depend on intracellular Ca^{2+} since about 50% of the TMP-induced current could be inhibited by pretreatment with a membrane-permeable Ca^{2+} chelator, BAPTA-AM. Intracellular Ca^{2+} measurements also confirm a stimulatory effect of TMP. These results suggest that the TMP-enhanced intracellular Ca^{2+} may directly or indirectly activate the apical cAMP-dependent Cl^- channel, CFTR. It has been reported previously that intracellular Ca^{2+} can directly activate CFTR and Cl^--dependent HCO_3^- transport in pancreatic duct cells (Namkung et al., 2003). The present results have ruled out this possibility since the TMP-activated apical conductance, as observed in basolateral membrane-permeabilized cells, was not sensitive to treatment with BAPTA-AM. Alternatively, Ca^{2+}-activated Cl^- secretion in human colonic epithelium has been suggested to be due to direct activation of basolateral Ca^{2+}-activated K^+ conductance which induces epithelial hyperpolarization and consequently increases the electrical driving force for luminal Cl^- exit via apical CFTR (MacVinish et al., 1998; Mall et al., 1998). Our data do seem to indicate the coupling of basolateral K^+ channels and apical CFTR in mediating

the effect of TMP since the TMP-induced I_{SC} response could be completely blocked by basolateral addition of $BaCl_2$, a non-specific blocker of K^+ channels.

It is well known that there are two categories of K^+ channels, Ca^{2+}-dependent (Moon et al., 1997) and cAMP-dependent (Devor et al., 1999), localized to the basolateral membrane, which play an important role in providing a driving force for anion secretion in most epithelia (MacVinish et al., 2001). Our present data tend to suggest that cAMP-dependent K^+ channels are not involved since 293B, a blocker of cAMP-dependent K^+ channels, does not have any effect on the TMP-induced current increase in both intact and apical permeabilized Caco-2 monolayers. On the contrary, the present results suggest that Ca^{2+}-dependent K^+ channels may play a role in providing the driving force for TMP-induced anion secretion, since ChTX, a blocker of Ca^{2+}-dependent K^+ channels inhibited TMP-induced current increase in intact Caco-2 monolayers by about 40%. However, the present study also indicates that the activation of the Ca^{2+}-dependent K^+ channel requires the integrity of the apical membrane since ChTX does not exert any inhibitory effect on apically permeabilized Caco-2 monolayer. Similarly, studies by others also suggest that the integrity of the apical membrane may be necessary for the activation of basolateral K^+ channels by cAMP. For example, forskolin and EBIO were found to be unable to activate cAMP-dependent K^+ channels in apical permeabilized monolayer due to inability to generate cellular cAMP in the presence of apical nystatin (MacVinish et al., 2001). In fact, chelating $[Ca^{2+}]_i$ or inhibiting the production of intracellular cAMP by MDL 20 μM in our study did not affect the TMP response in the apically permeabilized cells. Therefore, results from the apically permeabilized cells are inconclusive as to which type of K channels is involved. It does, however, suggest that under apically permeabilized condition, TMP may have direct effect on the basolateral K channels or indirect effect through pathways that are independent of cAMP and Ca^{2+}. Based on the results obtained in intact cells, Ca^{2+}-activated K^+ channels appear to be involved. However, we cannot exclude the involvement of other basolateral K^+ channels since non-specific blockers of K^+ channel ($BaCl_2$ and TEA) can partly inhibit TMP-induced current increase in apically permeabilized Caco-2 monolayers and totally blocked it in intact Caco-2 monolayers. While the identity of K^+ channel(s) involved remains to be elucidated, it is clear that the opening of the K^+ channel(s) involved in the TMP response requires the operation of Na–K-ATPase since inhibition of this pump in apically permeabilized cells drastically altered the basolateral K^+ channel-mediate TMP response, from sustained to transient, even in the presence of an imposed K^+ gradient.

Ca^{2+} and cAMP have been implicated in the biphasic characteristic of I_{SC} observed in many epithelia (Merlin et al., 1998; Zhu et al., 2002a; Fukuda et al., 2000; Mall et al., 1998), with Ca^{2+} being associated with the first transient peak and cAMP with the sustained plateau (Reenstra, 1993; Zhu et al., 2002a; Zhu et al., 2002b; Son et al., 2003; Sugi et al., 2001). Although the present study has demonstrated the ability of TMP to activate both Ca^{2+} and cAMP, our data do not suggest an association of Ca^{2+} with the transient peak since the biphasic characteristic of the TMP-induced I_{SC} was not changed by pretreatment with BAPTA-AM and thapsigargin nor blocking apical CACC by pretreatment with DIDS. The observation that inhibition of Na–K-ATPase resulted in a transient TMP response in apically permeabilized cells (see above) suggests that the kinetics of the TMP may be affected by the Na–K-ATPase activity. Further studies along this line may provide better understanding of the action of TMP.

TMP has been used for treatment of cardiovascular disorders (Kwan et al., 1990; Lin et al., 1997; Sutter and Wang, 1993) and acute ischemic cerebrovascular diseases (Cai et al., 2000). Several actions of TMP, such as vasodilation, antiplatelet aggregation, antithrombosis, have been proposed to improve microcirculation by blocking Ca^{2+} entry or inhibiting Ca^{2+} releasing from ER (Liu and Sylvester, 1994). However, the effect of TMP on the intracellular Ca^{2+} in Caco-2 cells appears to be different from that observed in the cardiovascular system. Our data show that acute administration of TMP, either in Ca^{2+} containing or Ca^{2+} free solution, increases $[Ca^{2+}]_i$, which may lead to the activation of basolateral K^+ channels, providing the driving force for anion secretion. TMP may increase intracellular Ca^{2+} in Caco-2 cells either by inhibiting Ca^{2+} uptake pump in the ER, similar to the action of thapsigargin, or by activating Ca^{2+} release from the intracellular Ca^{2+} pool. Further studies are required to elucidate the detailed mechanism.

In conclusion, TMP stimulates anion secretion in Caco-2 cells by activating both intracellular cAMP and Ca^{2+} signaling pathways coupled to activation of apical CFTR and basolateral K^+ channels including Ca^{2+}-dependent K^+ channels. However, the activation of the basolateral K channels requires the integrity of apical membrane and the operation of Na–K-ATPase. The detailed mechanisms as to how the TMP-stimulated Ca^{2+} signaling pathway coupled to activation of CFTR, as well as the identity of the specific type of K channels involved, remain to be elucidated. The present study provides pharmacological evidences for the stimulatory effect of TMP on the gastrointestinal secretion, which may be further explored for development of alternative treatment for gastrointestinal disorders.

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