Appearance of Segmental Discrepancy of Anion Transport in Rat Distal Colon

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The present study investigated the segmental discrepancy of the rat distal colonic anion transport induced by luminal forskolin and the possible underlying mechanisms using short-circuit current recording technique and comparative quantity real-time PCR analysis. Forskolin-induced ISc in the segment next to lymph node (DC1) and the segment 4 cm away from lymph node (DC2) were 4.09±0.66 μA/cm2 and 18.84±3.18 μA/cm2 (n=13), respectively, which were blocked by luminal Cl− channel blocker, glybenclamide (1 μM) (n=5, p<0.01), as well as removal of extracellular Cl− and HCO3− in both DC1 and DC4 (n=5, p<0.001). Furthermore luminal pretreatment with K+ blockers, TEA (5 mM) and glybenclamide (100 μM) didn’t affect forskolin and bumetanide-enhanced ISc. Reducing serosal Cl− concentration increased forskolin-induced ISc by 90% in DC1, but decreased forskolin-induced ISc in DC4 by 50%. Furthermore, pretreatment with serosal bumetanide, an inhibitor of Na+-K+-2Cl− cotransporter, enhanced forskolin-induced ISc by 87% in DC1, from 4.09±0.66 μA/cm2 to 7.65±0.53 μA/cm2 (n=6, p<0.01), but inhibited forskolin-induced ISc by 50% in DC4, from 29.19±4.51 μA/cm2 to 15.06±4.10 μA/cm2 (n=6, p<0.05). Pretreatment with luminal amiloride (10 μM), an inhibitor of ENaC, and serosal 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (200 μM), an inhibitor of NBC, significantly inhibited the forskolin-induced ISc in DC1 (n=6, p<0.05), but not in DC4. Real-time PCR analysis did not show the significant differences between the two segments in the expression amounts of CFTR and NKCC mRNAs, however the expression of NBC mRNA in DC4 was significantly higher than that in DC1. In conclusion, the rat distal colon manifests a segmental discrepancy in anion transport stimulated by luminal forskolin. HCO3− might be predominantly involved in the forskolin-induced anion secretion in DC1, but Cl− might be the main component of the anion secretion in DC4.

Key words segmental difference; HCO3−; Cl−; distal colon

Anion secretion in mammalian distal colon plays an important role in body electrolyte and water balance under physiological and pathophysiological conditions. Electrogenic Cl− secretion is the fundamental means of intestinal mucosal surface hydration as a primitive defense mechanism which can prevent the mucosa from noxious stimuli, and several human diseases including secretory diarrhea and cystic fibrosis result from its defective regulation. HCO3−, similar to Cl−, is not only an important component of normal colonic fluid, contributing to the maintenance and regulation of the pH microclimate observed at the luminal surface of the colon, but also a major participator of the secreted fluid colonic fluid, contributing to the maintenance and regulation of gastrointestinal, and also a very distal part of the rat colon is the part many colonic diseases predominated and easy to be affected or attacked under stress.

MATERIALS AND METHODS

Preparation of Colonic Mucosa Animal protocols followed guidelines established by the NIH and were approved by Animal Care and Use Committee, Capital Medical University. Adult male Sprague-Dawley rats (Laboratory Animal Services Center, Capital Medical University) ranging in weight from 200 to 300 g fed with a standard rat diet and tap water, were killed by cervical dislocation, and a segment of distal colon of approximately ca. 7 cm in length was removed, rinsed with ice-cold Krebs–Henseleit solution (KHS). The distal colon was defined as the 7-cm-long segment proximal to the lymph node, (typically situated 3 cm away from the anus) as reported elsewhere. The distal colon was split into four segments termed DC1 (next to lymph node), DC2, DC3, and DC4, respectively. Because the effect of forskolin on the segment around lymph node (DC1) is very different from the other three segments and also the variation from DC2 to DC4 is gradually prominent, the DC1 and DC4 were therefore chosen to investigate the segmental discrepancy of the rat distal colonic anion transport and the possible underlying mechanism.

Each segment was cut open along mesenteric border and pinned out on a silicon gel filled Petri dish with mucosa facing downwards. The serosa, muscularis and submucosa were...
stripped with fine forceps away to obtain the mucosa preparation of the distal colon. Stripped mucosa was mounted in a modified Ussing chamber with a tissue holder (Easy-Mount Chamber; Physiologic Instruments, San Diego, CA, U.S.A.) having an aperture surface area of 0.5 cm² and was bathed bilaterally in a solution. The chambers were filled with 5 ml Krebs–Henseleit solution (KHS) on both sides of the mucosa. The bathing solution was continuously gassed with a mixture of 95% O₂–5% CO₂ and had a pH of 7.4. The temperature was kept at 37 °C by water bath. The mucosa was allowed to equilibrate for 30 min before the measurements were taken.

### Short-Circuit Current Measurement
The short-circuit current (I_SC) was measured in vitro in Ussing Chambers. Electrodes for measuring transepithelial potential difference (PD) and passing current were connected to the chambers. The transepithelial PD was then clamped at 0 mV and the I_SC was recorded with VCC MC6 voltage-current clamp amplifier (Physiologic Instruments, San Diego, CA, U.S.A.). Transepithelial resistance (Ω cm²) was measured by altering the membrane potential stepwise (−0.1 mV) and applying the Ohmic relationship.

### Real-Time Polymerase Chain Reaction Analysis of Gene Expression
Distal colonic tissues were harvested from animal and washed with KHS. Briefly, tissue was flash-frozen in liquid nitrogen and stored at −80 °C until further processing. Total RNA was isolated from DC₁ and DC₄, respectively, using Trizol™ reagent. Two microliters of total RNA was first transcribed into cDNA in 20 μl reagent mix and 1/20 of the cDNA was subjected to comparative quantification via SYBR Green PCR kit (STRATAGENE, U.S.A.) following the manufacturer’s protocols. Primer sequences and annealing temperature for the above chemicals were dissolved in DMSO. Final DMSO concentrations never exceeded 0.1% (v/v). Preliminary experiments indicated that the vehicle did not alter any baseline of electrophysiological parameters.

### Statistical Analysis
The data were presented as means±S.E.M.; “n” refers to the number of experiments undertaken using different tissue preparations. Comparisons between groups of data were made via Student’s paired or unpaired “t” test. p value of less than 0.05 was considered statistically significant.

### RESULTS

#### Effects of Luminal forskolin on the I_SC of Different Segments in Rat Distal Colon
After an equilibration of 30—45 min, the basal I_SC in DC₁ was 21.05±2.13 μA/cm², which was significantly lower than that in DC₄, 40.25±4.18 μA/cm² (n=13, p<0.001). The basal TR between DC₁, 89.77±6.57 Ω cm² and DC₄, 79.38±7.25 Ω cm², had no significant difference (n=13). Before the subsequent experiments were performed, the freshly isolated distal colonic segments, DC₁ and DC₄, were pretreated with indomethacin (10 μM) in serosal side to suppress endogenous prostaglandin production.

Luminal addition of forskolin (1 μM) evoked an I_SC increase in both DC₁ and DC₄ (Fig. 1). The I_SC increase in DC₁, however, was much smaller, 4.09±0.66 μA/cm² (n=13) than that in DC₄, 18.84±3.18 μA/cm², (n=13) (p<0.001),

### Table 1. Primer Sequences and Annealing Temperatures Used to Investigate the Expression of CFTR, NKCC₁ and NBC at the Level of mRNA in the Colonocytes of in DC₁ and DC₄

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense primer</th>
<th>Antisense primer</th>
<th>T_a (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFTR</td>
<td>TGG AAC ACA TAC CTT CGA TAT TTC A</td>
<td>AAA AAC CAG CAC GCA CCA A</td>
<td>60</td>
</tr>
<tr>
<td>NKCC</td>
<td>GIG ATG AGCATG GTG TCA GGA T</td>
<td>TGC CAG TGC TGA GAG GAG TGT</td>
<td>60</td>
</tr>
<tr>
<td>NBC</td>
<td>GAA GCT AAT TGT GCC AAG TGA GTT C</td>
<td>CAA AAG GTG GGA CAA ACC AA</td>
<td>60</td>
</tr>
<tr>
<td>beta</td>
<td>TTC AAC ACC CCA GCC ATG T</td>
<td>GTG GTA CGA CCA GAG GCA TAC A</td>
<td>60</td>
</tr>
</tbody>
</table>

### Fig. 1. Effect of Luminal Forskolin-Induced Anion Secretion in Two Segments
Representative I_SC recordings in response to amiloride (10 μM, luminal), forskolin (1 μM, luminal), bumetanide (10 μM, serosal) and glybenclamide (1 μM, luminal) on transepithelial ion transport of DC₁ (A) and DC₄ (B). Arrowheads indicate the time of drug addition. Comparison of the effect of luminal forskolin-induced I_SC between two segments (C). *** p<0.001.
and the $I_{SC}$ response in DC$_1$ could not be inhibited, but further enhanced by serosal administration of bumetanide (10 μM) (Figs. 1A, C), which is very different from that in DC$_4$ (Figs. 1B, C). The following studies were focused on the underlying mechanism of luminal forskolin-induced $I_{SC}$ difference between DC$_1$ and DC$_4$.

**Involvement of Anion Transport in Luminal Forskolin-Induced $I_{SC}$ Response**  
Luminal pretreatment with glybenclamide (1 mM), a sulfonylurea and a known CFTR inhibitor induced a significant decrease of basal current in DC$_1$ (A) and DC$_4$ (B). Values are means±S.E.M.; *p<0.05, **p<0.01. Forskolin-induced $I_{SC}$ in two segments can be blocked by addition of glybenclamide (C and D). Values are means±S.E.M.; ***p<0.001.

**Serosal Mechanisms Underlying the Segmental Difference of $I_{SC}$ between DC$_1$ and DC$_4$**  
As Fig. 4A shown that when serosal side exposed to a low Cl$^-$ KHS with 15 μM Cl$^-$, forskolin-induced $I_{SC}$ in DC$_4$ was not reduced, but significantly increased from 4.09±0.66 μA/cm$^2$ to 7.80±1.69 μA/cm$^2$ (p<0.05, n=12); the $I_{SC}$ in DC$_4$ was significantly decreased from 18.84±3.18 μA/cm$^2$ to 9.28±1.84 μA/cm$^2$ (n=9, p<0.05). Serosal pretreatment with bumetanide (10 μM) did not inhibit, but significantly inhibit forskolin-induced $I_{SC}$, from 4.09±0.66 μA/cm$^2$ (n=13) to 7.65±0.53 μA/cm$^2$ (n=6, p<0.01) in DC$_1$ (Fig. 4B), whereas, in DC$_4$, forskolin-induced $I_{SC}$ was inhibited by serosal pretreatment with bumetanide (10 μM), from 29.19±4.51 μA/cm$^2$ (n=7) to 15.06±4.10 μA/cm$^2$ (Fig. 4B, n=6, p<0.05). Pretreatment of the tissue with luminal amiloride (10 μM) and serosal DIDS (200 μM), an inhibitor of NBC and anion exchanger (AE), significantly inhibition the forskolin-induced $I_{SC}$, from 18.84±3.18 μA/cm$^2$ to 11.68±1.66 μA/cm$^2$ to 4.80±1.98 μA/cm$^2$ in the DC$_1$ (n=6, p<0.05), conversely, forskolin-induced $I_{SC}$ was significantly higher than that without being pretreated with DIDS in DC$_4$ (Fig. 5, n=7, p<0.01). According to above results, we assumed that the forskolin-activated $I_{SC}$ increase in DC$_4$ is mostly carried by...
Cl⁻, but that in DC₄ could be HCO₃⁻, through the luminal anion channel (probably CFTR).

**Real-Time PCR Analysis of CFTR, NKCC₁ and NBC**

The mRNA expression levels of CFTR, NKCC₁ and NBC were determined by comparative quantity Real-time PCR analysis (Fig. 6). The results indicated that there were no significant differences of CFTR and NKCC₁ between DC₁ and DC₄ (p=0.15 and p=0.96, respectively). But the NBC mRNA expression level in DC₄ was significant higher than that in DC₁ (p<0.05).

**DISCUSSION**

It has been reported that serosal forskolin could induce intestinal epithelial Cl⁻ secretion in human¹¹ and rabbit.²² Few studies were focused on the effect of luminal administration of forskolin on colonic ion transport and the segmental difference within distal colon, although many clinical trials have shown the alteration of luminal environment hold the balance during many distal colonic diseases.¹³,¹⁴ The present study firstly demonstrated that the rat distal colon manifests a segmental difference in anion transport qualitatively.

It has been known that anion secretion involves two-step processes: 1) Cl⁻ and HCO₃⁻ enter cells across basolateral membrane with the help of bumetanide-sensitive Na⁺-K⁺-2Cl⁻ cotransporter (NKCC₁) and Na⁺-HCO₃⁻ cotransporter (NBC), respectively, and HCO₃⁻ influx can also be generated inside the cells through carbonic anhydrase.¹⁵ 2) Electrogenic Cl⁻ and HCO₃⁻ efflux are mediated by cyclic AMP-dependent, protein kinase A-activated CFTR,¹⁶,¹⁷ and there might also be other channel or transporter proteins involved. Firstly we demonstrated that luminal forskolin-induced Iₛₜc increase in both DC₁ and DC₄ was mediated by anion (Cl⁻ and HCO₃⁻) secretion, since the Iₛₜc response was totally abolished by removal of Cl⁻ and HCO₃⁻ from bathing solution, and also mostly blocked by luminal administration of glybenclamide, a well-known inhibitor of Cl⁻ channels including
CFTR, which is permeable to both Cl− and HCO3−,18) but not inhibited by luminal pretreatment with amiloride, an epithelial ENaC blocker. Luminal pretreatment with putative K+-blockers such as TEA (5 mM) or glybenclamide (100 μM), a concentration known to block K+-channel, didn’t affect forskolin and bumetanide induced ISc increase, implying the ISc may not be associated with K+.

Then we demonstrated that the segmental discrepancy of anion secretion existed within rat distal colon since luminal forskolin-induced ISc increase in DC4 was much higher than that in DC1 (p<0.001), and inhibiting NKCC or reducing serosal Cl− concentration decreased forskolin-induced ISc increase in DC4, indicating that luminal forskolin predominantly induces Cl− secretion in DC4. As to why inhibiting serosal NBC (or AE exchanger) with DIDS did not decrease, but increase luminal forskolin-induced ISc response in DC4 is still unknown. However, it is interesting to note that luminal forskolin-induced ISc increase in DC4 can not be reduced, but further enhanced by inhibiting serosal NKCC with bumetanide. Moreover, both forskolin-induced and bumetanide-enhanced response in DC4, after pretreatment with inhibitor of ENaC, can be blocked by luminal glybenclamide, indicating that an anion secretion are involved in. A previous study19) reported that bumetanide-resistant part of serosal forskolin-induced ISc in rat proximal colon was mainly mediated by a basaloteral Cl−-HCO3− exchanger, which contributes to the forskolin-evoked Cl− secretion, and additionally small amount of HCO3− secretion. In our study, however, reducing serosal Cl− could not decrease, but rather increase luminal forskolin-induced ISc response in DC4, suggesting that luminal forskolin-induced ISc increase in DC4 was different from that serosal forskolin-induced anion secretion in proximal colon, and it was also irrelevant to serosal anion exchanger (AE). Serosal pretreatment with DIDS, an inhibitor of AE and NBC significantly decreased luminal forskolin-induced ISc response in DC4, indicating that the current increase in DC4 might be carried by HCO3− mediated by serosal NBC and luminal CFTR.

CFTR is predominantly expressed in airways, sweat duct and adult colon, and at highest level in the crypts.20) Luminal Cl− transport is predominantly via CFTR, which plays a crucial role in colonic ion transport. CFTR can also mediate cAMP-dependent HCO3− secretion,21,22) which is tightly associated with Cl− secretion in rat colonic crypt.23) As mentioned above, serosal Na+-K+-2Cl− cotransporter and Na+-HCO3− cotransporter are involved in Cl− and HCO3− secretion, we therefore consider that the different distribution of CFTR, NKCC1 and NBC might be existed in the two segments. And this might be responsible for the observed quantitative difference of forskolin-induced anion transport. Interestingly however, mRNA expression level of CFTR and NKCC1 between two segments, as measured by real-time PCR, has no significant difference, and furthermore the NBC mRNA expression in DC4 is significant higher than that in DC1, which appears contradictory to our ISc results above. However Seidler et al.24) have also reported the low HCO3− secretion, but higher expression of NBC in proximal colon. Therefore, we speculate that there might be similar mechanism in NBC-mediated HCO3− secretion between proximal colon and DC4. Because there are no significant differences in expression levels of CFTR and NKCC1 between DC4 and DC1, the qualitative difference of forskolin-induced ISc between the two segments also originate from the differential expression of other unexamined channels or cotransporters excepting the morphological differences. Moreover, the reason of qualitative difference of anion transport of two segments still needs to be investigated.

DC1 is closer to rectum which is a high-incident part suffered from colorectal cancer (CRC), ulcerative colitis and inflammatory bowel diseases (IBDs). The present finding of predominant HCO3− secretion by DC1 is of physiological interest. Colonic bicarbonate secretion can significantly reduce exposure of the epithelium to H2S through conversion to anionic sulfide produced by sulfate-reducing bacteria being members of the normal colonic microbiota and recognized to be linked with IBDs, CRC, and ulcerative colitis.12) Our findings thus may be relevant to understanding pathophysiology of these diseases and may have implication for luminal cAMP elevator contributing to those diseases conversion.

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REFERENCES
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