Role of Ca\(^{2+}\)-activated K\(^+\) channels in duodenal mucosal ion transport and bicarbonate secretion

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The mammalian duodenal epithelium has both absorptive and secretory functions. Although the duodenum is not a major site for nutrient absorption under normal conditions, its ability to secrete, particularly mucus and HCO\(_3\)\(^-\), is critical to defending the vulnerable duodenal epithelium against the aggressive gastric factors of acid and pepsin (3, 41). Indeed, the importance of duodenal mucosal bicarbonate secretion (DMBS) can readily be demonstrated in patients with duodenal ulcers, in whom acid-stimulated DMBS is attenuated by 41% compared with healthy subjects (1, 2, 17, 27). It has been widely accepted that the cystic fibrosis transmembrane conductance regulator (CFTR) and Cl\(^-\)/HCO\(_3\)\(^-\) exchangers are primary apical HCO\(_3\)\(^-\) transporters involved in DMBS and that cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_{cyt}\)], cAMP, and cGMP each play an important role in the regulation of DMBS (8, 17, 26, 41). However, many details regarding the precise cellular and molecular mechanisms responsible for DMBS and their regulation remain to be elucidated.

Several plasma membrane ion channels, such as those conducting Na\(^+\), Ca\(^{2+}\), K\(^+\), or Cl\(^-\), are functionally expressed in gastrointestinal epithelia and may be involved in intestinal absorptive and secretory functions (34, 41). In intestinal epithelial cells from segments distal to the duodenum, K\(^+\) channels play an important role in the stabilization of membrane voltage and maintenance of the driving force for electrogenic Cl\(^-\) transport (4, 34, 54). It is now generally believed that cholinergic agents stimulate intestinal Cl\(^-\) secretion, at least in part, via the primary activation of membrane K\(^+\) conductances, which are involved in maintaining cellular energetics favorable for Cl\(^-\) transport (4, 41, 54). Cholinergic agents such as acetylcholine or carbachol activate muscarinic receptors in colonic epithelia and thereby elevate [Ca\(^{2+}\)\(_{cyt}\)] (48). Thus cholinergic agents are widely considered as Ca\(^{2+}\)-dependent secretagogues. This increase in [Ca\(^{2+}\)\(_{cyt}\)] then activates Ca\(^{2+}\)-activated K\(^+\) (K\(_{Ca}\)) conductances (49) and secondarily stimulates Cl\(^-\) secretion through the CFTR (9, 23, 47).

Three subtypes of K\(_{Ca}\) channels have been identified in colonic surface and crypt cells: large-conductance (BK\(_{Ca}\)), intermediate-conductance (IK\(_{Ca}\)), and small-conductance (SK\(_{Ca}\)) channels (5, 6, 30, 54). Among these three subtypes of K\(_{Ca}\) channels, IK\(_{Ca}\) channels seem to play an important role in epithelial Cl\(^-\) secretion, because blockade of IK\(_{Ca}\) channels by clotrimazole inhibited Cl\(^-\) secretion in intact colonic epithelia and human colonic T84 cells (14, 37). In addition, direct activation of IK\(_{Ca}\) channels stimulates Cl\(^-\) secretion in a variety of epithelial tissues (12–14, 20). It has also been demonstrated that activation of CFTR alone is insufficient to evoke transepithelial Cl\(^-\) secretion and that basolateral membrane K\(^+\) channels are also necessary components of the secretory response. Therefore, basolateral membrane K\(_{Ca}\) channels may represent novel pharmacological targets that could be exploited to augment Cl\(^-\) secretion in patients suffering from cystic fibrosis. Some of the symptomatology in cystic fibrosis likely relates to abnormalities in duodenal ion transport. Surprisingly, the expression and function of K\(_{Ca}\) channels in duodenal epithelium, as well as their role in the regulation of duodenal epithelial ion transport and DMBS, have not been explored in detail. Moreover, although it has

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long been accepted that \([Ca^{2+}]_{cyt}\) plays an important role in epithelial ion transport (16, 17, 52), relatively little is known about the molecular targets that account for the effect of \([Ca^{2+}]_{cyt}\) on duodenal \(HCO_3^-\) secretion, or indeed other ion transport mechanisms in this intestinal segment. This is important to study directly because our studies to date have suggested that regulatory mechanisms for ion transport identified in the colon do not necessarily extrapolate to the duodenum. In the present study, therefore, our aim was to explore whether \(K_{Ca}\) channels are molecularly and functionally expressed in the duodenal epithelium and whether they play a role in the regulation of duodenal mucosal ion transport. By combining molecular approaches, Ussing chamber experiments in vitro, and whole animal studies, we now present evidence that \(IK_{Ca}\) channels are functionally expressed on the basolateral side of duodenal epithelial cells and play an important role in \(Ca^{2+}\)-mediated duodenal mucosal ion transport and DMBS.

**MATERIALS AND METHODS**

**Animal preparation.** This study was approved by the University of California, San Diego, Committee on Investigations Involving Animal Subjects. All experiments were performed with adult NIH Swiss or C57 black mice. Swiss mice were used for in vitro experiments. C57 black mice were used for in vivo experiments. The mice were housed in a standard animal care room with a 12:12-h light-dark cycle and were allowed free access to food and water. Before each experiment, mice were deprived of food and water for 1 h.

**Ussing chamber experiments in vitro.** Ussing chamber experiments were performed as previously described (16). After Swiss mice were anesthetized by halothane, the abdomen was opened with a midline incision. The proximal duodenum was removed and immediately placed in ice-cold isosmolar mannitol and indomethacin (10 \(\mu M\)) solution (to suppress trauma-induced prostaglandin release). The duodenal tissue from each animal was stripped of seromuscular layers, divided, and examined in three chambers (window area 0.1 cm\(^2\)). Experiments were performed under continuous short-circuited conditions (voltage-current clamp, VCC 600; Physiologic Instruments, San Diego, CA), and luminal pH was maintained at 7.40 by the continuous infusion of 5 mM HCl under the automatic control of a pH-stat system (ETS 822; Radiometer America, Westlake, OH). The volume of the titrant infused per unit time was used to quantitate \(HCO_3^-\) secretion. Measurements were recorded at 5-min intervals, and mean values for consecutive 5- or 10-min periods were averaged. The rate of luminal \(HCO_3^-\) secretion is expressed as micromoles per square centimeter per hour. The short-circuit current (\(I_{sc}\)) was measured in microamperes and converted into microequivalents per square centimeter per hour.

After a 30-min period when basal parameters were measured, inhibitors were added for another 30 min, as dictated by the experimental design, followed by additions of carbachol to the serosal side, forskolin or heat-stable enterotoxin (STa) to the mucosal side, or 1-ethyl-2-benzimidazolinone (1-EBIO) and A-23187 to both sides of the tissue. In some experiments, 1-EBIO was added to only one side of the tissue. Inhibitors were added for another 30 min, as dictated by the experimental design, followed by additions of carbachol (CCh) to the serosal side, forskolin or heat-stable enterotoxin (STa) to the mucosal side, or 1-ethyl-2-benzimidazolinone (1-EBIO) and A-23187 to both sides of the tissue. In some experiments, 1-EBIO was added to only one side of the tissue to assess the sidedness of its effect. Electrophysiological parameters and \(HCO_3^-\) secretion were then recorded for 60 min. As shown in Fig. 1A, in control experiments, addition of 10 \(\mu L\) of vehicle (DMSO or distilled water) to both sides of the duodenal tissue in 3-ml Ussing chambers. \(I_{sc}\) and \(HCO_3^-\) secretion were then recorded for 90 min. Clotrimazole (30 \(\mu M\)) or TRAM-34 (10 \(\mu M\)) was added to both sides, and CCh (100 \(\mu M\)) was added serosally at the times indicated by the arrows. Values that differ significantly from those in the absence of clotrimazole or TRAM-34: *\(P < 0.05\), **\(P < 0.01\) vs. control.

**Acid-stimulated duodenal \(HCO_3^-\) secretion in vivo.** In vivo experiments were performed with a well-validated technique, as described previously (22), on C57 black mice. Mice were anesthetized by intraperitoneal injection of Hypnorm-midazolam cocktail (25% Hypnorm, 25% midazolam, and 50% distilled water) at a dose of 10 mg/kg. Anesthesia was maintained throughout the experiment via the administration of smaller doses (20% of initial) of the same cocktail as needed, determined through the careful monitoring of both respiratory rate and response to toe pinch. After anestheticization, the abdomen was opened and the duodenum was accessed through two small incisions, one just below the rib cage on the left side and the other just below the sternum. Through the first incision the stomach was located, and a tiny incision was made just proximal to the pyloric sphincter. Through this incision a soft polyethylene catheter was inserted into the stomach, gently pushed through the pyloric sphincter, and tied firmly into position with silk suture thread around the outside of the pyloric sphincter, isolating the proximal duodenum (5–10 mm) from the stomach. Through the incision below the sternum, the
junction of the pancreatic duct and the duodenum was located. A tiny incision was made in the duodenum, and a second polyethylene catheter was inserted and tied into place just proximal to the junction with the pancreatic duct but distal to the duodenal blood supply. Thus the pancreatic contributions were occluded from the isolated duodenal segment, while the blood supply remained intact. Throughout the duration of the experiment, the duodenum maintained a healthy pink color and was kept moist within the abdominal cavity.

After surgery, the proximal duodenum was perfused with isotonic saline for 20 min. After this initial washout and recovery period, basal HCO₃⁻ secretion was measured for 20 min. The mouse was then given an intraperitoneal injection of either clomizol (20 mg/kg) or control vehicle (DMSO), and HCO₃⁻ secretion was measured for 6 min. The duodenal segment was then perfused with 10 mM HCl in isotonic saline for 5 min. After acidification, the segment was gently flushed to remove any residual acid and allowed a 5-min washout period. HCO₃⁻ secretion was then measured for an additional 42 min. After each experiment, the length of the duodenal test segment was measured in situ to the nearest millimeter. As shown previously (23), animals could be sustained under these experimental conditions for 2 h. Sample volumes were measured by weight to the nearest 0.01 g/ml. The amount of HCO₃⁻ in the effluents was quantitated through use of a CO₂-sensitive electrode (Thermo Orion, Beverly, MA). The electrode was calibrated before each day’s use by constructing a semilogarithmic standard curve using known HCO₃⁻ concentrations.

HCO₃⁻ outputs were determined for 6-min periods and expressed as micromoles per square centimeter per hour. Stimulated HCO₃⁻ outputs are presented as HCO₃⁻ output over time and as net HCO₃⁻ output (peak minus average basal output).

RT-PCR analysis. Total RNA from Swiss mouse duodenal mucosae was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The total RNA thus extracted was resuspended in water. Five micrograms of total RNA were converted into cDNA with reverse transcriptase. After inactivation at 70°C for 10 min, 1 µl of the reaction mixture was then incubated in buffer containing 0.2 mM dATP, dCTP, dGTP, and dTTP, 0.2 µM oligonucleotide primers as shown below, 3 mM MgCl₂, 500 mM KCl, and a 10× buffer consisting of 200 mM Tris-HCl (pH 8.0) together with 1 U of Taq polymerase (Invitrogen). Primers were synthesized by Invitrogen. Mouse KCNN3 (SK3)-specific sense and antisense primers (GenBank accession no. NM_080466) were 5'-CACCAGACTCTGCTCCATCA-3' and 5'-ACAAACCCCCAGAGTAGTGCG-3'. The predicted size of the PCR-amplified product for KCNN3 was 238 bp. Mouse KCNN4 (SK4)-specific sense and antisense primers (GenBank accession no. NM_008433) were 5'-AAGCACACTCGAAGGAAGGA-3' and 5'-CCGTCGATTCCTCTTCCAG-3'. The predicted size of the PCR-amplified product for KCNN4 was 215 bp. Mouse GAPDH sense and antisense primers as described by Ijichi et al. (24) were 5'-ACCACTGTCATGCTCATAC-3' and 5'-TCCACACACCCTTTGCTGTA-3'. The samples were amplified in an automated thermal cycler (GeneAmp 2400; Applied Biosystems). DNA amplification conditions included an initial 3-min denaturation step at 94°C, 35 cycles of 30 s at 94°C, 30 s at 57°C, 40 s at 72°C, and a final elongation step of 10 min at 72°C. The products were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide (0.5 µg/ml), and then photographed under UV light. To confirm band identity, the RT-PCR products were subjected to restriction enzyme analysis.

Western blotting analysis. Duodenal tissues from C57 black mice and Swiss mice were isolated and stripped to obtain mucosal layers. The mucosae were immediately homogenized (PowerGen 125, Fisher Scientific) in lysis buffer (150 mM NaCl, 10 mM Tris·HCl pH 7.8, 1 mM EDTA, 0.5% Triton X-100, 1 mM sodium orthovanadate, 0.1% SDS, 1 µg/ml leupeptin, and 100 µg/ml PMSF) at 30,000 rpm on ice. The lysates were then centrifuged at 12,000 rpm for 10 min at 4°C to remove insoluble materials. The protein in supernatants were mixed with 2× loading buffer (50 mM Tris pH 6.8, 2% SDS, 100 mM dithiothreitol, 0.2% bromphenol blue, 20% glycerol) and boiled for 5 min. The proteins were then separated by SDS-PAGE (7.5%). Resolved proteins were transferred overnight at 4°C onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA) and blocked with a 5% solution of skim milk for 30 min at room temperature, followed by further incubation with polyclonal antibodies against KCNN3 (SK3) and KCNN4 (SK4). After the membrane was washed with phosphate-buffered saline with 1% Tween (PBST), the secondary antibody was applied to the membrane. After the membrane was washed with PBST, the membrane was treated with a chemiluminescent solution according to manufacturer’s instructions and exposed to X-ray film.

Chemicals and solutions. KCNN4 antibody was purchased from Alomone Labs (Jerusalem, Israel), and KCNN3 antibody was from Alomone labs or Abcam (Cambridge, MA). Carbachol, forskolin, chlotrimazole, A-23187, tetraethylammonium (TEA), 4-aminopyridine (4-AP), BaCl₂, and indomethacin were purchased from Sigma Chemical (St. Louis, MO). 1-EBIO was from Tocris (Ellisville, MO). The other chemicals were obtained from Fisher Scientific (Santa Clara, CA). The mucosal solution used in Ussing chamber experiments contained the following (in mM): 140 Na⁺, 5.4 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, 120 Cl⁻, 25 glucose, and 10 mannitol. The serosal solution contained the following (in mM): 140 Na⁺, 5.4 K⁺, 1.2 Ca²⁺, 1.2 Mg²⁺, 120 Cl⁻, 25 HCO₃⁻, 2.4 HPO₄⁻, 2.4 H₂PO₄⁻, 10 glucose, and 0.01 indomethacin. The osmolarities for both solutions were ~284 mosmol/kgH₂O.

Statistical analysis. Results are expressed as means ± SE. Differences between means were considered to be statistically significant at P < 0.05, using Student’s t-test for paired or unpaired values or analysis of variance, as appropriate.

RESULTS

Effects of K⁺ channel blockers on carbachol-mediated Isc and HCO₃⁻ secretion. K⁺ channels have been functionally demonstrated in colonic epithelia, where they are believed to play an important role in the regulation of Cl⁻ and K⁺ secretion induced by muscarinic agonists (30, 49). Therefore, in our initial studies, Ussing chamber experiments were conducted to test whether K⁺ channels are also functionally expressed in duodenal mucosae and involved in duodenal mucosal ion transport and DMBS. After duodenal mucosal tissues were equilibrated in Ussing chambers for 30 min, basal Isc and HCO₃⁻ secretion were recorded for an additional 30 min. Subsequently, duodenal mucosal tissues were pretreated with different K⁺ channel blockers for another 30 min by adding them, or their vehicle (DMSO or distilled water), to both sides of the tissues, because K⁺ channels in intestinal epithelial cells may not be restricted to only one side of the cell (46, 47, 54). Finally, carbachol (100 µM), a well-known Ca²⁺-dependent agonist (8), was added to the serosal side of the tissue. As shown in Fig. 1, B and C, carbachol induced a significant increase in Isc in a biphasic manner: an initial transient peak and a second sustained plateau, which reached their maxima 2 and 15 min after carbachol addition, respectively. Therefore, the first transient phase of net peak Isc, calculated as the difference between the baseline and the peak value at 2 min after carbachol addition, and the second plateau phase, calculated as the difference between the baseline and the peak value at 15 min, were used to describe carbachol-induced Isc (Fig. 1, B and C). However, carbachol induced only a monophasic increase in HCO₃⁻ secretion, reaching a peak value at 10 min after carbachol addition and persisting thereafter (16). Therefore, net peak HCO₃⁻ secretion, calculated as the
difference between the baseline and the peak value at 10 min, was used to describe carbachol-mediated HCO$_3^-$ secretion (Fig. 1D). Clotrimazole (30 μM), a selective blocker of IK$_{	ext{Ca}}$ (14, 43), significantly inhibited both phases of carbachol-mediated $I_{sc}$ and HCO$_3^-$ secretion (Fig. 1, B and D). The first transient phase of net peak $I_{sc}$ was decreased by 37% ($n = 7; P < 0.05$), and the second plateau phase was totally abolished by clotrimazole, which also attenuated carbachol-mediated net peak HCO$_3^-$ secretion by 53% ($n = 7; P < 0.01$). Clotrimazole is also an inhibitor of cytochrome P-450 enzymes (18, 57), metabolites of which have been shown to activate KC$_{Ca}$ channels (35). To assess the possibility that clotrimazole may block KC$_{Ca}$ channels by inhibiting activity of cytochrome P-450 enzymes, we used 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), which is a more potent and selective IK$_{	ext{Ca}}$ blocker that does not inhibit cytochrome P-450 enzymes (57). As shown in Fig. 1, C and D, TRAM-34 (10 μM) reproduced the action of clotrimazole on carbachol-mediated $I_{sc}$ and HCO$_3^-$ secretion. The first transient phase of net peak $I_{sc}$ was decreased by 25% ($n = 7; P < 0.05$), and the second plateau phase was totally abolished by TRAM-34, which also attenuated carbachol-mediated net peak HCO$_3^-$ secretion by 68% ($n = 7; P < 0.01$). Furthermore, to assess the specificity of clotrimazole for IK$_{	ext{Ca}}$, forskolin or STa was used in Ussing chamber experiments. Addition of clotrimazole (30 μM) to both side of the chambers failed to alter forskolin- or STa-chamber experiments. Addition of clotrimazole (30 μM) to both sides of the duodenal tissue, and carbachol (100 μM) was added serosally. $I_{sc}$, short-circuit current. *P < 0.05 vs. control.

Table 1. Effects of 4-AP, BaCl$_2$, and TEA on carbachol-stimulated duodenal mucosal net peak $I_{sc}$ and net peak HCO$_3^-$ secretion

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>4-AP</th>
<th>BaCl$_2$</th>
<th>TEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>First phase of $I_{sc}$, μeq-cm$^{-2}$-h$^{-1}$</td>
<td>8.1 ± 0.5 (9)</td>
<td>8.4 ± 0.9 (5)</td>
<td>7.2 ± 0.4 (6)</td>
<td>5.6 ± 0.5* (5)</td>
</tr>
<tr>
<td>Second phase of $I_{sc}$, μeq-cm$^{-2}$-h$^{-1}$</td>
<td>3.1 ± 0.2 (9)</td>
<td>3.0 ± 0.5 (5)</td>
<td>2.6 ± 0.2 (6)</td>
<td>2.4 ± 0.3 (5)</td>
</tr>
<tr>
<td>HCO$_3^-$ secretion, μmol-cm$^{-2}$-h$^{-1}$</td>
<td>0.59 ± 0.10 (6)</td>
<td>0.81 ± 0.12 (5)</td>
<td>0.69 ± 0.10 (6)</td>
<td>0.65 ± 0.11 (6)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE for the number of tissues (in parentheses) in each series. 4-Aminopyridine (4-AP; 3 mM), BaCl$_2$ (5 mM), and tetraethylammonium (TEA; 10 mM) were added to both sides of the duodenal tissue, and carbachol (100 μM) was added serosally. $I_{sc}$, short-circuit current.

Effects of K$^+$ channel blockers on KC$_{Ca}$ opener-mediated $I_{sc}$ and HCO$_3^-$ secretion. Our data therefore suggested that IK$_{	ext{Ca}}$ is involved in Ca$^{2+}$-dependent $I_{sc}$ and HCO$_3^-$ secretion. To confirm this, we examined whether a KC$_{Ca}$ channel opener can increase $I_{sc}$ and HCO$_3^-$ secretion and whether any such responses can be inhibited by selective IK$_{	ext{Ca}}$ channel blockers. l-EBIO has been demonstrated to stimulate Cl$^-$ secretion in T84 cells and colonic epithelial tissues via the direct activation line $I_{sc}$ and significantly inhibited the first transient phase of net peak $I_{sc}$ of carbachol-mediated $I_{sc}$ by 31% ($n = 5; P < 0.05$) but did not significantly inhibit the second plateau phase of carbachol-mediated $I_{sc}$ or carbachol-mediated HCO$_3^-$ secretion. A-23187, a Ca$^{2+}$ ionophore, also induced an increase in $I_{sc}$ and HCO$_3^-$ secretion in a monophasic manner, and clotrimazole (30 μM) significantly inhibited A-23187-mediated net peak HCO$_3^-$ secretion (Fig. 2).

![Fig. 2. Inhibitory effect of clotrimazole on the time course of A-23187-stimulated duodenal mucosal $I_{sc}$ (A) and net peak HCO$_3^-$ secretion (B). Clotrimazole (30 μM) was added to both sides, and A-23187 (10 μM) was also added to both sides at the time indicated by the arrow. Values are means ± SE; $n = 5$ in each series. Values that differ significantly from those in the absence of clotrimazole: *P < 0.05 vs. control.](http://ajpgi.physiology.org/ by 10.220.32.246 on June 20, 2017)
of IKCa channels (13, 20). As shown in Fig. 3, bilateral addition of 1-EBIO at 1 mM induced a significant increase in $I_{sc}$ and $\text{HCO}_3^-$ secretion. Both clotrimazole (30 μM) and TRAM-34 (10 μM) significantly inhibited both $I_{sc}$ and $\text{HCO}_3^-$ secretory responses evoked by 1-EBIO (Fig. 3). These data indicate that IKCa opening is likely sufficient to mediate $I_{sc}$ and $\text{HCO}_3^-$ secretory responses in the duodenum.

Because a portion of the enteric nervous system might still remain in the duodenal mucosae mounted in Ussing chambers, 10 μM indomethacin was added to serosal solutions throughout all experiments to rule out the possible involvement of PGE2. Pretreatment with 10 μM atropine, which abolished carbachol-induced duodenal $I_{sc}$ and $\text{HCO}_3^-$ secretion in our previous study (16), did not affect basal duodenal $I_{sc}$ and $\text{HCO}_3^-$ secretion (data not shown), also ruling out the possible involvement of acetylcholine release from the remaining enteric nervous system in the duodenal mucosa in an unstimulated state. To further study whether 1-EBIO acts on duodenocytes directly or via the enteric nervous system, the effect of 10 μM atropine on the response to 1 mM 1-EBIO was assessed. Net peak $I_{sc}$ was 8.1 ± 0.6 μeq·cm$^{-2}$·h$^{-1}$ for 1-EBIO only vs. 8.0 ± 0.4 μeq·cm$^{-2}$·h$^{-1}$ for 1-EBIO plus atropine ($n = 5$; $P > 0.05$). Net peak $\text{HCO}_3^-$ secretion was 1.20 ± 0.10 μmol·cm$^{-2}$·h$^{-1}$ for 1-EBIO only vs. 1.10 ± 0.20 μmol·cm$^{-2}$·h$^{-1}$ for 1-EBIO plus atropine ($n = 5$; $P > 0.05$). Therefore, these data suggest that 1-EBIO acts directly on duodenocytes to stimulate duodenal ion transport and $\text{HCO}_3^-$ secretion.

To determine whether IKCa expression is polarized in duodenal epithelial cells, 1-EBIO was added to either side of the tissues. As shown in Fig. 4, application of 1-EBIO caused significant increases in duodenal $I_{sc}$ and $\text{HCO}_3^-$ secretion when applied to the basolateral side of the tissues but essentially no response when applied apically. These data indicate that the...
1-EBIO-induced changes in duodenal $I_{sc}$ and DMBS are due to activation of basolateral IKCa channels.

Effects of $K^+$ channel blockers on acid-stimulated duodenal $HCO_3^-$ secretion. Our in vitro Ussing chamber experiments clearly showed that IKCa channels are likely functionally expressed in the basolateral aspect of duodenal epithelial cells and are involved in duodenal ion transport. To test further whether IKCa channels in the duodenal epithelium play a physiological role, acid-stimulated duodenal $HCO_3^-$ secretion was measured in whole animal experiments. As shown in Fig. 5, duodenal luminal perfusion of 10 mM HCl resulted in a robust increase in DMBS in control animals, which reached a maximum at around 30 min after HCl stimulation and then after declined. However, administration of clotrimazole (20 mg/kg ip) completely abolished acid-stimulated DMBS. Net peak $HCO_3^-$ secretion, calculated from the difference between the baseline and the peak value, was also used to describe acid-stimulated $HCO_3^-$ secretion (Fig. 5B). Clotrimazole inhibited net peak $HCO_3^-$ secretion by 77%, suggesting that the IKCa channels in duodenal epithelium play a physiological role in acid-stimulated duodenal $HCO_3^-$ secretion in vivo.

mRNA and protein expression of KCa channels in duodenal epithelium. Three subtypes of KCa channels, BKCa, IKCa, and SKCa, have been identified in colonic surface and crypt cells, but the extent to which this extends to the duodenum was unknown. We therefore attempted to identify the type(s) of KCa channels present in duodenal epithelium. This was done first by using RT-PCR analysis to determine the expression of mRNA specific for the three types of these channels. Figure 6A shows that transcripts for KCNN4 were readily detected in Swiss mouse duodenal epithelium. KCNN3 products were also detected but only at low levels in the duodenal epithelium. Afterwards, Western blot analysis was performed to determine the expression of KCa channel proteins in the duodenal mucosae freshly isolated from both C57 and Swiss mice. As illustrated in Fig. 6B, an anti-KCNN4 antibody recognized a prominent band with molecular mass of 45 kDa, corresponding to previous reports of the native KCNN4 proteins (21, 30). However, KCNN3 proteins were undetectable in both C57 and Swiss mice with anti-KCNN3 antibodies purchased from two different companies (data not shown). Therefore, on the basis of the protein expression pattern, KCNN4 gene is the most likely molecular candidate for IKCa channels functionally identified above.

**DISCUSSION**

Although it has been demonstrated that IKCa channels are abundantly expressed on the plasma membrane of human colonic epithelial cells and play an important role in the Cl$^-$ secretory mechanism, little is known about the expression of IKCa channels in duodenal epithelium and the function of these channels in duodenal ion transport mechanisms and, in particular, DMBS. In our previous study (16), we showed that Ca$^{2+}$-dependent agonists, such as carbachol, can increase [Ca$^{2+}$]$_{cyt}$ via activation of the reversed mode of plasma membrane Na$^+$/Ca$^{2+}$ exchangers and thereby mediate DMBS. However, the downstream molecular mechanisms of Ca$^{2+}$-mediated DMBS have not been well elucidated. IKCa, CFTR, Cl$^-$/$HCO_3^-$ exchanger, and Ca$^{2+}$-activated Cl$^-$ channels (CLCA) are candidates that might account for the ability of
eral IKCa channels have long been thought to be direct loci of 
Ca2+-mediated duodenal HCO3− secretion, as they do in 
Ca2+-mediated colonic Cl− secretion (4, 12, 13).

The expression and function of intestinal K+ channels have primarily been evaluated in colonic epithelial cells. In this segment, KCa channels in particular have been postulated to play an important role in intestinal K+ homeostasis and colonic ion transport (46, 49, 55). Three subtypes of KCa channels have been identified by molecular approaches, patch-clamp techniques, and selective pharmacological tools in colonic surface and crypt cells (5–7, 30, 34, 45, 56): 1) a large-conductance channel (BKCa), which has a conductance >100 pS and is sensitive to TEA and iberiotoxin; 2) an intermediate-conductance channel (IKCa), which has a conductance of 25–36 pS and is sensitive to trilomazine and charybdotoxin; and a small-conductance channel (SKCa), which has a conductance <20 pS and is sensitive to apamin. Likewise, at least two types of K+ channels involved in colonic Cl− secretion have been identified on functional grounds (19, 36, 40). One of these, relevant here, is activated by an increase in [Ca2+]cyt and is relatively insensitive to the K+ channel blocker barium but is blocked by trilomazine and charybdotoxin and can be opened directly by 1-EBIO (28, 29, 53). Data also suggest that this K+ channel is activated by the Ca2+-dependent agonist carbachol via G protein-dependent mechanisms and perhaps via an increase in the sensitivity of the channel to [Ca2+]cyt (10). Emerging data suggest that the recently characterized hIK1 channel (variously termed KCNN4 by the Human Genome Organization or hSK4, hKCa4, and rSK4 by individual authors) may be a candidate for this conductance, based on its electrophysiological properties, sensitivity to submicromolar Ca2+ concentrations, and pharmacology (28, 29, 53). Basolat- eral IKCa channels have long been thought to be direct loci whereby the regulation of the overall process of Cl− secretion is accomplished. Thus a classic view of Ca2+-dependent Cl− secretion held that a basolateral K+ channel was the only site of regulation for this process (11, 15). In this scenario, K+ channel opening would cause cell hyperpolarization and hence promote Cl− efflux across the fraction of Cl− channels found open in the apical membrane at any given time.

In the present study, we have demonstrated that IKCa channels are not only expressed abundantly in duodenal mucosa but also play a physiological role in the regulation of duodenal Cl− and HCO3− secretion. First, carbachol-induced duodenal Isc and HCO3− secretion were significantly inhibited by trilomazine, a selective IKCa blocker (43, 57). The specificity of trilomazine for IKCa was confirmed by its failure to inhibit forskolin- and STa-induced duodenal Isc and HCO3− secretion, which act via the cAMP and cGMP signaling pathways, respectively. Our findings are consistent with those obtained from T84 cells, demonstrating that trilomazine has no effect on cAMP-regulated CFTR while blocking IKCa channels (14). Moreover, TRAM-34, another more selective IKCa blocker without an effect on cytochrome P-450 enzymes (57), also significantly inhibited both carbachol-mediated Isc and HCO3− secretion. Second, 1-EBIO, a selective IKCa opener (13, 20), increased both duodenal Isc and HCO3− secretion, which were again significantly inhibited by trilomazine and TRAM-34. Because of the direct opening of KCa, 1-EBIO increased Isc in a monophasic manner and induced duodenal ion transport in a sustained fashion rather than the transient effect of carbachol. Furthermore, our findings from Ussing chamber studies suggest that the expression of IKCa channels is polarized and restricted to the basolateral aspect of the duodenal epithelial cells. These results are consistent with those in colonic epithelium and in T84 cells, in which IKCa channels are also baso- lateral (13, 30, 42). Third, use of A-23187, a Ca2+ ionophore, to directly transport Ca2+ into the cells increased duodenal Isc and HCO3− secretion, which were also significantly inhibited by trilomazine. Fourth, pharmacological blockers for K+ channel subfamilies other than IKCa did not significantly affect carbachol-induced duodenal Isc or HCO3− secretion. Fifth, abundant expressions of KCNN4 mRNA and proteins were demonstrated by RT-PCR and Western blot analysis in murine duodenal mucosa. Finally, trilomazine significantly inhibited acid-stimulated DMBS in vivo, demonstrating a physiological role for IKCa channels in DMBS.

It has long been accepted that DMBS is an important factor in protection of the duodenal mucosa against acid-induced injury (27). In the proximal duodenum, the ability to neutralize gastric acid is due largely to surface epithelial HCO3− secretion. In addition to protecting the duodenal mucosa from injury, DMBS promotes a nonacidic environment necessary for pancreatic enzyme activity (1, 17, 25). Whereas [Ca2+]cyt has been widely considered as an important mediator in DMBS (16, 17, 52), relatively little is known about the molecular targets that account for the effect of [Ca2+]cyt on DMBS. On the basis of our studies at the levels of mRNA, proteins, tissues, and whole animals, we proposed a model for the role of duodenal epithelial IKCa in acid-stimulated DMBS. Under physiological conditions, luminal acid stimulates duodenal mucosal enterochromaffin cells and/or nerve endings to release Ca2+-dependent agonists, such as acetylcholine, 5-HT, and/or histamine (1, 31–33). These Ca2+-dependent agonists bind to G protein-coupled receptors on the plasma membrane and increase Ca2+-mediated colonic Cl− secretion (4, 12, 13). In turn, [Ca2+]cyt activates the basolateral IKCa channels (39). K+ channel opening would cause cell hyperpolarization, which provides a driving force for HCO3− secretion via CFTR and/or Cl−/HCO3− exchangers on the apical membrane of duodenocytes (22, 23, 50, 51). Thereafter, extracellular K+ is brought into the cells by the basolateral Na+/K+-ATPase (4, 17, 41). Thus our findings strongly suggest that basolateral IKCa channels play an important role not only in the regulation of colonic epithelial Cl− secretion but also in the regulation of duodenal epithelial HCO3− secretion. Our functional data also suggest that the expression of IKCa is polarized in duodenal epithelial cells, because 1-EBIO caused significant increases in duodenal Isc and HCO3− secretion only when applied to the basolateral side of the tissues (Fig. 4). At the moment, there is no evidence for the presence of the apical IKCa channels in the duodenal epithelium. However, further studies are needed to confirm the polarized expression of IKCa proteins in the duodenal epithelium by immunohistochemistry and to clarify the electrophysiological properties of these channels by patch-clamp techniques. It will also be interesting to explore the clinical relevance of IKCa in duodenal disorders, such as cystic fibrosis, in which CFTR is defective but IKCa response to intracellular Ca2+ likely persists (12, 38).

In summary, IKCa channels are functionally expressed on the basolateral side of duodenal epithelial cells and play a physi-
ological role in Ca$^{2+}$-mediated duodenal mucosal Cl$^{-}$ and HCO$_3^-$ secretion. KCN4 could be one of the downstream molecular targets accounting for the ability of the Ca$^{2+}$ signaling pathway to mediate duodenal epithelial ion transport. A full understanding of the structure and function of KCa channels in the duodenum has the potential to illuminate mechanisms involved in duodenal disorders, such as duodenal ulcers and cystic fibrosis.

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