Inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange stimulates CCK secretion in STC-1 cells

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Inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange stimulates CCK secretion in STC-1 cells. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G689–G695, 1998.—It has been demonstrated that K\textsuperscript{+} channel regulation of membrane potential is critical for control of CCK secretion. Because certain K channels are pH sensitive, it was postulated that pH affects K channel activity in the CCK-secreting cell line STC-1 and may participate in regulating CCK secretion. The present study examines the role of electroneutral Na\textsuperscript{+}/H\textsuperscript{+} exchange on extracellular acidification and hormone secretion. Treatment of STC-1 cells with the amiloride analog ethylisopropyl amiloride (EIPA) to inhibit Na\textsuperscript{+}/H\textsuperscript{+} exchange inhibited Na\textsuperscript{+}-dependent H\textsuperscript{+} efflux and increased basal CCK secretion. Substituting choline in the extracellular medium elevated basal intracellular Ca\textsuperscript{2+} concentration and stimulated CCK release. Stimulatory effects on hormone secretion were blocked by the L-type Ca\textsuperscript{2+} channel blocker diltiazem, indicating that secretion was dependent on the influx of extracellular Ca\textsuperscript{2+}. To determine whether the effects of EIPA and Na\textsuperscript{+} depletion were due to membrane depolarization, we tested graded KCl concentrations. The ability of EIPA to increase CCK secretion was inhibited by depolarization induced by 10–50 mM KCl in the bath. Maneuvers to lower intracellular pH (pH\textsubscript{i}), including reducing extracellular pH (pH\textsubscript{o}) to 7.0 or treatment with sodium butyrate, significantly increased CCK secretion. To examine whether pH directly affects membrane K permeability, we measured outward currents carried by K\textsuperscript{+}, using whole cell patch techniques. K\textsuperscript{+} current was significantly inhibited by lowering pH\textsubscript{o} to 7.0. These effects appear to be mediated through changes in pH\textsubscript{i}, because intracellular dialysis with acidic solutions nearly eliminated current activity. These results suggest that Na\textsuperscript{+}/H\textsuperscript{+} exchange and membrane potential may be functionally linked, where inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange lowers pH\textsubscript{o} and depolarizes the membrane, perhaps through inhibition of pH-sensitive K channels. In turn, K channel closure and membrane depolarization open voltage-dependent Ca\textsuperscript{2+} channels, leading to an increase in cytosolic Ca\textsuperscript{2+} and CCK release. The effects of pH\textsubscript{i} on K channels may serve as a potent stimulus for hormone secretion, linking cell metabolism and secretory functions.

cholceystokinin; radioimmunoassay; hormone; proton exchange; STC-1

CCK is a gastrointestinal peptide hormone secreted into the circulation on ingestion of a meal. Circulating CCK binds to its receptors on target tissues, including the pancreas, gallbladder, stomach, and various nerves to stimulate pancreatic enzyme secretion and gallbladder contraction and to regulate satiety (18). Accordingly, CCK is an important physiological regulator of the ingestion and digestion of essential nutrients.

Studies of the cellular mechanisms regulating CCK secretion have been limited by difficulties in isolating native CCK-secreting cells, which comprise <1% of the mucosal cell population of the intestine (2, 12). More recently, an intestinal cell line that secretes CCK (STC-1) has been used in our laboratory and in others (4, 15, 17, 25) to delineate the molecular mechanisms regulating CCK secretion.

Previous studies (25) have demonstrated that CCK release is dependent on Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels, because removal of extracellular Ca\textsuperscript{2+} or addition of specific L-type Ca\textsuperscript{2+} channel blockers inhibits agonist-stimulated CCK release. Moreover, maneuvers that depolarize the membrane, including inhibition of K channels, result in Ca\textsuperscript{2+} influx through voltage-dependent opening of Ca\textsuperscript{2+} channels (16). Thus there is close coupling between membrane potential, intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]), and secretory activity.

Electroneutral Na\textsuperscript{+}/H\textsuperscript{+} exchangers (NHEs) are expressed in the plasma membrane of mammalian cells and contribute to regulation of intracellular pH (pH\textsubscript{i}), cell volume, and perhaps cell growth (1, 27). These effects are mediated directly through H\textsuperscript{+} efflux or indirectly through effects of changing [H\textsuperscript{+}] or pH\textsubscript{i} on ion channels or other effector pathways. The NHE-1 subtype can be activated by stimuli such as growth factors, hyperosmolarity, hormones, and neurotransmitters (5, 10, 19). However, little is known about the relationship between NHEs and hormone secretion. The current study was designed to determine whether NHE activity in STC-1 cells is involved with CCK release. The findings of the present study, in which H\textsuperscript{+} efflux is used as a measure of NHE activity, suggest important functional interactions between NHE activity and membrane potential in CCK secretion.

METHODS

Cell culture. The STC-1 cell line was derived from an intestinal endocrine tumor that developed in mice carrying the transgenes for the rat insulin promoter linked to the simian virus 40 large T antigen and the poliovirus small t antigen (22). The cell line was kindly provided by Dr. Douglas Hanahan (University of California, San Francisco, CA).

Cells were grown in solution containing 150 ml/l horse serum, 25 ml/l FCS, high-glucose DMEM, 100 kU/l penicillin,
and 100 mg/l streptomycin. Cells were incubated at 37°C and aerated with a 95% O₂-5% CO₂ gas mixture.

CCK secretion. CCK concentrations were determined using antisera no. 9322, according to a previously described RIA technique (15). Antisera no. 9322 was kindly provided by Dr. John Walsh (University of California, Los Angeles, CA). Studies examining CCK secretion were performed on 24-well plates with cells at 80–90% confluence. Cells were pretreated with test agents or control vehicles for 15 min. Secretion was measured after the 15-min treatment period unless otherwise stated.

Microphysiometry. Na⁺/H⁺ exchange activity was measured in real time as the rate of decrease in extracellular pH (pHₑᵢ) in intact cell monolayers placed in an eight-chamber Cytosensor microphysiometer (Molecular Devices, Sunnyvale, CA) (23). The microphysiometer uses a light addressable silicon sensor to detect extracellular protons, which can be derived primarily from Na⁺/H⁺ exchange, glycolysis, and other metabolic pathways. Data are presented as microvolts per second, which roughly correspond to millipH units per minute. The night before experimentation, STC-1 cells were replated onto polycarbonate membrane inserts (3 μm pore size, 12 mm size) at a density of 300,000 cells/inset. On the day of the experiment, cells were washed with serum-free, bicarbonate-free Ham's medium, placed into the microphysiometer chambers, and perfused at 37°C with the same medium or with a balanced salt solution. For most studies, the pump cycle was set to perfuse cells for 60 s, followed by a 30-s "pump-off" phase, during which proton efflux was measured from the 6th through the 28th s. Cells were exposed to the test agent for two or three cycles (180–270 s). Valve switches (to add or remove test agents) were usually performed at the beginning of the pump cycle. In some cases in which the response was expected to be both rapid and very transient (i.e., recovery from Na⁺-free conditions), the valve switch was performed 55 or 58 s into the perfusion phase, allowing 7–10 s for solution mixing before rate measurements. Rate data were then calculated every 90 s. The peak effect during stimulation was expressed as the percentage increase for solution mixing before rate measurements. Rate data were then calculated every 90 s. The peak effect during stimulation was expressed as the percentage increase for solution mixing before rate measurements.
lular buffer caused a substantial increase of 266 ± 14% in CCK secretion.

$\text{Na}^+/\text{H}^+$ exchange is known to participate in the control of both pH$_i$ and pH$_o$ and appears to be involved in a number of critical cellular processes, such as regulation of cell volume and growth (10, 27). If the effects of EIPA on CCK secretion are mediated by inhibition of $\text{Na}^+/\text{H}^+$ exchange, it would be anticipated that EIPA would decrease H$^+$ efflux and the rate of extracellular acidification. Consequently, the rate of extracellular acidification in STC-1 cells was measured as the decrease in pH$_o$ using a Cytosensor microphysiometer. Data were expressed as the percentage of basal acidification rate, which was 120 ± 12 µV/s. It would be expected that $\text{Na}^+/\text{H}^+$ exchange activity would be reduced not only by treatment with an amiloride analog but would be eliminated in the absence of extracellular Na$^+$. Cells were perfused with 1) NaCl-containing buffer, 2) NaCl-containing buffer to which EIPA had been added for 120 s, or 3) buffer in which choline chloride had been substituted for NaCl. Incubation of cells in Na$^+$-free, choline chloride-containing medium produced a low basal extracellular rate of acidification. (There was a small degree of acidification that resulted from proton accumulation during the brief period of pump switching.) On return to NaCl-containing medium, a rapid burst of proton efflux was observed. As shown in Fig. 2, the maximal acidification rate was significantly reduced by EIPA (20 µM). Figure 2 shows that under the same conditions in which CCK secretion occurs, an EIPA-inhibitable, $\text{Na}^+/\text{H}^+$-dependent proton efflux pathway exists and supports the involvement of $\text{Na}^+/\text{H}^+$ exchange in regulation of CCK secretion.

We have previously determined (3, 17, 20) that [Ca$^{2+}$] is an important component for secretagogue-stimulated CCK secretion. We, therefore, sought to determine whether the effects of NHE blockade, as demonstrated by removal of extracellular Na$^+$ (choline chloride substitution), were associated with a change in [Ca$^{2+}$]. If choline substitution increases CCK release through effects on [Ca$^{2+}$], then choline substitution would be expected to increase [Ca$^{2+}$] levels. As shown in Fig. 3, cells in choline chloride buffer consistently demonstrated higher basal [Ca$^{2+}$] compared with cells in standard medium. EIPA interfered technically with the fluorescence measurements of [Ca$^{2+}$], precluding its use for these studies.

If this increase in [Ca$^{2+}$] is a trigger for CCK secretion and is due to an influx of Ca$^{2+}$ from the outside of the cell, it may be sensitive to the L-type Ca$^{2+}$ channel blocker diltiazem. To address this possibility, we found that the increase in basal CCK release produced by EIPA and the removal of extracellular Na$^+$ was significantly (~60%) reduced by 10 µM diltiazem (Table 1). This finding suggests that inhibition of $\text{Na}^+/\text{H}^+$ exchange stimulates CCK secretion through the opening of Ca$^{2+}$ channels.

The relationship between NHE activity and Ca$^{2+}$ influx could result from a direct effect of pH$_i$ on Ca$^{2+}$ influx or an indirect effect of pH$_i$ on membrane potential, because L-type Ca$^{2+}$ channels in STC-1 cells are opened by membrane depolarization (14). pH-sensitive K$^+$ channels that are closed by intracellular acidification have been identified in several cell types (6, 7, 26). Consequently, additional studies were performed to assess the possible relationship between changes in membrane potential, [Ca$^{2+}$], and CCK release.
It is expected that inhibition of Na\(^+/\)H\(^+\) exchange would lower pHi. If STC-1 cells possess a pH-sensitive K\(^{+}\) conductance, accumulation of H\(^+\) would inhibit K\(^{+}\) efflux and result in membrane depolarization, which we have previously demonstrated is a potent stimulus for CCK secretion (13, 14, 16, 17, 24). If the effects of EIPA on CCK release are related to membrane depolarization, then it would be anticipated that elevating extracellular KCl concentrations to depolarize the membrane would inhibit EIPA-stimulated CCK release. The results of these experiments are shown in Fig. 4, in which the KCl concentrations of the extracellular buffer were increased from 5 to 50 mM. Depolarization caused a significant decrease in EIPA-stimulated CCK secretion, consistent with functional coupling between NHE activity, membrane potential, and Ca\(^{2+}\) influx.

It is likely that the changes in pHi are modest, yet have substantial effects on other intracellular processes, including hormone secretion. As further evidence that intracellular acidification is critical in regulating CCK release, STC-1 cells were exposed to low pH\(_{0}\). Acutely decreasing pH\(_{0}\) from 7.4 to 7.0 caused a 232% increase in CCK release from basal levels (Fig. 5).

To address this issue, we exposed cells to sodium butyrate (5 mM). This reagent serves as a proton donor on rapid uptake by cells and causes intracellular acidification (9). As shown in Fig. 5, exposure of STC-1 cells to sodium butyrate stimulated CCK release by 150% (P < 0.05).

Taken together, these findings suggest that intracellular acidification stimulates CCK release through effects on membrane K\(^{+}\) permeability and potential difference. To examine whether pH directly affects membrane K\(^{+}\) permeability, outward currents carried by K\(^{+}\) were measured using whole cell patch-clamp techniques (Fig. 6). With physiological solutions (140 mM Na\(^{+}\) and 5 mM K\(^{+}\) in the bath), net outward membrane currents were present at 0 mV (Fig. 7). With these solutions, outward currents at 0 mV are likely to reflect movement of K\(^{+}\) out of the cell since K\(^{+}\) is the only ion with a more negative reversal potential. Under basal conditions (pH\(_{0}\) 7.4, pHi 7.2), K\(^{+}\) current measured at 0 mV averaged 902 ± 118 pA (mean ± SE, n = 6). K\(^{+}\) current was significantly inhibited by lowering pH\(_{0}\) to 7.0 (K\(^{+}\) current = 325 ± 132 pA, n = 5) or 6.5 (K\(^{+}\) current = 180 ± 152 pA, n = 4). These effects appear to be mediated through changes in pHi, because intracellular dialysis with acidic solutions (pH\(_{0}\), 7.4, pHi 7.0) nearly eliminated current activity (6 ± 13 pA; P < 0.002; n = 5) in the absence of any changes in pH\(_{0}\).

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<th>Table 1. Effect of Ca(^{2+}) channel blockade on CCK secretion induced by inhibition of Na(^+/)H(^+) exchange</th>
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<td>CCK Secretion, %control</td>
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Values are means ± SD. STC-1 cells were plated and treated as described in METHODS. CCK secretion was measured after exposure to ethylisopropyl amiloride (EIPA) (20 µM) or buffer in which Na\(^{+}\) was replaced with choline chloride or with or without pretreatment with 10 µM diltiazem for 5 min. Extracellular fluid was collected after 15 min and assayed for CCK (n = 6). *P < 0.05 vs. control. †P < 0.05 vs. corresponding treatment with diltiazem.

Fig. 4. Effect of KCl-induced depolarization on EIPA-stimulated CCK secretion. STC-1 cells were plated and treated as described in legend, except that cells were placed in medium containing 5, 10, or 50 mM KCl, with or without 10 µM EIPA. After 15-min incubation, extracellular medium was collected for RIA. Data are presented as the difference between corresponding basal and EIPA-stimulated CCK secretion (n = 3).

Fig. 5. Effects of extracellular acidification or sodium butyrate treatment on CCK release. STC-1 cells were placed in medium adjusted to pH 7.4 or 7.0. After a 15-min incubation, medium was collected for measurement of CCK by RIA. Alternatively, at pH 7.4, cells were treated with 0 or 5 mM sodium butyrate for 5 min after which CCK was assayed in the medium. Data are representative of 3 experiments each. *P < 0.05 vs. corresponding control condition.
DISCUSSION

The present study was designed to determine whether Na⁺/H⁺ exchange is integral to stimulation of CCK secretion. Blocking Na⁺/H⁺ exchange was achieved by EIPA treatment and substitution of Na⁺ salts with choline chloride, both of which increased basal CCK release. Because Ca²⁺ influx through L-type Ca²⁺ channels is critical for CCK release stimulated by many secretagogues such as phenylalanine or glucose (14), we evaluated the effects of NHE blockade on [Ca²⁺]ᵢ. Although EIPA interfered technically with the fluorescence measurements of [Ca²⁺]ᵢ, precluding its use for these studies, removal of extracellular Na⁺ clearly raised [Ca²⁺]ᵢ. Moreover, the stimulatory effects of extracellular Na⁺ removal and EIPA on CCK secretion were blocked by the L-type Ca²⁺ channel blocker diltiazem. In aggregate, these findings suggest that the increase in basal secretion caused by inhibition of NHE is dependent on an influx of extracellular Ca²⁺. It is emphasized, however, that Na⁺ removal is likely to have other effects as well. The observation that diltiazem did not completely block CCK secretion induced by Na⁺/H⁺ exchange activity (19). It has been well established that acute

Fig. 6. Effect of acidification on membrane K⁺ permeability. In the whole cell configuration the current-voltage (I-V) relationship during 20-mV steps was designed to determine potentials between -120 and +100 mV (B, inset: 400-ms duration every 10 s). Recordings from 2 different cells are shown (A and B). An outwardly rectifying current that did not diminish over time was demonstrated in both. Exposure to pH 7.0 (A) and 6.5 (B) caused a rapid decrease in K⁺ current. Intracellular dialysis at intracellular pH (pHi) 7.0 nearly completely inhibited K⁺ current. Currents reversed near -40 mV and were measured 5 min after intracellular access to allow equilibration with intracellular space. A and B are each representative of 4–6 experiments.
inhibition of proton efflux leads to intracellular accumulation of H\textsuperscript{+} and a decrease in pH\textsubscript{i} (27). Our observations that maneuvers that could lower pH\textsubscript{i}, such as EIPA administration and removal of extracellular Na\textsuperscript{+}, reduced pH\textsubscript{o}, or sodium butyrate treatment, are consistent with the existence of pH-sensitive K\textsuperscript{+} channels in STC-1 cells. Similar to studies of K\textsubscript{ATP} channels in STC-1 cells, it is likely that these pH-sensitive K\textsuperscript{+} channels contribute to membrane potential and, in turn, modulate hormone secretion (6, 7, 26). If this mechanism was participating in EIPA-stimulated CCK release then it would be expected that CCK release would be no greater than that seen with depolarizing concentrations of KCl alone. EIPA-stimulated CCK secretion was reduced with higher concentrations of extracellular KCl, indicating that the effects of Na\textsuperscript{+}/H\textsuperscript{+} exchange are closely linked to membrane potential and are not observed under conditions in which the membrane is already depolarized.

To test this hypothesis, we measured the effects of pH changes on K\textsuperscript{+} current activity by whole cell electrophysiological recordings. Lowering pH\textsubscript{o} (and presumably pH\textsubscript{i}) to 7.0 or 6.5 significantly stimulated CCK release and concomitantly reduced the amplitude of outward K\textsuperscript{+} currents. These effects appeared to be mediated through changes in pH\textsubscript{i}, because intracellular dialysis with acidic solutions nearly eliminated current activity. These findings indicate that K\textsuperscript{+} permeability of STC-1 cells is tightly regulated by changes in pH\textsubscript{i}.

Intestinal endocrine cells are open to the gut lumen and, being downstream from the stomach, are exposed to solutions of low pH. If sufficient acidification of mucosal cells occurs, it is conceivable that this could serve to stimulate hormone secretion. The molecular mechanisms for such regulation have not previously been considered, but the present studies offer a possible explanation.

In summary, these studies suggest that stimulation of CCK secretion is functionally linked with activation of Na\textsuperscript{+}/H\textsuperscript{+} exchange through a mechanism that may involve pH-sensitive changes in membrane potential (Fig. 8). We propose that inhibition of Na\textsuperscript{+}/H\textsuperscript{+} exchange lowers pH\textsubscript{i}, and causes membrane depolarization presumably through effects of H\textsuperscript{+} on K\textsuperscript{+} permeability. K\textsuperscript{+} channel closure, in turn, depolarizes the plasma membrane and activates L-type Ca\textsuperscript{2+} channels, leading to an increase in [Ca\textsuperscript{2+}]. This increase in [Ca\textsuperscript{2+}] ultimately causes CCK secretion. These findings imply that CCK secretion from native cells in the intestine may be modified by secretagogues or nutrients that influence cellular proton exchange.

A role for pH\textsubscript{i} in the regulation of hormone secretion has implications for how potential secretagogues may influence CCK release. It is possible that agents that directly lower pH\textsubscript{i} as H\textsuperscript{+} donors or indirectly influence pH\textsubscript{i} through NHE blockade could affect basal CCK secretion. At the present time, the long-term conse-
quences of such agents are unknown, but this mechanism for regulation of hormone secretion provides a potential therapeutic target.

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