Serotonin Inhibits Na^+/H^+ Exchange Activity via 5-HT4 Receptors and Activation of PKCα in Human Intestinal Epithelial Cells

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Background & Aims: **Increased serotonin levels have been implicated in the pathophysiology of diarrhea associated with celiac and inflammatory diseases. However, the effects of serotonin on Na^+/H^+ exchange (NHE) activity in the human intestine have not been investigated fully. The present studies examined the acute effects of 5-hydroxytryptamine (5-HT) on NHE activity using Caco-2 cells as an in vitro model.** ***Methods:*** **Caco-2 cells were treated with 5-HT (.1 μmol/L, 1 h) and NHE activity was measured as ethyl-isopropyl-amiloride (EIPA)-sensitive ^{22}Na uptake. The effect of 5-HT receptor–specific agonists and antagonists was examined. The role of signaling intermediates in 5-HT–mediated effects on NHE activity was elucidated using pharmacologic inhibitors and immunoblotting.** ***Results:*** **NHE activity was inhibited significantly (~50%–75%, $P < .05$) by .1 μmol/L 5-HT via inhibition of maximal velocity (V_{max}) without any changes in apparent affinity (K_m) for the substrate Na^+. NHE inhibition involved a decrease of both NHE2 and NHE3 activities. Studies using specific inhibitors and agonists showed that the effects of 5-HT were mediated by 5-HT4 receptors. 5-HT–mediated inhibition of NHE activity was dependent on phosphorylation of phospholipase C γ 1 (PLCγ1) via activation of src-kinases. Signaling pathways downstream of PLCγ1 involved increase of intracellular Ca^{2+} levels and subsequent activation of protein kinase C α (PKCα). The effects of 5-HT on NHE activity were not cell-line specific because T84 cells also showed NHE inhibition.** ***Conclusions:*** **A better understanding of the regulation of Na^+ absorption by 5-HT offers the potential for providing insights into molecular and cellular mechanisms involved in various diarrheal and inflammatory disorders.**

Serotonin or 5-hydroxytryptamine (5-HT) is an endogenous amine that exerts a wide range of biological effects on mammalian physiology. About 90% of the whole body content of serotonin is present in the gastrointestinal tract, mainly stored in granular vesicles in the enterochromaffin cells.[1] Although enterochromaffin cells of the digestive tract release 5-HT across the basal cell membrane into the blood vessels,[2] a variety of stimuli including luminal acidification,[3] increased luminal pressure,[4] and food intake[5] cause the release of serotonin in the intestinal lumen. Serotonin plays an important role in the regulation of both gut motility and electrolyte and fluid transport across the apical membrane.[6,7] In addition, 5-HT inhibits the intestinal absorption of sugars and amino acids.[8–10] These effects are mediated via specific 5-HT receptors present throughout the gastrointestinal mucosa.[11] At least seven 5-HT receptor types have been described and classified into 14 subtypes.[12] With the exception of 5-HT3 receptors, which form a ligand-gated cation-binding channel, all other 5-HT receptors are G-protein–coupled receptors, containing a 7-transmembrane domain structure.[12]

5-HT has long been recognized as an intestinal secretagogue. High levels of 5-HT have been implicated in the pathophysiology of celiac disese,[13] irritable bowel syndrome,[14] dumping syndrome,[15] and diarrhea associated with carcinoid syndrome.[16] The effects of 5-HT on electrolyte transport have been reported to vary by species and regions of the gut studied. For example, 5-HT evoked net chloride secretion in rabbit small intestine[17,18] and rat jejunum.[19] However, others have reported an inhibition of NaCl absorption in rabbit ileum and gallbladder without changes in electrogenic Cl secretion. Sundaram et al.[20] observed decreased Cl^-/HCO_3^- exchange without any effect on Na^+/H^+ exchange activity in rabbit small intestinal villus cells, whereas Na^+/H^+ exchange (NHE) was stimulated in

Abbreviations used in this paper: CAM, calmodulin; EIPA, ethyl-isopropyl-amiloride; 5-HT, 5-hydroxytryptamine; NHE, Na^+/H^+ exchange; PKA, protein kinase A; PKC, protein kinase C.

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crypt epithelial cells. Finally, the only report of human tissue, a study of isolated human jejunal mucosa, suggested 5-HT increased Cl secretion, but had no significant effect on NaCl absorption in response to 5-HT.[7] In light of these contradictory reports, we investigated the effects of 5-HT on human intestinal sodium absorption in detail.

We also planned to define the intracellular signal transduction mechanisms responsible for 5-HT–mediated effects on NHE. The effects of serotonin on electrolyte transport have been suggested to require or be independent of increases in intracellular Ca^{2+} levels,[21,22] depending on the tissue studied. However, to date, the effects of 5-HT and the cellular mechanisms underlying 5-HT actions on the Na^+/H^+ exchange activity in the human intestine have been limited. Caco-2 cells, a well-established model of human enterocytes that express the appropriate intestinal NHE isoforms, also express proteins necessary for synthesis and degradation of serotonin.[23] Therefore, these cells were used in the present studies.

The data show that acute treatment of Caco-2 cells with serotonin (.1 μmol/L, 1 h) decreases the activity of both NHE2 and NHE3 via the involvement of 5-HT4 receptors. The observed inhibition in NHE activity occurs via a signal transduction pathway involving src-family kinases, phospholipase C γ 1 (PLCγ1), protein kinase C (PKC), and Ca^{2+}-calmodulin (CAM)–mediated pathways. Further, the data reveal tyrosine phosphorylation and activation of PLCγ1 leading to activation of PKCα and an increase in the level of intracellular Ca^{2+}.

Materials and Methods

Materials

Serotonin (5-HT) was obtained as creatinine sulfate complex from Sigma Chemicals Co. (St. Louis, MO). Radionuclide ^{22}Na was obtained from NEN Life Science Products, (Boston, MA). Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA). Chelerythrine chloride, calphostinC, calmidazolium Cl, Herbimycin A, PP1, and BAPTA-AM were obtained from Biomol (Plymouth Meeting, PA). RS 39604 and SB203186, 5-HT receptor antagonists, and the 5-HT4 agonist 2-[1-(4-pieronyl)piperazinyl]benzothiazole were procured from Tocris (Ellisville, MO). Antiphosphotyrosine antibody (4G10) was obtained from Upstate Biotechnology (Lake Placid, NY). Phosphospecific-PLC γ 1 and PKC isoform antibodies were obtained from Santa Cruz (Santa Cruz, CA). Anti-actin antibody was obtained from Sigma Chemicals Co. HOE-694 was a generous gift from Dr. Hans-J. Lang (Aventis, Pharma Deutschland GmbH; Frankfurt/main, Germany). All other chemicals were of at least reagent grade and were obtained from Sigma Chemicals Co. or Fisher Scientific (Pittsburg, PA).

Cell Culture

Caco-2 cells were grown routinely in T-75 cm^2 plastic flasks at 37°C in a 5% CO_2-95% air environment. The culture medium consisted of high-glucose Dulbecco's modified Eagle medium, 20% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Cells reached confluence after 4–5 days in culture. They were used for these studies between passages 25 and 55 and plated in 24-well plates at a density of 2×10^4 cells/mL. Cells were used for experiments on the 10th–12th day postplating and were fed with fresh incubation media on alternate days. Unless stated otherwise, cells were treated with .1 μmol/L concentration of 5-HT for a time period of 60 minutes. In a separate set of experiments, Caco-2 cells were pretreated with 5-HT–receptor antagonists or different inhibitors of PKC (chelerytherine Cl, calphostin C), Ca^{2+} (BAPTA-AM), CAM (calmidazolium Cl), tyrosine kinase (herbimycin), Src-kinases inhibitor (PP1), or PLC (Et-18) in the Caco-2 medium for 1 hour before the addition of serotonin and then co-incubated along with 5-HT for another hour as indicated in various figure legends.

T84 cells were grown as monolayers in a 1:1 mixture of Dulbecco-Vogt modified Eagle's medium and Ham's F-12 supplemented with 6% newborn calf serum, 14 mmol/L $NaHCO_3$, 15 mmol/L HEPES, 65 IU/mL penicillin, 8 μg/mL ampicillin, and 60 μg/mL streptomycin in a 5% CO_2 atmosphere at 37°C. Cells were plated at a density of 8×10^4 cells/well and required a minimum of 1 week to reach confluence. Cells were used for experiments at 2–3 weeks after plating. T84 cells represent secretory crypt cells derived from the colon and NHE3 typically is not present in colonic crypt cells.[24,25] Also, previous functional studies suggested the presence of NHE2 isoform on the apical membranes of T84 cells.[24,26]

Assay of Na^+/H^+ Exchange

The activity of Na^+/H^+ exchange was determined in acid-loaded Caco-2 cells as ethyl-isopropyl-amiloride (EIPA)-sensitive ^{22}Na uptake as previously described by us.[27] The activity of NHE isoforms was measured by using EIPA (50 μmol/L) and HOE-694 (NHE2 isoform–specific inhibitor at 50 μmol/L concentration). The activity of NHE2 was calculated as Na^+/H^+ exchange activity sensitive to 50 μmol/L HOE-694. NHE3 activity was calculated by subtracting the 50 μmol/L HOE-694–sensitive NHE activity from the total NHE activity (50 μmol/L EIPA-sensitive NHE activity).

For these studies, the cells were allowed to equilibrate by placing them at room temperature for a period of 15–20 minutes before the ^{22}Na uptake was performed. Briefly, confluent cell monolayers were preincubated for 30 minutes at room temperature in an acid-load solution containing (in mmol/L) 50 NH_4Cl, 70 Choline Cl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 5 glucose, and 15 MOPS (pH 7.0). The cells then were washed with a solution containing (in mmol/L) 120 choline chloride and 15 Tris-HEPES, pH 7.5. The earlier-described solution

then was aspirated and the cells were incubated in uptake buffer containing (in mmol/L) 10 NaCl, 110 Choline Cl, 1 $MgCl_2$, 2 $CaCl_2$, 20 HEPES (pH 7.4), and 1 mCi/mL of ^{22}Na, with or without 50 μmol/L EIPA or 50 μmol/L HOE-694. After 5 minutes, the ^{22}Na-containing uptake solution was aspirated and the cells were washed twice with ice-cold phosphate-buffered saline (PBS). The cells then were solubilized by incubation with .5 N NaOH for at least 4 hours and the incorporated radioactivity was determined. The protein content of cell lysates was estimated by the method of Bradford.[28] ^{22}Na uptake normally was measured at 5 minutes (because this was in the linear range of time course) and expressed as nmol/mg protein/5 min.

Assay of ^{3}H-Taurocholate Uptake and ^{14}C-D–Glucose

Sodium-dependent Taurocholate cotransport was measured in Caco-2 cells as described previously.[29] Briefly, Caco-2 cells (untreated or treated with 5-HT) were washed twice with uptake buffer containing 116 mmol/L NaCl (or choline chloride), 5.3 mmol/L KCl, 1.1 mmol/L KH_2PO4, .8 mmol/L $CaCl_2$, 11 mmol/L d-glucose, 10 mmol/L Hepes, at pH 7.4. The cells were incubated with uptake buffer containing 10 μmol/L ^{3}H-taurocholate for 5 minutes. Uptake of ^{14}C-d-glucose in Caco-2 cells was measured as described previously[30] with modifications. The cells were incubated with Hanks' balanced salt solution[27] containing ^{14}C-d–glucose at a final concentration of 50 μmol/L for a time period of 1 minute. The uptake solution was aspirated and the cells were washed twice with ice-cold PBS. The cells then were solubilized by incubation with .5 N NaOH for at least 4 hours and the incorporated radioactivity was determined.

Apical or Basolateral Membrane Unidirectional ^{22}Na Uptake Using Permeable Supports

For the experiments using permeable supports, Caco-2 cells were plated on Transwell inserts (Costar, Corning, NY) at a density of 4×10^4 cells/Transwell and used on the 12th–14th days postplating. Cells reached confluency 6–8 days postplating. Cells were treated with 5-HT (.1 μmol/L) from either the apical or the basolateral side for 60 minutes as indicated. Unidirectional apical or basolateral membrane ^{22}Na uptake was measured as described[31] for 24-well plates. After termination of uptake by washing twice with ice-cold 1× PBS, the filters were cut from the Transwell and cells on filters were solubilized by incubation with .5 N NaOH for at least 4 hours. The incorporated radioactivity was determined and the protein content was estimated by the method of Bradford.[28] Data are presented as the EIPA (50 μmol/L)-inhibitable component in pmol/mg protein/5 min for apical NHE activity and pmol/mg protein/10 min for basolateral NHE1 activity.

Membrane Translocation of PKC Subtypes Subcellular Fractionation

Caco2 cells were grown to confluence in 6-well plates (10×10^4 cells/mL; Costar) and treated with 5-HT (.1 μmol/L) for 15-, 30-, and 60-minute time periods. Cells were washed with ice-cold PBS 3 times and scraped into 400 μL of the cold homogenization buffer containing 20 mmol/L Tris-HCl, pH 7.5, 250 mmol/L sucrose, 4 mmol/L ethylenediaminetetraacetic acid, 2 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and complete protease inhibitor cocktail tablets. The cells were homogenized on ice with 25 strokes of a glass tissue homogenizer. The homogenate was ultracentrifuged at 59,000 rpm for 50 minutes at 4°C (Optima TLX Ultracentrifuge; Beckman, Fullerton, CA). The supernatant was designated the cytosolic fraction. The pellet was resuspended in 150 μL of the homogenization buffer containing .5% (vol/vol) Triton X-100 by brief sonication and incubated on ice for 30 minutes. The samples were centrifuged at 14,000 rpm for 20 minutes at 4°C. The resulting supernatant was designated the membrane fraction.

Gel Electrophoresis and Western Blotting

Equal amounts (75 μg/sample) of protein, as determined by the Bradford assay,[28] were combined with Laemmli's sample buffer containing 5% (vol/vol) β-mercaptoethanol and boiled for 5 minutes. Proteins were separated by electrophoresis on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels and transblotted to nitrocellulose membranes. The protein-bound nitrocellulose membranes were first incubated for 1 hour at room temperature in blocking buffer containing 1× PBS, .1% Tween 20, and 5% nonfat dry milk. Nitrocellulose membranes then were incubated with the polyclonal antibody specific to PKCα or β1 (1:800 dilution) in the blocking buffer containing 1× PBS, .1% Tween 20, and 1% nonfat dry milk for 1 hour at room temperature and rinsed for 30 minutes with a wash buffer containing 1× PBS and .1% Tween 20. Finally, the membranes were incubated with horseradish peroxidase–conjugated goat anti-rabbit immunoglobulin G antibody (1:2000 dilution) for 1 hour at room temperature and washed for 45 minutes with agitation, during which the wash buffer was changed every 5 minutes. PKC bands were visualized with enhanced chemiluminescence detection reagents.

Phosphorylation of PLCγ1

Phosphorylation of PLCγ1 in response to 5-HT was assessed by using a phospho-specific PLCγ1 antibody (Tyr residue-783). Briefly, Caco-2 cells were treated with .1 μmol/L of 5-HT for different time intervals and were lysed in the lysis buffer containing protease cocktail tablets and phosphatase inhibitor cocktail. The lysates were subjected to 6% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (100 μg protein per lane), blotted onto nitrocellulose membrane, and blocked with a Blotto buffer (Santacruz Biotech, Santa Cruz, CA) and incubated with phospho-specific–PLCγ1 antibody

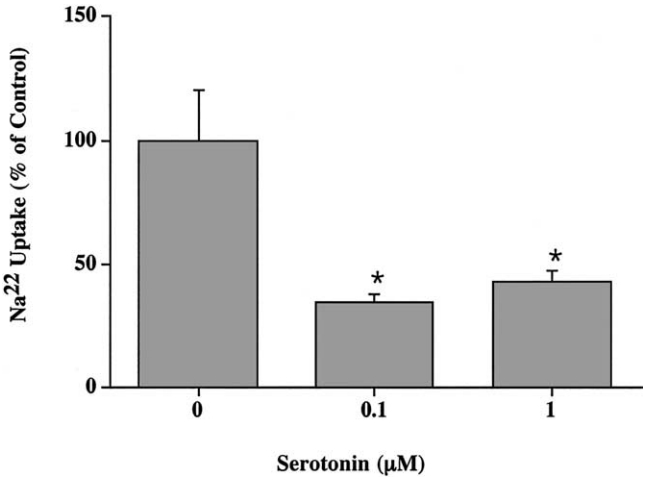

Figure 1. Effect of 5-HT on apical Na^+/H^+ exchange in Caco-2 cells. Caco-2 cells grown on plastic supports were incubated with .1 μmol/L or 1.0 μmol/L concentrations of 5-HT in the cell culture medium for 60 minutes. Cells then were washed with 1× PBS and were acid loaded with an NH_4Cl prepulse for 30 minutes. ^{22}Na uptake was measured as EIPA-sensitive (50 μmol/L) uptake. Results are expressed as percent of control value and represent the mean ± SEM of 4–7 separate experiments performed in triplicate. $^*P < .005$ compared with control. The absolute values for EIPA-sensitive ^{22}Na uptake for control (in nmol/mg protein/5 min) were 2.02 ± .42.

(1:50) overnight. After incubation with bovine anti-goat horseradish peroxidase–conjugated secondary antibody, immunoreactive bands were visualized by enhanced chemiluminescence detection kit. To confirm that an equal amount of protein was loaded in each lane, the blots were stripped and reprobed with PLCγ1 antibody, which recognizes PLCγ1 independent of its phosphorylation status.

Statistical Analysis

Results are expressed as mean ± SEM of 3–6 separate observations performed in triplicate. The Student *t* test was used in statistical analysis. A *P* value of .05 or less was considered statistically significant.

Results

5-HT Inhibits Na^+/H^+ Exchange Activity

To determine the effect of 5-HT on NHE activity, Caco-2 cells grown on plastic supports were treated with .1 μmol/L of serotonin for 60 minutes and NHE activity was measured as EIPA-sensitive ^{22}Na uptake. As shown in Figure 1, incubation with 5-HT resulted in a significant decrease in NHE activity (~60%; $P < .05$). Further experiments showed that this represented maximal inhibition because results were similar at 1 μmol/L 5-HT. Thus, .1 μmol/L 5-HT was used for all subsequent experiments. Because 5-HT was added in the form of creatinine sulfate complex to the Caco-2 cells, we also examined whether creatinine sulfate alone has any effect on NHE activity. The results showed that under similar conditions, creatinine sulfate did not affect NHE activity (data not shown).

Table 1. 5-HT Inhibits NHE Activity in T84 Cells

5-HT (*μmol/L*)	^{22}Na uptake (*nmol/mg protein/5 min*)
0	1.52 ± .28
.1	.64 ± .10[a]
1.0	.43 ± .34[a]

NOTE. Overnight serum-starved T84 cells grown on plastic supports were incubated with .1 and 1.0 μmol/L of 5-HT for 60 minutes in the cell culture medium. NHE activity was measured as EIPA-sensitive (50 μmol/L) ^{22}Na uptake at 5 minutes. Values represent the mean ± SEM of 3 separate experiments. n = 6.
[a]$P < .05$ compared with control.

To determine whether the effects of 5-HT on NHE activity were cell-line–specific, NHE activity also was measured in overnight serum-starved T84 cells treated with 5-HT (.1–1.0 μmol/L) for 1 hour. As shown in Table 1, 5-HT treatment of T84 cells decreased the NHE activity. These results suggest that the effects of 5-HT on intestinal epithelial cells are not cell-line–specific.

5-HT Inhibits NHE by Decreasing Maximal Velocity

To examine the mechanism of inhibition of NHE activity by 5-HT, we measured ^{22}Na uptake in the presence of increasing concentrations of Na^+ ranging from 5–20 mmol/L in Caco-2 cells. As shown in Figure 2, NHE activity exhibited saturation at 15–20 mmol/L in both control and 5-HT–treated cells, and 5-HT decreased the NHE activity at all Na^+ concentrations. Analysis of kinetic parameters (maximal velocity [V_{max}] and apparent affinity [K_m]) showed that 5-HT affected

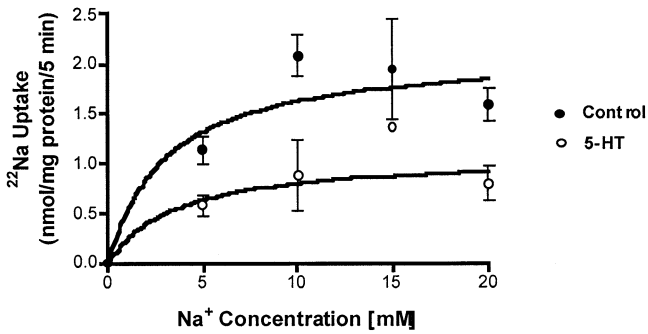

Figure 2. Kinetics of 5-HT–mediated inhibition on NHE activity. Caco-2 cells grown on plastic supports were treated with 5-HT (.1 μmol/L, 1 hour) and ^{22}Na uptake was measured in the presence of increasing concentrations of Na^+ ranging from 5–20 mmol/L. Results are expressed as the mean ± SEM of 3–4 separate experiments performed in triplicate. $^*P < .05$ compared with control. ●, Control; ○, 5-HT.

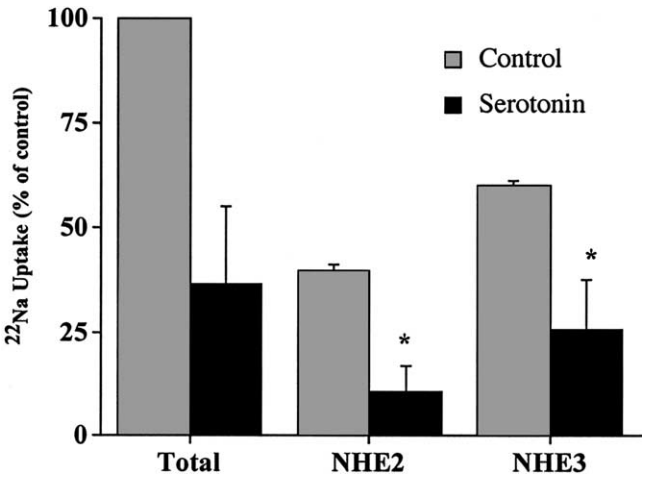

Figure 3. 5-HT decreases the activity of both NHE2 and NHE3 in Caco-2 cells. Caco-2 cells grown on plastic supports were incubated with 5-HT (.1 μmol/L) in the cell culture medium for 60 minutes. After acid loading the cells, Na^+/H^+ exchange was determined in the presence of either EIPA (50 μmol/L) or HOE-694 (50 μmol/L). Results are expressed as percent of control values and represent the mean ± SEM of 3 separate experiments performed in triplicate. *$P <$ .05 compared with control. ▒, Control; ■, serotonin.

the NHE isoforms by altering the V_{max} of the transporters, with no changes in the value of K_m (V_{max}: 2.13 ± .15 nmol/mg protein/5 min in the control vs 1.08 ± .35 nmol/mg protein/5 min in 5-HT–treated cells; K_m 4.68 ± .23 mmol/L in control vs 3.9 ± .45 mmol/L).

5-HT Decreases Both NHE3 and NHE2 Activities

Amiloride analogs EIPA and HOE-694 were used to determine the effects of 5-HT on NHE2 and NHE3. NHE2 was defined as 50 μmol/L HOE-694–sensitive NHE. NHE3 activity was defined as 50 μmol/L HOE-694–insensitive NHE activity. Under control conditions, 42% ± 6% of total NHE was contributed by NHE2, and 58% ± 8% was contributed by NHE3 (Figure 3). Treatment of Caco-2 cells with 5-HT (.1 μmol/L) decreased the activities of both NHE3 and NHE2 compared with the respective control values. The NHE3 activity was decreased by 58%, whereas the effect on NHE2 was more pronounced (73%).

Effects of 5-HT Are Specific for Apical NHE Activity

To determine whether the effects of 5-HT are primary in nature or secondary to changes in other transporters, we also examined the effect of luminally applied 5-HT on the basolaterally expressed isoform, NHE1. Caco-2 cells grown in Transwell inserts on the 14th day postplating were treated with .1 μmol/L of 5-HT for 1 hour from the apical side and NHE1 activity was assessed by performing ^{22}Na uptake (50 μmol/L EIPA-inhibitable) from the basolateral side. 5-HT treatment did not cause any alteration in NHE1 activity (NHE1 activity in pmol/Transwell insert/10 min: control, 3.9 ± .2; 5-HT, 3.6 ± .15). These results suggest that 5-HT inhibits the apical NHE2 and NHE3 activity independent of changes in NHE1 activity.

Also, the contribution of NHE1 in the observed effects of 5-HT on apical NHE activity was ruled out by measuring NHE activity from the apical side in Caco-2 cells grown on permeable supports, which have restricted basolateral access. The results showed that luminal 5-HT significantly inhibited the apical NHE activity (NHE activity in pmol/Transwell insert/5 min: control, 66.4 ± 3.4; 5-HT, 31.6 ± 1.3). Because enterochromaffin cells have the ability to release 5-HT into the mucosa as well as in the blood circulation,[2] it was also considered of interest to examine the effect of 5-HT applied from the basolateral side on the apical NHE activity. The results showed that in Caco-2 cells grown on permeable supports, basolateral application of 5-HT (.1 μmol/L, 1 h) also inhibited the apical NHE activity, similar to the results obtained with luminal 5-HT application (NHE activity in pmol/Transwell insert/5 min: control, 59.8 ± 4.5; 5-HT, 25.0 ± 1.1).

Further, the effects of 5-HT on other apical transporters in Caco-2 cells grown on plastic supports were investigated to determine the specificity of the observed effects of 5-HT. For these studies, Na^+-dependent d-glucose uptake (^{14}C) and ^{3}[H] Taurocholic acid uptake via the apical sodium-dependent bile acid transporter was measured in response to 5-HT treatment in Caco-2 cells. As shown in Table 2, 5-HT at a concentration of .1 μmol/L had no effect on the activity of the d-glucose uptake. It should be noted that d-glucose uptake in Caco-2 cells was Na^+-dependent and was measured at a 1-minute time period to prevent its metabolism in the cells. Similary, taurocholic acid uptake also essentially was unaltered by 5-HT treatment.

Table 2. Effects of 5-HT on ^{14}[C] d-Glucose and ^{3}[H] Taurocholic Acid Uptake in Caco-2 Cells

Treatment	d-Glucose uptake (pmol/mg protein/1 min)	Taurocholic acid uptake (pmol/mg protein/5 min)
Control	5.59 ± .05	15.25 ± .25
5-HT (.1 μmol/L, 1 h)	5.21 ± .25	18.21 ± .29

NOTE. Values represent the mean ± SEM of 3 separate experiments performed in triplicate. Caco-2 cells grown on plastic supports were incubated with 5-HT (.1 μmol/L) for a time period of 60 minutes and the Na-dependent ^{14}[C] d-glucose uptake and Na-dependent ^{3}[H] taurocholic acid uptake were assessed.

Table 3. Role of 5-HT4 Antagonists on 5-HT–Induced Inhibition of NHE Activity in Caco-2 Cells

Treatment	NHE activity (nmol/mg protein/5 min) SB203186 (300 nmol/L)	RS39604 (300 nmol/L)
Control	2.10 ± .19	2.91 ± .80
5-HT	.56 ± .24[a]	.82 ± .40[a]
Antagonist	2.14 ± .02	2.55 ± .07
5-HT + antagonist	2.11 ± .40	1.96 ± .42

NOTE. Caco-2 cells grown on plastic supports were preincubated with 5-HT4 antagonists: SB203186 (300 nmol/L) or RS39604 (300 nmol/L) for 60 minutes in the cell culture medium and then co-incubated with .1 μmol/L of 5-HT for another 1 h. Cells then were washed with 1× PBS and were acid loaded with NH_4Cl prepulse for 30 minutes. ^{22}Na uptake was measured as EIPA (50 μmol/L)-sensitive uptake for a time period of 5 minutes. Results are the mean ± SEM of 3–4 separate experiments performed in triplicate.

[a]$P < .05$ compared with control.

5-HT4 Receptors Mediate the Effects of 5-HT on NHE

5-HT activates multiple cell surface receptors. The receptors mediating the 5-HT–induced inhibition of NHE activity were defined using specific receptor agonists and antagonists. As shown in Table 3, the specific 5-HT4–receptor antagonist SB203186 blocked 5-HT effects on NHE activity. RS39604, another specific 5-HT4–receptor antagonist, also completely blocked the effects of 5-HT (Table 3). In contrast, 5-HT–mediated inhibition of NHE was intact in the presence of specific antagonist of 5-HT3 receptors, Y2513 (300 nmol/L) (control NHE activity [in nmol/mg protein/5 min], 2.01 ± .17; 5-HT, .89 ± .05; Y2513 [300 nmol/L], 2.1 ± .25; 5-HT + Y2513 [300 nmol/L], 1.07 ± .17). These results suggest that 5-HT4 receptors mediate the effects of 5-HT on NHE activity.

To confirm that the effects of 5-HT on NHE were mediated by 5-HT4 receptors, we examined the effects of the 5-HT4–receptor specific agonist 2-(1-[4-pieronyl] piperazinyl)benzothiazole on apical Na^+/H^+ exchange. As shown in Figure 4, incubation of Caco-2 cells for 60 minutes with the agonist resulted in a dose-dependent decrease in NHE activity ($P < .05$). In contrast, incubation of Caco-2 cells with 5-HT3–specific agonist, m-chlorophenylbiguanide (.1–1 μmol/L, 1 h), did not affect NHE activity (data not shown). Thus, the effects of 5-HT on NHE are mediated via the 5-HT4 receptor.

Tyrosine Kinases Are Necessary for 5-HT–Mediated Inhibition of NHE

To evaluate the potential role of tyrosine kinase pathways on the effects of 5-HT on NHE activity, Caco-2 cells were pretreated with general tyrosine kinase inhibitor, Herbimycin A. As shown in Figure 5*A*, the inhibitory effects of 5-HT on NHE activity were abolished completely by Herbimycin A. Additionally, the src-kinase inhibitor, PP1 (1 μmol/L), also prevented 5-HT–mediated inhibition of NHE activity (Figure 5*B*). To identify potential targets of 5-HT–mediated tyrosine kinase activation, lysates of Caco-2 cells incubated in the absence or presence of 5-HT were analyzed by immunoblots with phospho-specific tyrosine kinase antibodies. Increased phosphorylation of proteins in the range of 85–200 kilodaltons was induced by 5-HT (Figure 6).

One target of these kinases might be PLCγ1, which is activated by src-kinase–mediated tyrosine phosphorylation.[32–34] Thus, we measured PLCγ1 activation using a phospho-specific anti-PLCγ1 antibody. A representative blot is shown in Figure 7. 5-HT treatment increased the phosphorylation of PLCγ1 in a time-dependent manner (Figure 7, upper panel). The densitometric analysis of the blot revealed an ~6-fold increase in PLCγ1 phosphorylation (normalized to total PLC levels; Figure 7, lower panel), peaking at 60 minutes of 5-HT treatment, compared with control. Consistent with the specific role of src-kinases, the src-kinase inhibitor PP1 prevented phosphorylation of PLCγ1 after 5-HT stimulation. These results indicate that PLCγ1 can be phosphorylated by 5-HT via src kinase activation. To determine whether this PLCγ1 activation is involved in NHE inhibition,

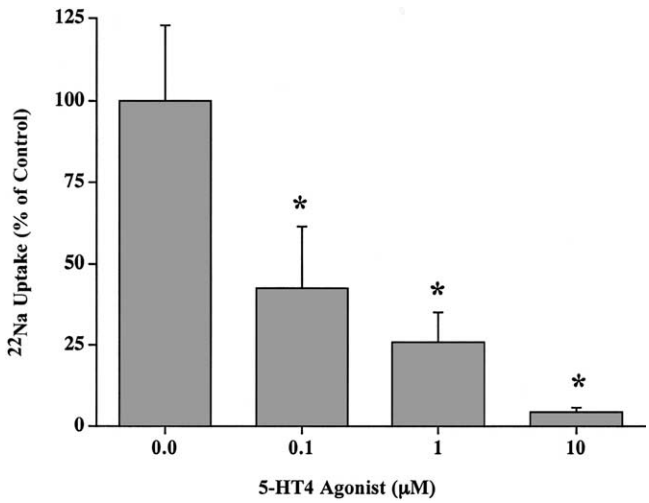

Figure 4. Effect of 5-HT4 receptor–specific agonist on Na^+/H^+ exchange in Caco-2 cells. Caco-2 cells grown on plastic supports were incubated with different concentrations of 5-HT4–receptor agonist 2-(1-[4-pieronyl]piperazinyl)benzothiazole (.1–10 μmol/L) in the cell culture medium for 60 minutes. Cells then were washed with 1× PBS and were acid loaded with an NH_4Cl prepulse for 30 minutes. ^{22}Na uptake was measured as EIPA (50 μmol/L)-sensitive uptake. Results are expressed as percent control value and represent the mean ± SEM of 3 separate experiments performed in triplicate. *$P < .05$ compared with control. The absolute values for EIPA-sensitive ^{22}Na uptake for control (in nmol/mg protein/5 min) were 2.09 ± .45.

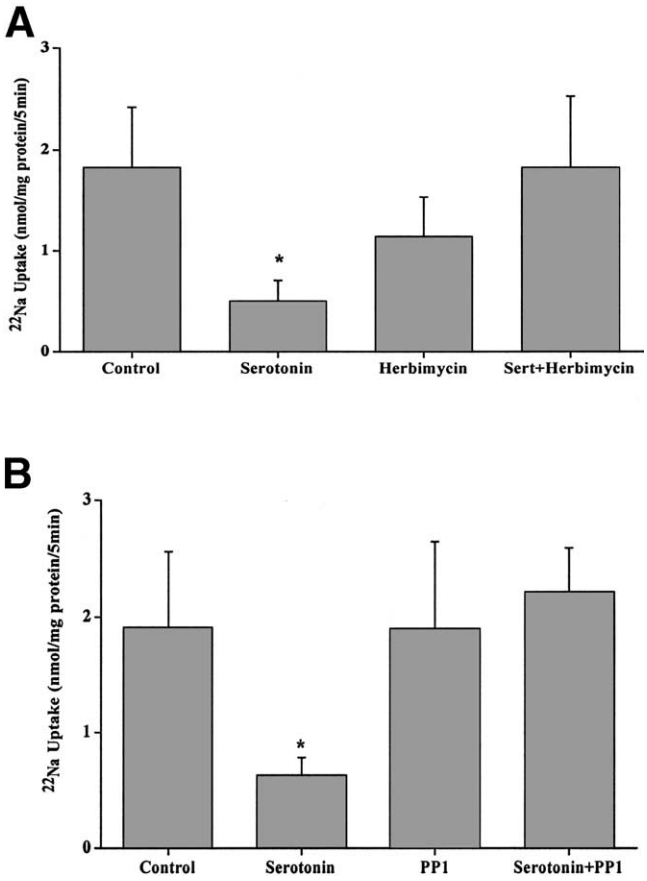

Figure 5. Role of tyrosine kinase inhibitors on 5-HT–mediated effects on Na^+/H^+ exchange in Caco-2 cells. Caco-2 cells grown on plastic supports were preincubated with (*A*) Herbimycin A, the general tyrosine kinase inhibitor (1 μmol/L), or (*B*) PP1 (10 μmol/L), the Src-kinase inhibitor for 60 minutes in the cell culture medium, and then co-incubated with .1 μmol/L of 5-HT for another 1 hour. Cells then were washed with 1× PBS and were acid loaded with NH_4Cl prepulse for 30 minutes. ^{22}Na uptake was measured as EIPA (50 μmol/L)-sensitive uptake for a time period of 5 minutes. Results are the mean ± SEM of 3–4 separate experiments performed in triplicate. *$P < .05$ compared with control.

the specific PLC inhibitor ET-18 (10 μmol/L)[35] was used. ET-18 significantly inhibited the 5-HT–mediated decrease in NHE activity in Caco-2 cells (Figure 8), confirming that PLCγ1 is part of the signaling cascade that leads to 5-HT–mediated inhibition of NHE.

PKCα Mediates 5-HT–Mediated Inhibition of NHE Activity

PLCγ1-mediated hydrolysis of inositol phospholipids produces 2 second messengers: inositol 1,4,5-triphosphate and diacylglycerol. Inositol 1,4,5-triphosphate mobilizes calcium from intracellular stores, whereas diacylglycerol is a well-known activator of PKC.[34] Thus, the role of PKC in the inhibition of NHE by 5-HT was investigated using 2 different PKC inhibitors: chelerythrine and calphostin C. Both PKC inhibitors completely prevented NHE inhibition (Table 4). To determine whether conventional PKC subtypes were those responsible for 5-HT–mediated effects on NHE activity, the conventional PKC inhibitor, Go6976, at a concentration of 5 nmol/L was used. Go6976 abolished the 5-HT–mediated decrease in NHE activity (Table 4).

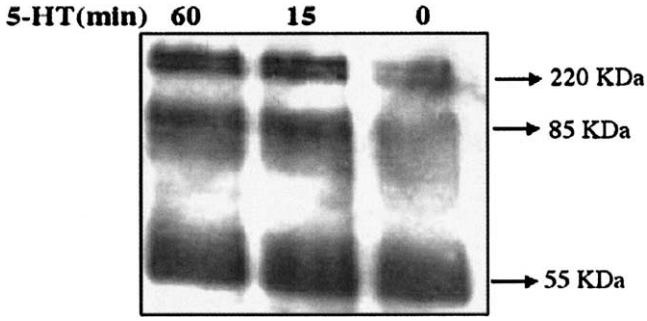

Figure 6. 5-HT causes the tyrosine phosphorylation of total proteins in Caco-2 cells. Caco-2 cells grown on plastic supports were incubated with 5-HT (.1 μmol/L) in the cell culture medium for 15 or 60 minutes. After washing the cells with 1× PBS, extracted proteins (75 μg) were subject to Western blot analysis on 8% sodium dodecyl sulfate–polyacrylamide gel using horseradish-peroxidase–conjugated antiphosphotyrosine antibody. 5-HT treatment to cells induced phosphorylation of total proteins in the size range of 85–200 kilodaltons.

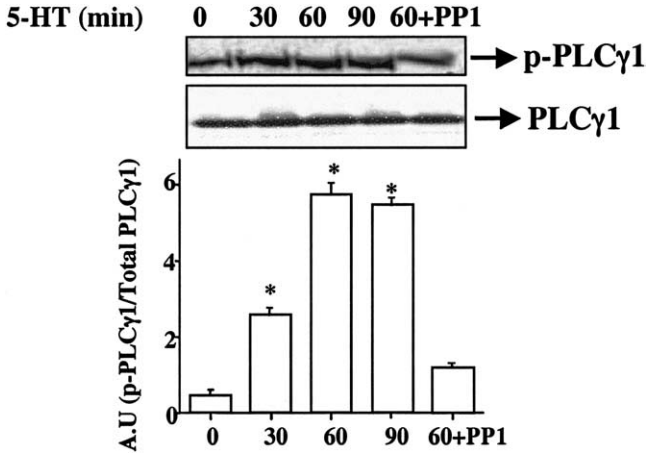

Figure 7. Src-kinases induce the tyrosine phosphorylation of PLCγ1 in Caco-2 cells in response to 5-HT. Caco-2 cells grown on plastic supports were incubated with 5-HT (.1 μmol/L) in the cell culture medium for different time intervals ranging from 30–60 minutes. Cells also were pretreated with the src-kinase inhibitor PP1 (10 μmol/L) for 60 minutes and then co-incubated with 5-HT (.1 μmol/L) for another 60 minutes. Cells were washed with 1× PBS, extracted proteins (100 μg) were subject to Western blot analysis on 6% sodium dodecyl sulfate–polyacrylamide gel using phospho-specific PLCγ1 antibody. A representative blot is shown. 5-HT–induced phosphorylation of PLCγ1 was blocked by the src-kinase inhibitor PP1. The blots were restripped and probed with total PLCγ1. The data were quantified by densitometric analysis and expressed as arbitrary units. Bars represent the mean ± SEM of 3–5 determinations. *$P < .05$ compared with untreated control.

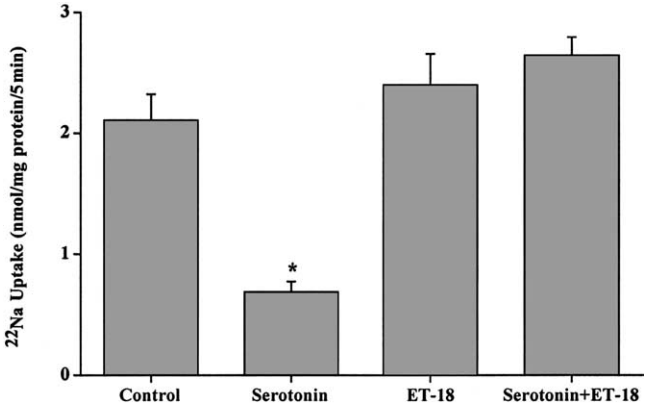

Figure 8. Role of PLC in 5-HT–mediated inhibition of Na^+/H^+ exchange activity in Caco-2 cells. Caco-2 cells grown on plastic supports were preincubated with the PLC inhibitor, Et-18 (10 μmol/L), for 60 minutes in the cell culture medium and then co-incubated with .1 μmol/L of 5-HT for another 1 hour. Cells then were washed with 1× PBS and were acid loaded with NH_4Cl prepulse for 30 minutes. ^{22}Na uptake was measured as EIPA (50 μmol/L)-sensitive uptake for a time period of 5 minutes. Results represent the mean ± SEM of 3 separate experiments performed in triplicate. *$P < .05$ compared with control.

Ca^{2+} Signaling is Necessary for 5-HT–Mediated NHE Inhibition

Because PLCγ1-generated inositol 1,4,5-triphosphate can release intracellular Ca^{2+}, increased intracellular Ca^{2+} is necessary for activation of PKCα, and 5-HT is reported to increase intracellular Ca^{2+},[22] we asked whether chelation of intracellular Ca^{2+} could prevent NHE inhibition by 5-HT. In cells loaded with the Ca^{2+} chelator BAPTA-AM, NHE inhibition by 5-HT was blocked completely (Table 5). Thus, 5-HT triggers both increases in cytosolic Ca^{2+} levels and activation of PLCγ1, both of which appear to be necessary for 5-HT–mediated NHE inhibition. In the presence of BAPTA-AM (20 μmol/L), the inhibitory effect of 5-HT was blocked completely. Increased Ca^{2+} levels in the cells can cause activation of CAM, which regulates a variety of kinases, phosphodiesterases, and other effectors. CAM also has been shown to influence the activities of different NHE isoforms.[36,37] The role of CAM in the observed decrease in NHE activity in response to 5-HT was examined by using the specific CAM inhibitor, calmidazolium Cl, at a concentration of 1 μmol/L. As shown in Figure 9, pretreatment of Caco-2 cells with calmidazolium blocked the decrease in NHE activity on 5-HT treatment. Taken together, these observations suggest an important role of Ca^{2+}/CAM in mediating the inhibitory effects of 5-HT on NHE activity.

Table 4. Role of PKC Antagonists on 5-HT–Induced Inhibition of NHE Activity in Caco-2 Cells

	NHE activity (nmol/mg protein/5 min)		
Treatment	Chelerythrine Cl, PKC inhibitor	Calphostin C, PKC inhibitor	GO6976, conventional PKC inhibitor
Control	1.29 ± .56	1.75 ± .12	1.51 ± .45
5-HT	.33 ± .12[a]	.50 ± .04[a]	.51 ± .25[a]
Antagonist	1.19 ± .2	1.90 ± .21	1.61 ± .15
5-HT + antagonist	1.01 ± .12	2.01 ± .50	1.65 ± .21

NOTE. Caco-2 cells grown on plastic supports were preincubated for 60 minutes in the presence of chelerythrine chloride (2 μmol/L), calphostin C (200 nmol/L), or GO6976 (5 nmol/L) in the cell culture medium and then co-incubated with 5-HT (.1 μmol/L) for a time period of 60 minutes and the Na^+/H^+ exchange was measured as EIPA (50 μmol/L) sensitive ^{22}Na uptake. Results represent the mean ± SEM of 3–4 observations.
[a]$P < .05$ compared with control.

Table 5. Effects of 5-HT on NHE Activity in Caco-2 Cells Are Ca^{2+} Dependent

Treatment	NHE activity (nmol/mg protein/5 min)
Control	1.14 ± .07
5-HT	.55 ± .18[a]
BAPTA-AM (20 μmol/L)	.97 ± .30
5-HT + BAPTA-AM	1.19 ± .26

NOTE. Caco-2 cells grown on plastic supports were preincubated with BAPTA-AM (20 μmol/L), an intracellular chelator of Ca^{2+} for 60 minutes in the cell culture medium and then co-incubated with .1 μmol/L of 5-HT for another 1 hour. Cells then were washed with 1× PBS and were acid loaded with NH_4Cl prepulse for 30 minutes. ^{22}Na uptake was measured as EIPA (50 μmol/L)-sensitive uptake for a time period of 5 minutes. Results are the mean ± SEM of 3–4 separate experiments performed in triplicate.
[a]$P < .05$ compared with control.

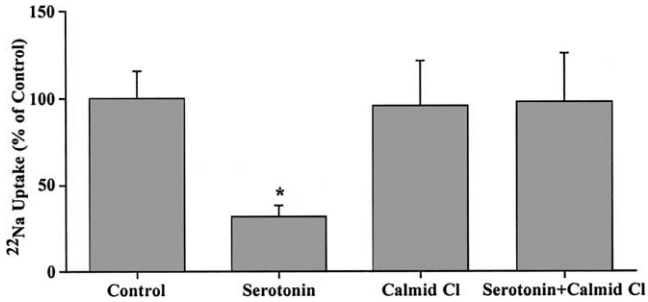

Figure 9. Involvement of CAM in 5-HT–mediated inhibition of Na^+/H^+ exchange activity in Caco-2 cells. Caco-2 cells grown on plastic supports were preincubated with the CAM inhibitor, calmidazolium chloride (Calmida, 1 μmol/L), for 60 minutes in the cell culture medium and then co-incubated with .1 μmol/L of 5-HT for another 1 hour. Cells then were washed with 1× PBS and were acid loaded with NH_4Cl prepulse for 30 minutes. ^{22}Na uptake was measured as EIPA (50 μmol/L)-sensitive uptake for a time period of 5 minutes. Results are expressed as percent of control value and represent the mean ± SEM of 3 separate experiments performed in triplicate. *$P < .05$ compared with control. The absolute values for EIPA-sensitive ^{22}Na uptake for control (in nmol/mg protein/5 min) were 2.66 ± .32.

PKCα Activation Requires src Kinases and PLCγ1

The data presented earlier suggest a signaling pathway in which engagement of 5-HT4 receptors lead to activation of src kinases, PLCγ1, and PKCα. Because inhibitors of each of these steps prevent NHE inhibition by 5-HT, we directly evaluated whether 5-HT activates PKCα and whether this could be prevented by inhibition of src kinase or PLCγ1. Because activation of PKC subtypes is associated with translocation from the cytosol to the membrane, we studied the movement of PKCα to the membrane in response to 5-HT. 5-HT caused a significant increase in membrane association of PKCα (Figure 10). This was blocked by PP1 (Figure 10). PKCβ1 was not found to be activated by 5-HT in Caco-2 cells (not shown). Inhibition of PLCγ1 also prevented translocation of PKCα to the membrane after 5-HT treatment, whereas CAM inhibitor (calmidazolium Cl) failed to block this translocation (not shown). Therefore, PKCα is activated by 5-HT and this activation depends on src kinases and PLCγ1. The blots were stripped and reprobed with anti-actin antibody to indicate equal loading of protein in all lanes.

Potential Role of Protein Kinase A (PKA) in Inhibition of NHE Activity

5-HT4 receptors also have been linked to adenylate cyclase and thus might stimulate adenosine 3′,5′-cyclic monophosphate (cAMP) formation. Therefore, it was of interest to investigate the role of PKA in 5-HT–mediated effects on NHE activity in Caco-2 cells. Our results using PKA inhibitor, RpcAMP (25 μmol/L), however, showed no significant effects on the inhibition of NHE activity by 5-HT (Table 6). Thus, PKA-mediated pathways are not involved in 5-HT–mediated inhibition of NHE activity.

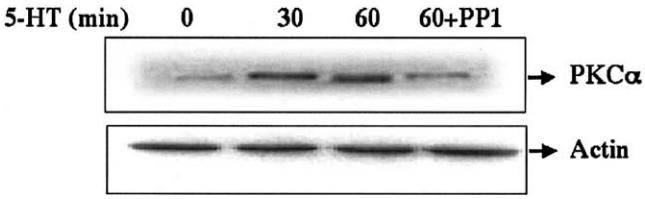

Figure 10. PKCα translocates to membrane fraction in response to 5-HT. Caco-2 cells grown on plastic supports were treated with .1 μmol/L of 5-HT for a 30- or 60-minute time period in the absence or presence of src-kinase inhibitor PP1 (10 μmol/L). The membrane fractions were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and probed with specific PKCα antibody. (Upper panel) A representative of 3 blots is shown. (Lower panel) The blots were stripped and reprobed with actin antibody to indicate equal loading of proteins.

Table 6. Effects of PKA Inhibitor on 5-HT–Mediated Inhibition on NHE Activity in Caco-2 Cells

Treatment	NHE activity (nmol/mg protein/5 min)
Control	.75 ± .08
5-HT	.49 ± .06[a]
RpcAMP (25 μmol/L)	.69 ± .02
5-HT + RpcAMP	.42 ± .03[a]

NOTE. Values represent the mean ± SEM of 3 separate experiments performed in triplicate. Caco-2 cells grown on plastic supports were preincubated for 60 minutes in the presence of PKA inhibitor, RpcAMP (25 μmol/L) in the cell culture medium and then co-incubated with 5-HT (.1 μmol/L) for a time period of 60 minutes and the Na^+/H^+ exchange was measured as EIPA (50 μmol/L)-sensitive ^{22}Na uptake. Results represent the mean ± SEM of 3–4 observations.
[a]$P < .05$ compared with control.

Discussion

The present studies were performed to investigate the effects of 5-HT on Na^+/H^+ exchange activity using Caco-2 cells as an in vitro model. Our results showed that incubation of Caco-2 cells with 5-HT at a concentration of .1 μmol/L for 60 minutes decreased the activity of NHEs. 5-HT also showed a similar response in the T84 cell line, indicating that the effects of 5-HT were not cell-line–specific. Our kinetic studies showed that 5-HT–mediated inhibition occurred via decreased NHE V_{max}, with no significant changes in K_m. The results also suggested the involvement of 5-HT4 receptors and different signaling intermediates in the observed inhibition of NHE activity by 5-HT.

5-HT Receptor Subtypes

In the gut, 5-HT1A, 5-HT1B, 5-HT2, 5-HT3, and 5-HT4 receptor subtypes are expressed.[38] Previous studies on the 5-HT–mediated chloride secretion in different animal models have shown the involvement of 5-HT2, 5-HT3, and 5-HT4–receptor subtypes. However, functional studies have shown contradictory effects of 5-HT antagonists on intestinal fluid transport and ion secretion, depending on the species and gastrointestinal segment studied.[11,39–41] In the present studies, we showed, by using both inhibitors and agonists, that the 5-HT4 receptor is the receptor responsible for NHE inhibition. This recently classified receptor subtype is expressed in brain, gastrointestinal tract, and heart. In the gastrointestinal tract, it is expressed in esophagus, stomach, small intestine, and colon of rodents and other mammals, including humans.[12] The functions of 5-HT4 receptors relate to modulation of intestinal fluid transport,[42–44] motility,[45] release of 5-HT from enterochromaffin cells,[46] and induction of Cl secretion in rat colon[44] and human jejunum.[7] Previous studies from our group

and others[47,48] described that Caco-2 cells grown on plastic support under the same conditions described in the current studies show the early morphologic parameters of differentiation in at least 2–5 days postconfluence (eg, formation of domes, development of well-defined brush-border membranes possessing microvilli, and expression of the alkaline phosphatase enzyme as indicated by the increase in its activity in these cells over the indicated time after reaching confluence). Hence, the Caco-2 cells grown on plastic support closely resemble the native epithelium. In light of these facts, the use of specific 5-HT4 agonists and agonists in response to 5-HT treatment from the apical side in the current studies provides evidence for the expression of the 5-HT4 receptors on the apical membrane. Future studies, however, will be aimed at characterizing the 5-HT–receptor subtypes on the purified luminal plasma membrane vesicles isolated from human organ donors. Consistent with these observations, Alcalde et al.[49] reported that ^{3}H–5-HT binds specifically to the 5-HT4 receptors in the brush-border membranes of rabbit jejunal enterocytes.

NHE2 Versus NHE3

Previous studies from our laboratory and others have provided strong evidence that Caco-2 cells are a valid in vitro model of small intestinal NHE regulation.[27,47,50] A variety of agents have been shown to regulate differentially the activity of apical isoforms of NHEs, namely NHE2 and NHE3.[50,51] More recently, we have shown that nitric oxide selectively inhibits NHE3, but not NHE2.[27] In contrast, the present studies show that both NHE2 and NHE3 are inhibited by 5-HT, although there was a slightly more pronounced effect on NHE2 activity. The activity of the basolaterally expressed NHE1 isoform was not changed in response to 5-HT treatment. The effects of 5-HT on NHE2 and NHE3 isoform activities appear to be specific and direct because D-glucose uptake and apical sodium-dependent bile acid–transporter activity in Caco-2 cells remained unaltered under these conditions.

Signal Transduction and Intracellular Mediators

Depending on the species and gastrointestinal segment studied, 5-HT receptors have been shown to activate characteristic intracellular signaling cascades. 5-HT4 receptors are known to activate adenylate cyclase and stimulate cAMP synthesis. However, previous studies have failed to show increased mucosal cAMP in response to 5-HT in rabbit and cat intestine.[17,52] Consistent with this, our data with a specific PKA inhibitor also suggest that cAMP and PKA are not involved in NHE inhibition in response to 5-HT. In contrast, our studies show that tyrosine kinases, including src kinases, are involved in 5-HT–mediated NHE inhibition. Immunoblots of lysates from 5-HT–stimulated cells detected several phosphorylated proteins from 85–220 kilodaltons, with 1 phosphoprotein consistent with PLCγ1. The present data show that PLCγ1 is activated by 5-HT and that this activation can be inhibited by a specific src kinase inhibitor. Consistent with these sequential roles of src kinase and PLCγ1, inhibitors of either enzyme blocked 5-HT effects on NHE. These data are in contrast with previous studies by Khurana et al.[53] and Kobayashi et al.[54] that showed activation of NHE3 and NHE2 by tyrosine kinases. These differences likely represent differing downstream target proteins in the specific experimental system used. For example, Khurana et al.[55] suggested that epidermal growth factor–stimulated tyrosine phosphorylation activated NHE3 via PI3 kinase. The current studies suggest that 5-HT induces tyrosine phosphorylation of PLCγ1.

We also found that NHE inhibition required 5-HT–induced increases in intracellular Ca^{2+} levels. These results are consistent with release of intracellular Ca^{2+} by PLCγ1-mediated inositol 1,4,5-triphosphate. The Ca^{2+}-dependent actions of 5-HT on intestinal secretion are consistent with those observed in rodent and dog intestine and chicken enterocytes,[11,56,57] but contrast sharply with results in hen colon, where 5-HT evoked secretion independent of Ca^{2+}.[11] Increases in intracellular Ca^{2+} levels in turn stimulate different signaling targets inside the cell. One possibility could be CAM, which in turn can cause phosphorylation/dephosphorylation of proteins. CAM directly and indirectly regulates Na and Cl transport in the small intestine and colon. Although direct interactions between CAM and NHE3 have not been observed, there could be a possibility of other downstream effectors inhibiting NHE3. However, Nath et al.[37] showed that NHE2 contains some conserved amino acid residues in its C-terminal that can bind CAM.

Because PLCγ1 induces increased intracellular Ca^{2+} release, via IP3, and also causes release of diacylglycerol, we asked if this resulted in activation of conventional PKC isoforms, which require both Ca^{2+} and diacylglycerol for activation. The data show that PKCα is activated by 5-HT and that this PKC activation is necessary for 5-HT–mediated decreases in NHE. We also found that both src kinase and PLCγ1 inhibitors, which blocked 5-HT–mediated NHE inhibition, also blocked PKCα activation. The site of action of CAM in the sequence of events induced by 5-HT to inhibit NHE activity is not yet clear. The CAM inhibitor, calmidazolium chloride,

failed to block the serotonin-induced PKCα activation (data not shown). These results, therefore, suggest that activation of PKCα is dependent on src kinase and PLCγ1, but is CAM independent. Also, our data showing tyrosine phosphorylation of total cellular proteins in the range of 85–220 kilodaltons rule out the possibility of CAM (~17 kilodaltons) being phosphorylated by src kinases. However, CAM also has been shown to be phosphorylated at serine and threonine residues.[58] Recent studies showed that melatonin, a secretory product of the pineal gland, increased phosphorylation of CAM by PKCα.[59] It therefore is possible that CAM might act downstream to PKCα to inhibit NHE activity in response to 5-HT. Further studies are needed to investigate this issue.

Luminal Versus Serosal Effects of 5-HT

5-HT in humans is distributed predominantly in the gastrointestinal tract with the majority of it being stored in enterochromaffin cells, neurons, and mast cells in lamina propria.[1] Besides the release of 5-HT into the lumen,[3–5] enterochromaffin cells also release 5-HT across the basal cell membrane into the circulation to exert endocrine effects on distant targets.[2] Interestingly, the present studies using Transwell inserts showed that both the luminal and basolateral application of 5-HT decreased the apical NHE activity to a significantly similar extent. These results might suggest that 5-HT can function as a hormone, as well as a local paracrine modulator in the gastrointestinal tract to modulate electrolyte transport processes. Whether the effects of the luminal or serosal 5-HT on NHE activity differ in terms of signaling and/or type of receptors mediating their effects is of interest and will be addressed in future investigations.

In summary, our results show that 5-HT decreases V_{max} of apical NHE activity via 5-HT4–receptor activation. The intracellular signaling pathway mediating this effect includes activation of src kinases and subsequent activation of PLCγ1, increased intracellular Ca^{2+} levels, and activation of PKCα (Figure 11). In addition, the data provide increased understanding of the role of 5-HT on regulation of NHE and the cell signaling mechanism(s) underlying this process. Because the 5-HT–mediated inhibition occurred via alterations in the value of V_{max} of the NHEs, but not K_m, it could be speculated that membrane trafficking events, for example, increased endocytic retrieval or decreased exocytic insertion of NHEs triggered by different signaling intermediates, might be involved in the observed inhibition of NHE activity. Further understanding of the regulation of NHE by serotonin may lead to the development of new approaches to the therapy of disturbances in intestinal fluid and electrolyte transport, particularly in severe diarrheal conditions, such as chronic watery diarrhea associated with carcinoid syndrome.

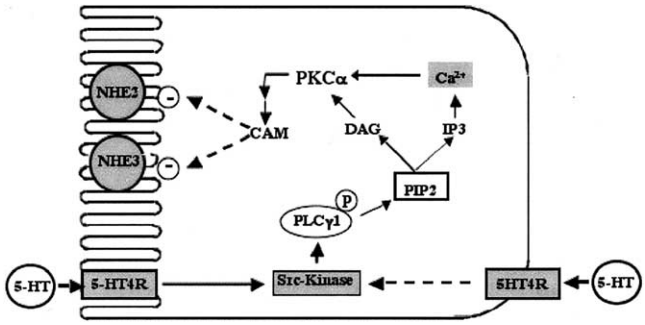

Figure 11. Speculative model of putative signal transduction mechanisms involved in 5-HT–mediated inhibition of NHE2 and NHE3 in Caco-2 cells. P, phosphorylation.

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