α 2-Adrenergic Receptors Attenuate Secretagogue-Induced Endocytosis and Promote Exocytosis of Intestinal NHE2 and NHE3

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Received February 4, 2009; accepted June 23, 2009

ABSTRACT

Adrenergic agonists, through activation of intestinal epithelial α 2-adrenergic receptors (α 2AR), inhibit electrolyte secretion and promote absorption. The mechanisms of action to promote basal Na^+ absorption and inhibit stimulated secretion are not understood completely. The effects of α 2-agonists on Na^+ transport were studied in a cell line, Caco2-3B, derived from the Caco2 cell line engineered to permanently express human α 2A-adrenergic receptors. Serosal, but not mucosal, addition of the α 2AR agonist *N*-(2,6-dichlorophenyl)-4,5-dihydro-1*H*-imidazol-2-amine (clonidine) increased Caco2-3B apical $^{22}\text{Na}^+$ uptake, an effect not seen in the Caco2 parent line that lacks α 2AR expression. This effect was blocked by the α 2AR antagonist 17 α -yohmban-16 α -carboxylic acid methyl ester (yohimbine). Increased Na^+ uptake was paralleled by increased apical surface abundance of the sodium/hydrogen exchangers NHE2 and NHE3. No changes in total cell NHE2 and NHE3 expression

were observed. Clonidine also inhibited both cAMP and Ca^{2+} -induced decreases in apical Na^+ uptake and apical membrane NHE2 and NHE3 endocytosis stimulated by these agents. α 2AR actions were mediated via stimulation of phospholipase C, and metabolism of arachidonic acid by an epoxygenase activity followed epidermal growth factor release and activation of the epidermal growth factor receptor, resulting in phosphatidylinositol-3-kinase and Akt stimulation. In summary, activation of intestinal epithelial α 2AR significantly blocks the inhibition of apical Na^+ transporters by cAMP- and Ca^{2+} -mediated pathways and also directly increases apical sodium/hydrogen exchange activities. By both blocking electrolyte secretion and promoting absorption, α 2-agonists could be potent antidiarrheal agents that could directly counteract the actions of toxigenic pathogens and other secretagogues causing secretory diarrhea.

Intestinal apical membrane sodium/hydrogen exchangers (NHE), NHE2 and NHE3, are major pathways for nutrient-independent, electroneutral Na^+ absorption. The activity and regulation of both exchangers have received some attention, although more is known about NHE3. NHE3 activity in the brush border is regulated in part by insertion or retrieval of the NHE3 protein in the apical membrane. Stimulated NHE3 insertion into the apical membrane (exocytosis)

(Khurana et al., 1996; Li et al., 2001; Lee-Kwon et al., 2003) seems to be regulated by several accessory NHE regulatory factors (Zachos et al., 2008) and involves the activation of phosphatidylinositol 3-kinase (PI3K) and Akt (Khurana et al., 1996; Lee-Kwon et al., 2003). Stimulated NHE endocytosis that blocks NaCl absorption, (McSwine et al., 1998; Kim et al., 2002; Lee-Kwon et al., 2003; Musch et al., 2007) seems to involve trafficking proteins, including clathrin, the adaptor protein complex, synaptotagmin 1, EPAC, ezrin, and NHERF-1 and NHERF-2 (Chow et al., 1999; Weinman et al., 2000; Hu et al., 2001; Kim et al., 2002; Cha et al., 2006; Honegger et al., 2006; Musch et al., 2007).

Treatment of diarrhea remains a major global health problem. Bacterial enterotoxins such as cholera toxin (Subra-

This work was supported by the National Institutes of Health [Grants DK38510, DK47722] (to E.B.C.); the National Institutes of Health [Grant DK42086] (to the Digestive Disease Research Center of the University of Chicago); and the Gastrointestinal Research Foundation of Chicago.

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.109.151910.

ABBREVIATIONS: NHE, sodium hydrogen exchange; α 2AR, α 2-adrenergic receptor; 8-CPT-cAMP, 8-chlorophenylthio-cAMP; M, mucosal; S, serosal; EGF, epidermal growth factor; HOE 694, 3-methylsulfonyl-4-piperidinobenzoyl, guanidine hydrochloride; MESNA, 2-mercaptoethane-sulfonate; PI3K, phosphatidylinositol 3-kinase; sulfo-NHS-SS-biotin, sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; AG-1478, 4-(3'-chloroanilino)-6,7-dimethoxy-quinazoline; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; PD123319, *S*-(+)-1-([4-(dimethyl-amino)-3-methylphenyl]methyl)-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1*H*-imidazo(4,5-*c*)pyridine-6-carboxylic acid; PD98059, 2'-amino-3'-methoxyflavone; U-73122, 1-[6-[[17 β -methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; TAPI-1, TNF- α protease inhibitor-1, *N*-(*R*)-[2-(hydroxyaminocarbonyl)methyl]-4-methylpentanoyl-L-naphthylalanyl-L-alanine, 2-aminoethyl amide.

manya et al., 2007) and *Escherichia coli* heat-stable toxin (Gill et al., 2007) inhibit apical membrane Na⁺ uptake by decreasing abundance and function of NHE2 and/or NHE3 and stimulating anion secretion. Oral replacement solutions have benefited many of these patients; however, these solutions do not stop fluid losses, they only replace them. Octreotide has been used for endocrine tumor-related secretory and cancer treatment-induced diarrhea (Cascinu et al., 1993; Mystakidou et al., 2006). Octreotide may also act directly on intestinal water and electrolyte transport (Roberts et al., 1988; Högenauer et al., 2002), but its cost and means of delivery make it infeasible as a treatment of global infectious diarrheal diseases. In this regard, few clinically used anti-diarrheal agents directly counteract the end-organ effects of bacterial enterotoxins and intestinal secretagogues.

In vitro studies of intestinal ion transport have demonstrated that α 2-adrenergic receptor (α 2AR)-agonists, such as clonidine, inhibit basal and stimulated intestinal secretion and promote NaCl absorption (Chang et al., 1982, 1983). By binding studies, α 2A-adrenergic receptors seem to be located on the basolateral membranes of intestinal epithelial cells (Chang et al., 1983; Paris et al., 1990; Valet et al., 1993) along the entire villus to crypt axis, but more abundantly in crypt cells. The α 2AR transduction pathways have been investigated in renal cells (Cussac et al., 2002), neuronal cells (Karkoulas et al., 2006), and intestinal epithelial cells (Buffin-Meyer et al., 2007). α 2AR stimulation inhibits adenylyl cyclase through the G_{i2} and G_{i3} adaptor proteins (Schaak et al., 2000) and is associated with activation of phospholipase C, resulting in release of arachidonic acid. When arachidonic acid is metabolized by a cytochrome P450 epoxygenase, its metabolites stimulate two pathways. The first involves release of epidermal growth factor (EGF) that stimulates EGF receptors that activate PI3K and Akt. EGF receptor stimulation also results in activation of the extracellular regulated kinases ERK1/2 (p44/42 mitogen-activated protein kinases). The second pathway involves activation of src activity that independently stimulates PI3K and Akt.

Clonidine's usefulness as an antidiarrheal agent was shown in the treatment of diabetic diarrhea (Fedorak et al., 1985; Chang et al., 1986) and diarrhea induced by castor oil (Lal et al., 1981). However, the significant side effects of clonidine and related agonist have limited their usefulness as antidiarrheal agents.

The mechanism(s) of action of α 2-agonists to alter ion transport have not been determined because of the lack of an intestinal cell line that expresses these receptors. In renal proximal tubules, clonidine has also been noted to increase basal NaCl absorption, but the mechanism was not investigated (Nord et al., 1987; Gesek et al., 1989). We have used a model derived from the human colonic epithelium cell line Caco2 that expresses α 2AR (Schaak et al., 2000) to investigate the mechanisms underlying clonidine's effects on intestinal electroneutral Na⁺ transport mediated by NHE2 and NHE3. Our findings show that α 2AR stimulation inhibits cAMP- and Ca²⁺-induced decreases in apical NHE activity and membrane endocytosis. These actions are mediated through the activation of a cascade of signaling pathways similar to those reported for clonidine's action in stimulating intestinal epithelial cell proliferation (Buffin-Meyer et al., 2007).

Materials and Methods

Cell Culture. Human colonic epithelial Caco2BBE cells were used between passages 57 and 82. The generation of the Caco2-3B line has been described previously (Schaak et al., 2000). The Caco2-3B clone was obtained by permanent transfection of the parental Caco2 cell line with a bicistronic plasmid containing the gene encoding the human α 2AR. Caco2-3B cells were used between passages 4 and 25 after transfection, a period when radioligand binding studies indicate that the level of α 2AR expression remains constant. Both cell lines were grown in high-glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, and 50 μ g/ml streptomycin in a humidified atmosphere of air containing 5% CO₂. For the Caco2BBE, 10 μ g/ml human transferrin was added. Cells were seeded on collagen-coated polycarbonate Transwell permeable supports (Costar, Cambridge, MA) at 10⁵ cells/cm² and allowed to differentiate for 10 to 14 days before use. The transepithelial electrical resistances increased by this time and were stable in both the Caco2BBE (approximately 250 Ω -cm²) and the Caco2-3B (approximately 175 Ω -cm²) monolayers. Differentiation of the monolayers was similar, assessed by the degree of villin protein expression, which is a marker of epithelial maturation (data not shown).

Apical Membrane Unidirectional ²²Na⁺ Influx as a Measure of NHE Activity. For influx studies, cells were grown on Transwell permeable supports (Corning, Inc.) for 14 days as described above. Monolayers were washed once in 150 mM choline Cl, 10 mM HEPES, pH 7.4, and then unidirectional apical membrane Na⁺ uptake was determined in flux buffer (130 mM choline Cl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 15 mM HEPES, pH 7.4, 20 mM NaCl with 1 μ Ci/ml [²²NaCl]). After 10 min at 37°C, the incubation was stopped by four washes in ice-cold buffer (140 mM NaCl, 5 mM KCl, 15 mM HEPES, pH 7.4, and 1 mM Na₃PO₄) and Na⁺ influx was calculated. Dimethylamiloride (500 μ M) and HOE 694 (30 μ M) were used to distinguish NHE2 and NHE3 activities as described previously (McSwine et al., 1998). NHE2 activity was defined as the HOE 694-inhibitable portion of the flux and NHE3 activity as the difference between the dimethylamiloride- and HOE694-inhibitable portions of the unidirectional ²²Na⁺ influx.

Mouse Jejunum Transmural ²²Na⁺ Fluxes. The use of mouse intestinal tissue was approved by the institutional animal care and use committee of the University of Chicago. Mouse jejunum was excised, cut longitudinally, placed immediately in Krebs-Ringer bicarbonate solution (114 mM NaCl, 5 mM KCl, 25 mM NaHCO₃, 1.25 mM CaCl₂, 1.25 mM MgCl₂, 1.65 mM Na₂HPO₄, 0.3 mM NaH₂PO₄) at room temperature, and gassed with 95% oxygen/5% carbon dioxide (resulting in a pH of 7.4 at 37°C). One-centimeter segments were prepared beginning 3 cm distal to the ligament of Treitz. These segments were then mounted in Ussing chambers (Physiological Instruments, San Diego, CA) with an aperture of 0.3 cm². To prevent edge damage, a small ring of parafilm was used around the opening for the tissues. Tissues were maintained at 37°C in the Krebs-Ringer bicarbonate solution. After 10 min, tissue electrical resistances were measured so that tissues with similar resistances (differing by less than 20%) could be paired for mucosal-to-serosal and serosal-to-mucosal unidirectional transmural fluxes. After an additional 10 min, clonidine (100 μ M) was added to the serosal medium when appropriate, and 15 min later 8-chlorophenylthio-cAMP (100 μ M) was added to the serosal side when appropriate. At this time, 1 μ Ci of ²²NaCl was added to either the mucosal or serosal medium. Fluxes were initiated 15 min later and samples taken again at 30 min from both fluid reservoirs. Fluxes were calculated with use of samples taken for the specific activity of ²²Na⁺ on the side where the radioactivity was added. Transepithelial electrical resistance was measured in 15-min intervals for the duration of the experiment.

Apical (Brush-Border) Membrane NHE Expression. For studies on apical NHE exocytosis, cell monolayers were stimulated with clonidine with or without pretreatment with inhibitors as

noted. Monolayers were cooled rapidly by placing them on ice, changing apical compartment medium to PBS containing 0.5 mg/ml sulfo-NHS biotin. During the 30-min incubation with the biotinylation solution, apical membrane proteins were labeled. No basolateral or intracellular protein labeling was observed during this period (Musch et al., 2007). Biotinylation was terminated by the addition of 10 μ l of 1 M Tris, pH 8.0, because Tris has a free amine group. Cell monolayers were scraped off the filters, pelleted, and resuspended in immunoprecipitation buffer (IP buffer: 150 mM NaCl, 10 mM HEPES, pH 7.4, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS, 0.5% deoxycholate, and 1% Triton X-100). Samples were solubilized, an aliquot was removed to measure protein and total NHE3 expression, and streptavidin-agarose was added to the remainder. Samples were rotated for 120 min, washed three times with IP buffer, and eluted by boiling in Laemmli buffer. Biotinylated apical surface proteins and total NHE3 were analyzed by use of Western blotting as described below. For total protein expression, 50 μ g were used for analysis and 500 μ g were used for isolation of biotinylated apical proteins.

To specifically measure endocytosed apical membrane proteins, a disulfide-sensitive form of biotin (Sulfo-NHS-SS-Biotin) was used. Apical membrane proteins were labeled at 37°C in HEPES-buffered saline (10 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) with 1 mg/ml Sulfo-NHS-SS-Biotin. The monolayers were washed twice in Tris-buffered saline to inactivate and remove free biotin and then incubated for 10 min at 37°C in HEPES-buffered saline with 8-chlorophenylthio-cAMP (8-CPT-cAMP; 100 μ M) or thapsigargin (100 ng/ml). This saline was removed and replaced with HEPES-buffered saline containing 50 mM sodium 2-mercaptoethanesulfonate (MESNA) to cleave off biotin from apical membrane proteins. Because MESNA is not cell permeant, biotinylated apical membrane proteins that have been internalized will remain biotinylated. Three consecutive 10-min treatments with MESNA containing saline were necessary to remove all apical biotin. Cells were then solubilized, and biotinylated proteins were identified as described above.

For studies of mouse jejunum, loops of 2 cm were created and filled with phosphate-buffered saline where 25 mM $NaHCO_3$ was used to replace 25 mM NaCl. Filled loops were placed in tissue culture dishes in HCO_3^- -containing isotonic saline and treated with clonidine (100 μ M) or saline control for 15 min. Control and clonidine-pretreated loops were then exposed to 8-CPT-cAMP (100 μ M) for another 30 min, opened, and filled with ice-cold saline containing 0.5 mg/ml Sulfo-NHS-biotin. Loops were placed in saline on ice, incubated for 30 min, and then opened. Tris was added to react with free biotin and mucosa was then scraped off. Samples were solubilized in IP buffer, an aliquot removed for total NHE2 and NHE3 determinations, and biotinylated brush-border proteins isolated with streptavidin agarose as described previously for cell culture monolayers. Samples were then analyzed on SDS-polyacrylamide gel electrophoresis and Western blotting procedures.

Western Blotting. Total cellular protein (35 μ g) or IP samples were separated on 7.5% SDS-polyacrylamide gel electrophoresis and immediately transferred to polyvinylidene difluoride membranes in Towbin's buffer (25 mM Tris, 192 mM glycine, pH 8.8, 15% methanol). Membranes were blocked for 1 h at room temperature in T-TBS (Tween 20-Tris-buffered saline; 150 mM NaCl, 5 mM KCl, 10 mM Tris, pH 7.2, 0.1% Tween 20) containing 5% nonfat dry milk. Blots were incubated overnight at 4°C with affinity-purified specific anti-NHE3 polyclonal antisera (developed and characterized in our laboratory; Bookstein et al., 1994) diluted in T-TBS containing 1% BSA. Blots were developed by use of an enhanced chemiluminescence system with species-appropriate horseradish peroxidase-conjugated secondary antibodies.

Materials. All cell culture reagents were from Invitrogen (Carlsbad, CA). Chemicals were from Thermo Scientific (Waltham, MA). Clonidine, U73122, ketoconazole, PD98059, wortmannin, LY294002, tyrphostin AG-1478, 8-CPT-cAMP, and thapsigargin were purchased

from Alexis Biochemicals (San Diego, CA). PD123319 was from Tocris Bioscience (Ellisville, MO) and API-9 and TAPI-1 from EMD Bioscience (La Jolla, CA). MESNA was obtained from Sigma-Aldrich (St. Louis, MO). Polyscreen PVDF membranes were from PerkinElmer Life and Analytical Sciences (Waltham, MA). Sulfo-NHS-Biotin, sulfo-NHS-SS-Biotin, supersignal enhanced chemiluminescence reagents, and streptavidin agarose were obtained from Thermo Scientific (Rockford, IL). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Statistical Analysis. Comparisons between multiple groups and between paired groups were performed, respectively, by use of analysis of variance with a Bonferroni correction and a paired Student's *t* test. Both analyses were performed by use of Instat software for the Macintosh (GraphPad Software Inc., San Diego, CA)

Results

Clonidine Increases Basal and cAMP-Inhibited Intestinal Na^+ Absorption. To determine the effects of the α_2 -agonist clonidine on intestinal Na^+ transport, mouse small intestinal strips were mounted in Ussing chambers and mucosal-to-serosal (M-to-S) or serosal-to-mucosal (S-to-M) $^{22}Na^+$ fluxes were measured. As shown in Fig. 1, the addition of clonidine (100 μ M) to the serosal solution increased the M-to-S $^{22}Na^+$ flux. Conversely, exposure to the cell-permeant, stable cAMP analog (8-CPT-cAMP, 100 μ M) decreased M-to-S $^{22}Na^+$ flux significantly. In tissues pretreated with clonidine, the cAMP analog caused a much smaller decrease in the M-to-S flux. However, neither clonidine nor cAMP analog alone or in combination affected the S-to-M flux (Fig. 1). Therefore, stimulation of intestinal α_2ARs promotes basal and attenuates cAMP inhibition of intestinal Na^+ absorption.

Fig. 1. Clonidine increases basal and reverses cAMP-mediated inhibition of Na^+ absorption. Mouse jejunum strips were mounted in Ussing chambers, and clonidine (100 μ M) was added to the serosal bathing solution 10 min after mounting. Fifteen minutes later, cAMP (8-CPT-cAMP, 100 μ M) was added to the serosal bathing solution along with 1 μ Ci/ml $^{22}Na^+$ added to either the mucosal or serosal solution. Samples for determination of transmural mucosal-to-serosal (M-S) and serosal-to-mucosal (S-M) sodium fluxes (JNa) were taken 15 and 30 min later. Fluxes values are means $\pm$ S.E. for four separate experiments. *, $p < 0.05$ and +, $p < 0.01$ by analysis of variance with a Bonferroni correction.

α 2AR Stimulation Increases NHE2 and NHE3 Activity. The effect of α 2AR stimulation on the activity of sodium-hydrogen exchangers NHE2 and NHE3 was investigated with use a cultured intestinal epithelial cell line derived from Caco2 (Caco2-3B) which expresses α 2ARs at a density similar to that found in human intestinal mucosa (Valet et al., 1993; Schaak et al., 2000). In these experiments, another Caco2-derivative cell line, Caco2BBE, which does not express α 2ARs, was used as a control. As shown in Fig. 2A, Caco2-3B cells respond to clonidine with an increase in basal apical membrane $^{22}\text{Na}^+$ influx, an effect that was evident for both NHE2- and NHE3-mediated components. In contrast, no increase in $^{22}\text{Na}^+$ influx was seen in Caco2BBE cells stimulated with clonidine or when NHE2 and NHE3 measurements in Caco2-3B were performed in the presence of the α 2-antagonist, yohimbine (Fig. 2B). Yohimbine by itself had no effect on the apical $^{22}\text{Na}^+$ influx. These observations add further support for the role of α 2ARs in mediating the absorptive action effects of clonidine.

In agreement with similar observations in the Caco2BBE cell line (McSwine et al., 1998), activation of cAMP- or Ca^{2+} -dependent pathways by 8-CPT-cAMP and thapsigargin significantly inhibited basal $^{22}\text{Na}^+$ influx through NHE2 and NHE3 in Caco2-3B monolayers (Fig. 3) (+, $p < 0.01$ by analysis of variance for both NHE2 and NHE3 for the No Addition group). As in Fig. 1, clonidine increased basal NHE2- and NHE3-mediated ^{22}Na influxes across the apical membrane (α , $p < 0.05$). Clonidine also significantly attenuated the cAMP- and thapsigargin-induced inhibition of NHE2 and NHE3 activities (Fig. 3; *, $p < 0.05$ compared with clonidine treated alone or cAMP or thapsigargin addition for the No Addition group). Yohimbine by itself did not affect

Fig. 3. Clonidine blocks cAMP- and Ca^{2+} -mediated inhibition of apical membrane NHE activity. Caco2-3B monolayers, grown for 14 days on Transwell permeable supports, were pretreated with clonidine (100 μM , 15 min) before stimulation with 8-CPT-cAMP (100 μM) or thapsigargin (Thaps; 100 ng/ml). Apical NHE2 and NHE3 Na^+ flux activities were measured 15 min later, as described under *Materials and Methods*. Values are means $\pm$ S.E. for four separate experiments. *, $p < 0.05$ and +, $p < 0.01$ compared with control in each group; α , $p < 0.05$ comparing clonidine addition (Con) with No Addition control (Con). All comparisons were performed by analysis of variance with a Bonferroni correction.

Fig. 2. Clonidine increases basal apical membrane Na^+ influx in Caco2-3B, but not in Caco2BBE monolayers. Caco2BBE and Caco2-3B cells were grown on Transwell permeable supports for 14 days. Apical membrane $^{22}\text{Na}^+$ influxes were measured as described under *Materials and Methods* after treatment with clonidine (100 μM for 15 min) and pretreatment or not with yohimbine (300 μM) 15 min before clonidine. Values are means $\pm$ S.E. for four separate experiments. *, $p < 0.05$ by analysis of variance with a Bonferroni correction.

basal NHE2 or NHE3 activity or block the effects of cAMP or thapsigargin. However, it did antagonize the effects of clonidine on basal, cAMP- and thapsigargin-inhibited NHE2 and NHE3 activities (Fig. 3). Clonidine also had no effect on cAMP- or Ca^{2+} -inhibited $^{22}\text{Na}^+$ influx in Caco2BBE monolayers that did not express the α 2AR receptor (data not shown).

Clonidine Increases Apical Membrane NHEs and Decreases cAMP-Induced Endocytosis of Apical NHEs in Vitro and in Vivo. To determine the mechanisms underlying the stimulatory effects of clonidine on NHE activity, the trafficking of apical membrane NHEs was investigated by surface biotinylation as described previously in Caco2BBE (Musch et al., 2007). As shown in Fig. 4A, treatment of Caco2-3B cell monolayers with 8-CPT-cAMP (100 μM) or thapsigargin (100 ng/ml) enhanced endocytosis of apical NHE2 and NHE3, as evidenced by the decrease in biotinylated apical membrane NHEs 15 min after stimulation (Fig. 4A). Conversely, exposure to clonidine (100 μM for 15 min) caused a rapid increase in the abundance of both NHE2 and NHE3 in the apical membrane in the absence of any change in total abundance of these isoforms. Under these conditions, only apical membrane proteins are labeled and no biotinylation of basolateral or cytosolic proteins is observed (Musch et al., 2007). Pretreatment of cell monolayers with clonidine completely abolished the cAMP- or thapsigargin-induced endocytosis of NHE2 and NHE3.

To examine whether the same effects occur in intact intestine, biotinylation of luminal membranes was performed in clonidine-treated mouse small intestine loops. Under basal conditions, clonidine (100 μM for 15 min) induced a twofold

Fig. 4. Clonidine inhibits cAMP- and Ca^{2+} -stimulated endocytosis of NHE2 and NHE3 and promotes their exocytosis. A, Caco2-3B cells monolayers were pretreated with clonidine (100 μM , 15 min), and then treated with 8-CPT-cAMP (100 μM) or thapsigargin (Thaps; 100 ng/ml) for 15 min. Cells were immediately iced and surface proteins were biotinylated as described under *Materials and Methods*. A representative Western blot of four separate experiments is shown. Densitometry was performed by use of National Institutes of Health Image software and data are means $\pm$ S.E., setting the “no addition” to 100% for each experiment. *, $p < 0.05$; +, $p < 0.01$; ++, $p < 0.001$ compared with no addition in each set, by analysis of variance with a Bonferroni correction.

increase in apical NHE2 and NHE3 (Fig. 4B). In contrast, 8-CPT-cAMP and thapsigargin caused a large decrease in apical membrane NHE2 and NHE3. In agreement with *in vitro* findings, clonidine pretreatment significantly attenuated the cAMP- or Ca^{2+} -induced endocytosis of NHE2 and NHE3 (Fig. 4B). From these studies, however, it cannot be determined whether the action of clonidine is independent of cAMP- or Ca^{2+} -evoked inhibition of NHE activity/trafficking and/or whether it directly counteracts the stimulated endocytosis of apical membrane NHEs.

Clonidine Stimulates NHE2 and NHE3 Exocytosis through Stimulation of a Cascade of Intracellular Events. To investigate the cellular pathways that mediate clonidine-stimulated exocytosis, cells were pretreated with various inhibitors for 120 min. The inhibitors used were U73122 (30 μM , phospholipase C inhibitor), ketoconazole (30 μM , cytochrome P450/arachidonate epoxygenase inhibitor), TAPI-1 (100 μM , metalloproteinase 9 inhibitor), tyrphostin AG-1478 (100 μM , EGF receptor tyrosine kinase inhibitor), wortmannin (10 μM , PI3K inhibitor), LY294002 (30 μM , PI3K inhibitor), API-9 (30 μM , Akt inhibitor), PD98059 (30 μM , MEK-1 inhibitor), and colchicine (10 $\mu\text{g/ml}$, microtubule inhibitor). None of the inhibitors alone affected basal apical NHE2 or NHE3 flux activity [Fig. 5, top, fluxes; middle,

Western blot (–Clonidine)]. Clonidine increased $^{22}\text{Na}^{+}$ uptake by stimulating both NHE2 and NHE3 activities and promoting their apical surface abundance [Fig. 5, top, fluxes; and Fig. 5, bottom, Western blots for apical surface protein abundance (+ Clonidine)]. Pretreatment with certain inhibitors differentially affected clonidine’s stimulation of $^{22}\text{Na}^{+}$ influx (Fig. 5, top) and effects on apical surface NHE2 and NHE3 protein abundance (Fig. 5, bottom, two Western blots). Notable was the lack of inhibition by PD-98059, a MEK-1 inhibitor, and partial inhibition by the metalloproteinase and EGF receptor tyrosine kinase inhibitors. These results suggest that the clonidine stimulation of MEK-1/ERK1/2 does not play a role in regulation of apical NHE trafficking and that PI3K/Akt stimulation may occur in part through EGF receptor transactivation. However, a second pathway involving src stimulation by epoxygenase arachidonic acid metabolites could contribute to the overall effect (Karkoulas et al., 2006; Buffin-Meyer et al., 2007).

The second set of experiments was designed to determine whether clonidine’s ability to inhibit NHE endocytosis was mediated by similar transduction pathways. Cyclic AMP decreases NHE2 and NHE3 activities ($^{22}\text{Na}^{+}$ influxes; Fig. 6, left, bar graphs). To determine NHE2 and NHE3 protein endocytosis, a disulfide-sensitive form of biotin was used. As

Fig. 5. Clonidine stimulates apical NHE-mediated Na⁺ influx via a multistep transduction pathway. Caco2-3B monolayers were treated with various inhibitors shown to play a role in α 2-receptor-induced increases in cell growth (Buffin-Meyer et al., 2007). The inhibitors used for these studies and their targets were: U73122, phospholipase C; ketoconazole, cytochrome P450/arachidonate epoxidase; TAPI-1, metalloproteinase 9; tyrphostin AG-1478, EGF receptor tyrosine kinase; wortmannin and LY294002, PI3K; API-9, Akt inhibitor; and PD98059, MEK-1 inhibitor. Caco2-3B monolayers were pretreated with inhibitors for 2 h before stimulation with clonidine (100 μ M for 15 min). All samples for determination of apical membrane ²²Na⁺ influxes were taken immediately thereafter. Two sets of monolayers were treated for apical surface NHE2 and NHE3. Both sets were treated with inhibitors for 2 h, the first set was chilled, and the apical surface proteins were biotinylated (middle, Western blots, -Clonidine) and the second set was stimulated with clonidine for 15 min, chilled, and apical surface proteins biotinylated (bottom, Western blots, +Clonidine). Fluxes are means $\pm$ S.E. and the Western blot is representative of four separate experiments. Clonidine-stimulated fluxes were compared with untreated control and with inhibitors plus clonidine as indicated. For NHE2 and NHE3 apical abundance in Western blots, control (unstimulated) was set to 100% and all comparisons were made relative to this by analysis of variance by use of National Institutes of Health Images densitometric values. *, $p < 0.05$; +, $p < 0.01$; ++, $p < 0.001$.

noted in the Western blots, cAMP stimulation seems to promote endocytosis of NHE2 and NHE3, an effect blocked by clonidine pretreatment (Fig. 6, right, Western blots). Data in Fig. 6 support these findings by use of a complementary approach for assessing membrane trafficking of apical NHEs (Fig. 6, right, Western blots). Therefore, the same transduction pathways seem to mediate effects of clonidine on NHE endo- and exocytosis.

Discussion

Of the antidiarrheal agents that are currently being used clinically, few are capable of directly counteracting the ac-

tions of bacterial enterotoxins and/or of other intestinal secretagogues that cause secretory diarrhea. Octreotide, a stable analog of the peptide somatostatin, had been used for treatment of endocrine-tumor-related secretory diarrhea, but most of its action may be through the reduction of plasma levels of secretagogues. Octreotide has also been shown to have some proabsorptive actions in the normal human jejunum and ileum (Högenauer et al., 2002). Octreotide has also been used to treat diarrhea of varying etiologies, including dumping syndrome, diabetic enteropathy, short-bowel syndrome, and HIV-1 related diarrhea (Cascinu et al., 1993; Li-Ling and Irving, 2001; Mystakidou et al., 2006), but its

Fig. 6. Clonidine blocks cAMP-stimulated endocytosis of NHE2 and NHE3 via a multistep transduction pathway. Caco2-3B monolayers were treated with the inhibitors used for the studies in Fig. 5. Caco2-3B monolayers were pretreated with inhibitors for 2 h before stimulation when appropriate with clonidine (100 μ M, 15 min) and subsequently when appropriate with 8-CPT-cAMP (100 μ M, 15 min). One set of monolayers was treated for 60 min with Sulfo-NHS-S-S-biotin before treatments for fluxes, and after fluxes, monolayers were treated with three washes of MESNA-containing buffer so that only the biotinylated material internalized remain biotinylated. Samples for fluxes were treated as described previously. Fluxes are means $\pm$ S.E., and images representative of four separate experiments. For fluxes, statistical comparisons were as indicated, and for internalized NHE2 and NHE3, comparisons were made by setting the cAMP-stimulated (internalized) densitometric value to 100% and comparisons with this were made by analysis of variance. *, $p < 0.05$; +, $p < 0.01$; ++, $p < 0.001$.

major action in these disorders may be mediated by its effects on motility. Inhibiting motility promotes enteropooling, increasing the contact time for luminal contents with the absorptive intestinal mucosa. The practical use of octreotide for treatment of global infectious diarrheal disease, however, is limited because of its cost and the necessity for subcutaneous delivery via injection. Thus, development of effective treatments that can directly counteract the actions of enterotoxigenic agents would be desirable.

Oral rehydration solutions, which are effective in treating patients with diarrhea, act by enhancing fluid absorption pathways that are not impaired by intestinal secretagogues. Oral rehydration, in general, is achieved with a solution that contains glucose (or a polymer such as partially hydrolyzed rice) with a balanced electrolyte solution. Providing glucose and sodium promotes water absorption by stimulating electrogenic Na^+ absorption via SGLT1. These therapies have been effective in treating secretory diarrhea such as cholera, but they do so by reducing metabolic losses through enhanced Na^+ absorption (Harrell and Chang, 2006). They have little effect in reducing diarrheal volume and may actually increase overall stool output.

The current results demonstrate that $\alpha 2\text{AR}$ effects on apical NHE in the intestine are similar to those found for $\alpha 2\text{AR}$ in renal and neural cells (Cussac et al., 2002; Karkoulas et al., 2006). The dissection of $\alpha 2\text{AR}$ pathways relevant to pro-

liferation of Caco2-3B cells have been published recently (Buffin-Meyer et al., 2007). Whereas $\alpha 2\text{AR}$ stimulation of ERK-1/2 is pivotal for effects on proliferation, this does not seem to be the case for $\alpha 2\text{AR}$ effects on apical NHE activities. Stimulation of PI3K and Akt by clonidine, however, is pivotal to clonidine's effect on apical NHEs (Figs. 5 and 6). The roles of PI3K and Akt as regulators of NHE3 exocytosis and endocytosis have been demonstrated previously (Janecki et al., 2000; Lee-Kwon et al., 2001). Whether the $\alpha 2\text{AR}$ signaling pathway observed in Caco2-3B cell also occurs in normal intestinal epithelium remains to be established. Studies of $\alpha 2\text{AR}$ distribution along the villus-crypt axis of intestinal mucosa from different species including human (Paris et al., 1990; Valet et al., 1993) have shown that $\alpha 2\text{AR}$ s are abundant in proliferative crypt cells but rare in differentiated villus cells where Na^+ absorption via NHE takes place. Although the expression of $\alpha 2\text{AR}$ in villus epithelial cells is less abundant, the current studies and past reports clearly show that these receptors are sufficient to promote active Na absorption.

Clonidine clinical use in diarrheal diseases has been limited to a few anecdotal reports of secretory and diabetic diarrhea where its effects seem to be mediated by correction of intestinal water and electrolyte disturbances (Fedorak et al., 1985; Chang et al., 1986). Clonidine and lofexidine (another $\alpha 2$ -agonist) have also been demonstrated to reduce

castor oil-induced diarrhea (Lal et al., 1981). However, more extensive use of α_2 -agonists for diarrheal diseases has been limited by their hypotensive effects that, for the most part, are mediated through the central nervous system. There is limited information on whether smaller, minimally hypotensive doses of clonidine or related agents can be used that are still effective in promoting net Na absorption. The development of α_2 -agonists with effects that are more gut-restricted would provide a potent antidiarrheal agent that could be clinically useful. One possibility is to modify the structure of currently available α_2 AR agents to confine their actions to the gut, either through enhanced first-pass hepatic clearance or exploitation of regional metabolizing pathways. Enhanced first-pass hepatic clearance, for instance, could be achieved through glucuronidation or sulfation to an inactive form. Another approach would be to develop α_2 AR agents that do not cross the blood-brain barrier. Loperamide, a commonly used antidiarrheal agent, is an example of such an approach which does not have the CNS-associated side effects of its counterpart, diphenoxylate (for review see Niemgeers et al., 1981).

In summary, activation of intestinal epithelial α_2 ARs can significantly attenuate the inhibition of apical member Na⁺ transporters by cAMP- and Ca²⁺-mediated pathways. The development of new α_2 -agonists, like clonidine, provide the potential for clinically useful antidiarrheal agents that can directly counteract or prevent the actions of toxigenic pathogens and other secretagogues that cause secretory diarrhea.

Acknowledgments

We thank Michael Flavin for advice and consultation on potential approaches for development of future α_2 -adrenergic agents.

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