Stimulation of apical $\text{Cl}^-/\text{HCO}_3^-(\text{OH}^-)$ exchanger, SLC26A3 by neuropeptide Y is lipid raft dependent

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Saksena S, Tyagi S, Goyal S, Gill RK, Alrefai WA, Ramaswamy K, Dudeja PK. Stimulation of apical $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchanger, SLC26A3 by neuropeptide Y is lipid raft dependent. Am J Physiol Gastrointest Liver Physiol 299: G1334–G1343, 2010. First published September 30, 2010; doi:10.1152/ajpgi.00039.2010.—Neuropetide Y (NPY), an important proabsorptive hormone of the gastrointestinal tract has been shown to inhibit chloride secretion and stimulate NaCl absorption. However, mechanisms underlying the proabsorptive effects of NPY in the human intestine have not been fully understood. The present study was designed to examine the direct effects of NPY on apical $\text{Cl}^-/\text{HCO}_3^-(\text{OH}^-)$ exchange activity and the underlying mechanisms involved utilizing Caco2 cells. Our results showed that NPY (100 nM, 30 min) significantly increased $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange activity (∼2-fold). Selective NPY/Y1 or Y2 receptor agonists mimicked the effects of NPY. NPY-mediated stimulation of $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange activity involved the ERK1/2 MAP kinase-dependent pathway. Cell surface biotinylation studies showed that NPY does not alter DRA (apical $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchanger) surface expression, ruling out the involvement of membrane trafficking events. Interestingly, DRA was found to be predominantly expressed in the detergent-insoluble (DI) and low-density fractions (LDF) of human colonic apical membrane vesicles (AMVs) representing lipid rafts. Depletion of membrane cholesterol by methyl-β-cyclodextrin (MβCD, 10 mM, 1 h) remarkably decreased DRA expression in the D1 fractions. Similar results were obtained in Triton-X 100-treated Caco2 plasma membranes. DRA association with lipid rafts in the DI and LDF fractions of Caco2 cells was significantly enhanced (∼45%) by NPY compared with control. MβCD significantly decreased $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange activity in Caco2 cells as measured by DIDS- or niflumic acid-sensitive 36Cl$^-$ uptake (∼50%). Our results demonstrate that NPY modulates $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange activity by enhancing the association of DRA with lipid rafts, thereby resulting in an increase in $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange activity. Our findings suggest that the alteration in the association of DRA with lipid rafts may contribute to the proabsorptive effects of NPY in the human intestine.

chloride absorption; human intestine; downregulated in adenoma; ERK 1/2 MAP kinase

Electroneutral NaCl absorption in the human ileum and colon occurs via coupled operation of luminal Na$^+$/H$^+$ (NHE) and $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange activities (13, 20). Disturbances in electrolyte absorption across the luminal membrane of the intestinal epithelial cells are implicated in the pathophysiology of diarrhea associated with several intestinal disorders including inflammatory bowel diseases (15). Diarrhea occurs due to either increased secretion or decreased absorption of water and electrolytes or both. Extensive studies recently have focused on the mechanisms involved in NHE isoform regulation (23–26, 37, 45); however, very little is known about the regulation of $\text{Cl}^-$ absorption. In this regard, previous studies have shown that DRA or SLC26A3 plays a major role in apical $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange process in the intestine (43). Genetic analysis studies have shown that mutations in DRA result in congenital chloride diarrhea (CLD), associated with metabolic alkalosis and voluminous diarrhea with high chloride content (27). The phenotype of DRA-deficient mice was similar to CLD in humans (55).

Previous studies from our laboratory utilizing human intestinal epithelial cell lines have demonstrated the short-term effect of various inflammatory agents, e.g., nitric oxide (50), serotonin (49), reactive oxygen species (51), and enteropathogenic Escherichia coli infection (21) in the inhibition of luminal $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange activity. We have recently demonstrated that the bioactive lipid lysophosphatidic acid stimulated apical $\text{Cl}^-/\text{HCO}_3^-$(OH$^-$) exchange activity via an increase in the surface levels of DRA in Caco2 cells (59a). However, to date, almost nothing is known about the mechanisms underlying the short-term regulation of the proabsorptive peptide hormone neuropeptide Y (NPY) on chloride absorption and DRA expression in human intestinal epithelium. NPY is a 36-amino acid hormone extensively expressed in myenteric and submucosal neurons throughout the gastrointestinal tract of rat, mouse, and human (17, 46, 52) and has been shown to inhibit gastric emptying and intestinal and colonic motility (54). NPY has also been shown to be a potent inhibitor of small intestinal and colonic ion secretion and thus has been suggested to act as a proabsorptive hormone in various animal models and humans (3, 7, 10, 28, 29, 48, 60). NPY binds to a family of six G-protein-coupled receptors named the “Y receptor family.” Y1, Y2, and Y5 receptors bind NPY with high affinity; Y4 receptor binds to NPY with a lower affinity, and the existence of Y3 remains controversial (9).

Functional studies showed that a combination of Y1 and Y2 receptors mediate the antisecretory effects of NPY in rat, mouse, and human colon (11, 48, 61). The receptors are located on the basolateral epithelial surfaces of the human colon (Y1 > Y2 receptors) (39, 63), rat jejunum (Y2 receptors) (10) and polarized layers of human colonic adenocarcinoma cell line, Colon-6 cells (Y1 > Y2 receptors) (11), and HT29 cells (Y1 receptors) (39). NPY receptors are well known to be linked negatively to adenyl cyclase through G proteins, likely Gi, and Gs (9). The transduction mechanism elicited by activation of epithelial Y1 or Y2 receptors is an inhibition of adenylate

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cyclase and resultant reduction in intracellular cAMP concentration (39, 56) leading to attenuated Cl− secretion.

The present studies were undertaken to examine in detail the direct effects of NPY on apical Cl−/HCO3− (OH−) exchange activity in Caco2 cells and the potential involvement of DRA. Our studies demonstrated that NPY stimulated apical Cl−/HCO3− (OH−) exchange activity in human intestinal epithelial Caco2 cells via a NPY/Y1 receptor subtype and ERK1/2 MAP kinase-dependent pathway. Moreover, this stimulation of Cl−/HCO3− (OH−) exchange activity in response to NPY was due to an enhanced association of DRA with lipid rafts (specialized microdomains of the membrane). These findings provide novel evidence of the functional role of lipid rafts in regulating DRA protein levels in these membrane domains and increase function in the intestine.

**MATERIALS AND METHODS**

Materials. Caco2 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). 36Chloride (HCl) radioisotope was procured from American Radiolabeled Chemicals (St. Louis, MO). NPY and NPY/Y1 receptor agonist [Leu3]Pro4 were obtained from Sigma (St. Louis, MO). ERK1/2 MAP kinase inhibitor U0126 was obtained from Biomol (Plymouth Meeting, PA). Sulfo-NHS-SS-biotin and streptavidin agarose were from Pierce (Rockford, IL). Goat anti-mouse and goat anti-rabbit antibody conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of at least reagent grade and were obtained from either Sigma Chemical (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Cell culture and treatment. Caco-2 cells were grown in modified Eagle’s medium supplemented with 4.5g/l glucose, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 10 mM HEPES, 1% essential and nonessential amino acids, and 20% fetal bovine serum, pH 7.4 in 5% CO2-95% O2 at 37°C. For the uptake experiments, cells were co-cultured with essential and nonessential amino acids, and 20% fetal bovine serum, Eagle’s medium supplemented with 4.5g/l glucose, 2 mM glutamine, and 150NaCl, 10 EDTA, 1 DTT, 1% Triton X-100, and 1 Complete protease inhibitor cocktail. Membrane vesicles were then incubated with MES buffer on a rotary shaker for 30 min at 4°C.

Our studies demonstrated that NPY stimulated apical Cl−/HCO3− (OH−) exchange activity in Caco2 cells. For the uptake experiments, cells were treated with NPY (1–100 nM) from days 10–12 postplating because Caco2 cells grown under these conditions (medium containing 20% FBS) were found to be fully differentiated and exhibit maximal DRA expression at this time point. To study the effect of NPY on Cl−/HCO3− (OH−) exchange activity, cells were acutely exposed to NPY (1–100 nM) from the basolateral side in serum-free cell culture medium for different time points (15–120 min). In separate sets of experiments, cells were pretreated with the specific ERK1/2 MAP kinase inhibitor U0126 (10 μM) for 1 h and then coincubated with NPY (100 nM) for another 30 min. For methyl-β-cyclodextrin (MβCD) and MβCD + cholesterols, MβCD and water-soluble cholesterol (49 mg cholesterol balanced by 561 mg of MβCD per gram) were obtained from Sigma. Caco2 cells were treated with different concentrations of MβCD (1–10 mM) for 60 min. Solutions containing 1.25 mM cholesterol were supplemented with additional MβCD to reach a final concentration of 10 mM of the latter.

Cl−/HCO3− (OH−) exchange activity. Cl−/HCO3− (OH−) exchange activity was determined by measuring DIDS-sensitive 36Cl− uptake in base-loaded cells as previously described by us (49, 51). We have also used another anion exchange inhibitor, niflumic acid, that has been shown to inhibit DRA (Cl−/HCO3− (OH−) exchange) more potently than DIDS in the heterologous expression systems (8). Cl−/HCO3− (OH−) exchange activity (DIDS- or niflumic acid-sensitive 36Cl− uptake) was expressed as nanomoles per milligram protein per 5 min.

Biotinylation of cell surface proteins. Cell surface biotinylation studies to measure surface protein expression of DRA were done as described previously (21). The apical surface of untreated or NPY-treated Caco-2 cells were exposed to sulfo-NHS-SS-biotin (1.5 mg/ml in borate buffer pH 9.0; Pierce) for 60 min at 4°C in horizontal motion to stop endocytosis and internalization of antigens. After immunoprecipitation of biotinylated antigens with streptavidin agarose, biotinylated proteins were released by incubation in 50 mM DTT, and reconstituted in Laemmli buffer. Immunoprecipitates were separated on 8–10% SDS-PAGE gels and probed with human anti-DRA (21). Bands were visualized with enhanced chemiluminescence reagent.

Isolation of DS and DI fractions from human colonic AMVs or Caco2 cells. Colon from healthy adult organ donors was obtained immediately after harvest of transplantation organs (Gift of Hope, IL). Purified apical membrane vesicles (AMVs) were prepared from mucosa as previously described (22). Detergent-soluble (DS) and insoluble (DI) fractions of colonic AMVs or Caco2 cell lysates were prepared essentially as previously described (2). Briefly, 3 mg of AMVs were incubated in the presence or absence of 10 mM MβCD for 1 h at 37°C and then were centrifuged for 30 min at 100,000 g at 4°C and resuspended in MES buffer containing (in mM) 50 MES (pH 5.5), 60 NaCl, 3 EGTA, 5 MgCl2, 1% Triton X-100, and 1 Complete protease inhibitor cocktail. Membrane vesicles were then incubated with MES buffer on a rotary shaker for 30 min at 4°C. At the end of the incubation, AMVs were centrifuged at 100,000 g at 4°C for 30 min and supernatant was designated as DS fraction. The pellet was resuspended in buffer containing (in mM) 15 HEPES (pH 7.4), 150 NaCl, 10 EDTA, 1 DTT, and 1 Complete protease inhibitor cocktail. Membrane vesicles were then loaded on a discontinuous Optiprep density gradient. Initial experiments were performed in Caco2 cells in the presence or absence of NPY (100 nM) for 30 min.

**Flotation on a discontinuous Optiprep density gradient.** Lipid rafts were isolated by flotation on Optiprep density gradient as previously described (2, 33). Equal amount of protein (−3 mg) from colonic AMVs or untreated or NPY-treated Caco2 membranes were used for the Optiprep gradient studies. Membrane preparations were then centrifuged at 100,000 g for 30 min at 4°C, resuspended, and incubated for 30 min at 4°C in TNE buffer containing (in mM) 25 Tris (pH 7.4), 150 NaCl, 5 EDTA, and 1 Complete protease inhibitor cocktail. The membranes were then incubated on ice for 40% final concentration of Optiprep (Nycomed, Oslo, Norway) and layered at the bottom of density gradient with steps of final concentrations of 35, 30, 25, and 20% of Optiprep in TNE buffer. TNE buffer was laid on the top of the gradient, which was then centrifuged at 48,000 rpm for 4 h at 4°C. Fractions (−13–14) were collected from the top to the bottom of the gradient and then analyzed by Western blotting. The specific activity of alkaline phosphatase was measured in each fraction of gradient. Alkaline phosphatase has been previously shown as a marker of lipid rafts (33). Protein concentrations in each fraction were assessed by the method of Bradford (5).

Western blotting. Equal amounts of protein from the DS and DI fractions of colonic AMVs or Caco2 cells (75 μg) or equal volumes from each fraction of the Optiprep gradient were reconstituted in Laemmli buffer and separated on 8% SDS PAGE. DRA expression was detected utilizing human anti-DRA antibody as previously described (21, 51).

**Tissue accumulation studies in mice.** C57BL/6J mice (8–10 wk) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at the Veterinary Medical Unit at the Jesse Brown Veteran Affairs Medical Center. Mice had free access to food and water and were fed regular chow diet. The effect of NPY on Cl−/HCO3− (OH−) exchange activity was examined in these mice under ex vivo conditions by an internal sac method as previously described (41, 64). It should be noted that due to the nonavailability of radioactive 36Cl−, we used 125I− instead of 36Cl− since DRA is also known to transport iodide. We further established that iodide uptake in Caco2 cells represent Cl−/HCO3− (OH−) activity as similar to chloride uptake, iodide uptake was also inhibited by DIDS or niflumic acid (−50–60%). Also, similar results were obtained showing significant stimulation or inhibition of iodide.
uptake in Caco2 cells by *Lactobacillus acidophilus* or enteropathogenic *E. coli* (unpublished observations) as previously described (4, 21). Distal colon from mice was removed, flushed with Krebs-Ringer buffer (KRB), and everted by using tips of thin glass rods. Everted distal colon segments were tied at one end by nylon thread (000 Ethilon Black braided nylon), and the serosal side was filled with either KRB alone (control) or with NPY (100 nM). The filled everted distal colon segments were then tied at the other end forming sacs and placed in tubes containing KRB for 30 min at 37°C. After 30 min, the sacs were then placed in Krebs Ringer buffer containing unlabeled NaI (3 mM) and labeled 125I (1 μCi/ml, Perkin Elmer, Waltham, MA) in the presence or absence of the anion exchange inhibitor DIDS (1 mM), and 125I uptake was measured by the tissue accumulation method (14). The reaction was stopped by washing in ice-cold 1× PBS solution and the radioactivity retained was assessed after dissolving the tissues in 10% KOH; activity was expressed as nanomoles per milligram protein per 10 min. Animal studies were approved by the Animal Care Committee of the Jesse Brown VA Medical Center.

Statistical analysis: Results are expressed as means ± SE. Each independent set represents means ± SE of data from at least nine wells used on three separate occasions. Student’s *t*-test was used for statistical analysis. *P* < 0.05 or less was considered statistically significant.

RESULTS

NPY increases Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells. NPY is known to function as a proabsorptive peptide by stimulating NaCl absorption (28) and inhibiting Cl⁻ secretion (12, 48, 53, 61) in the intestine. Therefore, we examined the direct effects of NPY on Cl⁻/HCO₃⁻ (OH⁻) exchange activity in polarized Caco2 cells. The optimal time and dose of NPY on Cl⁻/HCO₃⁻ (OH⁻) exchange activity was evaluated. Cells were treated with NPY added from the basolateral side for 30 min, and apical Cl⁻/HCO₃⁻ (OH⁻) exchange activity was measured as DIDS-sensitive 36Cl uptake after base loading the cells. Dose-response studies showed that NPY significantly increased Cl⁻/HCO₃⁻ (OH⁻) exchange activity at the maximum dose of 100 nM (~2-fold; *P* < 0.05, Fig. 1A), whereas time course studies showed that effect of NPY was observed as early as 30 min, which persisted till 120 min (~2-fold; *P* < 0.05, Fig. 1B). Similar effects of NPY (100 nM, 30 min) were also observed with the other anion exchange inhibitor, niflumic acid (100 μM, Fig. 1C).

Effect of NPY/Y1 or Y2 receptor agonists on Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells. Our initial studies showed that both NPY/Y1 and Y2 but not NPY/Y5 receptor mRNAs are present in differentiated Caco2 cells (data not shown). Also, NPY/Y1 and 2 receptor subtypes have been shown to be involved in mediating the inhibitory effects of NPY on Cl⁻ secretion in animal and cell culture models (11, 12, 39, 48, 53, 61). We next examined the effect of [Leu3¹,Pro3⁴] NPY, selective Y1 receptor agonist or NPY [3–36], selective Y2 receptor agonist on apical Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells. As shown in Fig. 2, A and B, basolateral addition of the Y1 receptor agonist [Leu3¹,Pro3⁴] NPY or the Y2 receptor agonist NPY [3–36] (100 nM) for 30 min resulted in a significant increase in apical Cl⁻/HCO₃⁻ (OH⁻) exchange activity. These observations suggest that both Y1 and Y2 receptor agonists mimicked the effects of NPY in stimulating Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells.

Role of ERK1/2 MAP kinase in NPY-induced stimulation of Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells. NPY is well known to trigger a number of intracellular pathways including activation of MAP kinases in different cell types (38, 40). Therefore, we next determined the role of MAP kinases in the NPY-mediated stimulation of apical Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells. The effect of U0126A (a specific inhibitor of ERK1/2, 10 μM, 1 h) and SB203580 (a
specific p38 MAP kinase inhibitor, 30 μM, 1 h) on Cl⁻/HCO₃⁻ (OH⁻) exchange activity in response to NPY was examined. Specific ERK1/2 MAP kinase inhibitor U0126 blocked the stimulatory effects of NPY on Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells (Fig. 3), suggesting the involvement of ERK1/2 MAP kinase in NPY-stimulated effects. However, SB203580 failed to show any effect, indicating that p38 MAP kinase is not involved (data not shown).

**NPY does not alter surface DRA expression.** Since the observed stimulation of Cl⁻/HCO₃⁻ (OH⁻) exchange activity by NPY occurred within minutes (short-term), this type of regulation might be due to an increase in the number of active transporter molecules at the membrane (increased exocytic insertion) or decreased retrieval of the transporter into the intracellular pools (42, 45). We, therefore, examined whether NPY regulates DRA expression via recycling of the transporter from the intracellular pools to the apical compartment. Cell surface biotinylation studies were performed to determine the changes in cell surface levels of DRA protein or the biotinylated fraction of total cellular protein. Biotinylated proteins from control and NPY-treated cells were separated from the cell lysate by avidin and proteins were probed with human DRA antibody. As shown in Fig. 4, cell surface biotinylation studies demonstrated that the surface expression of DRA remained unaltered in response to NPY, thereby ruling out the involvement of membrane trafficking events.

**DRA is associated with lipid rafts.** Since changes in surface membrane levels of DRA were not involved in NPY-mediated regulation of DRA, we next investigated whether NPY regulates DRA by redistribution between different specialized domains of the plasma membrane. Previous studies have shown that the regulation of membrane transporter NHE3 is dependent on its association with specific microdomains of the plasma membrane such as lipid rafts (33). Lipid rafts are enriched with cholesterol and sphingolipids and are resistant to detergent solubilization such as Triton X-100 (35). DRA encodes for the apical Cl⁻/HCO₃⁻ (OH⁻) exchanger in the human intestine (43) and has been shown to play an important role in coupled NaCl absorption. However, to date, no studies are available showing the association of DRA with lipid rafts. Thus it was considered to be of interest to investigate the association of DRA with lipid rafts by determining the partitioning of DRA between DS and DI fractions of human colonic apical membranes prepared from organ donors as well as purified Caco2 cell membranes.

Human colonic AMVs were treated with Triton-X 100 at 4°C and the DI fraction was collected by high-speed sedimentation, whereas the supernatant contained the DS fraction of plasma membrane (as described in MATERIALS AND METHODS). Figure 5A shows that in parallel to the high abundance of β-actin of the cytoskeleton detected in the DI fraction of human colonic AMVs, DRA was also found to be expressed predominantly (~97 kDa) in the DI fraction. The detection of DRA in the DI fraction of plasma membrane suggests its...

![Fig. 2](http://ajpgi.physiology.org/)

**Fig. 2.** NPY/Y1 or Y2 receptor agonists mimic effects of NPY on Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells. Overnight serum-starved postconfluent Caco2 cells grown on permeable supports were incubated with 100 nM NPY/Y1 (A) or NPY/Y2 receptor (B) agonists in serum-free cell culture medium for 30 min. Cl⁻/HCO₃⁻ (OH⁻) exchange activity was measured in base-loaded cells as DIDS-sensitive ³⁶Cl uptake at 5 min as described in MATERIALS AND METHODS. Results are expressed as % of control and represent means ± SE of 3 separate experiments performed in triplicate. *P < 0.05 compared with control.

![Fig. 3](http://ajpgi.physiology.org/)

**Fig. 3.** Effect of ERK1/2 MAP kinase inhibitor U0126 on NPY-induced stimulation of Cl⁻/HCO₃⁻ (OH⁻) exchange activity in Caco2 cells. Overnight serum-starved postconfluent Caco2 cells grown on permeable supports were preincubated with U0126 (10 μM), a specific inhibitor of ERK1/2 MAP kinase, for 60 min in the serum-free cell culture medium and then coincubated with 100 nM of NPY for another 30 min. Cl⁻/HCO₃⁻ (OH⁻) exchange activity was measured in base-loaded cells as DIDS-sensitive ³⁶Cl uptake at 5 min as described in MATERIALS AND METHODS. Results are expressed as % of control and represent means ± SE of 3 separate experiments performed in triplicate. *P < 0.05 compared with control.
NPY enhances DRA membrane expression in the detergent-resistant fraction of Caco2 cells. Since membrane trafficking was not involved in NPY-mediated regulation of DRA, we next investigated whether NPY caused any alterations in the association of DRA with lipid rafts. Control and NPY-treated (100 nM, 30 min) membranes of Caco2 cells were incubated with Triton-X 100 at 4°C and the DI and DS fractions were collected. As shown in Fig. 6A, DRA was expressed in the DI fraction of both the control and NPY-treated Caco2 cells. However, DRA expression in the membrane DI fraction was significantly enhanced in the presence of NPY compared with control. As expected, β-actin was detected in the DI fraction and used as an internal control to indicate equal loading of protein. Optiprep density gradient (40–20%) studies also showed that DRA was predominantly expressed in upper low-density (high-buoyancy) fractions representing lipid rafts in both control and NPY-treated cells. As seen in Fig. 6B, DRA is distributed in fractions ranging from fractions 4 to 14; however, compared with the alkaline phosphatase activity in these fractions (control), the high-buoyancy fractions 4–7 showed high alkaline phosphatase activity, indicating that fractions 4–7 represent lipid rafts. Moreover, in response to NPY, a significant increase in DRA protein levels in fractions 3–5 was observed compared with control (Fig. 6C). These results suggest that NPY enhanced the redistribution of DRA in the lipid raft fractions, thereby increasing the amount of DRA within lipid rafts. The increased levels of DRA in the DI fraction of plasma membrane of NPY-treated Caco2 cells suggest that NPY-mediated stimulation of Cl-/HCO3-(OH-) exchange activity in Caco2 cells is due to enhanced association of DRA with the lipid rafts.

Depletion of plasma membrane cholesterol affects Cl-/HCO3-(OH-) exchange activity in Caco2 cells. Previous studies have shown that the association of membrane transporters with lipid rafts is essential for both their regulation and optimal activity (36). Since the integrity of lipid rafts depends on their content of cholesterol (58), we examined the influence of cholesterol depletion of plasma membrane on Cl-/HCO3-(OH-) exchange activity in Caco2 cells. Cells were incubated with different concentrations of MβCD (cholesterol sequestering agent) for 60 min at 37°C and Cl-/HCO3-(OH-) exchange activity was measured as DIDS- or niflumic acid-sensitive 36Cl- uptake. As shown in Fig. 7A, parallel to a decrease in DRA expression, DRA function (Cl-/HCO3-(OH-) exchange activity) was significantly reduced in a dose-dependent manner with a maximal inhibition at 10 mM (~50%). Also, similar results were observed as MβCD (10 mM) significantly decreased niflumic acid sensitive 36Cl- uptake by ~55% (Fig. 7B). To determine whether the observed reduction in Cl-/HCO3-(OH-) exchange activity was specifically due to cholesterol depletion, Caco2 cells were treated with 10 mM MβCD alone or along with 1.25 mM cholesterol. As depicted in Fig. 8, the presence of cholesterol prevented the MβCD-induced inhibition of Cl-/HCO3-(OH-) exchange activity, indicating that the observed reduction is indeed due to cholesterol depletion of the plasma membrane and is critical for the optimal activity of the Cl-/HCO3-(OH-) exchanger, DRA.

NPY increases iodide uptake in native tissues of mice. Our in vitro cell culture results were validated by use of an ex vivo mouse model to obtain a comprehensive view about the potential proabsorptive effects of NPY in the native intestine. The
direct effects of NPY on $^{125}$I uptake in the distal colon of mice were examined by everted sac technique. As shown in Fig. 9, NPY (100 nM, 30 min) significantly increased DIDS-sensitive $^{125}$I uptake in the distal colon by approximately twofold; $P < 0.05$. These results are consistent with the in vitro studies and further demonstrate the potential proabsorptive role of NPY that may contribute to increased anion absorption in the lumen.

**DISCUSSION**

NPY is an important hormone and neurotransmitter produced by the intrinsic neurons, which directly innervate the epithelial cells of the intestinal mucosa in different species, including human (9). NPY has been shown to be a potent inhibitor of intestinal Cl$^-$ secretion. For example, NPY was found to reduce basal secretion in porcine and mouse jejunum (7, 48) and antagonize VIP (3) and cAMP-stimulated secretion in rabbit distal colon and rat jejunum (10). In the present study, NPY (100 nM) increased apical Cl$^-$/HCO$_3^-$/(OH$^-$) exchange activity by approximately twofold in Caco2 cells. The data are in accordance with the previous in vivo perfusion studies in the human small intestine, showing that NPY increased net absorption of water, Na$^+$, K$^+$, and Cl$^-$ and inhibited prostaglandin E$_2$-induced intestinal fluid and electrolyte secretion (28). Our ex vivo studies in mice showed that NPY (100 nM, 30 min) increased $^{125}$I uptake (~2-fold) in the distal colon, further suggesting a proabsorptive/antisecretory action of NPY.
concentration of NPY used in our present study is in the physiological range (100 nM), since a similar dose of NPY was used in previous studies to inhibit VIP-induced increase in short-circuit current in human adenocarcinoma cell line, Colony-6 epithelial cells (11). NPY/Y1 and Y2 receptors are responsible for mediating the antisecretory effects of NPY in rat, mouse, and human colon (12, 48, 61). Our receptor mRNA expression studies utilizing real-time PCR have shown that NPY/Y1 and 2 but not 5 receptors are expressed in Caco2 cells (data not shown). We showed that NPY receptor subtypes 1 and 2 (NPY/Y1 and Y2) are responsible for the stimulatory effects of NPY on Cl⁻/HCO₃⁻ (OH⁻)⁻ exchange activity since [Leu³¹,Pro³⁴] NPY (NPY/Y1 receptor agonist) and NPY [3–36] (NPY/Y2 receptor agonist) increased Cl⁻/HCO₃⁻ (OH⁻)⁻ exchange activity in Caco2 cells. However, the observed effects are more profound with the Y1 receptor agonist (6-fold) compared with Y2 receptor agonist (2-fold), suggesting that the NPY-induced effects on Cl⁻/HCO₃⁻ (OH⁻)⁻ exchange activity in Caco2 cells are predominantly Y1 receptor dependent rather than Y2 receptor. We speculate that NPY/Y1 agonist has a high binding affinity to Y1 receptor compared with NPY since it contains a rigid imino acid residue, proline. The presence of proline in NPY/Y1 agonist but not NPY has been previously shown to be responsible for the difference in receptor binding (18) and could be the reason for the observed dramatic stimulatory effect on Cl⁻/HCO₃⁻ (OH⁻)⁻ exchange activity in Caco2 cells.

Y1 receptor has been shown to couple to MAP kinase activation via a number of different signaling molecules in intestinal epithelial cells (38) and fibroblasts (40). We showed that ERK1/2 MAP kinase via the NPY/Y1 receptor is involved in mediating the effects of NPY, since the specific ERK1/2 MAP kinase inhibitor U0126 blocked NPY-induced effects on Cl⁻/HCO₃⁻ (OH⁻)⁻ exchange activity in Caco2 cells. It is well known that, under short-term conditions, multiple plasma membrane transport proteins are regulated via recycling mechanisms (19, 21, 33). However, NPY failed to increase the surface levels of DRA, thereby ruling out the involvement of membrane trafficking. Recent studies have shown the importance of lipid rafts in the regulation of membrane protein functions (33, 36). Lipid rafts are specialized microdomains of the plasma membrane enriched with cholesterol and sphingolipids and are resistant to detergent solubilization such as Triton X-100 (35). In polarized epithelial cells, lipid rafts are predominantly localized in the apical domains. Lipid rafts are implicated in a variety of dynamic cellular processes such as signal transduction (59), apical membrane protein targeting in

Fig. 6. NPY enhances DRA association with lipid rafts in Caco2 cells. A: NPY enhances DRA expression in DI fractions of Caco2 cells. Untreated or NPY (100 nM, 30 min)-treated cells were solubilized in buffer containing Triton X-100 and then DS and DI fractions were isolated as described in MATERIALS AND METHODS. Equal amounts of proteins (~75 μg) from DI and DS fractions were separated on 8% PAGE and then analyzed by Western blotting for DRA and actin expression. The data were quantified by densitometric analysis and expressed as arbitrary units and represent means ± SE of 3 determinations. *P < 0.05 compared with untreated control. The y-axis indicates the density of DI fractions. B and C: NPY enhances redistribution of DRA in lipid raft fraction (fractions 5-9) compared with untreated control. Untreated or NPY (100 nM, 30 min)-treated Caco2 cells were solubilized in buffer containing Triton X-100 for 30 min at 4°C and were then layered at the bottom of a discontinuous density gradient of OptiPrep as described in MATERIALS AND METHODS. After high-speed centrifugation, fractions were collected from the top to the bottom of the gradient and run on 2 separate 8% SDS-PAGE and probed simultaneously with anti-hDRA antibody. The blots shown are a representative of 4 separate experiments. Alkaline phosphatase activity (marker of lipid rafts) for each fraction was assessed. Specific activity is expressed as nmol·mg protein⁻¹·min⁻¹ and data are means ± SE of 4 separate determinations from different occasions.
epithelial cells (31, 57), and regulation of the activity of membrane proteins (2, 6, 32). Our present studies also showed that DRA was predominantly expressed in the Triton X-100 insoluble fractions of the plasma membrane of the human colonic AMVs. Moreover, MβCD (cholesterol-depleting agent) significantly decreased DRA expression in the DI fractions, further suggesting its association with lipid rafts. We have previously utilized MβCD to disrupt lipid rafts in Caco2 cells and human ileal brush-border membrane vesicles (2). The association of DRA with lipid rafts in human colonic AMVs was further confirmed by its presence in the low-density fractions (high buoyancy) of the Optiprep density gradient. Similar results of association of DRA with lipid rafts were also observed in human intestinal epithelial Caco2 cells. Therefore, the presence of DRA in lipid rafts of Caco2 membranes and human colonic AMVs indicates that the basal activity of DRA is dependent on the lipid composition of these microdomains. Moreover, previous studies demonstrated that depletion of plasma membrane cholesterol alters the basal activity of several intestinal transporters such as the apical sodium/hydrogen exchanger NHE3 (44), sodium-dependent apical bile acid transporter (2), and basolateral calcium-activated potassium channels (32). Interestingly, incubation of Caco2 cells with MβCD in present studies significantly decreased association of DRA with lipid rafts (as seen with Optiprep gradient studies; data not shown) parallel to a decrease in DRA function. In addition, MβCD-induced inhibition of Cl-/HCO₃⁻/OH⁻ exchange activity was reversed in the presence of cholesterol, suggesting that plasma membrane cholesterol is essential for optimal DRA function. We showed that NPY significantly increased DRA protein levels in the DI or low-density raft fractions of total Caco2 membranes compared with untreated control. Also, Optiprep density gradient studies showed increased redistribution of DRA in the lipid raft fractions (fractions 3-5) in response to NPY.
compared with untreated control, further suggesting an enhanced association of DRA with lipid rafts. This enhanced association was accompanied with an increase in Cl\(^{-}/\text{HCO}_3^{-}(\text{OH}^-)\) exchange activity in Caco2 cells by NPY compared with untreated control. Moreover, our studies showed that ERK1/2 MAP kinase activation is responsible for the stimulatory effects of NPY on DRA function in Caco2 cells. Plasma membrane lipid raft domains are known to function as centers for the assembly of signaling complexes. Such assembly is suggested to increase both the specificity and efficiency of signaling event by providing a restricted spatial compartment for multiple proteins involved in signal transduction (1, 47, 62). Thus lipid rafts play an important role in enhancing receptor signaling in response to an agonist (30, 62).

We believe that, in the presence of NPY, lipid rafts may serve as signaling platforms to allow DRA to be in close proximity with ERK1/2 MAP kinase and other regulatory proteins for more efficient DRA regulation. We further speculate that modifications in DRA due to direct or indirect phosphorylation by ERK1/2 MAP kinase and/or association with other downstream effector proteins within lipid rafts may be important for NPY-induced stimulation of DRA protein levels in the lipid rafts and its increased function in the intestine. Future studies will be needed to address this important aspect regarding the role of ERK1/2 MAP kinase in the phosphorylation of the apical Cl\(^{-}/\text{HCO}_3^{-}(\text{OH}^-)\) exchanger, DRA, resulting in the stimulation of its protein level and function [Cl\(^{-}/\text{HCO}_3^{-}(\text{OH}^-)\) exchange activity] by NPY.

It appears that a similar mechanism of regulation involving lipid rafts was also observed in the stimulation of NHE3 activity by EGF in rabbit ileal brush border membranes and Caco2 cells (33, 34). These studies showed that increased NHE3 along with the signaling mediators PI3 kinase and Akt2 in lipid rafts led to a stimulation of NHE3 activity.

In summary, our results demonstrated that DRA is associated with lipid rafts in the plasma membrane of intestinal epithelial cells and disruption of lipid rafts by cholesterol depletion results in an inhibition of DRA function and its association with lipid rafts. We also showed that NPY enhanced the association of DRA with lipid rafts, leading to an increase in Cl\(^{-}/\text{HCO}_3^{-}(\text{OH}^-)\) exchange activity in intestinal epithelial cells. Our findings demonstrate an important role of lipid rafts in the optimal function of DRA and provide novel mechanistic insights underlying the proabsorptive peptideergic regulation of DRA in the human intestine.

NOTE ADDED IN PROOF

During the period of peer review for this research article, a new study was published regarding the association of DRA with lipid rafts in HEK293 and Caco2/BBE cells. They demonstrated that disruption of lipid rafts by MβCD decreased DRA activity and its association with lipid rafts. These studies further confirm our findings that DRA function depends on its association with lipid rafts. This study was published as Lissner S, Nold L, Hsieh CJ, Turner JR, Gregor M, Graeve L, Lamprecht G. Activity and PI3-kinase dependent trafficking of the intestinal anion exchanger downregulated in adenoma depend on its PDZ interaction and on lipid rafts. Am J Physiol Gastrointest Liver Physiol 299: G907–G920, 2010.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


