Brush Border Phosphatidylinositol 3-Kinase Mediates Epidermal Growth Factor Stimulation of Intestinal NaCl Absorption and Na+/H+ Exchange*

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In terminally differentiated ileal villus Na+/H+-absorptive cells, epidermal growth factor (EGF) stimulates NaCl absorption and its component brush border Na+/H+ exchanger, acting via basolateral membrane receptors, and as we confirm here, a brush border tyrosine kinase. In the present study we show that brush border phosphatidylinositol 3-kinase (PI 3-kinase) is involved in EGF stimulation of NaCl absorption and brush border Na+/H+ exchange. In rabbit ileum studied with the Ussing chamber-voltage clamp technique, EGF stimulation of active NaCl absorption is inhibited by the selective PI 3-kinase inhibitor wortmannin. PI 3-kinase, a largely cytosolic enzyme, translocates specifically to the brush border of ileal absorptive cells following EGF treatment. This translocation occurs as early as 1 min after EGF treatment and remains increased at the brush border for at least 15 min. EGF also causes a rapid (1 min) and large (4-5-fold) increase in brush border PI 3-kinase activity. Involvement of PI 3-kinase activity in intestinal Na+ absorption is established further by studies done in the human colon cancer cell line, Caco-2, stably transfected with the intestinal brush border isoform of the Na+/H+ exchanger, NHE3 (Caco-2/NHE3 cells). Brush border Na+/H+ exchange activity was measured using the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)5(6)-carboxyfluorescein. EGF added to the basolateral surface but not apical surface of Caco-2/NHE3 cells increased brush border Na+/H+ exchange activity. The EGF-induced increase in brush border Na+/H+ exchange activity was completely abolished in cells pretreated with wortmannin. EGF treatment caused increased tyrosine phosphorylation of PI 3-kinase in both ileal brush border membranes and Caco-2/NHE3 cells, suggesting that a tyrosine kinase upstream of the PI 3-kinase is involved in the EGF effects on Na+ absorption. In conclusion, the present study provides evidence in two separate intestinal models, the ileum and a human colon cancer cell line, that PI 3-kinase is an intermediate in EGF stimulation of intestinal Na+ absorption.

We reported previously that in terminally differentiated rabbit ileal villus cells, epidermal growth factor (EGF) increases NaCl absorption acting via basolateral membrane (BLM) receptors (1). Biochemically very little is known about the steps involved between binding of EGF to its receptor at the BLM and stimulation of NaCl absorption, other than the EGF effects involve tyrosine kinases at both the brush border membranes (BBMs) and BLMs (1). In addition, EGF increased tyrosine phosphorylation of an ileal brush border protein of 85 kDa, and the tyrosine kinase inhibitor genistein inhibited tyrosine phosphorylation of several ileal brush border proteins, including one of 85 kDa (1).

Phosphatidylinositol 3-kinase (PI 3-kinase) consists of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit that is tyrosine phosphorylated in vivo (2–5). Since the enzyme forms a complex with growth factor receptors and with oncogene products, it has been implicated in the growth signaling mechanism of receptors and oncogenes that act through tyrosine kinases. In addition, PI 3-kinase is emerging as a critical enzyme in intracellular signaling pathways in nonmitogenic stimuli (6–8). However, in nonmitogenic cells the nature and site of interaction of PI 3-kinase with other signaling molecules have not been established.

The majority of PI 3-kinase activity is found in the soluble fraction of resting cells, although a significant fraction is associated with the membrane in growth factor-stimulated and/or oncogene-transformed fibroblasts (9, 10) and following the addition of agonists such as thrombin (7). Recruitment of PI 3-kinase from the cytosol to the plasma membrane, where its substrate phosphatidylinositol is phosphatidylinositol 3-kinase, is enriched, is part of a complex with growth factor receptors and with oncogene products, it has been implicated in the growth signaling mechanism of receptors and oncogenes that act through tyrosine kinases. In addition, PI 3-kinase is emerging as a critical enzyme in intracellular signaling pathways in nonmitogenic stimuli (6–8). However, in nonmitogenic cells the nature and site of interaction of PI 3-kinase with other signaling molecules have not been established.

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Ileal Na+/H+-absorbing cells are polarized epithelial cells in the...
villus and upper crypt. Their cell surface is divided into two domains, the luminal facing apical domain which is separated by tight junctions from the basolateral domain. We have shown that the signal transduction processes stimulated by BLM receptors in these cells are distinct in these two domains (18, 19). We now demonstrate a role for PI 3-kinase in regulation of $Na^+$ absorption in a polarized epithelial cell. We show that EGF acts on ileal BLM receptors to increase selectively the amount and activity of apical membrane PI 3-kinase and that PI 3-kinase is involved in EGF stimulation of apical membrane Na$$^+$/$H^+$$$ exchange.

EXPERIMENTAL PROCEDURES

Materials

 Sodium orthovanadate, $\beta$-glycerophosphate, phenylalanine, phosphoramidone, aprotinin, leupeptin, EGF, PI, Triton X-100, phenylmethylsulfonyl fluoride (PMSF), phosphatidylinositol 4-phosphate, wortmannin, potassium oxalate, butylated hydroxytoluene, ethyoxquin, and dimethyl sulfoxide (MeSO) were obtained from Sigma. Pansorbin and gentisin were from Calbiochem. Gentisin was from Apo-Pharma Chemicals. Percoll was from Pharmacia Biotech Inc. Sodium deoxycholate was from Fisher. Anti-PI 3-kinase polyclonal and monoclonal antibodies were from Upstate Biotechnology, Inc. Anti-phosphotyrosine polyclonal antibody was from Zymed. Prestained protein molecular weight standards were from Bio-Rad (high molecular weight range). Chemilumininescent reagents were from DuPont NEN. Silica Gel 60 plates were from Merck. Fetal bovine serum was from HyClone Laboratories. Dulbecco’s modified Eagle’s medium was from Life Technologies, Inc. Tetramethylammonium (TMA) was from Fluka Chemical Corp. Nigericin and the acetoxyethyl ester of BICEF (BICEF-AM) were from Molecular Probes. [(\gamma-$^3$C)ATP (3,000 Ci/mmol) was from Amersham Corp.

Methods

Cell Culture—Caco-2 cells obtained from American Type Culture Collection were stably transfected with the full-length cDNA for the rabbit intestinal brush border Na$$^+$/$H^+$$$ exchanger isoform, NHE3 (Caco-2/NHE3 cells) and grown as reported previously (20). These cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 26 mM NaHCO$_3$, 50 units/ml penicillin, 50 $\mu$g/ml streptomycin, and 1% nonessential amino acids (Life Technologies, Inc.), pH 7.4, in 5% CO$_2$, 95% O$_2$. Ten mM glucose was added to the serosal and 10 mM NaCl to the mucosal bathing fluids at the time of mounting the tissue or cells were preincubated for 10–15 min with 0.5% ethanol control; or genistin (100 $\mu$m) added to the ileal mucosal plus serosal surfaces.

In Vitro Electrolyte Transport in Intact Tissue—The methods used to measure active ileal electrolyte transport have been described previously (19). In brief, ileal mucosa, with serosa and muscularis propria removed, was mounted as a flat sheet between two Lucite-modified tissue culture membranes, Becton Dickinson), which covered an oval aperture of 13 mm. The tissue was mounted on a cuvette (supplied by the manufacturer) and incubated at 37°C. The mucosal bathing fluid was composed (in mM): 145 NaCl, 2.4 Tris-HCl, pH 7.1, 0.8 K$_2$HPO$_4$, 0.4 MgCl$_2$, 12 CaCl$_2$, 1.2 MgSO$_4$, 0.4 KH$_2$PO$_4$, 0.8 K$_2$CO$_3$, 5% O$_2$, 5% CO$_2$. Ten mM glucose was added to the serosal and 10–14 fold; and these membrane fractions were essentially free of BBM, as measured by sucrose specific activity (18).

In Vivo Electrolyte Transport in Intact Tissue—For electrolyte transport experiments, NaCl transport and preparation of BBMs, ileal tissue was used. For preparation of BBMs, ileal villus cells were used. The tissue or cells were preincubated for 10 min at 37°C gassed with 95% O$_2$, 5% CO$_2$ in Ringer’s HCO$_3$ containing 10 mM glucose. 200 ng/ml EGF was then added to one set of tissues, and the incubation was continued for the indicated times. The tissues were then chilled, and control and EGF-stimulated samples were processed in parallel; all steps were at 4°C unless stated otherwise.

Preparation of Brush Border Membranes—At the end of the above incubation, ileal sheets were chilled on ice and homogenized in cold saline with a glass slide, and BBM vesicles were prepared for transport studies as described previously (23, 24). Cell membranes were isolated from 13- to 15-mm segments of ileum maintained at 37°C. Transmural potential difference, short circuit current (Isc), conductance, and unidirectional fluxes of Na$$^+$ and Cl$$^-$$ were determined.

Usual.$^{*}$ually six pieces of ileum from a single animal were studied simultaneously. Unless specified, the bathing solution consisted of Ringer’s HCO$_3$ composed (in mM): 115 NaCl, 25 NaHCO$_3$, 2.4 K$_2$HPO$_4$, 0.4 MgCl$_2$, 12 CaCl$_2$, 1.2 MgSO$_4$, 0.4 KH$_2$PO$_4$, 0.8 K$_2$CO$_3$, 5% O$_2$, 5% CO$_2$. Ten mM glucose was added to the serosal and 10–14 fold; and these membrane fractions were essentially free of BBM, as measured by sucrose specific activity (18).

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In Vivo Ileal Villus Cell Brush Border Na$$^+$/$H^+$$$ Exchange and Na$$^+$$ and Cl$$^-$$ Transport and preparation of BBMs, ileal tissue was used. For preparation of BBMs, ileal villus cells were used. The tissue or cells were preincubated for 10 min at 37°C gassed with 95% O$_2$, 5% CO$_2$ in Ringer’s HCO$_3$ containing 10 mM glucose. 200 ng/ml EGF was then added to one set of tissues, and the incubation was continued for the indicated times. The tissues were then chilled, and control and EGF-stimulated samples were processed in parallel; all steps were at 4°C unless stated otherwise.

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In Vivo Ileal Villus Cell Brush Border Na$$^+$/$H^+$$$ Exchange and Na$$^+$$-Dependent Glucose Uptake—Ileal segments were exposed in vitro to EGF (200 ng/ml) or control for 15 min. Villus cells were then scliced on ice with a glass slide, and BBM vesicles were prepared for transport studies as described previously (23, 24). Cells were homogenized with a glass slide in Ringer’s HCO$_3$, washed with cold saline, and resuspended in a solution containing (in mM): 60 mannitol, 2.4 Tris-HCl, pH 7.1, 1 EGTA, 0.32 PMSF, 0.006 phosphoramidone, and 0.3 aprotinin tryspin inhibitor units/ml. BBMs were prepared by double Mg$$^{2+}$$ precipitation as reported previously (23, 24). The final brush border pellet was resuspended in (in mM): 200 mannitol, 40 MOPS, 11.4 Tris, pH 6.5, 0.5 20-butanolic acid, 100 mM genistein (100 $\mu$m) dissolved in ethanol or 0.5% ethanol control; or genistin (100 $\mu$m) dissolved in MeSO or 0.5% MeSO control vesicles. Batches were rinsed in liquid N$_2$ overnight and next day thawed gradually on ice; under these conditions these vesicles have been shown to be opened by freeze thaw (25). Na$$^+$$ transport buffers consisted of either (in mM): 187 mannitol, 37 MOPS, 10.6 Tris, pH 6.5, 0.5 20-butanolic acid, 1.0 sodium gluconate (Na$$^+$$ = 33–35 $\mu$equiv/ml, DuPont NEN); or 187 mannitol, 28 Tris, 9.62 MES, pH 8.0, 4.7 Mg(gluconate)$$^2$$, 1.0 sodium gluconate, with both solutions containing 100 $\mu$m genistin, or 0.5% ethanol; or 100 $\mu$m genistin or 0.5% MeSO control.
FIG. 1. EGF stimulates ileal villus active NaCl absorption. Ileal mucosa was exposed under voltage-clamped conditions to 200 ng/ml EGF on the serosal surface and the effect on mucosal-to-serosal (Jm) and serosal-to-mucosal (Jsm) fluxes of 22Na+ and 36Cl− transport 15–30 min after EGF addition compared with same parameters during two 20-min control (black bars) flux periods in the same tissue before the addition of EGF. Results are means ± S.E.; n = 7, n = number of animals studied. Jm and Jsm are fluxes expressed in pmol/cm2-h, and conductance (G) is expressed in millisiemens/cm2. p values represent a comparison of EGF and control periods in the same tissue (paired t test). NS, not significant.

The glucose transport buffer contained (in mM): 80 mannitol, 40 MOPS, 11.4 Tris, pH 6.5, 2 EGTA, 50 Mg[glutamate], 60 NaCl, 0.1 μM D-glucose ([3H]glucose, 20 μCi/ml, DuPont NEN). Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, 5 mM MgCl2, 30 μM Na3VO4, 50 mM NaF, 1 mM PMSF, 25 μM D-glucose ([3H]glucose, 20 μCi/ml, DuPont NEN). 22Na+ initial uptake was stopped electronically after 3, 5, or 8 s by injection of 1 ml of ice-cold stop solution. [3H]Glucose uptake was stopped after 90, a time not during the linear phase of uptake. Glucose equilibrium values were determined after a 90-min incubation at 25°C. Reaction mixture was then vacuum filtered through nitrocellulose filters, 0.45-μm pore size (Millipore, Bedford, MA) and rinsed with 6 ml of stop solution. Stop solutions contained (in mM) either 40 mannitol, 90 potassium glutonate, 15.6 MES, 20 Tris, pH 8.0; or 40 mannitol, 90 potassium glutonate, 20 MOPS, 5.6 Tris, pH 6.5. The filters were dissolved in 4 ml of scintillant, and radioactivity was determined in a liquid scintillation spectrometer (Beckman LS 7500). Initial uptake rates of Na+/H+ exchange were expressed in pmol/mg of protein/s using linear regression analysis; glucose uptake was expressed as pmol/mg of protein.

Immunodetection of PI 3-Kinase—Fifty μg of membrane proteins were separated by SDS-PAGE (7%), transferred to nitrocellulose filters (Bioblot, Costar), and probed with monoclonal antibodies to the p85 subunit of PI 3-kinase (Upstate Biotechnology, Inc., 05-217). Immunostained proteins were visualized using the enhanced chemiluminescence detection system according to the instructions of the supplier. Quantitation of PI 3-kinase was by computerized densitometry (ImageQuant, Molecular Dynamics, Sunnyvale, CA) using enhanced chemiluminescence under conditions in which the PI 3-kinase signal was not saturated.

Immunoprecipitation and Kinase Assay for PI 3-Kinase in Caco-2/NHE3 Cells—Caco-2/NHE3 cells were seeded on Falcon cell culture inserts and serum deprived overnight prior to the experiment. Serum-starved cells were treated with EGF (100 ng/ml, 1 or 15 min) added to the basolateral surface in control cells or cells preincubated with wortmannin (100 nM, 30 min). The wortmannin was added to both the apical and basolateral surfaces. The cells were washed twice with ice-cold phosphate-buffered saline and lysed with RIPA buffer containing 0.15 M NaCl, 15 mM HEPES, pH 7.5, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM PMSF, 25 μg/ml aprotinin, 0.01% leupeptin, and 4 μg/ml phosphorhamidine. Tyrosine-phosphorylated proteins were immunoprecipitated using a polyclonal anti-phosphotyrosine antibody (Zymed, 61-5800). The immunoprecipitates were washed and the PI 3-kinase activity determined on the immunoprecipitates as described above.

RESULTS

EGF Stimulates Ileal NaCl Absorption, Which Is Inhibited by Wortmannin—The effects of EGF (200 ng/ml) on active ileal Na+ and Cl− transport were studied using the Ussing chamber-voltage clamp technique. Serosal addition of EGF stimulated NaCl absorption (Fig. 1) in rabbit ileum and caused a short lived increase in Iosc, as reported previously (1). Starting 15 min after EGF addition, a 15-min flux study was performed, a time during which ileal Iosc was constant and similar to that during two 20-min basal flux periods in the same tissue before the addition of EGF. Serosal addition of EGF caused a statistically significant increase in mucosal-to-serosal and net Na+ and Cl− fluxes (Fig. 1). It also caused a small but statistically significant increase in the serosal-to-mucosal Cl− flux (p < 0.01) (Fig. 1). These data show that EGF increases ileal active Na+ and Cl− absorption.

The PI 3-kinase inhibitor wortmannin added to the ileal mucosal and serosal surfaces (100 nM) did not alter basal active electrolyte transport (Fig. 2). Also, the d-glucose-stimulated increase in Na+ absorption was not altered by wortmannin (3.0 ± 0.5 versus 2.9 ± 0.5 μEq/cm2-h) in control and wortmannin treated tissue, respectively, n = 7; not significant).
The effects of wortmannin on the transport effects caused by serosal EGF were determined (Fig. 3). The addition of wortmannin prevented the EGF effect to increase the mucosal-to-serosal and net Na\(^+\) and Cl\(^-\) fluxes. Wortmannin did not alter the magnitude of the increase in serosal-to-mucosal Cl\(^-\) flux or the short lived increase in I\(_o\) seen with EGF (0.3 ± 0.1 versus 0.3 ± 0.05 μEq/cm\(^2\)-h in the absence versus in the presence of wortmannin, respectively, n = 7, n.s.), suggesting that wortmannin only affects the EGF-stimulated NaCl-absorptive process.

EGF Stimulates Ileal Brush Border Na\(^+\)/H\(^+\) Exchange via Brush Border Tyrosine Kinase—Studies were undertaken to determine whether EGF acting via BLM receptors causes prolonged effects on apical membrane Na\(^+\)/H\(^+\) exchange and whether an apical membrane tyrosine kinase is involved.

Brush border vesicles made from ileum exposed in vitro to EGF exhibited increased Na\(^+\)/H\(^+\) exchange compared with control vesicles (Fig. 4). This was exerted only on Na\(^+\) uptake in the presence of an acid inside pH gradient and not in the absence of a pH gradient. Genistein (100 μM) freeze thawed into control vesicles inhibited basal Na\(^+\)/H\(^+\) exchange, again via an effect only on Na\(^+\) uptake in the presence of an acid inside pH gradient. When genistein was freeze thawed into brush border vesicles made from ileum exposed in vitro to EGF, Na\(^+\)/H\(^+\) exchange was reduced even more than the decrease in control vesicles caused by genistein, to a rate not significantly different from control/genistein vesicles. In contrast, genistein did not alter basal Na\(^+\)/H\(^+\) exchange or the EGF-stimulated brush border Na\(^+\)/H\(^+\) exchange (data not shown).

In contrast to these results, Na\(^+\)-dependent D-glucose uptake was not affected by EGF treatment or by genistein (Table I). Thus, brush border vesicles made from EGF-exposed ileum maintain a memory of in vitro exposure to EGF with a prolonged stimulation of Na\(^+\)/H\(^+\) exchange. Also, a brush border tyrosine kinase(s) involved in control of brush border basal Na\(^+\)/H\(^+\) exchange and the EGF-induced increase in brush border Na\(^+\)/H\(^+\) exchange.

Effect of EGF on Rabbit Ileal Brush Border and Basolateral Membrane PI 3-Kinase Amount, Activity, and Tyrosine Phosphorylation—We first determined whether PI 3-kinase was an intestinal brush border or BLM protein. Rabbit ileum was exposed in vitro to EGF before BLMs were prepared as described under "Methods." BLM and BLM (50 μg) protein were separated by 7% SDS-PAGE, and Western analysis done with monoclonal antibodies...
overshoots. Vesicles were prepared as in Fig. 4 at pHin 6.5 and were controlled for 15 min. Ileal villus cells were then prepared by scraping and both BBM and BLM contain similar to the 85-kDa subunit of PI 3-kinase. Under basal conditions exchange (14.4 pmol/mg protein–s, n = 5) versus Na+ uptake with no pH gradient (pHout 6.5/pHin 6.5). Results from a representative experiment are shown with similar data having been obtained in five identical experiments. Slopes of this single experiment are in parentheses. EGF increased brush border Na+ /H+ exchange by approximately 25% (control Na+ /H+ exchange 18.4 ± 4.3 pmol/mg protein–s, n = 5 versus EGF Na+ /H+ exchange 22.6 ± 5.1, n = 5, p < 0.02). Genistein caused an approximately 20% decrease in control Na+ /H+ exchange (14.4 ± 3.9 pmol/mg protein–s, n = 5, p < 0.02 compared with control); with Na+ /H+ exchange rate in EGF + genistein (15.9 ± 4.9 pmol/mg protein–s, n = 5) and control + genistein not being significantly different.

Table I

| Condition               | [3H]Glucose uptake | Overshoot | Equilibrium volumes |
|-------------------------|--------------------|-----------|---------------------|
|                         | pmol/mg protein    | %         | pmol/mg protein     |
| Control                 | 165 ± 16           | 211 ± 16  | 0.78 ± 0.003        |
| EGF                     | 180 ± 14           | 208 ± 20  | 0.72 ± 0.04         |
| Genistein               | 159 ± 14           | 206 ± 19  | 0.78 ± 0.01         |
| Genistein + EGF         | 188 ± 19           | 213 ± 22  | 0.74 ± 0.04         |

PI 3-Kinase and Intestinal NaCl Absorption

Effects of EGF and genistein on glucose uptake

Values are means ± SE; n, no. of vesicle preparations. Shown are the effects of EGF (200 ng/ml), genistein (100 μM), or EGF + genistein on [3H]glucose uptake at 90 s, glucose equilibrium volumes, and percent overshoots. Vesicles were prepared as in Fig. 4 at pHin 6.5 and were then exposed to glucose transport buffer consisting of (in mM) 80 mannitol, 40 MOPS, 11.1 Tris, pH 6.5, 2 EGTA, 5 Mg(glucocinate), 60 NaCl, 0.1 α-glucose (14H)glucose, 20 μCi/ml, Dupont NEN) plus genistein or ethanol control or EGF plus genistein. Then 15 μl of membrane buffer was mixed with 30 μl of transport buffer, and transport was allowed to occur for 90 s or 90 min at 22°C before being stopped by injection of 1 ml of ice-cold stop solution. p values, comparison of uptake in the presence of EGF, genistein, or EGF + genistein versus control (paired t test) were all not significant.

EGF and genistein effects on ileal brush border Na+/H+ exchange. Sheets of ileal mucosa were exposed to EGF (200 ng/ml) or control for 15 min. Ileal villus cells were then prepared by scraping and BBM vesicles prepared by Mg precipitation under conditions such that intravesicular ATP was approximately 50 μM (34). Vesicles were snap frozen in membrane buffer with and without genistein (100 μM) and studied the next day. Na+ uptake was performed over a period of linear uptake (3, 5, and 8 s) with Na+ /H+ exchange determined as Na+ uptake with an intravesicular acid pH gradient (pHout 8.0/pHin 6.5) minus Na+ uptake with no pH gradient (pHout 6.5/pHin 6.5). Results from a representative experiment are shown with similar data having been obtained in five identical experiments. Slopes of this single experiment are in parentheses. EGF increased brush border Na+ /H+ exchange by approximately 25% (control Na+ /H+ exchange 18.4 ± 4.3 pmol/mg protein–s, n = 5 versus EGF Na+ /H+ exchange 22.6 ± 5.1, n = 5, p < 0.02). Genistein caused an approximately 20% decrease in control Na+ /H+ exchange (14.4 ± 3.9 pmol/mg protein–s, n = 5, p < 0.02 compared with control); with Na+ /H+ exchange rate in EGF + genistein (15.9 ± 4.9 pmol/mg protein–s, n = 5) and control + genistein not being significantly different.

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to the 85-kDa subunit of PI 3-kinase. Under basal conditions both BBM and BLM contain similar amounts of PI 3-kinase (Fig. 5A). One min of EGF treatment caused a 2-fold increase (234 ± 40%, n = 3, p < 0.01) in the amount of BBM PI 3-kinase, whereas a 15-min exposure showed a 40 ± 14% (n = 3, p < 0.05) increase (Fig. 5B). No change was seen in the amounts of PI 3-kinase in the BLM 30 s after EGF treatment (data not shown). Thus EGF specifically causes an increase in amount of PI 3-kinase in the BBM of rabbit ileal villus cells.

Studies were done to determine whether the BBM PI 3-kinase associated with the cytoskeleton or plasma membrane under both basal and EGF-treated conditions and whether EGF treatment caused a rearrangement of the BBM PI 3-kinase from one compartment to the other. 150 μg of BBM protein was lysed using a buffer containing 1% Triton X-100. The detergent-soluble and -insoluble fractions were separated on 7% SDS-PAGE, transferred to nitrocellulose, and probed with monoclonal antibodies to PI 3-kinase. The total BBM PI 3-kinase activity from control and EGF-exposed tissue was present in the detergent-soluble fraction (Fig. 6), suggesting that PI 3-kinase is not associated with the villus cell cytoskeleton as defined as the Triton X-100-insoluble fraction. No PI 3-kinase was detected in the detergent-insoluble fraction either in BBM prepared from control or EGF-exposed ileum.

To determine if BBM PI 3-kinase is tyrosine phosphorylated in response to EGF, we separately immunoprecipitated PI 3-kinase and tyrosine-phosphorylated proteins from BBM from control and EGF-treated ileum (1 min). The immunoprecipitated proteins were separated by SDS-PAGE, and Western analysis done with an anti-phosphotyrosine antibody. There was approximately a 5-fold increase in the tyrosine phosphorylation of PI 3-kinase in EGF-treated tissue compared with simultaneously studied control tissue (Fig. 7). When immunoprecipitated with anti-phosphotyrosine antibody, increased tyrosine phosphorylation of an 85-kDa protein was seen. These results show that the p85 subunit of brush border PI 3-kinase has increased tyrosine phosphorylation in response to EGF (Fig. 7).

We next measured the PI 3-kinase activity in both BBM and BLB to determine whether exposure to EGF activated this enzyme. A 4–5-fold increase was seen in the PI 3-kinase activity as early as 1 min in BBM prepared from EGF-treated tissue (Fig. 8A), and the activity remained elevated (2-fold) 15 min after the addition of EGF. No change was seen in the BBM PI 3-kinase activity 30 s following EGF treatment (Fig. 8B).
EGF

p85

P-Tyr

PI 3-K

PI 3-P

FIG. 6. PI 3-kinase is present only in the ileal BBM Triton X-100-soluble fraction. BBM from control and EGF-treated tissue were solubilized in a buffer containing Triton X-100 (as described for immunoprecipitation of PI 3-kinase under "Methods"), and the detergent-soluble and -insoluble fractions were probed by Western analysis for PI 3-kinase. This experiment is representative of three with similar results.

FIG. 7. EGF increases tyrosine phosphorylation of ileal BBM PI 3-kinase. 250 μg of BBM from control and EGF-exposed ileum were solubilized as described under "Methods," and immunoprecipitations (Ip) were done with polyclonal antibodies to either PI 3-kinase (PI 3-K) or phosphotyrosine (P-Tyr). The immunoprecipitates were separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-phosphotyrosine antibodies. This experiment is representative of three with similar results. First lane from left, BBM from control ileum immunoprecipitated with anti-phosphotyrosine polyclonal antibody; second lane, BBM from ileum exposed to EGF (200 ng/ml, 1 min) immunoprecipitated with anti-phosphotyrosine antibody; third lane, BBM from control ileum immunoprecipitated with anti-PI 3-kinase polyclonal antibody; fourth lane, BBM from ileum exposed to EGF (200 ng/ml, 1 min) immunoprecipitated with anti-PI 3-kinase antibody. The arrow on the right indicates the position of p85 subunit of PI 3-kinase.

The EGF-stimulated increase in PI 3-kinase activity was inhibited in the presence of 100 nM wortmannin (Fig. 9). These results show that stimulation of intestinal absorbing cells with EGF increases the amount, activity, and tyrosine phosphorylation of BBM PI 3-kinase, whereas the BLM PI 3-kinase amount and activity remain unchanged.

EGF Stimulates Na+/H+ Exchange in Caco-2/NHE3 Cells, an Effect Inhibited by Wortmannin. The brush border isoform of the Na+/H+ exchanger involved in NaCl absorption is NHE3 (26). Caco-2 is a colon adenocarcinoma cell line that lacks an endogenous apical Na+/H+ exchanger under the conditions studied (20). Caco-2 cells, stably transfected with the brush border isoform of the Na+/H+ exchanger NHE3, have been shown to gain a brush border Na+/H+ exchanger compared with untransfected cells measured as Na+-dependent, amiloride-sensitive intracellular alkalization using the pH-sensitive dye BCECF (21). EGF (100 ng/ml) added to the basolateral surface but not apical surface stimulates brush border Na+/H+ exchange in Caco-2/NHE3 cells (Fig. 10A).

We measured the Na+/H+ exchange in transfected Caco-2/NHE3 cells pretreated with wortmannin for 30 min at room temperature. Wortmannin (100 nM) was added to both the apical and basolateral surfaces. Wortmannin by itself did not alter the Na+/H+ exchange activity (data not shown). Subsequent addition of EGF to these cells demonstrated that the stimulation of the Na+/H+ exchange by EGF (Fig. 10A) was completely inhibited when the cells were pretreated with wortmannin (Fig. 10B).

EGF Stimulates PI 3-Kinase Activity in Caco-2/NHE3 Cells. The PI 3-kinase activity in Caco-2/NHE3 cells was measured. Cells were cultured under conditions similar to those used for the measurement of Na+/H+ exchange activity. PI 3-kinase activity in Caco-2/NHE3 cells was measured using anti-phosphotyrosine immunoprecipitates of whole cell lysates. The PI 3-kinase activity was elevated 2-fold by 1 min of EGF treatment (Fig. 11A). In contrast, PI 3-kinase activity in untransfected Caco-2 cells was unchanged following EGF treatment (Fig. 11B).

DISCUSSION

In terminally differentiated (nondividing) intestinal epithelial cells, EGF does not act as a mitogen but, as shown previously (1) and confirmed here, stimulates active NaCl absorption. Ileal NaCl absorption is stimulated by serosal but not...
mucosal EGF (1). This supports other evidence that EGF receptors are present on the basolateral but not apical membranes of adult intestinal epithelial cells. Thompson and others have shown by immunofluorescence that EGF binds to the BLM and not BBM of rat small intestinal epithelial cells (27–29). Similar separation of intestinal cell EGF receptors to one cell pole and the regulated function involving a brush border Na+/H+ exchange. EGF stimulated ileal Na+/H+ exchange activity. EGF in TMA+/H+ medium was added to the basolateral chamber of the cuvette after cells reached steady-state pH. The EGF-induced alkalization represents stimulation of brush border Na+/H+ exchange. Panel B, wortmannin inhibits the EGF-induced increase in Na+/H+ exchange activity. Cells were pretreated with wortmannin (100 nM, 30 min) during the dye loading period. The cells were perfused with Na+ medium from the apical side until a steady-state pH was reached, and then EGF was added as described above. These traces are representative of three separate experiments.

We provide several types of evidence linking brush border PI 3-kinase to EGF stimulation of NaCl absorption and brush border Na+/H+ exchange. (i) Studied with the Ussing chamber-voltage clamp technique, wortmannin, a selective, potent, and irreversible inhibitor of PI 3-kinase (31), inhibited the increase in neutral NaCl absorption induced by EGF (Fig. 3) and the EGF stimulation of brush border Na+/H+ exchange in Caco-2/NHE3 cells (Fig. 10). (ii) EGF stimulation of PI 3-kinase activity and amount occurred only at the BBM. (iii) EGF stimulation of PI 3-kinase amount and activity in the BBM was a very rapid event occurring as quickly (1 min) as we could isolate brush borders. The wortmannin effect was not nonspecific or toxic since it did not alter ileal basal Na+ and Cl− fluxes, d-glucose stimulated increase in ileal Na+ absorption, or EGF-stimulated ileal Cl− secretion. It has been demonstrated previously that at concentrations up to 100 nM wortmannin does not directly inhibit any other enzyme activity so far investigated including insulin receptor tyrosine kinase or phosphatidylinositol 4-kinase (32), myosin light chain kinase, protein kinase A, protein kinase C, cyclic GMP-dependent protein kinase (33), the components of the mitogen-activated protein kinase cascade (34, 17), or S6 kinase (32).

Our studies have demonstrated an asymmetric plasma membrane activation of PI 3-kinase as part of EGF-induced signal transduction. Small but equal amounts of PI 3-kinase were present in both BBMs and BLMs under basal conditions. EGF treatment, however, caused a rapid increase in the amount and activity of PI 3-kinase specifically to the BBM and not BLM. The increase in PI 3-kinase activity was rapid, occurring by 1 min; was higher at 1 min than at 15 min; and correlated with a greater amount of BBM PI 3-kinase at 1 min than at 15 min of EGF treatment. PI 3-kinase is a cytosolic enzyme and is known to translocate following its activation by an agonist to the plasma membrane where its substrates are present (7). There was no change in the BLM PI 3-kinase activity following EGF treatment. This suggests that the transport effects seen with EGF do not involve the association of PI 3-kinase with the basolateral EGF receptor. This is consistent with a previous demonstration that the EGF receptor lacks the PI 3-kinase-binding tyrosine phosphorylated Tyr-X-X-Met motif and binds to p85 very weakly (35) or not at all (36).

EGF treatment for 1 min also increased the PI 3-kinase activity.
activity 2 fold in Caco-2/NHE3 (Fig. 11A). However, the increase in PI 3-kinase activity in Caco-2/NHE3 cells following EGF treatment could only be detected in anti-phosphotyrosine immunoprecipitates, suggesting activation of a subpopulation of PI 3-kinase, which was activated by tyrosine phosphorylation. Of note, EGF also increased the tyrosine phosphorylation of ileal brush border PI 3-kinase, suggesting that tyrosine phosphorylation activates BBM PI 3-kinase, which is involved in the regulation of Na⁺ absorption. In fact, the semiquantitative aspects of PI 3-kinase activation shown in this study are consistent with tyrosine phosphorylation being primarily responsible for brush border PI 3-kinase activation. At 1 min, EGF increases the amount of brush border PI 3-kinase by approximately 2-fold; activity is increased approximately 4–5 fold; and tyrosine phosphorylation approximately 5-fold. Thus the similarity in the EGF stimulation of tyrosine phosphorylation and the activity of brush border PI 3-kinase is consistent with the increase in activity, likely via tyrosine phosphorylation, being the major mechanism of PI 3-kinase activation, although translocation also contributes. Whether tyrosine phosphorylation of PI 3-kinase occurs at the brush border or in the cytosol and whether only tyrosine-phosphorylated PI 3-kinase moves to the brush border are unknown.

Of note is that EGF treatment did not alter the PI 3-kinase activity in untransfected Caco-2 cells (Fig. 11B). The significance of this observation is not known, although the results would seem to indicate a role for NHE3 in the increase in tyrosine-phosphorylated and activated PI 3-kinase present in the apical membrane.

We reported previously suggestive evidence that a brush border tyrosine kinase was involved in the EGF effects on Na⁺ and Cl⁻ transport transduced from BLM receptors (1). Genistein added separately to the ileal mucosal or serosal surfaces inhibited EGF stimulation of NaCl absorption. In contrast, carbachol effects on NaCl absorption, acting by a BLM M₂ receptor, were inhibited by mucosal but not serosal genistein. Thus the sidedness of genistein effects on this preparation can separate apical from BLM tyrosine kinase involvement. In this study we provide more direct evidence that a brush border tyrosine kinase is involved in the regulation of basal brush border Na⁺/H⁺ exchange and in the increase in brush border Na⁺/H⁺ exchange caused by EGF. The fact that brush borders of ileal villus cells made from EGF-exposed ileum maintain an increase in Na⁺/H⁺ exchange indicates that basolateral EGF acts via a prolonged biochemical mechanism at the apical membrane. That genistein but not the negative control, genitin, inhibits both the basal brush border Na⁺/H⁺ exchange (1) as well as the increase in Na⁺/H⁺ exchange caused by EGF indicates the involvement of a brush border tyrosine kinase in both. Whether the same tyrosine kinase is involved in the regulation of basal and the EGF-induced increase in brush border Na⁺/H⁺ exchange is not known, although the decrease of both processes to the same level of Na⁺/H⁺ exchange suggests an overlapping mechanism. Since these studies suggest that EGF-induced tyrosine phosphorylation of PI 3-kinase is what activates it to regulate brush border Na⁺/H⁺ exchange it is likely, but not proven, that the tyrosine kinase that mediates the EGF-induced increase in brush border Na⁺/H⁺ exchange is the same as that which activates PI 3-kinase. The identity of the tyrosine kinase that activates brush border PI 3-kinase is unknown.

Our data suggest that the ileal BBM PI 3-kinase may be restricted to the membrane and not associated with the cytoskeleton, although this interpretation is entirely based on definition of cytoskeleton as the Triton X-100-insoluble fraction of brush border. Ileal BBM was solubilized in a buffer containing 1% Triton X-100, and the Triton-soluble and -insoluble fractions were probed for PI 3-kinase. The BBM PI 3-kinase was found only in the Triton-soluble fraction under both control and EGF stimulated conditions. No PI 3-kinase was detected in the detergent-insoluble fraction in either control or EGF-stimulated conditions. This suggests that PI 3-kinase may not associate with the apical membrane cytoskeleton in ileal absorbing cells, as defined as the Triton X-100-insoluble fraction. The lack of any PI 3-kinase activity associated with ileal BBM cytoskeleton also suggests that cytoskeletal rearrangement may not be the mechanism by which PI 3-kinase regulates the Na⁺/H⁺ exchanger, which is also present in the detergent-soluble fraction of the BBM. This is relevant, since activated PI 3-kinase associates with cytoskeleton (Triton X-100 insoluble fraction) in multiple other cells. For instance, PI 3-kinase associates with membrane cytoskeleton in thrombin-exposed platelets (7). In stimulated neutrophils, there is a strong correlation between the production of phosphatidylinositol 3,4-diphosphate and phosphatidylinositol 3,4,5-trisphosphate and reorganization of actin filaments, suggesting that the D-3-phosphorylated polyphosphoinositides are involved in cytoskeletal rearrangement (37). However, it may be pointed out that none of these studies has actually demonstrated a physical association of PI 3-kinase and a cytoskeletal component.

Regulation of a Na⁺/H⁺ exchanger by PI 3-kinase in nonpolarized Chinese hamster ovary fibroblasts and mouse mammary gland cells was described in a recent study by Ma et al. (38). The PI 3-kinase pool involved in regulation was linked to the platelet-derived growth factor receptor. Mutations in the platelet-derived growth factor receptor COOH-terminal sequence involved in PI 3-kinase binding and inhibition of PI 3-kinase (using the inhibitor LY 294002) prevented platelet-derived growth factor stimulation of the Na⁺/H⁺ exchanger. Not clarified in this study was the number or identity of steps between PI 3-kinase stimulation and the regulation of the Na⁺/H⁺ exchange activity. Furthermore, the isoform of the Na⁺/H⁺ exchanger involved was not specified in this study, although it likely was NHE1, the housekeeper isoform that is involved in the regulation of intracellular pH and volume. The involvement of PI 3-kinase in regulation of NHE3 in the brush border and in the regulation of Na⁺ absorption shown in the study represents a different mechanism, not even being in the same plasma membrane domain as the receptor that initiates signal transduction. Thus PI 3-kinase regulates multiple NHE isoforms and can do so at steps removed from the involved plasma membrane receptor.

Our study demonstrates that PI 3-kinase regulates Na⁺ absorption in both the ileal villus absorbing cells and the colon cancer cell line Caco-2/NHE3. It remains to be determined whether the Na⁺/H⁺ exchanger is regulated directly by PI 3-kinase or through intermediate signaling molecules. Na⁺/H⁺ exchangers have been shown to contain prion-like sequences (39), which could allow a direct association of PI 3-kinase through its SH3 group to the exchanger. It is possible that the D-3 polyphosphoinositides themselves act as second messengers, such that these lipids can be the signals rather than precursors of signals. Since phospholipase C-γ and PI 3-kinase both use the same substrate, it is possible that, like one of the products of PLC-γ activation (inositol trisphosphate), the products of PI 3-kinase are themselves second messengers. However, another possibility is that PI 3-kinase activation may be an intermediate step in a cascade of signaling events that begins at the BLM with EGF binding to its receptor and leads to the activation of the brush border Na⁺/H⁺ exchanger several steps downstream from PI 3-kinase activation.
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Brush Border Phosphatidylinositol 3-Kinase Mediates Epidermal Growth Factor Stimulation of Intestinal NaCl Absorption and Na/H Exchange
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