Epidermal Growth Factor Up-Regulates Intestinal Na^+/H^+ Exchange Activity (43510)

FAYEZ K. GHISHAN,*[,1] KAZUHIRO KIKUCHI,[†] AND BRIAN RIEDEL[‡]

Departments of Pediatric Gastroenterology and Molecular Physiology and Biophysics, Vanderbilt Medical Center, Nashville, Tennessee 37232; Department of Medicine,[†] Tokai University, Boseidai, Isehara, Kanagawa, 259-11 Japan; and Department of Pediatrics,[‡] USAF Medical Center, Wright Patterson AFB, Ohio 45433*

Abstract. **The present studies were designed to examine the regulation of Na^+/H^+ exchange activity by epidermal growth factor (EGF) in an *in vitro* system. Na^+/H^+ exchange activity was determined in brush-border membranes isolated from rat jejunal enterocytes incubated with epidermal growth factor and a number of second messengers. EGF at physiological concentrations stimulated Na^+/H^+ exchange activity without affecting vesicle size. The stimulation of Na^+/H^+ activity was the result of increasing V_{max} of Na^+/H^+ (6.0 ± 0.4 compared with 3.3 ± 0.27 nmol/mg protein/5 sec, $P < 0.01$). K_m values of the Na^+/H^+ exchanger in brush-border membrane from cells stimulated with EGF and controls were similar (16.0 ± 3.0 vs 13.0 ± 3.0, respectively). Na^+/H^+ activity was inhibited by phorbol esters, calmodulin, and cyclic AMP. The effects of EGF, calmodulin, cyclic AMP, and phorbol esters were dependent on ATP, because depleting the cells from ATP masked the effects on Na^+/H^+ exchange activity. The results suggest that EGF stimulates Na^+/H^+ exchange activity in the enterocytes. This stimulation is most likely not via activation of the phosphatidylinositol pathway.**

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Epidermal growth factor (EGF) is a potent mitogen for several cell types, including cells of the gastrointestinal tract (1–4). EGF exerts its effects by binding to a specific high affinity receptor on the target cells (5, 6). Such receptors are present in the plasma membrane of the enterocyte (7, 8). Several cellular events are induced after binding of the EGF to its receptor. The receptor has been identified as a tyrosine-specific protein kinase with the capacity for autophosphorylation (9). Some of the consequences of receptor activation include hydrolysis of inositol phospholipids (10), a transient rise in cytoplasmic free calcium (11, 12), and stimulation of the Na^+/H^+ exchanger (13, 14). The evidence for growth factor stimulation of the Na^+/H^+ exchanger was originally demonstrated in neuroblastoma cells (13) and fibroblasts (14, 15). Stimulation of the Na^+/H^+ exchanger leads to a rapid and persistent increase in intracellular pH. A rise in intracellular pH has been shown to stimulate DNA synthesis in fertilized sea urchin eggs (16). Moreover, mutant fibroblasts that lack functional Na^+/H^+ exchange activity can grow by artificial elevation of their intracellular pH (17). Moreover, amiloride, an inhibitor of the Na^+/H^+ exchanger, blocks both growth factor-stimulated Na^+ influx and ribosomal protein phosphorylation (18). The present studies were designed to address the relationship between EGF and the Na^+/H^+ exchanger in enterocytes and the mechanisms by which EGF regulates the Na^+/H^+ exchanger utilizing a new *in vitro* system.

Materials and Methods

Materials. D-[^{3}H]Glucose and ^{22}Na were obtained from New England Nuclear (Boston, MA). Enzymes and substrates for leucine aminopeptidase were obtained from Sigma Chemical Co. (St. Louis, MO). EGF was a gift from David Orth, M.D., Vanderbilt University (Nashville, TN). Cellulose nitrate filters (0.45-μm pore size) were obtained from Satorius Filters (Hayward, CA). All other chemicals were of the highest purity available and were obtained from Sigma.

Methods. Brush-border membrane vesicles were isolated from freshly prepared enterocytes incubated

[1] To whom requests for reprints should be addressed at Department of Pediatric Gastroenterology, Vanderbilt University, Medical Center North, S-1111, 21st and Garland, Nashville, TN 37232-2576.

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with or without EGF in the following manner. After adequate anesthesia with an intraperitoneal injection of pentobarbital (40 mg/kg), the abdominal cavity was opened and the jejunal segment was isolated, cannulated, and flushed with a Krebs-Ringer solution that was oxygenated for 30 min. The jejunal segments were removed and then filled with oxygenated citrate buffer (1.5 mM KCl, 96 mM NaCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 27 mM Na_3 citrate, and 2 mM dithiothreitol [pH 7.2]) and allowed to incubate for 15 min in a shaking water bath (80 oscillations/min). The epithelial cells were then drained into a beaker on ice containing oxygenated Krebs-Ringer buffer. The cells were then pelleted at 120*g* for 10 min. The pellet was resuspended in 2–5 ml of incubation solution and divided into two equal portions. Half the cells were incubated for 30 min with the agent under study (i.e., EGF, 200 ng/ml; calmodulin, 10 μg/ml; 12-*O*-tetradecanoylphorbol 13-acetate [TPA]), 10 μM; cAMP, 100 μM). The other half of the cells were incubated in a similar manner without the agent. Both cell fractions were continuously gassed with 100% O_2.

After the incubation period, the cells were pelleted at 120*g* for 10 min. From this step onward the routine procedure for preparing brush-border membrane vesicles using $MgCl_2$ was followed, as detailed previously by our laboratory (19).

Transport Measurements. Uptake of radiolabeled glucose and sodium was measured by a rapid filtration technique as described by us previously (19). All incubations were done at 25°C and were initiated by addition of 20 μl of vesicle suspension to 80 μl of incubation solution. The composition of the incubation medium is noted in the legend of each figure.

At the desired time point, 1 ml of ice-cold stop solution containing, per liter, 185 mM potassium gluconate, 10 mM Tris, 16 mM HEPES, and 0.1 mM amiloride (for Na^+ studies) was added to the reaction mixture. The stop solution for D-glucose consisted of 100 mM NaCl, 100 mM mannitol, 10 mM K_3PO_4, and 0.2 mM phlorizin. The diluted reaction mixture was immediately pipetted onto prewetted filter (cellulose nitrate 0.45-μm pore size; Sartorius Filters) and kept under suction. The filter was then washed with 5 ml of ice-cold stop solution. Radioactivity was counted in a scintillation counter (model LS 4000; Beckman Instruments, Palo Alto, CA). The radioactivity remaining in the filters after pipetting incubation solution into the radioactive substrate in the absence of vesicles was considered as background and was considered in the calculations.

Analysis of Data. All values were expressed as nanomoles of sodium uptake per milligram of vesicle protein, and expressed graphically as the mean ± 1 SE. Each data point represents the mean values of three different experiments run in triplicate. An analysis of variance test was used to evaluate the statistical significance of differences among the groups. A P-value of $<$ 0.05 was considered statistically significant.

Results

Validation Studies. *Enrichment of marker enzymes.* Table I depicts marker enzyme activities in crude homogenate and brush-border membrane vesicles in the different treatment groups. Leucine aminopeptidase activity was enriched 10–12-fold in brush-border membranes compared with crude homogenate, whereas markers for basolateral membranes (Na^+-K^+-ATPase), mitochondria (cytochrome *c* oxidase), and endoplasmic reticulum (NADPH-cytochrome *c* reductase) were impoverished.

D-Glucose studies. Figure 1 depicts D-glucose uptake in brush-border membrane vesicles prepared from control enterocytes as well as from enterocyte incubated with EGF, as detailed in the Materials and Methods section. There was a typical "overshoot" phenomena in the presence of a sodium gradient suggesting functional vesicles; however, the overshoot was higher with EGF compared with control vesicles ($P < 0.05$–0.01). cAMP, calmodulin, and phorbol ester had no effect on glucose uptake (data not shown).

Effect of EGF on Na^+/H^+ Exchange Activity. Figure 2 depicts Na^+ uptake in the presence and absence of pH gradient conditions. Na^+ uptake under pH gradient condition was higher at each time point, except at equilibrium in the EGF group compared with controls ($P < 0.05$–0.01). In the absence of pH gradient, Na^+ uptake was similar in both EGF and controls. Equilibrium values at 180 min were similar. EGF added directly to the vesicles had no effect.

Effect of EGF on Kinetics of Amiloride-Sensitive Na^+ Uptake. To determine the effect of EGF on amiloride-sensitive Na^+ uptake, experiments were designed to examine Na^+ uptake in the presence or absence of 1 mM amiloride in the setting of outwardly directed H^+ gradient. V_{max} and K_m were analyzed using a nonlinear computerized model of Michaelis-Menten kinetics (Enzfitter, by Robin J. Leatherbarrow, published by Elsevier Science Publishers, distributed by Elsevier-Biosoft, Cambridge, UK).

As seen in Figure 3, V_{max} of Na^+/H^+ was significantly greater in EGF compared with control (6.0 ± 0.4 compared with 3.3 ± 0.27 nmol/mg protein/5 sec, $P < 0.02$). K_m values were not significantly different (16 ± 3.0 vs 13 ± 3.0 mM, respectively).

Effect of Phorbol Ester (TPA) on Na^+/H^+ Exchange Activity. Figure 4 depicts Na^+ uptake in the presence and absence of pH gradient conditions. Na^+ uptake values under pH gradient conditions were lower at each time, except at equilibrium in vesicles isolated from cells treated with TPA compared with controls. The percentage of decrease was approximately 30% (*P*

Table I. Specific Activity of Marker Enzymes in Crude Homogenate and Brush-Border Membrane Vesicles[a]

Marker enzyme	EGF		Calmodulin		TPA		cAMP	
	Homogenate	BBM	Homogenate	BBM	Homogenate	BBM	Homogenate	BBM
Leucine aminopeptidase (μmol βNPHA hr^{-1} mg protein^{-1})	870 ± 210	8900 ± 270 (10)	950 ± 170	9700 ± 350 (10)	850 ± 170	1050 ± 250 (12)	920 ± 180	8800 ± 150 (9.5)
Na-K-ATPase (μmol/P$_i$/hr^{-1}/mg protein^{-1})	12 ± 0.2	6.2 ± 0.3 (0.5)	12.5 ± 0.5	5.2 ± 0.4 (0.41)	12.5 ± 0.4	6.1 ± 0.3 (0.48)	13 ± 0.4	6.0 ± 0.2 (0.46)
Cytochrome c oxidase (μmol min^{-1} mg protein^{-1})	6.8 ± 0.5	3.0 ± 0.1 (0.4)	6.7 ± 0.2	3.2 ± 0.2 (0.47)	6.0 ± 0.5	2.8 ± 0.1 (0.46)	5.8 ± 0.6	2.9 ± 0.2 (0.5)
NADPH-cytochrome c reductase (μmol min^{-1} mg protein^{-1})	10 ± 2	4 ± 0.6 (0.4)	8.5 ± 0.9	4.2 ± 0.5 (0.49)	9.5 ± 1	5 ± 0.6 (0.52)	7.9 ± 0.5	3.5 ± 0.5 (0.44)

[a] Number in parentheses indicates enrichment or impoverishment factor. Values are mean ± SE of three preparations. BBM, brush-border membrane.

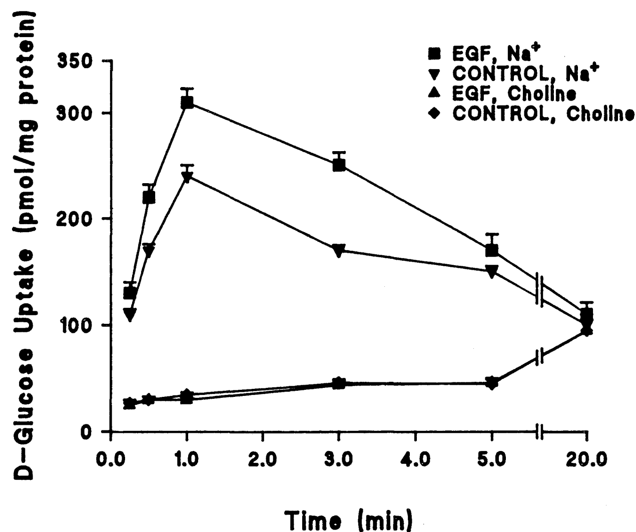

Figure 1. D-Glucose uptake in brush-border membrane vesicles isolated from jejunal enterocytes. Vesicles were incubated in mannitol buffer (300 m*M* mannitol, 20 m*M* HEPES/Tris [pH 7.4]) for 1 hr and diluted in a medium containing either (■) 100 m*M* NaCl, 100 m*M* mannitol, and 20 m*M* HEPES/Tris buffer (pH 7.4) or (▼) 100 m*M* choline chloride, 100 m*M* mannitol, and 20 m*M* HEPES/Tris buffer. The final glucose concentration was 0.1 m*M*.

< 0.05–0.01). α-Phorbol added to the cells at 10 μ*M* had no effect on 15-sec Na^+/H^+ exchange activity (values were 0.58 ± 0.05 and 0.6 ± 0.05 nmol/mg protein for α-phorbol and controls, respectively).

Effect of Calmodulin on Na^+/H^+ Exchange Activity. Figure 5 depicts Na^+ uptake in the presence and absence of pH gradient conditions. Na^+ uptake values under pH gradient condition were lower at each time, except at equilibrium in vesicles isolated from cells treated with calmodulin compared with controls ($P <$ 0.05–0.01). Calmodulin had no effect on Na^+ uptake under the no pH gradient condition. Calcium concentration was 1 μ*M*. In a separate experiment, *N*-(4-aminobutyl) 5-chloro-2-naphthalenesulphonamide (W-13) added to the cells at a concentration of 45 μ*M* reversed the effect of calmodulin on Na^+/H^+ exchange activity (values at 15 sec were 0.3 ± 0.05, 0.6 ± 0.04, and 0.62 ± 0.05 nmol/mg protein for calmodulin, control, and W-13 conditions, respectively). W-13 alone had no effect on Na^+/H^+ exchange activity (0.55 ± 0.03 nmol/mg protein/15 sec). Calmodulin added to freeze-thawed vesicles inhibited Na^+/H^+ exchange in a similar manner to that for vesicles from cells incubated and homogenized with calmodulin. To provide evidence that calmodulin enters the intravesicular space, we have incubated ^{131}I-calmodulin (gift from Dr. Martin Waterson, Vanderbilt University) with freshly isolated epithelial cells for 30 min and then prepared brush-border membrane vesicles. Radioactivity of ^{131}I was determined in vesicles subjected to detergent treatment (0.06% deoxycholate), in vesicles washed twice with mannitol buffer, and in vesicles after washing. The

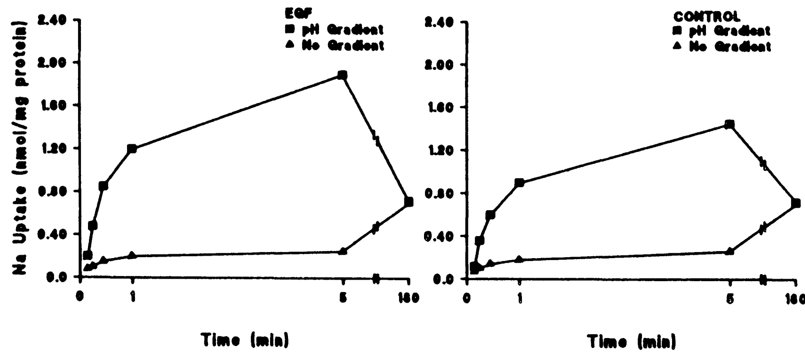

Figure 2. Effect of EGF (200 ng/ml) on 1-m*M* Na^+ uptake under outwardly directed pH gradient (pH_i/pH_o = 5.2/7.5) and no gradient condition (pH_i/pH_o = 7.5/7.5). Freshly isolated enterocytes were incubated for 30 min at room temperature with or without EGF. Brush-border membrane vesicles were preincubated for 1 hr at 25°C with various combinations of Tris, HEPES, and morpholino-ethane-sulfonic acid (MES) (130 m*M* total) to bring intravesicular pH to 5.2 or 7.5. The preincubation medium for the pH gradient condition contained, per liter, 100 m*M* tetramethylammonium (TMA) gluconate, 90 m*M* MES, and 40 m*M* HEPES/Tris (pH 5.2). For no gradient condition, the medium contained 100 m*M* TMA gluconate, 85 m*M* HEPES, and 45 m*M* Tris (pH 7.5). The reaction was started with the addition of 20 μl of vesicles to a medium containing, per liter, 100 m*M* TMA gluconate, 85 m*M* HEPES, 45 m*M* Tris (pH 7.5), and 1 m*M* Na with ^{22}Na as a tracer.

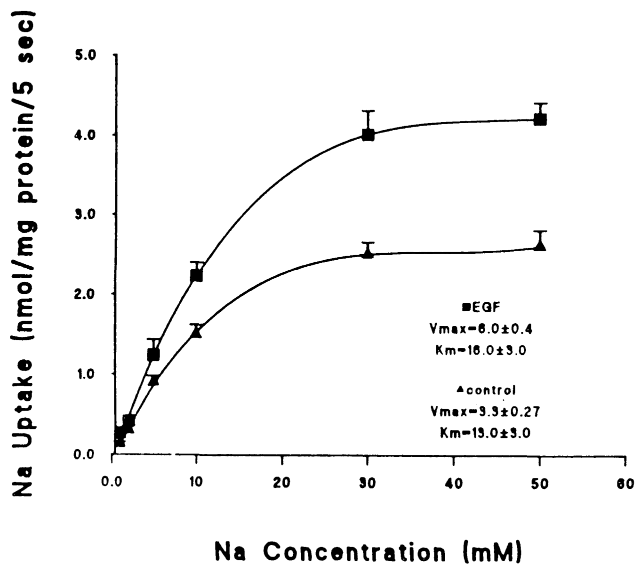

Figure 3. Effect of EGF on kinetics of Na^+/H^+ exchanger by rat jejunal brush-border membrane vesicles. Freshly isolated enterocytes were incubated with or without 200 ng/ml of EGF for 30 min. Vesicles were prepared in 100 m*M* tetramethylammonium gluconate, 40 m*M* HEPES, and 90 m*M* morpholino-ethane-sulfonic acid buffer (pH 5.2). The incubation pH was 7.5. The amiloride-sensitive component was plotted against Na^+ concentration. The reaction was stopped at 5 sec. K_m and V_{max} values were analyzed using a model of the Michaelis-Menten kinetics.

counts per minute in vesicles subjected to 0.06% deoxycholate was 1660 ± 100 cpm (n = 3), in vesicles after washing, 1060 ± 75 cpm (n = 3), and in vesicles washed twice with buffer, 550 ± 60 cpm (n = 3). These results indicate that approximately 65% of the ^{131}I-calmodulin was inside the vesicles, whereas approximately 35% was adherent to the outside of the vesicles.

Effect of cAMP on Na^+/H^+ Exchange Activity. Figure 6 depicts Na^+ uptake in the presence and absence of pH gradient conditions. Na^+ uptake under pH gradient condition was lower at each time, except at equilibrium under cAMP compared with controls ($P <$ 0.05–0.01). Cyclic AMP had no effect on Na^+ uptake under the no pH gradient condition. Equilibrium values were similar.

Effect of ATP Depletion on Na^+/H^+ Exchange Activity as Modulated by EGF, Calmodulin, cAMP and Phorbol Esters. To determine whether the effects of these agents depend on ATP, we carried out experiments in which cells were either depleted from ATP (incubation with 5 m*M* glucose and 1 unit/1 μmol of hexokinase for 30 min) or nondepleted. EGF, calmodulin, cAMP, and phorbol esters were tested with controls for each condition. As seen in Table II, the effects of these agents were not seen in cells depleted from ATP.

Discussion

Epidermal growth factor is a 6045-mol wt single-chain polypeptide consisting of 53 amino acid residues that was first isolated from the mouse submandibular gland by Cohen (20). The amino acid structure has three sulfide bonds that are necessary for biological activity (21). EGF is resistant to acid and trypsin (22) and is widely distributed in mammalian tissues and fluids. It is present in breast milk (23), Brunner's glands of the duodenum, and Paneth cells of the small intestine (24). The biological activity of EGF has been studied extensively in *in vitro* cell cultures (25), and *in vivo* in several mammalian species (26, 27). *In vivo* studies in the rat have shown that EGF stimulated DNA synthesis of the oxyntic gland of the stomach (26), pancreas, and small intestine (3). The activity of ornithine decarboxylase, an important enzyme in polyamine synthesis, was shown to be increased in the intestine of mice and rats injected with EGF (28). Similarly, EGF enhanced the transport of glucose (29, 30), amino acids (31), and calcium (32) across the intestinal tract. The above stud-

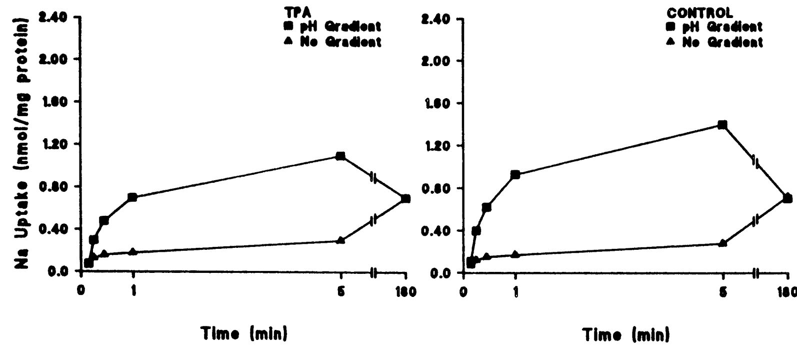

Figure 4. Effect of 10 μM TPA on 1-mM Na^+ uptake under outwardly directed pH gradient (pH_i/pH_o = 5.2/7.5) and under no pH gradient condition (pH_i/pH_o = 7.5/7.5). Freshly isolated enterocytes were incubated for 30 min at room temperature with or without TPA. Brush-border membrane vesicles were then preincubated for 1 hr at 25°C with various combinations of Tris, HEPES, and morpholino-ethane-sulfonic acid (130 mM total) to bring intravesicular pH to 5.2 or 7.5. The composition of solutions is similar to that described in the legend to Figure 2.

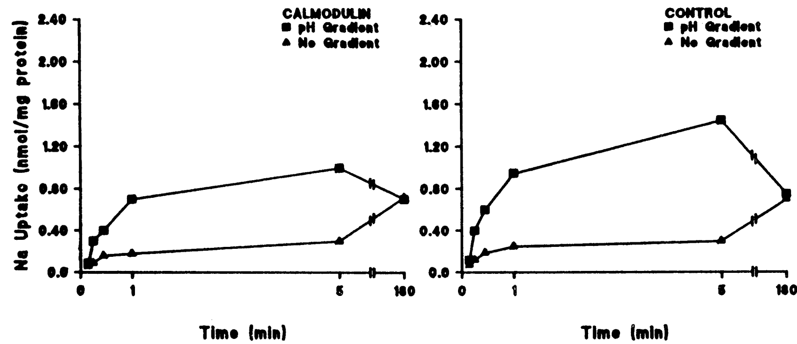

Figure 5. Effect of calmodulin (10 μg/ml) on 1-mM Na^+ uptake under outwardly directed pH gradient (pH_i/pH_o = 5.2/7.5) and under no pH gradient condition (pH 7.5/7.5). Freshly isolated enterocytes were incubated for 30 min at room temperature with or without calmodulin. Brush-border membrane vesicles were then preincubated for 1 hr at 25°C with various combinations of Tris, HEPES, and morpholino-ethane-sulfonic acid (130 mM total) to bring intravesicular pH to 5.2 or 7.5. The composition of solutions is similar to that described in the legend to Figure 2.

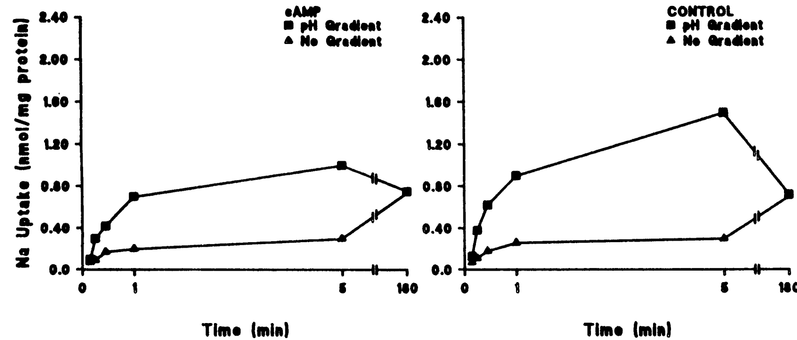

Figure 6. Effect of cAMP (100 μM) on 1-mM Na^+ uptake under outwardly directed pH gradient (pH_i/pH_o = 5.2/7.5) and under no pH gradient condition (pH 7.5/7.5). Freshly isolated enterocytes were incubated for 30 min at room temperature with or without 100 μM dibutyryl cAMP. Brush-border membrane vesicles were then preincubated for 1 hr at 25°C with various combinations of Tris, HEPES, and morpholino-ethane-sulfonic acid (130 mM total) to bring intravesicular pH to 5.2 or 7.5. The composition of solutions is similar to that described in the legend to Figure 2.

ies suggest that EGF exerts trophic effects on the gastrointestinal tract. The mechanisms by which growth factors induce their trophic effects in the intestinal tract are not well understood. However, it is well recognized that stimulation of Na^+/H^+ exchange is among the earliest detectable events induced by various mitogens, including EGF (12, 13), platelet-derived growth factor (33), vasopressin (34), and bradykinin (35). The stimulation of Na^+/H^+ by growth factors is believed to be via binding of growth factors to their specific receptors. Receptor occupancy alters the pH_i sensitivity of the Na^+/H^+ exchanger, allowing a rise in pH_i to a more alkaline level. This change is thought to be secondary to receptor-linked activation of phospholipase C (36). These events have been shown to occur in fibroblasts, neutrophils, B lymphocytes, MDCK kidney cell lines,

Table II. Effect of ATP Depletion on Na^+/H^+ Exchange Activity as Modulated by EGF, Calmodulin, cAMP, and Phorbol Esters[a]

	Control	EGF	Control	Calmodulin	Control	cAMP	Control	Phorbol esters
+ATP	0.56 ± 0.02	1.3 ± 0.1*,†	0.65 ± 0.03	0.4 ± 0.02*,†	0.7 ± 0.1	0.4 ± 0.02*,†	0.68 ± 0.02	0.4 ± 0.02*,†
−ATP	0.5 ± 0.03	0.48 ± 0.02	0.6 ± 0.04	0.65 ± 0.02	0.7 ± 0.06	0.65 ± 0.02	0.6 ± 0.05	0.6 ± 0.03

[a] Values are mean ± SE of three experiments. Symbols (*,†) indicate mean values in membranes prepared from (+ATP) cells are significantly different compared with corresponding mean values in (−ATP) cells and controls respectively, $P < 0.05$–0.01.

and in a human epidermoid carcinoma cell line (37). These events have not been explored in enterocytes. We utilized freshly isolated enterocytes and determined the effect of physiological doses of EGF (29) and other cellular mediators on Na^+/H^+ exchange activity. EGF stimulated Na^+/H^+ exchange without affecting equilibrium values, which are an indicator of vesicle size. The increase in Na^+/H^+ activity is secondary to an increase in V_{max} rather than K_m, indicating that the number and/or the activity of the exchanger is increased. EGF directly added to vesicles had no effect on Na^+/H^+ exchange activity, which suggests that the action of EGF requires viable cells to allow receptor binding and autophosphorylation. The effect of EGF does not appear to occur via stimulation of phospholipase C. Phorbol esters that, like TPA, bind to and directly stimulate protein kinase C inhibited rather than stimulated Na^+/H^+ exchange activity. The inhibition of Na^+/H^+ exchange by phorbol esters and calmodulin has also been shown in rabbit proximal colon (38) and rat colon (39). This effect on the small intestine and colon appears to be in contrast to the stimulatory effect of phorbol esters on Na^+/H^+ activity in many other cell systems such as fibroblasts, neutrophils, and MDCK cells (37). In support of these findings is the observation by Macara (40) showing activation of ^{45}Ca influx and Na^+/H^+ exchange by EGF in A431 cells via a pathway independent of phosphatidylinositol turnover. The mechanism for the differential effect of second messengers on Na^+/H^+ exchange activity in different cell types is not known, but may be related to differences in the Na^+/H^+ exchanger itself, its internal modifier site, or other regulatory proteins. The effect of calmodulin seen in our system conceivably relates to the access of calmodulin to the internal compartment of the vesicles during the preparative procedure.

In conclusion, our studies utilizing an *in vitro* approach suggest that EGF stimulates Na^+/H^+ activity possibly via a pathway independent of the activation of the hydrolysis of PIP_2. The stimulation of Na^+/H^+ activity may play a role in the trophic effect of EGF in the intestinal tract. To our knowledge, this is the first demonstration of EGF stimulation of Na^+/H^+ exchange activity in brush-border membrane vesicles isolated from cells pretreated with EGF.

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