

# Signaling of short- and long-term regulation of intestinal epithelial type 1 Na<sup>+</sup>/H<sup>+</sup> exchanger by interferon- $\gamma$

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**1** The present study evaluated the effect of interferon- $\gamma$  (IFN- $\gamma$ ) on intestinal Na<sup>+</sup>/H<sup>+</sup> exchange (NHE) activity and the intracellular signaling pathways set into motion after IFN- $\gamma$  receptor activation.

**2** Caco-2 cells express endogenous NHE1, NHE2 and NHE3 proteins, as detected by immunoblotting. Short- (0.5 h) and long- (24 h) term exposure of Caco-2 cells to IFN- $\gamma$  resulted in a concentration-dependent decrease in NHE activity. Inhibition of NHE activity by IFN- $\gamma$  was absent in cariporide-treated cells, but not in cells treated with S-3226. The long-term exposure to IFN- $\gamma$  was accompanied by a 20% increase in surface NHE1 abundance and no changes in total NHE1 abundance.

**3** Inhibition of Raf1, mitogen-activated protein kinase kinase (MAPKK/MEK) and p38 MAPK with, respectively, GW 5074, PD 98059 and SB 203580 and downregulation of protein kinase C (PKC) with phorbol-12,13-dibutyrate (100 nM for 24 h) prevented inhibition of NHE activity by IFN- $\gamma$  (0.5 and 24 h exposure). The signal transducer and activator transcription factor 1 (STAT1) inhibitor epigallocatechin-3-gallate (EGCG) prevented inhibition of NHE activity by long- but not the short-term treatment with IFN- $\gamma$ .

**4** Treatment with IFN- $\gamma$  activated phospho-p38 MAPK, this effect being detected as early as 1 h, persisting over 3 h and decreasing after 24 h. IFN- $\gamma$  produced a sustained action of phospho-STAT1 that was prevented by EGCG and partially attenuated by SB 203580 and insensitive to downregulation of PKC.

**5** In conclusion, short- and long-term inhibition of NHE1 activity by IFN- $\gamma$  involves a complex signaling pathway that includes PKC activation and STAT1 phosphorylation, respectively, but is not accompanied by downregulation of NHE1.

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**Abbreviations:** CD, Crohn's disease; EGC, (–)-epigallocatechin; EGCG, (–)-epigallocatechin-3-gallate; ERK, extracellular signal-regulated kinase; IFN- $\gamma$ , interferon- $\gamma$ ; IBD, inflammatory bowel disease; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; MEK, MAPK/ERK kinase; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; STAT1, signal transducer and activator transcription factor 1; UC, ulcerative colitis

## Introduction

The dominant symptom in both Crohn's disease (CD) and ulcerative colitis (UC) is diarrhea associated with autonomic nerve dysfunction (Magro *et al.*, 2002), and recent evidence suggests that this may relate to a reduction in electrolyte absorption rather than increases in electrolyte secretion (Sundaram *et al.*, 1997). In agreement with this view is the finding that the colonic mucosa of inflammatory bowel disease (IBD) patients responds poorly to secretagogues and that Na<sup>+</sup> absorption is diminished (Sandle *et al.*, 1990). Consistent with these findings is the observation that interferon- $\gamma$  (IFN- $\gamma$ ), a cytokine suggested to be involved in the pathophysiology of IBD, reduces agonist-induced and cAMP-induced Cl<sup>–</sup> intestinal secretion (Colgan *et al.*, 1994). IFN- $\gamma$  has also been shown to downregulate the cystic fibrosis conductance regulator

(Colgan *et al.*, 1994), the Na<sup>+</sup>-K<sup>+</sup>-2Cl<sup>–</sup> cotransporter (Zund *et al.*, 1996; Fish *et al.*, 1999), the Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) (Rocha *et al.*, 2001) and the Na<sup>+</sup>-K<sup>+</sup>-ATPase (Sugi *et al.*, 2001). IFN- $\gamma$  has direct effects on intestinal epithelial cells, such as changes in cell morphology and decreased levels of proteins involved in transport and barrier function (Madara & Stafford, 1989; Adams *et al.*, 1993; Besançon *et al.*, 1994; Colgan *et al.*, 1994; Fish *et al.*, 1999; Sugi *et al.*, 2001), but the overall cytoskeletal structure appears to be maintained (Madara & Stafford, 1989). CD is a Th1-mediated disease, and an increase in IFN- $\gamma$ -secreting cells and IFN- $\gamma$  mRNA levels has been described in intestinal lesions of patients with CD (Podolsky, 1991; Breese *et al.*, 1993; Niessner & Volk, 1995; Adorini & Francesco, 1997).

In general, the signal transduction pathway initiated by binding of IFN- $\gamma$  to its receptor leads first to Janus kinase (JAK) 1 and 2 activation and their association with the IFN- $\gamma$  receptor (Haque & Williams, 1998). JAK then phosphorylates IFN- $\gamma$  receptor on specific tyrosines, which serve as docking

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sites for the signal transducer and activator transcription factor 1 (STAT1). Recently, IFN- $\gamma$  was also shown to stimulate mitogen-activated protein kinase (MAPK) activity, namely extracellular signal-regulated kinases (ERK1 and ERK2) (Liu *et al.*, 1994) and p38 MAPK (Verma *et al.*, 2002). Activation of MAPKs is controlled *via* membrane-associated signaling complexes and involves a network that includes Ras protein(s), the Raf family (Raf-1, B-Raf, A-Raf and MEKK) of serine kinases and MAPK kinases, MEK1 and MEK2 as upstream regulators of ERK1 and ERK2 and MEK3 and MEK6 as upstream regulators of p38 MAPK (Pearson *et al.*, 2001). Recently, we have reported on the effects of IFN- $\gamma$  on intestinal Na<sup>+</sup>-K<sup>+</sup>-ATPase activity and the intracellular signaling pathways involved in cultured human intestinal epithelial Caco-2 cells, using probes that interact with Raf-1, MAPK/ERK kinase (MEK), ERK2, p38 MAPK, protein kinase C (PKC) and STAT1, and measuring the degree of activation of p38 MAPK and STAT1. It was shown that the decrease in Na<sup>+</sup>-K<sup>+</sup>-ATPase activity by IFN- $\gamma$  may involve the activation of PKC downstream STAT1 phosphorylation and Raf-1, MEK, ERK2 and p38 MAPK pathways, in a complex sequence of events (Magro *et al.*, 2004).

In the present study, the short- and long-term regulation of the intestinal NHE by IFN- $\gamma$  was evaluated and the intracellular signaling pathways set into motion after IFN- $\gamma$  receptor activation identified. For this purpose, human intestinal epithelial Caco-2 cells, which endogenously express NHE1, NHE2 and NHE3 isoforms, were used. A decrease in NHE1 activity after acute and chronic exposure to IFN- $\gamma$  involving different signaling pathways is reported. The short-term regulation of NHE activity by IFN- $\gamma$  involves mainly the activation of Raf-1, MEK, p38 MAPK and PKC with NHE1 as an effector protein for PKC. The transduction mechanisms set into motion by long-term exposure to IFN- $\gamma$  involve STAT1 activation with p38 MAPK playing a minor role in SER701 phosphorylation of STAT1.

## Methods

### Cell culture

Caco-2 cells (ATCC 37-HTB; passages 39–49) were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and maintained in a humidified atmosphere of 5% CO<sub>2</sub>-95% air at 37°C. Cells were grown in minimal essential medium (Sigma Chemical Company, St Louis, MO, U.S.A.) supplemented with 10<sup>6</sup> U l<sup>-1</sup> penicillin G, 250  $\mu$ g l<sup>-1</sup> amphotericin B, 100 ng l<sup>-1</sup> streptomycin (Sigma), 20% fetal bovine serum (Sigma) and 25 mmol l<sup>-1</sup> N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES; Sigma). For subculturing, the cells were dissociated with 0.05% trypsin-EDTA, split 1:3 and subcultured in Costar Petri dishes with 21 cm<sup>2</sup> growth area (Costar, Badhoevedorp, The Netherlands). For studies on NHE activity, the cells were seeded in 96-well plates. The cell medium was changed every 2 days, and the cells reached confluence after 5 days of initial seeding. For 24 h prior to each experiment, the cell medium was free of fetal bovine serum. Experiments were generally performed 2 days after cells reached confluence, usually 7 days after the initial seeding; each cm<sup>2</sup> contained about 100  $\mu$ g of cell protein.

### NHE activity

NHE activity was assayed as the initial rate of intracellular pH (pH<sub>i</sub>) recovery after an acid load imposed by 20 mM NH<sub>4</sub>Cl followed by removal of Na<sup>+</sup> from the Krebs' modified buffer solution (in mM: NaCl 140, KCl 5.4, CaCl<sub>2</sub> 2.8, MgSO<sub>4</sub> 1.2, NaH<sub>2</sub>PO<sub>4</sub> 0.3, HEPES 10, glucose 5 (pH 7.4)) in the absence of CO<sub>2</sub>/HCO<sub>3</sub>. In these experiments, NaCl was replaced by an equimolar concentration of tetramethylammonium chloride (TMA). pH<sub>i</sub> measurements were performed in cells cultured in 96-well plates, as previously described (Gomes *et al.*, 2001; Gomes & Soares-da-silva, 2002a; Pedrosa *et al.*, 2004). Briefly, cells were loaded in serum-free medium with 5  $\mu$ M BCECF/AM, the membrane-permeant acetoxymethyl ester derivative of 2',7'-bis (carboxyethyl)-5,6-carboxyfluorescein (BCECF), for 40 min at 37°C in 5% CO<sub>2</sub>-95% air atmosphere. The cells were washed free of dye and, unless stated otherwise, the test compounds were added to the extracellular fluid 0.5 h before starting the sodium-dependent pH<sub>i</sub> recovery. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini, Molecular Devices, Sunnyvale, U.S.A.) and fluorescence was monitored every 17 s alternating between 440 and 490 nm excitation at 535 nm of emission, with a cutoff filter of 530 nm. The ratio of intracellular BCECF fluorescence at 490 and 440 nm was converted to pH<sub>i</sub> by comparison with values from an intracellular calibration curve using the 10  $\mu$ M nigericin and high-K<sup>+</sup> method with pH<sub>i</sub> ranging from pH 6.6 to 7.8 (Gomes *et al.*, 2001; Gomes & Soares-da-silva, 2002a).

In experiments aimed to evaluate the sensitivity of the sodium-dependent pH<sub>i</sub> recovery to selective inhibitors of NHE isoforms, cells were treated with increasing concentrations of amiloride, EIPA, cariporide, S-3226 or vehicle for 0.5 h before starting the sodium-dependent pH<sub>i</sub> recovery. Amiloride and EIPA are both more effective in inhibiting NHE1 than NHE3, although EIPA is relatively more potent in inhibiting NHE1 (Noel & Pouyssegur, 1995). On the other hand, cariporide and S-3226 are relatively selective for inhibition of NHE1 and NHE3 isoforms, respectively (Schwark *et al.*, 1998). Differentiation between NHE1 and NHE2 can be achieved while employing selective concentrations of cariporide; NHE1 is fully inhibited by low concentrations (1  $\mu$ M), whereas inhibition of NHE2 requires much higher concentrations of cariporide (25  $\mu$ M) (Bachmann *et al.*, 1998).

In experiments in which the effect of IFN- $\gamma$  on NHE activity was tested, cells were treated with a given concentration of IFN- $\gamma$  (from 10 to 1000 U ml<sup>-1</sup>) or vehicle for 24, 3 or 0.5 h before starting the sodium-dependent pH<sub>i</sub> recovery. In some experiments, cariporide (300 nM) or S-3226 (300 nM) were coincubated with IFN- $\gamma$  (1000 U ml<sup>-1</sup>) for 0.5 h before starting the sodium-dependent pH<sub>i</sub> recovery. Assessment of signaling pathways used by IFN- $\gamma$  was conducted by using a single concentration (1000 U ml<sup>-1</sup>) and a 0.5 and 24 h exposure of Caco-2 monolayers to human IFN- $\gamma$ . Cells were pretreated with inhibitors of interest 0.5 h prior to the addition of IFN- $\gamma$ , which was subsequently added to the culture well (inhibitor not washed out).

In experiments aimed to evaluate the effect of PKC and p38 MAPK activation on NHE activity, cells were treated with increasing concentrations of phorbol-12,13-dibutyrate (PDBu) and anisomycin, respectively, for 30 min before starting the sodium-dependent pH<sub>i</sub> recovery. In some experiments, cells

were pretreated with inhibitors of interest 1 h prior to the addition of PDBu or anisomycin.

### Measurement of cell viability

Cell viability was measured as previously described (Pedrosa & Soares-da-silva, 2002) using calcein-AM (Molecular Probes, Eugene, OR, U.S.A.). The membrane-permeant calcein-AM, a nonfluorescent dye, is taken up and converted by intracellular esterases to membrane-impermeant calcein, which emits green fluorescence. After treatment with IFN- $\gamma$  (1000 U ml<sup>-1</sup>) or vehicle for 24 h, cells were washed twice with Hanks' medium (medium composition (in mM): NaCl 137, KCl 5, MgSO<sub>4</sub> 0.8, Na<sub>2</sub>HPO<sub>4</sub> 0.33, KH<sub>2</sub>PO<sub>4</sub> 0.44, CaCl<sub>2</sub> 0.25, MgCl<sub>2</sub> 1.0, Tris-HCl 0.15, sodium butyrate 1.0 (pH 7.4)) and loaded with 2  $\mu$ M calcein-AM in Hanks' medium at room temperature for 30 min. Fluorescence was measured at 485 nm excitation and 530 nm emission wavelengths in a multiplate reader (Spectromax Gemini, Molecular Devices). To determine minimum staining for calcein-AM (calcein<sub>min</sub>), six wells were treated with ethanol 30 min before calcein-AM addition. The percent viability was then calculated as [(calcein<sub>sample</sub> - calcein<sub>min</sub>)/(calcein<sub>control</sub> - calcein<sub>min</sub>)]  $\times$  100.

### NHE immunoblotting

Caco-2 cells cultured to 90% of confluence were washed twice with PBS and total cell protein was extracted for NHE1, NHE2 and NHE3 detection. Briefly, to obtain total cell extract, cells were lysed by brief sonication (15 s) in lysis buffer with protease inhibitors (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100  $\mu$ g ml<sup>-1</sup> PMSF, and aprotinin and leupeptin 2  $\mu$ g ml<sup>-1</sup> each) and incubated on ice for 1 h. After centrifugation (16,000  $\times$  g, 30 min, 4°C), the supernatant was mixed in 6  $\times$  sample buffer (0.35 M Tris-HCl, 4% SDS, 30% glycerol, 9.3% DTT (pH 6.8), 0.01% bromophenol blue) and boiled for 5 min. The proteins (50  $\mu$ g) were subjected to SDS-PAGE (10% SDS-polyacrylamide gel) and electrotransferred onto nitrocellulose membranes. The transblot sheets were blocked with 5% of nonfat dry milk in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20 overnight at 4°C. Then, the membranes were incubated with appropriately diluted antibodies: rabbit anti-NHE1, anti-NHE2 or anti-NHE3 polyclonal isoform specific antibodies (Alpha Diagnostics, Autogenbioclear, Wiltshire, U.K.) and the anti- $\beta$ -actin primary antibody (Upstate Biotechnologies). The reaction was detected by peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and ECL system (Amersham Life, Arlington Heights, IL, U.S.A.). The densities of the appropriate bands were determined using Quantity One imaging software (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Protein concentration was measured using the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and bovine serum albumin as standard.

### Cell surface biotinylation

Cell surface biotinylation was used to determine apical membrane NHE1 expression in Caco-2 cells treated with IFN- $\gamma$  (1000 U ml<sup>-1</sup>) or vehicle for 24 h. Briefly, confluent cells were rinsed twice with ice-cold PBS with 0.1 mM CaCl<sub>2</sub> and

1.0 mM MgCl<sub>2</sub> (PBS-Ca-Mg). The apical surface was then exposed to 500  $\mu$ g ml<sup>-1</sup> of Sulfo-NHS-Biotin (Pierce, Rockford, IL, U.S.A.) in biotinylation buffer (10 mM triethanolamine, 2 mM CaCl<sub>2</sub>, 150 mM NaCl (pH 7.4)) for 20 min with horizontal motion at 4°C. After labeling, the cells were rinsed with quenching solution (PBS-Ca-Mg with 100 mM glycine) and cells lysed with RIPA buffer with protease inhibitors (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 100  $\mu$ g ml<sup>-1</sup> PMSF, and aprotinin and leupeptin 2  $\mu$ g ml<sup>-1</sup> each), briefly sonicated and incubated on ice for about 1 h. After centrifugation (16,000  $\times$  g, 30 min, 4°C), the supernatant was adjusted to 3.4 mg ml<sup>-1</sup> and the biotinylated protein precipitated overnight at 4°C with 100  $\mu$ l of streptavidin-agarose beads (Pierce, Rockford, IL, U.S.A.) in a total volume of 500  $\mu$ l. The streptavidin-agarose beads were washed twice with PBS and the bound proteins solubilized with SDS sample buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol (pH 6.8)) and subjected to SDS-PAGE and blotting for NHE1 as described in NHE immunoblotting.

### p38 MAPK immunoprecipitation and immunoblotting

p38 MAPK activity in Caco-2 cells exposed to IFN- $\gamma$  (1000 U ml<sup>-1</sup>) and anisomycin (100 nM) for 1, 3 and 24 h was analyzed by immunoblotting using the p38 MAPK assay kit (Cell Signaling Technology™) according to the manufacturer's protocol. Briefly, cells were washed twice with PBS, lysed with cell lysis buffer and briefly sonicated (15 s). A monoclonal phospho-specific antibody to p38 MAPK (Thr180/Tyr182) was used to immunoprecipitate active p38 MAPK from cell lysates overnight at 4°C. Beads were sedimented by centrifugation (1300 r.p.m., 1 min, 4°C) and the pellet was washed with lysis buffer and kinase buffer. The pellet was then suspended in kinase buffer supplemented with 200  $\mu$ M ATP and 2  $\mu$ g ATF-2 fusion protein and incubated for 30 min at 30°C. The p38 active MAPK ATF-2-induced phosphorylation reaction was terminated by addition of SDS sample buffer (187.5 mM Tris-HCl (pH 6.8), 6% w v<sup>-1</sup> SDS, 30% glycerol, 150 mM DTT, 0.03% w v<sup>-1</sup> bromophenol blue). Samples were denatured at 95°C for 5 min and loaded onto 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The transblot sheets were blocked for 1 h with 5% of nonfat dry milk in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20 at room temperature. Then, the membranes were incubated with phospho-ATF-2 (Thr71) antibody (1 : 1000) and the immunocomplexes detected by the Phototope®-HRP Western detection kit.

### STAT1 immunoblotting

Total and phosphorylated STAT1 protein levels in Caco-2 cells exposed to IFN- $\gamma$  (1000 U ml<sup>-1</sup>) and anisomycin (100 nM) for 1, 3 and 24 h were analyzed using the PhosphoPlus® Stat1 (Tyr701) antibody kit (Cell Signaling Technology™) according to the manufacturer's protocol. In some experiments, cells were pretreated with inhibitors of interest 0.5 h prior to the addition of IFN- $\gamma$ , which was subsequently added to the culture well (inhibitor not washed out). Briefly, cells (9.5 cm<sup>2</sup>) were washed twice with PBS, lysed with 200  $\mu$ l SDS sample buffer (62.5 mM Tris-HCl (pH 6.8), 2% w v<sup>-1</sup> SDS, 10% glycerol, 50 mM DTT, 0.1% w v<sup>-1</sup> bromophenol blue) and

briefly sonicated (15 s). Samples were then denatured for 5 min at 95°C, loaded (5  $\mu$ l) onto 10% SDS-PAGE and electrophoretically transferred onto nitrocellulose membranes. The transblot sheets were blocked for 3 h with 5% of nonfat dry milk in 25 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20 at room temperature. Then, the membranes were incubated with phospho-Stat1 or Stat1 antibody (1:1000) and the immunocomplexes detected with the Phototope<sup>®</sup>-HRP Western detection kit.

### Data analysis

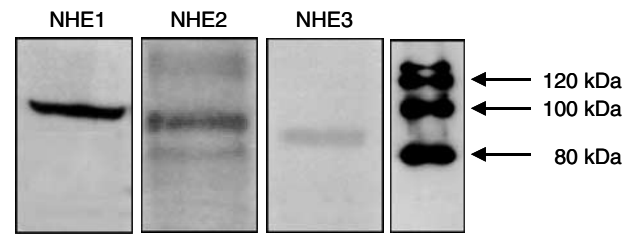
Geometric means are given with 95% confidence limits and arithmetic means are given with s.e.m. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the Student's *t*-test or the Newman-Keuls test for multiple comparisons. A *P*-value less than 0.05 was assumed to denote a significant difference.

### Drugs

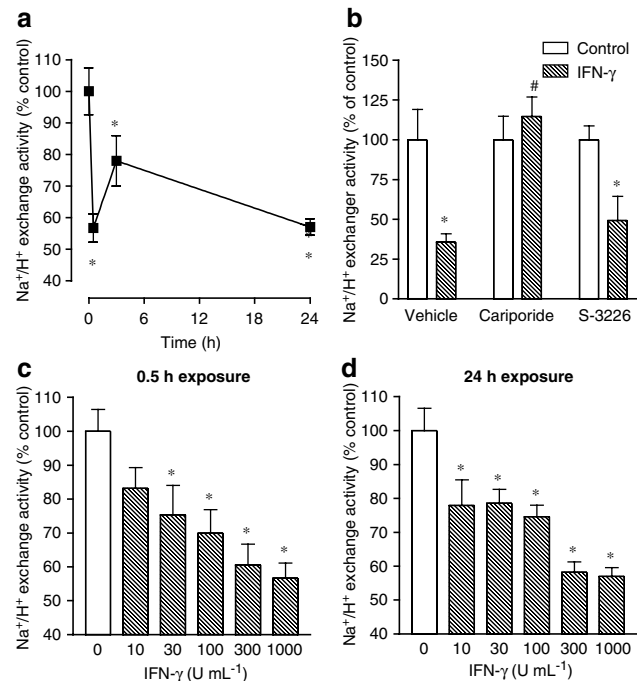
Anisomycin, (-)-epigallocatechin-3-gallate (EGCG), (-)-epigallocatechin (EGC) and human IFN- $\gamma$  were obtained from Sigma (Chemical Company, St Louis, MO, U.S.A.). GW 5074 (3-(3,5-dibromo-4-hydroxybenzylidene-5-iodo-1,3-dihydroindol-2-one) was purchased from Tocris (Tocris Cookson Inc., Avonmouth, U.K.). PD 98059 (2'-amino-3'-methoxyflavone) and SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole) were obtained from Calbiochem (Calbiochem-Novabiochem Corporation, San Diego, CA, U.S.A.). Cariporide (4-isopropyl-3-methylsulfonylbenzoylguanidine methanesulfonate) and S-3226 (3-(54)-*N*-isopropylidene-2-methyl-acrylamide dihydrochloride) were kindly provided Dr H.J. Lang from Aventis Pharma Deutschland (Frankfurt, Germany). Stock solutions of anisomycin, EGCG, EGC, GW 5074, PD 98059, SB 203580, cariporide and S-3226 were prepared in 100% DMSO and working solutions contained 0.1% DMSO. Stock solutions of IFN- $\gamma$  were prepared in water.

## Results

In the present study, NHE activity was assayed in Caco-2 cells loaded with a pH-sensitive dye (BCECF) as the Na<sup>+</sup>-dependent recovery of pH<sub>i</sub> measured after an acid load imposed by 20 mM NH<sub>4</sub>Cl followed by removal of Na<sup>+</sup> from the Krebs modified buffer solution in the absence of CO<sub>2</sub>/HCO<sub>3</sub>. After acidification, Caco-2 cells showed a rapid alkalization on addition of 140 mM Na<sup>+</sup>. This alkalization process was inhibited by amiloride (1 mM), EIPA (10  $\mu$ M) and cariporide (1  $\mu$ M), but was insensitive to S-3226 (1  $\mu$ M). Cariporide (IC<sub>50</sub> = 5 [2, 18] nM) was considerably more potent than EIPA (IC<sub>50</sub> = 24 [12, 49] nM) and amiloride (IC<sub>50</sub> = 11 [3, 34]  $\mu$ M) inhibiting NHE activity in Caco-2 cells. Differences in sensitivity to these inhibitors are in agreement with the observation that Caco-2 cells express different NHE subtypes, namely NHE1, NHE2 and NHE3 isoforms (Biemesderfer *et al.*, 1997; Karim *et al.*, 1999). The presence of NHE1, NHE2 and NHE3 isoforms was confirmed by immunoblotting, as shown in Figure 1. The anti-NHE1, anti-NHE2 and anti-NHE3 protein antibodies recognized single bands of about



**Figure 1** Expression of NHE1, NHE2 and NHE3 isoforms in Caco-2 cells. Immunoblots were repeated three times. NHE1 ~ 100 kDa, NHE2 ~ 90 kDa and NHE3 ~ 85 kDa.



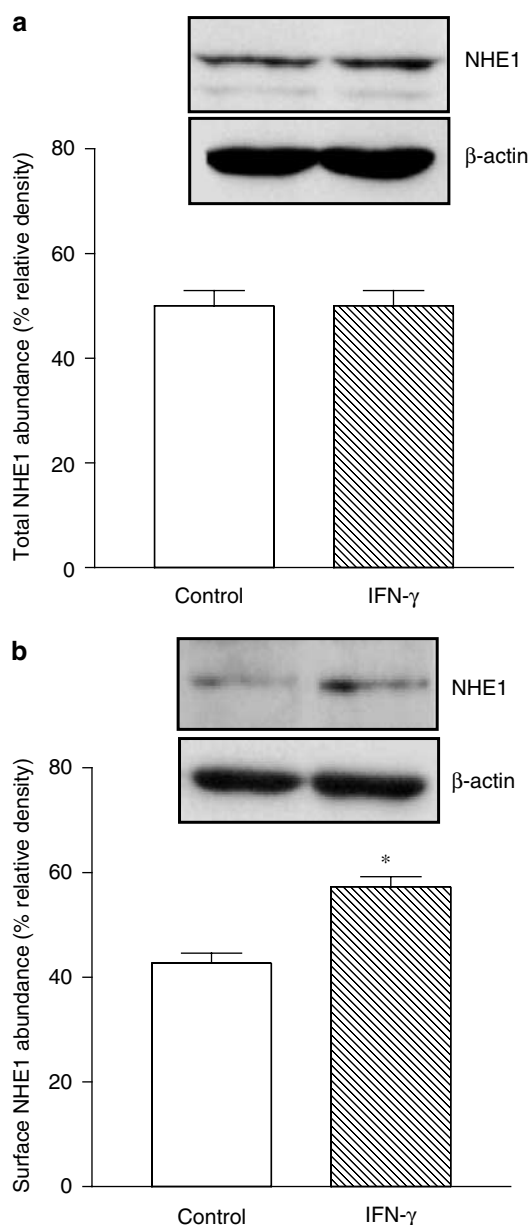
**Figure 2** (a) Time-dependent effect of IFN- $\gamma$  (1000 U ml<sup>-1</sup>) on NHE activity in cultured Caco-2 cells. (b) Effect of cariporide (300 nM) and S-3226 (300 nM) on the IFN- $\gamma$ - (1000 U ml<sup>-1</sup>) induced inhibition of NHE activity in cultured Caco-2 cells. Concentration-dependent effect of IFN- $\gamma$  (1000 U ml<sup>-1</sup>) after (c) 0.5 h and (d) 24 h of exposure. Columns or symbols represent means of seven experiments per group; vertical lines indicate s.e.m. values. \**P* < 0.05, values significantly different from control values using the Newman-Keuls test; #*P* < 0.05, values significantly different from values with IFN- $\gamma$  alone using the Newman-Keuls test.

100, 90 and 85 kDa in Caco-2 cells. The size of the NHE isoforms identified in this study is comparable to the size previously reported (Noel & Pouyssegur, 1995). In polarized cells in culture, the NHE3 isoform is exclusively restricted to the apical membranes; however, the NHE1 isoform is expressed in the apical and basal lateral membranes (Noel *et al.*, 1996).

As indicated in Figure 2a, the maximal inhibitory effect of IFN- $\gamma$  (1000 U ml<sup>-1</sup>) on NHE activity was obtained at 0.5 h with sustained inhibition up to 24 h exposure. Exposure of Caco-2 cells to 1000 U ml<sup>-1</sup> IFN- $\gamma$  during 24 h did not reduce cell viability when compared to vehicle-treated cells (102.7  $\pm$  4.1 vs 100  $\pm$  3.9% of live cells). To evaluate which NHE isoform was involved in the inhibitory effect of IFN- $\gamma$ , cells were coincubated with IFN- $\gamma$  (1000 U ml<sup>-1</sup>) plus caripor-

ide (300 nM) or S-3226 (300 nM) for 30 min before starting the sodium-dependent  $\text{pH}_i$  recovery. As shown in Figure 2b, inhibition of NHE activity by IFN- $\gamma$  (1000 U ml $^{-1}$ , 0.5 h exposure) was absent in cells treated with cariporide (300 nM), but not in cells treated with S-3226 (300 nM), suggesting that NHE1 is the predominant isoform inhibited by the cytokine (Figure 2b). The short- (0.5 h) and long-term (24 h) treatment with increasing concentrations of human IFN- $\gamma$  (10–1000 U ml $^{-1}$ ) resulted in a concentration-dependent decrease in NHE activity that was identical with either exposure time (Figure 2c and d). To evaluate whether inhibition of NHE1 by IFN- $\gamma$  was related to downregulation of NHE1, Caco-2 cells were treated with IFN- $\gamma$  (1000 U ml $^{-1}$ ) for 24 h and the abundance of total NHE1 and the abundance of biotinylated NHE1 quantified. As shown in Figure 3a, the abundance of total NHE1 was not altered after the long-term exposure (24 h) to IFN- $\gamma$ , but the abundance of biotinylated NHE1 was significantly increased (Figure 3b). The abundance of total or surface NHE1 was corrected for the amount of  $\beta$ -actin.

The next series of experiments were intended to explore the signaling pathways from IFN- $\gamma$  receptor stimulation downstream to NHE, namely the involvement of STAT, MAPK and PKC. STAT1, Raf-1, MEK1, ERK1/2 and p38 MAPK can be probed with specific inhibitors. EGCG specifically inhibits the tyrosine phosphorylation of STAT1, but not of STAT3 (Menegazzi *et al.*, 2001); EGC does not interfere with the tyrosine phosphorylation of STAT1 (Menegazzi *et al.*, 2001). GW 5074 is a selective Raf-1 kinase inhibitor (Lackey *et al.*, 2000). PD 98059 inhibits activation of MEK and therefore blocks the activation of ERK1/2 (Davies *et al.*, 2000). PD 98059 binds to MEK1 preventing its activation by upstream protein kinases, such as Raf (Alessi *et al.*, 1995). PD 98059 is therefore not a protein kinase inhibitor, but a compound that stops one protein kinase (Raf) from activating another (MEK1) (Davies *et al.*, 2000). p38 MAPK  $\alpha$  and  $\beta$  can be inhibited selectively with SB 203580 (Saklatvala *et al.*, 1996; Davies *et al.*, 2000). Assessment of signaling pathways used by IFN- $\gamma$  was conducted by using a single concentration (1000 U ml $^{-1}$ ) and a 0.5 and 24 h exposure of Caco-2 monolayers to human IFN- $\gamma$ . Cells were pretreated with inhibitors of interest 0.5 h prior to the addition of IFN- $\gamma$ , which was subsequently added to the culture well (inhibitor not washed out). As shown in Table 1, the Raf-1 inhibitor GW 5074, the MEK1 inhibitor PD 98059 and the p38 MAPK inhibitor SB 203580 effectively prevented the short-term inhibitory effect of IFN- $\gamma$  on NHE activity. By contrast, the STAT1 inhibitor EGCG (Table 1) failed to alter the short-term inhibitory effect of IFN- $\gamma$  on NHE activity. The long-term inhibitory effects of IFN- $\gamma$  (24 h exposure) were also evaluated, and the Raf-1 inhibitor GW 5074 (Table 1) markedly attenuated the long-term inhibitory effect of IFN- $\gamma$  (24 h exposure) on NHE activity. From the results shown above, one would expect the involvement of MAPK downstream MEK. To test this assumption, cells were treated with PD 98059 (10  $\mu\text{M}$ ) and SB 203580 (10  $\mu\text{M}$ ). As shown during the short-term treatment with IFN- $\gamma$ , both PD 98059 and SB 203580 completely attenuated the inhibition of NHE activity after long-term exposure to IFN- $\gamma$ . The STAT1 inhibitor EGCG (Table 1), but not the inactive analog EGC, prevented the long-term inhibitory effect of IFN- $\gamma$  (24 h exposure) on NHE activity. In the short-term series of experiments, NHE



**Figure 3** Abundance of (a) total NHE1 and (b) surface NHE1 in Caco-2 cells treated for 24 h with IFN- $\gamma$  (1000 U ml $^{-1}$ ). Western blots were repeated 4–6 times. Columns indicate relative density and represent the mean of five separate experiments; vertical lines indicate s.e.m. values. \* $P < 0.05$ , values significantly different from corresponding control values using the Student's *t*-test.

activity (in pH units s $^{-1}$ ) was not affected by GW 5074 (0.0033  $\pm$  0.0002 vs 0.0026  $\pm$  0.0002), EGC (0.0020  $\pm$  0.0003 vs 0.0030  $\pm$  0.0003) and EGCG (0.0020  $\pm$  0.0003 vs 0.0024  $\pm$  0.0003), whereas a significant ( $P < 0.05$ ) increase in NHE was observed after treatment with PD 98059 (0.0028  $\pm$  0.0003 vs 0.0053  $\pm$  0.0002) or SB 203580 (0.0028  $\pm$  0.0003 vs 0.0040  $\pm$  0.0003). In the long-term series of experiments, NHE activity (in pH units s $^{-1}$ ) was not affected by GW 5074 (0.0041  $\pm$  0.0003 vs 0.0050  $\pm$  0.0003), EGC (0.0033  $\pm$  0.0003 vs 0.0033  $\pm$  0.0002), EGCG (0.0033  $\pm$  0.0003 vs 0.0030  $\pm$  0.0003), PD 98059 (0.0050  $\pm$  0.0003 vs 0.0053  $\pm$  0.0002) or SB 203580 (0.0050  $\pm$  0.0003 vs 0.0058  $\pm$  0.0005).

**Table 1** Effect of GW 5074 (20 nM), PD 98059 (10  $\mu$ M), SB 203580 (10  $\mu$ M), EGCG (20  $\mu$ M) and EGC (20  $\mu$ M) on NHE activity after short- (0.5 h) and long-term (24 h) exposure to IFN- $\gamma$  (1000 U ml<sup>-1</sup>) in Caco-2 cells

	Short-term	Long-term
Vehicle	100 $\pm$ 7	100 $\pm$ 9
IFN- $\gamma$	69 $\pm$ 7*	57 $\pm$ 3*
GW 5074	100 $\pm$ 9	100 $\pm$ 8
GW 5074 + IFN- $\gamma$	112 $\pm$ 7	99 $\pm$ 7
Vehicle	100 $\pm$ 7	100 $\pm$ 10
IFN- $\gamma$	57 $\pm$ 4*	64 $\pm$ 9*
PD 98059	100 $\pm$ 14	100 $\pm$ 6
PD 98059 + IFN- $\gamma$	95 $\pm$ 9	104 $\pm$ 6
Vehicle	100 $\pm$ 10	100 $\pm$ 13
IFN- $\gamma$	64 $\pm$ 9*	58 $\pm$ 2*
SB 203580	100 $\pm$ 8	100 $\pm$ 5
SB 203580 + IFN- $\gamma$	116 $\pm$ 4	114 $\pm$ 10
Vehicle	100 $\pm$ 9	100 $\pm$ 13
IFN- $\gamma$	52 $\pm$ 6*	58 $\pm$ 2*
EGCG	100 $\pm$ 12	100 $\pm$ 10
EGCG + IFN- $\gamma$	62 $\pm$ 8*	103 $\pm$ 12
EGC	100 $\pm$ 8	100 $\pm$ 11
EGC + IFN- $\gamma$	68 $\pm$ 4*	52 $\pm$ 5*

Values indicate means of 12 experiments per group. \* $P$ <0.05, values significantly different from control values using the Student's *t*-test. The NHE activity (in pH units s<sup>-1</sup>) in vehicle-treated cells in the short- and long-term stimulation series was 0.0033 $\pm$ 0.0002 and 0.0050 $\pm$ 0.0003, respectively.

Because there is evidence that PKC may function as a serine kinase for STAT1 and as an upstream regulator of the p38 MAPK that plays an important role in biological responses to IFN- $\gamma$  (Watanabe *et al.*, 1995), it was decided to test whether PKC is involved in inhibition of NHE activity by IFN- $\gamma$ . Treatment of Caco-2 cells with PDBu (1–1000 nM) for 0.5 h resulted in marked inhibition of NHE activity (IC<sub>50</sub>=2.3 [0.1, 52.0] nM). Downregulation of phorbol ester-sensitive PKC isoforms, obtained with overnight (~18 h) incubation of cells in the presence of PDBu (100 nM) (Gomes & Soares-da-silva, 2002b; Pedrosa *et al.*, 2004), prevented the short-term (0.5 h exposure) inhibitory effects of PDBu on NHE activity (Table 2). NHE activity (in pH units s<sup>-1</sup>) was significantly ( $P$ <0.05) reduced after PKC downregulation (0.0030 $\pm$ 0.0002 vs 0.0020 $\pm$ 0.0001). On the other hand, the short-term inhibitory effects of PDBu on NHE activity were not altered by the Raf-1 inhibitor GW 5074 and the STAT1 inhibitor EGCG (Table 2) or the MEK1 inhibitor PD 98059 and the p38 MAPK inhibitor SB 203580 (Table 2). To inhibit the signaling pathway initiated with PKC activation that appears to be involved after short- and long-term exposure to IFN- $\gamma$ , it was decided to downregulate PKC activity, as described above. As shown in Figure 4, downregulation of phorbol ester-sensitive PKC isoforms also markedly attenuated the short-term (Figure 4a) and the long-term (Figure 4b) inhibitory effects of IFN- $\gamma$  on NHE activity.

The role of p38 MAPK in inhibiting NHE activity was also examined in Caco-2 cells treated for 0.5 h with the p38 MAPK agonist anisomycin. As shown in Figure 5a, anisomycin inhibited in a concentration-dependent manner NHE activity in Caco-2 cells. The inhibitory effects of anisomycin (100 nM)

**Table 2** Inhibitory effect of PDBu (100 nM) on NHE activity, effect of downregulation of phorbol ester-sensitive PKC isoforms (PKC dr, overnight treatment with 300 nM PDBu) on NHE activity after 0.5 h exposure to PDBu and effect of PD 98059 (10  $\mu$ M), SB 203580 (10  $\mu$ M), GW 5074 (150 nM) and EGCG (20  $\mu$ M) on NHE activity after 0.5 h exposure to PDBu

	Vehicle	PDBu
Vehicle	100 $\pm$ 6	45 $\pm$ 3*
PKC dr	100 $\pm$ 6	100 $\pm$ 8
PD 98059	100 $\pm$ 11	65 $\pm$ 4*
SB 203580	100 $\pm$ 5	67 $\pm$ 6*
GW 5074	100 $\pm$ 11	63 $\pm$ 4*
EGCG	100 $\pm$ 9	59 $\pm$ 6*

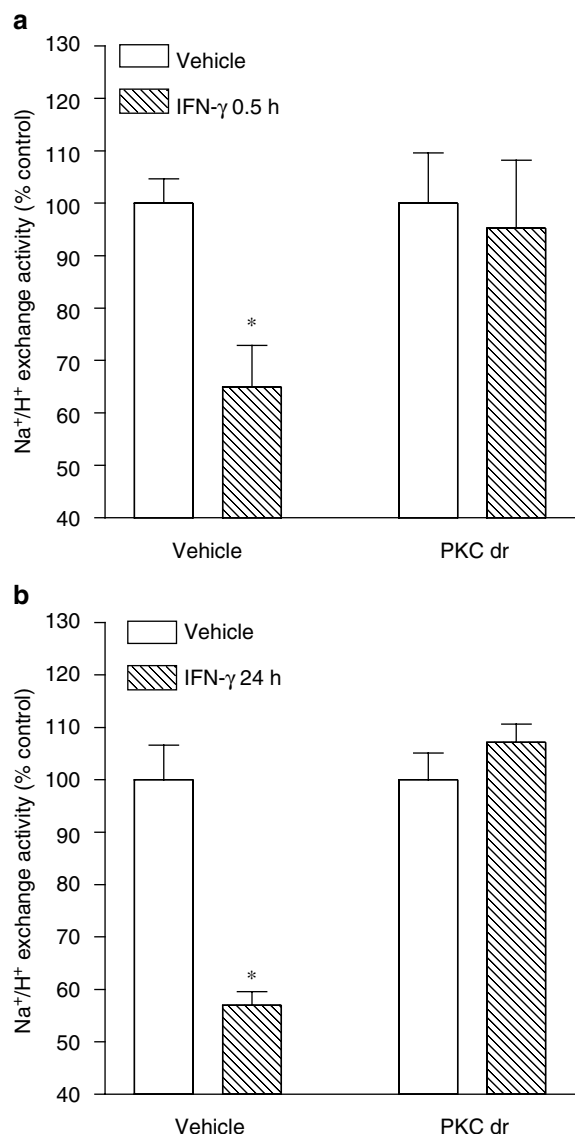
Values indicate means of 13 experiments per group. \* $P$ <0.05, values significantly different from control values using the Newman-Keuls test. The NHE activity in pH units s<sup>-1</sup> was as follows: vehicle, 0.0030 $\pm$ 0.0002; PKC dr, 0.0020 $\pm$ 0.0001; GW 5074, 0.0030 $\pm$ 0.0003; PD 98059, 0.0025 $\pm$ 0.0002; SB 203580, 0.0025 $\pm$ 0.0003; EGCG, 0.0035 $\pm$ 0.0003.

on NHE activity were not altered by the Raf-1 inhibitor GW 5074 and the STAT1 inhibitor EGCG, but were completely prevented by the p38 MAPK inhibitor SB 203580 (Figure 5b) and by the downregulation of phorbol ester-sensitive PKC isoforms (Figure 5b).

STAT, phospho-STAT and phospho-p38 MAPK activation was evaluated in Caco-2 cells exposed to IFN- $\gamma$  (1000 U ml<sup>-1</sup>) and anisomycin (100 nM) for 1, 3 and 24 h. Treatment with IFN- $\gamma$  for 24 h markedly increased total STAT and phospho-STAT (Figure 6a and c). However, 1 and 3 h treatment with IFN- $\gamma$  was found to increase phospho-STAT only (Figure 6a and c). Treatment with IFN- $\gamma$  resulted in marked activation of phospho-p38 MAPK, this effect being detected as early as 1 h, persisting over 3 h and decreasing after 24 h (Figure 6e). By contrast, the p38 MAPK activator anisomycin did not alter the expression of total STAT (Figure 6b) and phospho-STAT (Figure 6d) was not detected, although it markedly increased phospho-p38 MAPK (Figure 6f). The effects of anisomycin on phospho-p38 MAPK were evident as early as 1 h and faded after 3 h (Figure 6f). Activation of phospho-STAT after 24 h exposure to IFN- $\gamma$  (1000 U ml<sup>-1</sup>) was completely prevented by EGCG (20  $\mu$ M) (Figure 7b) and partially attenuated by SB 203580 (10  $\mu$ M) (Figure 7d). Downregulation of PKC following overnight exposure of cells to 100 nM PDBu failed to alter the activation of phospho-STAT after 24 h exposure to IFN- $\gamma$  (Figure 7f).

## Discussion

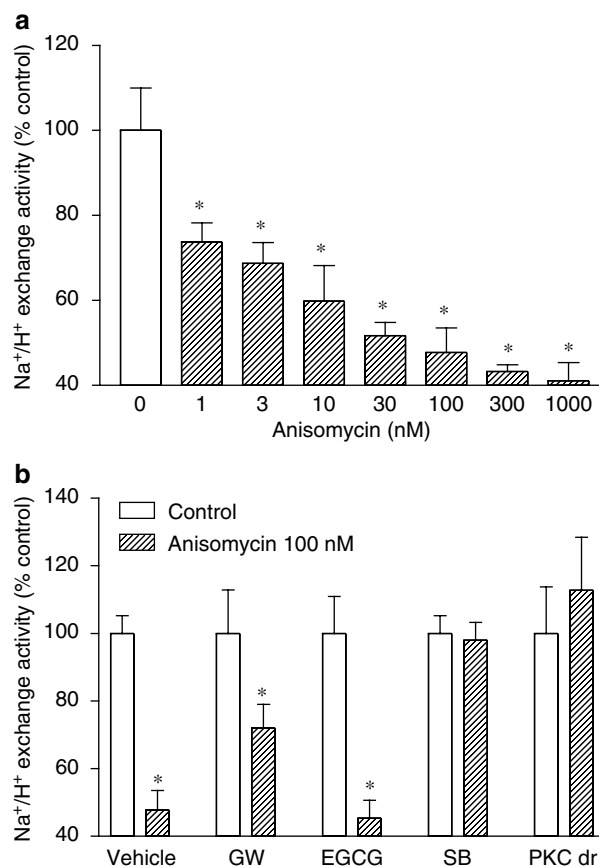
The present study was aimed at evaluate the effect of IFN- $\gamma$  on intestinal NHE activity and identify the intracellular signaling pathways set into motion after IFN- $\gamma$  receptor activation. A decrease in NHE1 activity after acute and chronic exposure to IFN- $\gamma$  that is not accompanied by downregulation of NHE1 and involves different signaling pathways is reported. The short-term regulation of NHE activity by IFN- $\gamma$  involves mainly the activation of Raf-1, MEK, p38 MAPK and PKC with NHE1 as an effector protein for PKC. The transduction mechanisms set into motion by long-term exposure to IFN- $\gamma$



**Figure 4** Effect of downregulation of phorbol ester-sensitive PKC isoforms (PKC dr) on (a) short-term and (b) long-term inhibitory effects of IFN- $\gamma$  (1000 U ml<sup>-1</sup>) on NHE activity. Columns represent means of seven experiments per group; vertical lines indicate s.e.m. values. \* $P < 0.05$ , values significantly different from control values using the Student's *t*-test. The NHE activity in pH units s<sup>-1</sup> was as follows: vehicle,  $0.0030 \pm 0.0002$ ; PKC dr,  $0.0020 \pm 0.0002$ .

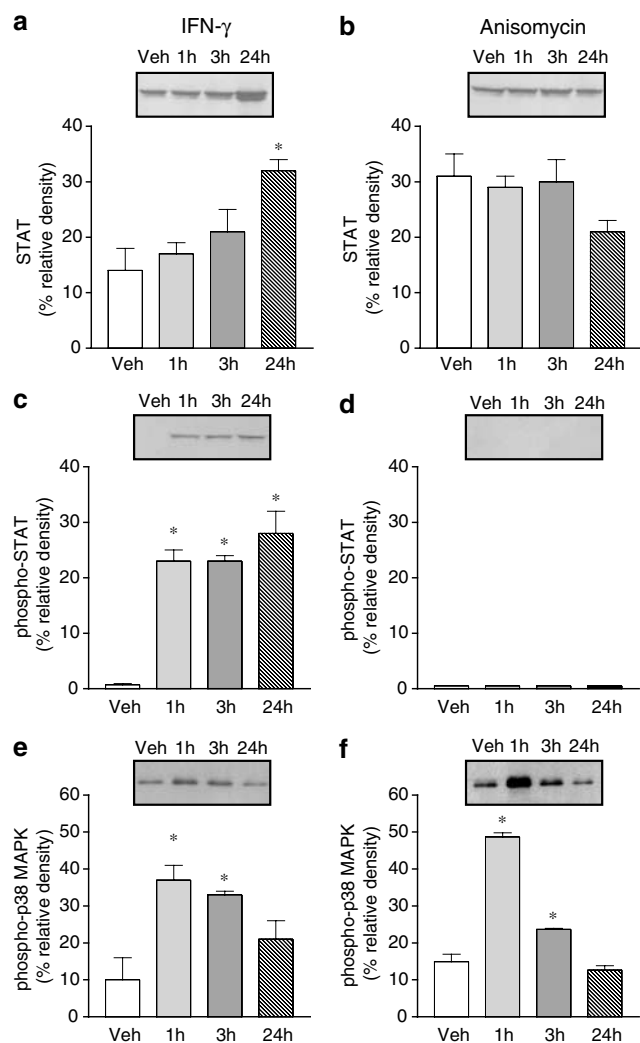
involve STAT1 activation with p38 MAPK playing a minor role in SER701 phosphorylation of STAT1.

Although inhibition of NHE activity by IFN- $\gamma$  has been reported before, the signaling pathways that initiate the IFN- $\gamma$ -induced inhibition and downregulation of NHE have not been examined. Some studies suggested that inhibition of NHE2 and NHE3 activities by IFN- $\gamma$  could be mediated by downregulation of NHE2 and NHE3 (Rocha *et al.*, 2001), but provided no insights into downstream events. On the other hand, these authors showed that downregulation of NHE2 and NHE3 occurred just after prolonged exposure to IFN- $\gamma$  (24 and 48 h), being devoid of effects as early as after 2 h exposure to the cytokine (Rocha *et al.*, 2001). The results in the present study provide evidence that inhibition of NHE activity by IFN- $\gamma$  occurs as early as 0.5 h exposure with a magnitude



**Figure 5** (a) Concentration-dependent effect of anisomycin (1–1000 nM) on NHE activity. (b) Effect of GW 5074 (150 nM), PD 98059 (10  $\mu$ M), SB 203580 (10  $\mu$ M), EGCG (20  $\mu$ M) and downregulation of phorbol ester-sensitive PKC isoforms (PKC dr) on NHE activity after 0.5 h exposure to anisomycin (100 nM). Columns represent means of seven experiments per group; vertical lines indicate s.e.m. values. \* $P < 0.05$ , values significantly different from control values using the Newman-Keuls test. The NHE activity (in pH units s<sup>-1</sup>) was as follows: vehicle,  $0.0030 \pm 0.0005$ ; GW 5074,  $0.0030 \pm 0.0005$ ; EGCG,  $0.0040 \pm 0.0004$ ; SB 203580,  $0.0033 \pm 0.0003$ ; PKC dr,  $0.0020 \pm 0.0003$ .

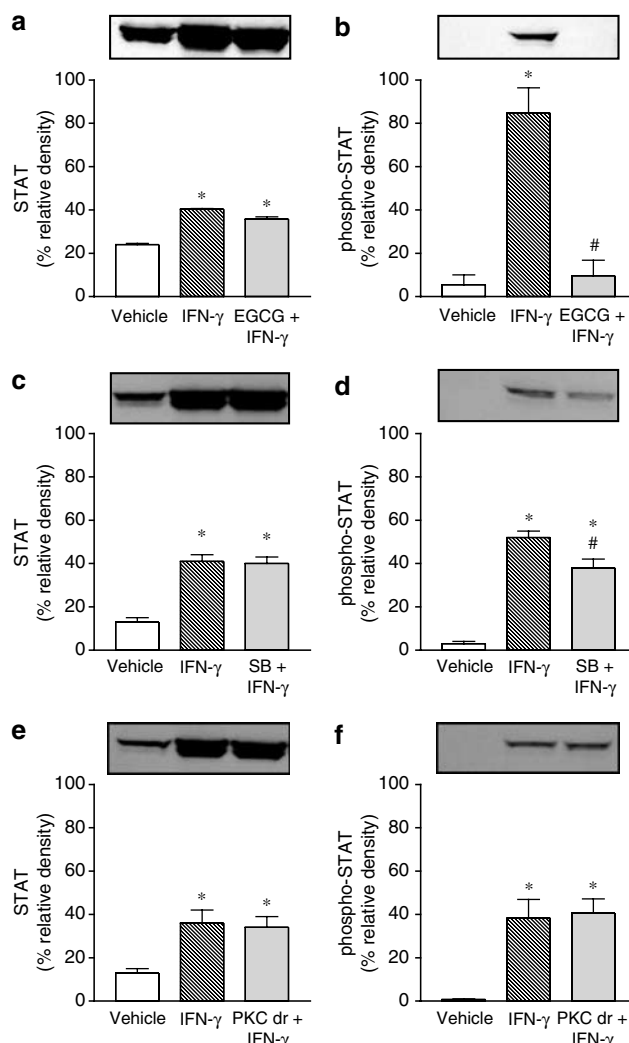
similar to that observed at 24 h. However, it is likely that the NHE isoform involved in IFN- $\gamma$ -mediated events is NHE1, although Caco-2 cells used in the present study expressed NHE1, NHE2 and NHE3, as previously described (Janecki *et al.*, 1999). There is no evidence in the literature for the presence of isoforms NHE4, NHE5 and NHE6 in Caco-2 cells. Differences between data provided by Rocha *et al.* (2001) and the present study may relate to differences in the origin of the cells; the present study was performed with the Caco-2 parental cell line, whereas the studies by Rocha *et al.* (2001) were performed in Caco-2BBE clonal cells (Peterson & Mooseker, 1992). NHE2 and NHE3 mainly serve the important purpose of acting as transport pathways whenever luminal Na<sup>+</sup> is present (Wormmeester *et al.*, 1998), the inhibition of which may be regarded as a potential cause of inflammation-associated diarrhea (Rocha *et al.*, 2001). The ubiquitously expressed NHE1 primarily serves the purpose of pH<sub>i</sub> and cell volume regulation, cellular growth and differentiation (Yun *et al.*, 1995; Wakabayashi *et al.*, 1997), the inhibition of which may also reduce the uptake of sodium chloride and water from the inflamed colonic lumen and



**Figure 6** Abundance of total STAT1, phospho-STAT1 and phospho-p38 MAPK in Caco-2 cells treated with vehicle and IFN- $\gamma$  (1000 U ml<sup>-1</sup>) or anisomycin (100 nM) for 1, 3 and 24 h.

thus contribute to diarrhea. On the other hand, it is interesting to underline the observations that levels of NHE1 protein and mRNA were significantly decreased in the colonic mucosa of patients afflicted with both CD and UC (Khan *et al.*, 2003); a decrease in  $pH_i$  in colonocytes from patients with CD was also described (Rowe & Bobar, 1995). This goes well with the suggestion that increases in NHE1 expression may contribute to  $pH_i$  and to cell growth and repair in colitis, and therefore, may be beneficial (Khan *et al.*, 1998). Recent studies have also implicated NHEs in the development of inflammatory response. It has been shown that amiloride reduced IL-8 production in response to IL-1 $\beta$  (Nemeth *et al.*, 2002a) and prevented the LPS-mediated I $\kappa$ B degradation (Nemeth *et al.*, 2002b). Furthermore, infection with *Escherichia coli* produced stimulation of NHE1 and NHE2, which was accompanied by significant reduction in NHE3 exchange activity (Hecht *et al.*, 2004).

Transduction mechanisms set into motion during short-term activation of IFN- $\gamma$  receptors in Caco-2 cells appear to involve the activation of Raf-1, MEK, p38 MAPK and PKC pathways. This is evidenced by the sensitivity to GW 5074,



**Figure 7** Effect of EGCG (20  $\mu$ M), SB 203580 (10  $\mu$ M) and PKC downregulation (PKC dr; overnight treatment with 100 nM PDBu) on the abundance of STAT1 and phospho-STAT1 in Caco-2 cells treated for 24 h in the absence (vehicle) and presence of IFN- $\gamma$  (1000 U ml<sup>-1</sup>).

PD 98059, SB 203580 and PKC downregulation of the IFN- $\gamma$ -induced inhibition of NHE1 activity. Although the sequence of events may be not entirely apparent, the findings reported here suggest different types of interactions between the intervening molecular entities with NHE1 as an effector protein for PKC. Firstly, the sensitivity to SB 203580, but not to GW 5074, of the anisomycin-induced inhibition of NHE activity supports the view that activation of p38 MAPK is downstream Raf-1 activation. Secondly, activation of p38 MAPK by IFN- $\gamma$  most likely occurs upstream PKC, as evidenced by the finding that downregulation of PKC prevents the anisomycin-induced inhibition of NHE activity. Thirdly, downregulation of PKC isoforms sensitive to PDBu prevents inhibition of NHE by IFN- $\gamma$ , anisomycin and PDBu. Finally, inhibition of NHE activity by short-term exposure to PDBu was insensitive to the Raf-1 inhibitor GW 5074, the MEK1 inhibitor PD 98059 and the p38 MAPK inhibitor SB 203580. Several other observations support the suggested sequence of events. In fact, MEK1 was found to scaffold the ERK2 cascade by binding



to each of the three protein kinases of the module, Raf-1, MEK1 and ERK2 (Karandikar *et al.*, 2000). The positions of the binding sites on MEKK1 suggest that the three kinases may be closely packed on the MEKK1 surface. Because MEK1 binds to both Raf-1 and ERK2 (Fukuda *et al.*, 1997), the proximity of their binding sites on MEKK1 might allow these kinases to interact with each other, thereby stabilizing the heteromeric MEKK1 scaffolded complex. Although the relationship between IFN- $\gamma$ , Raf/MEK/MAPK cascade and PKC is not apparent, several studies have suggested that IFN- $\gamma$  mediates its cellular effects *via* activation of PKC (Benveniste *et al.*, 1991; Lee *et al.*, 1995; Lin *et al.*, 1996; Flaishon *et al.*, 2001; Chang *et al.*, 2002). There is also evidence that IFN- $\gamma$  causes a rapid but transient increase in diacylglycerol, the endogenous activator of PKC (Benveniste *et al.*, 1991). Another finding that should be underlined concerns differences between NHE inhibition at 0.5 and 3 h exposure to IFN- $\gamma$ , which could be related to the degree of temporal activation of p38 MAPK.

The long-term regulation of NHE1 activity by IFN- $\gamma$ , in contrast to that observed during the short-term exposure to the cytokine, is suggested to primarily involve the activation of STAT1. In fact, the STAT1 inhibitor EGCG prevented both the inhibition of NHE activity and activation of phospho-STAT1 associated with the long-term exposure to IFN- $\gamma$ . Although preventable by the Raf-1 inhibitor GW 5074, the MEK1 inhibitor PD 98059 and the p38 MAPK inhibitor SB 203580, it is likely that activation of Raf-1, MEK and p38 MAPK during long-term exposure to IFN- $\gamma$  may correspond to events associated with the acute IFN- $\gamma$ -induced NHE1 inhibition. Alternatively, the binding of MEK1 to both Raf-1 and ERK2 and their vicinity with STAT1 might allow these kinases to interact with each other, which modulates both the Raf/MEK/MAPK and JAK/STAT signaling cascades (Stancato *et al.*, 1998). However, the results presented here may envision a relationship between p38 MAPK and STAT1 during long-term exposure to IFN- $\gamma$ . In fact, the prolonged exposure to SB 203580 partially prevented the increase in phospho-STAT1 expression after long-term exposure to IFN- $\gamma$ . This is compatible with the observation that when the IFN-

$\gamma$ -induced increase in phospho-STAT1 expression attained its maximum (24 h), the increase in phospho-p38 MAPK was no longer observed. On the other hand, the PKC-mediated events following the long-term exposure to IFN- $\gamma$  most likely correspond to events associated with the acute IFN- $\gamma$ -induced NHE1 inhibition and are not related to STAT1 phosphorylation. This possibility is supported by the observation that downregulation of phorbol ester-sensitive PKC isoforms, following overnight exposure of cells to 100 nM PDBu, failed to reduce the activation of phospho-STAT1 after 24 h exposure to IFN- $\gamma$ . Taken together, the results presented here strongly suggest that downstream IFN- $\gamma$  receptor activation to long-term regulation of NHE1 in Caco-2 cells, there is a chain of events comprising STAT1 activation with p38 MAPK playing a minor role in SER701 phosphorylation of STAT1. It is likely that STAT1 may have a role in the increased abundance of cell surface NHE1, which is presently under investigation. However, it is possible that increases in cell surface NHE1 may just relate to an adaptation to the prolonged IFN- $\gamma$ -induced inhibition of NHE1 activity.

In conclusion, the short- and long-term exposure to IFN- $\gamma$  results in decreases in NHE1 activity that do not result from downregulation of NHE1 and involves different signaling pathways. The short-term regulation of NHE activity by IFN- $\gamma$  involves mainly the activation of Raf-1, MEK, p38 MAPK and PKC with NHE1 as an effector protein for PKC. The transduction mechanisms set into motion by long-term exposure to IFN- $\gamma$  involve STAT1 activation with p38 MAPK playing a role in SER701 phosphorylation of STAT1. Keeping with the view that increases in IFN- $\gamma$ -secreting cells and IFN- $\gamma$  mRNA levels have been found in intestinal lesions of patients afflicted with IBD (Breese *et al.*, 1993; Niessner & Volk, 1995) and increases in NHE1 expression may contribute to cell growth and repair in colitis (Khan *et al.*, 1998), it might be then therapeutically adequate to prevent the IFN- $\gamma$ -mediated inhibition of NHE1 activity.

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