Short- and Long-Term Regulation of Intestinal Na\(^+\)/H\(^+\) Exchange Activity Associated with TLR2 Receptor Activation Is Independent of Nuclear Factor-κB Signaling

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ABSTRACT

Type 2 Toll-like receptors (TLR2s) are expressed in cell membranes and recognize a wide range of pathogen-associated molecular patterns derived from bacteria, such as lipoteichoic acid (LTA). The aim of this study was to evaluate the effect of TLR2 activation by LTA on the activity of type 1 Na\(^+\)/H\(^+\) exchanger (NHE) in T84 intestinal epithelial cells. Short-term (0.5 hour) and long-term (18 hours) TLR2 activation significantly inhibited NHE1 activity in a concentration-dependent manner (0.01–100 μg/ml; –7 ± 3 to –21 ± 3% and 3 ± 3 to –21 ± 3% of control values, respectively). S3226 [3-[(2-(3-guanidino-2-methyl-3-oxopropenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acylamide dihydrochloride], an NHE3-selective inhibitor, did not affect the inhibitory effect on NHE activity. LTA-induced NHE inhibition did not occur in the presence of ethylisopropylidene-2-methyl-acrylamide dihydrochloride, an NHE3-selective inhibitor. PKC, phospholipase C, and downregulation of protein kinase C prevented LTA-induced NHE1 inhibition, similar to that observed with the AC2 inhibitor KH7 [(2R)-2-(1H-benzimidazol-2-ylthio)propanoic acid 2-(5-bromo-2-hydroxyphenyl)methylene]hydrazide]. A significant increase in cAMP levels (32 ± 3% and 14 ± 2% after short- and long-term stimulation, respectively) was detected, and inhibition of protein kinase A (PKA), phospholipase C (PLC), and downregulation of protein kinase C (PKC) prevented NHE1 inhibition. Inhibition of nuclear factor-κB (NF-κB) failed to revert NHE1 inhibition. We concluded that activation of TLR2 reduces NHE1 activity in epithelial cells through an alternative pathway that is unrelated to NF-κB, which involves SCR, PI3K, AC3, PKA, PLC, and PKG.

Introduction

Maintenance of healthy intestinal epithelia is crucial to ensure optimal nutrient absorption, as well as an efficient immune barrier. Interactions between the intestinal microflora and the intestinal mucosal immune system have been described in the development of inflammatory bowel disease (IBD) (Kau et al., 2011; Khor et al., 2011). The balance between intestinal microflora, intestinal epithelium, and host immune system is crucial for normal cell work; therefore, changes in any of these three factors can influence intestinal epithelial function (Kau et al., 2011; Maloy and Powrie, 2011). Activation of innate immunity relies on the recognition of conserved microbial motifs known as pathogen-associated molecular patterns by pattern recognition receptors (PRRs). Abnormal activity of PRRs was suggested to play a key role in the development of IBD (Maloy and Powrie, 2011). The two major families of PRRs are the Toll-like receptors (TLRs) (Kawai and Akira, 2010) and the nucleotide-binding oligomerization domain (LeC et al., 2010) receptors. Type 2 TLRs (TLR2) are expressed in the cell membrane surface and participate in the recognition of a wide range of pathogen-associated molecular patterns derived from bacteria as lipoteichoic acid (LTA), a Gram-positive bacteria component (Akira and Takeda, 2004; Takeda and Akira, 2004a; Kawasaki and Akira, 2010).

ABBREVIATIONS: AC3, adenylyl cyclase; BCECF, 2/7-bis(carboxyethyl)-5,6-carboxyfluorescein; db-cAMP, N6,O2-dibutyl adenosine-3′-cyclic monophosphate sodium salt; FBS, fetal bovine serum; IBD, inflammatory bowel disease; H89, N-[2-[(4-bromophenyl)-2-propenyl]-aminomethyl]-5-isoquinoline sulfonamide dihydrochloride; IEC-6, intestinal epithelial cell line 6; IIK, IIK, IκB kinase; KH7, (2R)-2-(1H-benzimidazol-2-ylthio)propanoic acid 2-(5-bromo-2-hydroxyphenyl)methylene]hydrazide; LTA, lipoteichoic acid; LY294002, 2-morpholin-4-yl-8-phenylchromen-4-one; m-3M3, 2,4,6-trimethyl-N-[3-trifluoromethylphenyl] benzene sulfonamide; MLE, mouse lung epithelial (cell line); NF-κB, nuclear factor-κB; NHE, Na\(^+\)/H\(^+\) exchanger; PBS, phosphate-buffered saline; pHi, intracellular pH; PI3K, phosphatidylinositol 3-kinase; PP1, 1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amin; PP2, 3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; PRR, pattern recognition receptors; PLC, phospholipase C; PKA, protein kinase A; PKC, protein kinase C; siRNA, small interfering RNA; S3226, 3-[(2-(3-guanidino-2-methyl-3-oxopropenyl)-5-methyl-phenyl]-N-isopropylidene-2-methyl-acylamide dihydrochloride; SRC, tyrosine-protein kinase; T84, intestinal epithelial cell line; TLR, Toll-like receptor; U73,122, 1-[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]aminohexyl-1H-pyrole-2,5-dione.
The stability of cytoplasmic intracellular pH (pHi) within the physiologic range is critical for normal cell function, as many physiologic processes depend on it. Changes in pHi, namely, decreases in pHi, have been described in the inflammatory process as a requirement for internalization of pathogens and infection. Intracellular pHi is controlled mostly by two types of ion transporters: the Na+/H+ exchanger (NHE) and the Cl-/HCO3− exchanger. At present, nine different NHE isoforms (NHE1–9) have been identified in mammalian tissues. NHE isoforms differ in tissue and subcellular expression, drug sensitivity, and kinetics (Beltran et al., 2008). NHE1-5 and NHE8 are expressed in the cell surface, whereas NHE6, NHE7, and NHE9 are expressed in intracellular organelles. Among NHE isoforms found in the colon (NHE1, NHE2, NHE3, and NHE4), NHE1 is largely expressed and plays a key role in pH homeostasis and the control cell volume, whereas NHE3 primarily controls Na+ uptake and intracellular Na+ concentrations (Masereel et al., 2003).

The level of NHE protein and sodium pump activity was reduced (P < 0.05) in both untreated and treated patients with Crohn’s disease and ulcerative colitis (Khan et al., 2003; Siddique et al., 2009), and high TLR expression was reported (Campos et al., 2011). To understand the interplay between inflammation and NHE activity, the present study evaluated the short- and long-term regulation of intestinal Na+/H+ exchange during TLR2 activation induced by LTA and the intracellular signaling pathways set into motion. For this purpose, T84 intestinal epithelial cells, derived from a human colonic carcinoma, were used. T84 cells have been used as a model of ion transport and are known to express endogenously NHE1, NHE2, and NHE3 (Ramirez et al., 2000; Magro et al., 2007).

**Materials and Methods**

**Cell Cultures.** Human T84 cells (CCL-248), mouse lung epithelial (MLE)-12 cells (CRL-2110), and rat intestinal epithelial cell line-6 (IEC-6) cells (CRL-1592) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in a humidified atmosphere of 5% CO2, 95% air at 37°C. T84 and MLE-12 cells were grown in Dulbecco’s modified Eagle’s medium/ nutrient mixture F-12 (Corning Life Sciences, Tewksbury, MA), and IEC-6 cells were grown in Dulbecco’s modified Eagle’s medium. Growth media were supplemented with 10% FBS, 100 μg/ml streptomycin (Sigma-Aldrich, St Louis, MO), 10% FBS (Sigma-Aldrich) and 25 mM HEPES (Sigma-Aldrich). For subculturing, the cells were dissociated with 0.25% trypsin-EDTA, split 1:3, and subcultured in 21-cm2 growth area Petri dishes (Costar, Badhoevedorp, The Netherlands). The cell medium was changed every 2 days, and the cells reached confluence after 3 days of initial seeding. For studies on Na+/H+ exchanger activity, the cells were seeded in 96-well plates. For 24 hours before each experiment, the cell medium was free of fetal bovine serum (FBS). Experiments were generally performed 4 days after cells reached confluence, usually 7 days after the initial seeding.

**NHE Activity, NHE Kinetics, and pHi Levels.** NHE activity and NHE kinetics were assayed as the initial rate of pHi recovery after an acid load imposed by 20 mM NH4Cl, followed by the removal of Na+ from the Krebs’ modified buffer solution (140 mM NaCl, 5.4 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 0.3 mM NaH2PO4, 10 mM HEPES, 5 mM glucose, pH 7.4) in the absence of CO2/HCO3−. In these experiments, NaCl was replaced by an equimolar concentration of tetramethylammonium chloride. The cells were incubated in Krebs’ solution for 15 minutes, and then an acid load was imposed with NH4Cl during 5 minutes; subsequently, the NH4Cl was aspirated, and the cells were placed in tetramethylammonium chloride solution for an additional 5 minutes. Na+ (140 mM) then was added, and the pHi recovery was measured for 10 minutes. Intracellular pH measurements were performed in cells cultured in 96-well plates, as previously described (Gomes et al., 2001; Gomes and Soares-Da-Silva, 2004). Cell culture medium was 2%, 7% bis(carboxyethyl)-5,6-carboxyfluorescein aspirated, and the cell monolayers were incubated for 45 minutes with 5 μM 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein (BCECF/AM, the membrane-permeantacecytoxymethyl ester derivative of BCECF at 37°C in 5% CO2, 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 (short-term) or 18 hours (long-term) before starting the Na+-dependent pHi recovery. Na+ 140 mM for NHE activity assay, and Na+ 0–300 mM for NHE kinetics evaluation. Cells were placed in the sample compartment of a dual-scanning microplate spectrofluorometer (Spectramax Gemini; Molecular Devices, Sunnyvale, CA), and fluorescence was monitored every 17 seconds 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 pHi by comparison with values from an intracellular calibration curve using 10 μM nigericin and the high-K+ method, with pHi ranging from pH 6.6 to pH 7.8 (Gomes and Soares-Da-Silva, 2004).

In experiments aimed to evaluate the effect of protein kinase C (PKC), protein kinase A (PKA), or phospholipase C (PLC) activation or inhibition, cells were treated with the corresponding activators (100 nM PDBu [11αR,18βR,20S,22S]7β,7S,7β,8,9,9-[5α,10α]-16,14,4a,5,7a,8,8,9α-decalhydro-4a,7b-di-hydroxy-3-(hydroxymethyl)-1,1,6,8-tetra-methyl-5-oxo-1H-cyclopenta[3,4]benz[1,2-e]azulen-9,9-diyl butanoic acid ester) for PKC, 200 μM N6,02'-dibutylry adenosine-cyclic monophosphate sodium salt) (db-cAMP), or 3 μM forskolin [38R-3α,14β,15β,16α,17β,20α]-acetyl-3-ethenyl-dodecahydro-6,10,10b-trihydroxy-3,4,7,7,10α-pentamethyl-1H-naphtho [2,1-b]pyran-1-one] for PKA, 50 μM 2,4,6-trimethyl-N-[3-(triﬂuoromethyl)phenyl] benzene sulfonamide (m-3MS) FBS for PLC, or inhibitors [1 μM chelerythrine (1,2-dimethyl-12-methyl[1,3]benzodioxolo[5,6-c]phenanthridinium chloride) for PKC, 10 μM H89 [N-[2-[(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isooquinoline sulfonamide dihydrochloride] for PLC, and 3 μM U73,122 [1-β(2-[(17β-3-methoxy-3,5,10-trien-17-yl)amino]ethyl]-1H-pyrolo[2,5-dione] for PLC] on 0.5 hour or 18 hours before starting the sodium-dependent pHi recovery. To evaluate the influence of tyrosine-protein kinase (SCR), phosphatidylinositol 3-kinase (PI3K), and adenylyl cyclase 3 (AC3) in this signaling pathway, specific inhibitors were used [10 μM PP1 [1-(4,1,1-dimethyl-ethyl)-1-(4-methylphosphinyl)-1H-pyrazolo[3,4-d]pyrimidin-10-amine] and 10 μM PP2 [3-(4-chlorophenyl)1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine] for SCR, 20 μM LY294002 (2-morpholin-4-yl-5-phenylchromen-4-one) for PI3K, and 300 μM KH7 for AC3]. In experiments designed to evaluate the influence of NF-xB activation in this signal pathway, the NF-xB inhibitor PDTC (pyrrolidinedithiocarbamate; 10 or 100 μM) was used. In some experiments, to avoid the influence of NHE3, the experiments were performed in the presence of 100 nM S3226 [3-[2-(3-guanidino-2-pyrazolo[3,4-day]pyrimidin-4-amine] for SCR, 100 μM db-cAMP or 3 μM forskolin for PKA, 50 μM m-3MS FBS for PLC] or inhibitors [1 μM chelerythrine for PKC, 10 μM H89 for PKA, 3 μM U73,122 for PLC] and/or LTA for 0.5 or 18 hours before starting the experiments.

Cells were cultured in 96-well microplates and maintained until confluence at the same conditions as described already herein. PKA activity was evaluated with PKA kinase assay kit (ADI-EKS-390A) from Enzo (Farmingdale, NY), PKC activity was evaluated with PKC
kinase activity kit (ADI-EKS-420A) from Enzo, and PLC activity was evaluated with EnzChek Direct phospholipase C assay kit from Molecular Probes, Life Technologies S.A. (Madrid, Spain) according to each kit's specifications and the protocols provided.

**cAMP Activity.** To determine cAMP activity on LTA-induced TLR2 activation, cells were cultured in 96-well microplates and maintained until confluence at the same conditions as described already herein. Amersham cAMP Biotrak Enzyme Immunoassay System kit (RPN2251) from GE Healthcare (Buckinghamshire, UK) was used to determine cAMP activity according to the manufacturer's indications and protocol.

**Immunoblotting.** T84 cells cultured to confluence were washed twice with phosphate-buffered saline (PBS), and total cell protein was extracted for NHE1, NHE2, NHE3, TLR2, AC3, AC4, and AC6 detection. In brief, to obtain total cell extract, cells were lysed by sonication (15 seconds) 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 µg/ml phenylmethyl sulfonyl fluoride, and aprotinin and leupeptin 2 µg/ml each) and incubated on ice for 30 minutes. After centrifugation (13,200 g, 30 minutes, 4°C), the supernatant was mixed in 6× sample buffer (0.35 M Tris-HCl, 4% SDS, 30% glycerol, 9.3% dithiothreitol, pH 6.8, 0.01% bromphenol blue) and boiled

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**Fig. 1.** (A) Variation of pH in T84 cell line after an acute acid load in the presence or absence of S3226 (100 nM). (B) Variation of pH in T84 cell line after an acute acid load in the presence of LTA (10 µg/ml) and/or EIPA (10 µM). (C and D) Effect of different concentrations of LTA (0.01–100 µg/ml) in NHE activity in the presence (C) or absence (D) of S3226 (100 nM) in T84 cells.
for 5 minutes. The proteins (60 µg) were subjected to 10% SDS-PAGE and electrotransferred onto nitrocellulose membranes. The transblot sheets were blocked with 5% nonfat dry milk in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20 overnight at 4°C. The membranes then were incubated with appropriately diluted antibodies: rabbit anti-NHE1, anti-NHE2, or anti-NHE3 polyclonal specific antibodies (Alpha Diagnostics, Autogenbioclear, Wiltshire, UK); rabbit anti-TLR2 polyclonal antibody (Abcam, Cambridge, UK); rabbit polyclonal anti-AC3, anti-AC4, and anti-AC6; and the anti-GAPDH primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The reaction was detected by peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). The densities of the appropriate bands were determined using Quantity One imaging software (Bio-Rad Laboratories, Hercules, CA). Protein concentration was measured using the DC protein assay kit (Bio-Rad Laboratories) and bovine serum albumin as standard.

Cell-Surface Biotinylation. Cell-surface biotinylation was used to determine apical membrane NHE1, NHE2, NHE3, and TLR2 expression in T84 cells treated with LTA (10 µg/ml) or vehicle for 0.5 or 24 hours. In brief, confluent cells were rinsed twice with ice-cold PBS with 0.1 mM CaCl₂ and 1.0 mM MgCl₂ (PBS-Ca-Mg). The apical surface was then exposed to 500 µg/ml sulfo-NHS-biotin (Pierce, Rockford, IL) in biotinylation buffer [10 mM triethanolamine, 2 mM CaCl₂, 150 mM NaCl, pH 7.4] for 20 minutes with horizontal motion at 4°C. After labeling, the cells were rinsed with quenching solution (PBS-Ca-Mg with 100 mM glycine), and cells were lysed with radioimmunoprecipitation assay 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 µg/ml phenylmethyl sulfonyl fluoride, and aprotinin and leupeptin (2 µg/ml each)], briefly sonicated, and incubated on ice for approximately 1 hour. After centrifugation (13,200 g, 30 minutes, 4°C), the supernatant was adjusted to 3.4 mg/ml, and the biotinylated protein was precipitated overnight at 4°C with 100 µl of streptavidin-agarose beads (Pierce) in a total volume of 500 ml. 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].

Fig. 2. Effect of LTA (10 µg/ml) in NHE activity in the presence or absence of EIPA (10 µM). (A) Effect of LTA (10 µg/ml) in NHE activity in T84, MLE-12, and IEC-6 cells in the absence (vehicle) and the presence of LTA (10 µg/ml). (B) The experiments were performed in the presence of S3226 (100 nM). (C) Effect of short- and long-term administration of 10 µg/ml LTA on pHi in T84 cells. (D) [Na⁺] dependence of recovery from an acute acid load in the presence of 0.3 or 10 µg/ml LTA in the presence of S3226. Plots are means and vertical lines show S.E.M. of three experiments per group. *Significantly different from corresponding control values using the Student’s t test. #Significantly different from corresponding 10 µM EIPA values using the Student’s t test.
Finally, the cells were seeded in 96-well microplates with amino acid–free fresh medium in the presence of LTA (10 μg/ml) and siRNA pool. Cells were allowed to grow for another 24 hours in a humidified atmosphere of 5% CO₂, 95% air at 37°C. At the end of the experiment, NHE1 activity was assayed using the BCECF-AM assay, as previously described.

**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, followed by the Student’s t test or Newman-Keuls test for multiple comparisons. A P value less than 0.05 was assumed to denote a significant difference.

**Drugs.** Chelerythrine, db-cAMP, forskolin, H89, KT7, LY2994002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one hydrochloride], m-3M FBS, PDBu, P1P1, P2P2, P4T4, and U73,122 were obtained from Tocris Cookson Ltd (Bristol, UK). LTA from *Staphylococcus aureus* was obtained from Sigma-Aldrich. S3226 was kindly provided Dr. H. J. Lang from Aventis Pharma Deutschland (Frankfurt, Germany).

**Results**

**NHE Activity.** In the present study, NHE activity was assayed in cells loaded with a pH-sensitive dye (BCECF) as the Na⁺-dependent recovery of pH, measured after an acid load imposed by 20 mM NH₄Cl, followed by removal of Na⁺ from the Krebs’ modified buffer solution in the absence of CO₂/HCO₃. After acidification, cells showed rapid alkalization on the addition of 140 mM Na⁺ (Fig. 1A). As shown in Fig. 1B, the presence of 10 μM EIPA led to a strong inhibition of pH recovery. Ethylisopropylamiloride (EIPA) is more effective in inhibiting NHE1 than either NHE2 or NHE3 (Noel and Pouyssegur, 1995), and the results show a strong influence of NHE1 in the pH recovery in T84 cells. Short-term (0.5 hour) and long-term (18 hours) TLR2 activation significantly decreased NHE activity in a concentration-dependent manner (Fig. 1, C and D). Apical cellular expression of both NHE1 and NHE3 has been previously shown in polarized cells in culture (Noel and Pouyssegur, 1996; Magro et al., 2005). For these reasons, it seemed worthwhile to distinguish between the three major NHEs expressed in T84 (Noel et al., 1996; Magro et al., 2005). The presence of S3226 (100 nM), an NHE3 selective inhibitor, did not affect the inhibition of NHE activity after short-term and long-term TLR2 activation (Fig. 1, C and D). Thereafter, all subsequent experiments were performed in the presence of 100 nM S3226 to minimize the influence of NHE3, and the observed Na⁺-dependent S3226-insensitive pH recovery was assumed to reflect NHE1/NHE2 activity.

### TABLE 1

**K**ₐ and **V**ₐₐₛ values were determined using a Michaelis-Menten computer fit analysis.

<table>
<thead>
<tr>
<th>nM</th>
<th>ΔpH U/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.44 ± 0.54</td>
</tr>
<tr>
<td>LTA (0.3 μg/ml)</td>
<td>53.06 ± 1.80**</td>
</tr>
<tr>
<td>LTA (10 μg/ml)</td>
<td>64.98 ± 1.61**</td>
</tr>
</tbody>
</table>

**Significantly different from corresponding control values using the Newman-Keuls test.

**Significantly different from corresponding LTA (0.3 μg/ml) values using the Newman-Keuls test.

**TABLE 2**

NHE inhibition by LTA (10 μg/ml) for 0.5 or 18 hours in the presence of the corresponding inhibitors of SRC (PFP2, 10 μM), PI3K (LY294002, 20 μM), AC3 (KH7, 300 μM), PKA (H89, 10 μM), PLC (U73,122, 3 μM), and PKC (chelerythrine, 1 μM).

<table>
<thead>
<tr>
<th>Target</th>
<th>Inhibitor</th>
<th>Vehicle</th>
<th>LTA (10 μg/ml) 0.5 h</th>
<th>LTA (10 μg/ml) 18 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100.00 ± 3.24</td>
<td>75.57 ± 2.86*</td>
<td>83.83 ± 5.54*</td>
<td></td>
</tr>
<tr>
<td>NF-κB</td>
<td>PDTD (10 μM)</td>
<td>95.00 ± 4.57</td>
<td>75.5 ± 4.75*</td>
<td>78.8 ± 5.75*</td>
</tr>
<tr>
<td>NF-κB</td>
<td>PDTD (100 μM)</td>
<td>103.90 ± 5.01</td>
<td>81.8 ± 3.14*</td>
<td>82.9 ± 4.57*</td>
</tr>
<tr>
<td>SRC</td>
<td>PFP2 (10 μM)</td>
<td>101.40 ± 2.99</td>
<td>98.83 ± 3.88</td>
<td>100.09 ± 4.18</td>
</tr>
<tr>
<td>SRC</td>
<td>PFP2 (10 μM)</td>
<td>96.79 ± 3.96</td>
<td>96.98 ± 5.20</td>
<td>103.02 ± 3.08</td>
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<tr>
<td>PI3K</td>
<td>LY294002 (20 μM)</td>
<td>98.91 ± 5.66</td>
<td>101.67 ± 2.94</td>
<td>101.69 ± 4.23</td>
</tr>
<tr>
<td>AC3</td>
<td>KH7 (300 μM)</td>
<td>99.85 ± 3.45</td>
<td>97.65 ± 2.02</td>
<td>100.12 ± 3.57</td>
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<tr>
<td>PKA</td>
<td>H89 (10 μM)</td>
<td>100.07 ± 2.73</td>
<td>97.07 ± 3.75</td>
<td>95.65 ± 5.04</td>
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<tr>
<td>PLC</td>
<td>U73,122 (3 μM)</td>
<td>101.86 ± 2.07</td>
<td>96.26 ± 2.17</td>
<td>98.51 ± 2.26</td>
</tr>
<tr>
<td>PKC</td>
<td>Chelerythrine (100 nM)</td>
<td>98.84 ± 3.01</td>
<td>99.96 ± 3.64</td>
<td>94.19 ± 3.38</td>
</tr>
</tbody>
</table>

**Significantly different from corresponding vehicle values using the Newman-Keuls test.
differentiation between NHE1 and NHE2 activities was assessed by the use of 10 μM EIPA. As mentioned, EIPA is more effective in inhibiting NHE1 than NHE2 or NHE3 (Noel and Pouyssegur, 1995). Exposure to EIPA induced a reduction of ∼50% on NHE activity in T84 cells compared with corresponding control (Fig. 2A). As depicted in Fig. 2A, the NHE activity reduction induced by LTA alone was not observed when cells were exposed both to EIPA and LTA. These two findings suggest that NHE inhibition induced by LTA is mainly through the NHE1 isoform without involvement of NHE2 or NHE3. Inhibition of NHE1 activity by LTA was also observed in other cell lines, namely, in mouse lung epithelial MLE-12 and rat intestinal epithelial IEC-6 cells (Fig. 2B). Because NHE activity regulates pHᵢ (Masereel et al., 2003) and the stability of pHᵢ plays a critical role in normal cellular functions such as cell volume, proliferation, differentiation, survival, apoptosis, migration, and inflammation (De Vito, 2006; Malo and Fliegel, 2006), we assessed the pHᵢ in T84 cells after 10 μg/ml LTA, either short- or long-term. LTA concentrations determined to induce TLR2 activation are similar to those described in the literature (Canto et al., 2006; Satta et al., 2008). Both short- and long-term activation led to a reduction in pHᵢ (Fig. 2C). The smaller difference found in the long-term exposure to LTA might be due to possible compensation mechanisms set in motion by the cell to maintain pHᵢ. As a decrease in pHᵢ in T84 cells was seen after LTA exposure, we considered it worthwhile to evaluate the effect of TLR2 activation by LTA (0.3 or 10 μg/ml) on NHE kinetic parameters in human intestine epithelial T84 cells. Kinetic parameters determined in our experiments are similar to those observed in the literature (Raleysusman et al., 1991). As shown in Table 1, no differences in Vₘₐₓ were observed in cells exposed to 0.3 or 10 μg/ml LTA after 18 hours of treatment. On the other hand, Kₘ values were higher after 18 hours of 0.3 μg/ml LTA and even higher after 10 μg/ml LTA, although without significant statistical differences. These findings suggest a decrease in affinity for the substrate (Na⁺) in the presence of LTA.

The next series of experiments were designed to explore the signal pathways from TLR2 to NHE1, particularly the involvement of NF-κB, SRC, PI3K, AC3, PKA, PLC, and PKC. PDTC inhibits IKK phosphorylation and therefore prevents the consequent degradation by multiubiquitination; IKK remains attached to NF-κB, preventing its activation (Muriel, 2009). The contribution of SRC, PI3K, AC3, PKA, PLC, and PKC.

### TABLE 3

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activator</th>
<th>PKA [Forskolin (3 μM); db-cAMP (200 μM)]</th>
<th>PLC [m-3M3FBS (50 μM)]</th>
<th>PKC [PDBu (100 nM)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>78.4 ± 8.1°; 70.7 ± 2.3°</td>
<td>72.0 ± 4.0°</td>
<td>68.9 ± 2.5°</td>
<td></td>
</tr>
<tr>
<td>PKA (H89; 10μM)</td>
<td>96.7 ± 5.5; 105 ± 4.7</td>
<td>74.3 ± 3.1°</td>
<td>70.8 ± 3.8°</td>
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<tr>
<td>PLC (U73,122; 3μM)</td>
<td>95.1 ± 8.2; 92.1 ± 3.6</td>
<td>92.1 ± 3.4</td>
<td>70.9 ± 3.2°</td>
<td></td>
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<tr>
<td>PKC (Chelerythrine; 100 nM)</td>
<td>95.5 ± 4.6; 103.3 ± 4.3</td>
<td>94.1 ± 4.9</td>
<td>98.6 ± 3.7</td>
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*°Significantly different from corresponding control values using the Newman-Keuls test.
PKC was probed with specific inhibitors, respectively, PP1/PP2, LY294002, KH7, H89, U73,122, chelerythrine, and PDBu. KH7 is a selective AC inhibitor, being more selective to the AC-soluble isoforms at lower concentrations, but it is also possible to inhibit the AC membrane isoforms (such as AC3) at higher concentrations (i.e., >100 μM) (Langefeld et al., 2009). For this reason, and after testing different KH7 concentrations, we selected 300 μM to perform the experiments of interest. Cells were pretreated with the inhibitors 0.5 hour before the addition of LTA, which was subsequently added to the culture well (inhibitor not washed out). As depicted in Table 2, PDTC failed to reverse the inhibitory effect of LTA, which suggests that an alternative signaling to NF-κB might be involved in LTA-induced NHE1 inhibition. As shown in Table 2, SRC, PI3K, AC3, PKA, PLC, and PKC inhibitors successfully reversed the inhibition of NHE1 activity after short- and long-term treatment with LTA.

The influence of PKA, PLC, and PKC was determined using specific enzyme activators (forskolin for PKA, m-3M3FBS for PLC, and PDBu for PKC) and/or LTA in combination with the corresponding kinase inhibitors (H89 for PKA; U73,122 for PLC; and chelerythrine for PKC). As shown in Fig. 3, PKA, PLC, and PKC activities were increased after LTA exposure, as well as with the specific agonists (forskolin, m-3M3FBS, and chelerythrine, respectively) successfully reverted the effect of each kinase agonist and also reverted the increased LTA-induced activity, proving that PKA, PLC, and PKC activation is related to cell exposure to LTA. Subsequently, the sequence of events in PKA, PLC, and PKC signaling pathways was determined. In this set of experiments, cells were exposed to PKA-, PLC-, and PKC-specific activators and inhibitors of each of the three kinases. As shown in Table 3, all enzyme activators (forskolin and db-cAMP for PKA, m-3M3FBS for PLC, and PDBu for PKC) successfully reduced NHE1 activity. Thereafter, by combining these activators with the corresponding kinase inhibitors, it could be observed that inhibition of PKA by H89 did not prevent the inhibitory effects of PDBu and m-3M3FBS, which supports the view that PKA is upstream of PLC and PKC. PLC inhibition with U73,122 prevented the inhibition of NHE1 activity by db-cAMP and forskolin, but it had no effect on the inhibition of NHE1 activity induced by PDBu. This supports the concept that PLC might be, in this sequence of events, downstream of PKA but upstream of PKC. Finally, the inhibition of PKC by chelerythrine prevented the inhibition of NHE1 activity induced by PKA and PLC activators (Table 3), which is in line with the view that PLC is downstream of PKA and PLC. The relevance of cAMP as a second messenger in several cellular signaling events, including PKA-dependent responses, is a well-documented phenomenon (Pavan et al., 2009). Therefore, levels of cAMP were measured after 0.5 and 18 hours of exposure to LTA (10 μg/ml). cAMP levels increased after 0.5-or 18-hour exposure to LTA, being higher at 0.5 hour (32 ± 3% of control values) than at 18 hours (14 ± 2% of control values) (P < 0.05, Newman-Keuls).

SRC family kinases constitute one of the major kinase groups and are involved in a variety of signal transduction mechanisms (Bradshaw, 2010). The involvement of SRC kinase in the TLR2 phosphorylation and subsequent activation has also been reported (Chun and Prince, 2009). To determine the role of SCR on LTA-induced inhibition of NHE1 activity, two SRC inhibitors were used (PP1 and PP2). As depicted in Table 3, both PP1 and PP2 prevented the inhibitory effect of LTA. This finding supports the view that SRC is involved in TLR2 activation.

TLR2, AC3, AC4, AC6, and NHE1 Immunoblotting. Identification and quantification of TLR2, AC3, AC4, AC6, NHE1, NHE2, and NHE3 proteins were performed by means of Western blotting and quantified using densitometry. As shown in Fig. 4, abundance of NHE1 (A), NHE2 (B), and NHE3 (C) in T84 cell surfaces and cytoplasm after treatment with LTA (10 μg/ml) for 0.5 and 18 hours. Columns are means, and the vertical lines show S.E.M. of three experiments per group. *Significantly different from corresponding values for respective control using the Student’s t test.
of immunoblotting in protein extracts of T84 cells. As shown in Figs. 4 and 5, specific antibodies recognized the presence of all seven proteins in T84 cells. No statistically significant differences were found in the expression of NHE1, NHE2, or NHE3, both in the cell surface fraction and the cytoplasmic fraction, after treatment with LTA (10 μg/ml) (Fig. 4). The amount of TLR2 in the cell surface was significantly increased (17% ± 3% of corresponding control) after treatment with LTA (10 μg/ml) for 18 hours, but not after 0.5 hour (Fig. 5A). In terms of the cytoplasmic fraction of TLR2, no differences were found between treatments with vehicle or LTA (10 μg/ml) (Fig. 5A). Activation of adenylyl cyclase, and consequently cAMP production, has been described in many different regulatory processes (Sunahara and Taussig, 2002). AC is associated in numerous inflammatory processes, modulating cAMP levels (Pedrosa et al., 2004). To assess the influence of AC in this particular signaling pathway, the presence of three of the most widely expressed AC isoforms (AC3, AC4, and AC6) (Pierre et al., 2009) was evaluated. The total amount of AC3 was increased (23 ± 8% of control) after long-term treatment with LTA (10 μg/ml), but not after short-term activation of TLR2 (Fig. 5B). As shown in Fig. 5C, the expression of AC4 in T84 cells was not affected by long-term treatment with 10 μg/ml LTA. As depicted in Fig. 5D, long-term treatment with 10 μg/ml LTA reduced the expression of AC6.

Fig. 5. Abundance of TLR2 (A) and AC3 (B) in T84 cells surface and cytoplasm after treatment with LTA (10 μg/ml) for 0.5 and 18 hours. Abundance of AC4 (C) and AC6 (D) in T84 cells all cell lysate after 18 hours of exposure to LTA (10 μg/ml). Columns are means, and the vertical lines show S.E.M. of three experiments per group. *Significantly different from corresponding for values for respective control using the Student’s t test.
Fig. 6. (A) Effect of KH7 (300 μM) and/or PDBu (100 nM) in NHE1 activity. (B) Effect of KH7 (300 μM) and/or db-cAMP (200 μM) in NHE1 activity. (C) Effect of KH7 (300 μM) and/or m-3MFBS (3 μM) in NHE1 activity. (D) Effect of LTA (10 μg/ml), PDBu (100 nM), db-cAMP (200 μM), and m-3M3FBS (3 μM) on AC3 expression. Drugs exposure occurred 18 hours before the experiments. Columns are means, and the vertical lines show S.E.M. of three experiments per group. *Significantly different from corresponding for values for respective control using the Student’s t test.
Thereafter, to determine AC3 position in the cell-signal pathway initiated by TLR2 activation, two different sets of experiments were performed. First, T84 cells were treated with the AC inhibitor KH7, and PDBu, db-cAMP, or m-3M3FBS was added to cells to stimulate PKC, PKA, and PLC, respectively. As shown in Fig. 6, KH7 failed to prevent the inhibitory effect on NHE1 activity promoted by PKA, PLC, and PKC. Furthermore, AC3 blockade was also achieved by siRNA, as depicted in Fig. 7A. As observed in Fig. 7B, anti-AC3 siRNA prevented LTA-induced NHE1 inhibition, similarly to that observed with AC inhibitor KH7. In another set of experiments designed to evaluate the effect of PKA, PLC, and PKC activators on AC3 expression, none of the activators was able to mimic the increase in AC3 levels induced by LTA treatment (Fig. 6D). This finding suggests that AC3 may be activated before PKA, PLC, and PKC in the signal transduction pathway that leads to NHE1 inhibition during TLR2 activation (Fig. 8).

**Discussion**

Several studies correlated Na\(^+\)-impaired transport and IBD-associated diarrhea (Greig et al., 2004; Sullivan et al., 2009; Surawicz, 2010). NHE and Na\(^+\) transporters have been related to ion absorption deficiency in IBD (Greig et al., 2004; Sullivan et al., 2009; Surawicz, 2010). Activation of PRRs by microorganisms in the epithelial barrier was identified as one of possible triggers of IBD flares (Kau et al., 2011; Khor et al., 2011; Maloy and Powrie, 2011); however, the relationship between NHE and PRRS is largely unknown and poorly understood. For these reasons, the aim of this study was to evaluate the effect of TLR2 activation by LTA on intestinal NHE1 activity and identify the intracellular signaling pathway set into motion during TLR2 stimulation.

The activation of TLR2 led to significant decreases in NHE1 activity, after both short- and long-term exposure to LTA. This effect most likely involves only NHE1 as a result of its insensitivity to the NHE3 selective inhibitor S3226 and because it was no longer observed when the selective NHE1 inhibitor EIPA was used. The LTA-induced NHE inhibition was not limited to T84 cells, as this was also observed in other epithelial cell lines, such as MLE-12 and IEC-6.

Long-term LTA treatment induced an increase in the amount of TLR2 expressed in the cell surface without changes in NHE1, NHE2, and NHE3 membrane expression. Cell-surface biotinylation was performed to determine whether the inhibitory effect on NHE1 activity was due to the migration of NHE functional units to the cytoplasm or to a reduction in cell-membrane functional units. NHE1 kinetic parameters were evaluated, and it was hypothesized that the reduced NHE1 activity during TLR2 activation was probably due to a decrease in affinity to the NHE substrate (Na\(^+\)), rather than to loss of active exchanger units in the membrane surface. This suggestion is in line with that observed on NHE1 expression. Thereafter, the signal pathway between TLR2 and the effector proteins NHE1 exchanger was also evaluated. To evaluate the classic transduction mechanisms described after TLR2 activation by LTA (Akira and Takeda, 2004; Takeda and Akira, 2004b) PDTC, a potent NF-κB inhibitor (Muriel, 2009), was used. PDTC inhibits the phosphorylation of Ik-β kinase and therefore inhibits the activation of NF-κB (Traenckner et al., 1994). The results reported here show that the blockade of NF-κB failed to revert the inhibitory effect of LTA on NHE1 exchanger activity, which suggests that the transduction mechanism set in motion after TLR2 activation may correspond to an alternative signaling pathway.

![Figure 7](https://example.com/figure7.png)
Herein, we showed that activation of SRC, PI3K, AC3, PKA, PLC, and PKC pathways results in inhibition of NHE1 activity. This is evidenced by sensitivity of the LTA-induced inhibition of NHE1 activity to PP1/PP2, LY294002, KH7, H89, U73,122, and chelerythrine. The experiments reported suggest that SRC phosphorylation and PI3K recruitment do indeed occur after TLR2 activation. PI3Ks were found to be involved in mediating signals generated by bacterial recognition of various TLRs apart from TLR2 and is a tissue-specific event (Chun and Prince, 2009). Thereafter, AC3 is activated and appears to have a central role in LTA-induced inhibition of NHE1 activity. This suggestion is supported by AC3 blockade assays both with KH7 and siRNA anti-AC3 that prevented LTA-induced NHE1 inhibition during TLR2 activation by LTA. AC3 activation is suggested to take place upstream to activation of PKA, PLC, and PKC. Two types of findings support this suggestion: first, stimulation of PKC, PLC, and PKA with specific activators did not result in increases in AC3 expression; second, inhibition of AC3 with KH7 failed to affect the inhibition on NHE1 activity induced by PKA, PLC, and PKC activation. Activation of AC, and consequently cAMP production, has been described in many different regulatory processes (Sunahara and Taussig, 2002). ACs are associated with several inflammatory processes, modulating cAMP levels (Pedrosa et al., 2004). Therefore, it appears that there is a sequence of events initiated by PKA and cAMP, followed by recruitment of PLC and PKC. The PKA-, PLC-, and PKC-increased activity produced by LTA treatment was similar to the effect of each selective agonist: db-cAMP, 3-M3MFBs, and PDBu, respectively. Furthermore, the use of PKA, PLC, or PKC antagonists successfully revert the effect of LTA treatment.

Previous results from our group showed that stimulation of PKC induced NHE1 inhibition in colonic epithelial cell lines (Magro et al., 2005). Therefore, it appears that there is a sequence of events initiated by PKA and cAMP followed by recruitment of PLC and PKC. This is supported by the observations that the PKA inhibitor H89 did not prevent the inhibition by both PDBu and m-3M3FBs; the PLC inhibitor U73,122 prevented the inhibitory effect induced by db-cAMP and forskolin; no effect on the PDBu-induced inhibition of NHE1 activity was observed with PKA and PLC inhibitors; and the PKC inhibitor chelerythrine prevented the inhibition of NHE1 activity induced by both PKA and PLC activators.

This is in line with the findings that NHE1 appears to be an effector protein for PKC in epithelial intestinal cells (Akira and Takeda, 2004; Takeda and Akira, 2004a; Barton and Kagan, 2009; Langefeld et al., 2009; Kau et al., 2011; Khor et al., 2011; Maloy and Powrie, 2011).

Different protective processes have been described as involving TLR activation, such as intestinal epithelial restitution and strengthening of intercellular tight junctions (Maloy and Powrie, 2011). The results presented here on TLR2 overexpression after LTA treatment are in line with previous findings showing increased expression of TLR2 and TLR4 expression in macrophages of patients with IBD (Campos et al., 2011), suggesting an attempt to protect epithelial cells against pathogens. Furthermore, the importance of intracellular acidification in the development of the inflammatory process, as well as the role played by NHE1 as an important regulator in different immune responses, has been described (De Vito, 2006). NHE1 is the most expressed isoform in colonic lumen after bacterial exposition and serves mainly to maintain pH homeostasis and cell volume regulation, as well as cellular growth and differentiation (Yun et al., 1995; Wakabayashi et al., 1997; Magro et al., 2005). Inhibition of NHE1 may induce intracellular acidification and reduced Na\(^+\) uptake. Impaired ion absorption (namely, Na\(^+\)) and changes in intracellular pH values might contribute to ion malabsorption and consequently reduced water absorption, leading to diarrhea, which is a characteristic symptom in IBD flares (Kaser et al., 2010). Another interesting observation is related to the involvement of AC3 activation associated with cAMP production after treatment with LTA. This phenomenon was reported previously in human bladder epithelial cells and mast cells after IL-6 treatment (Song et al., 2007) but, to our knowledge, has not been previously described in intestinal epithelial cells.

In conclusion, short- and long-term exposure to LTA results in decreased activity of NHE1 exchange with no down-regulation of NHEs and with no changes in \(V_{\text{max}}\) values but with a decrease in affinity for Na\(^+\). The signal transduction pathway involves SCR activation and recruitment of PI3K and AC3, resulting in increased cAMP levels and activation of PKA. Activated PKA may lead to recruitment of PLC, the effect of which is activation of PKC as a final effector protein on NHE1 exchanger. This pathway is unrelated to the NF-\(\kappa\)B pathway, with TLR2 triggering a distinct and more rapid
signaling response involving AC3-generated cAMP. The understanding of this pathway may be of importance for the development of better therapeutic approaches in IBD.

Authorship Contributions

- Participated in research design: Cabral, Soares-da-Silva, Magro.
- Conducted experiments: Cabral, Magro.
- Performed data analysis: Cabral, Magro.
- Wrote or contributed to the writing of the manuscript: Cabral, Soares-da-Silva, Magro.

References


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