Na\(^+\)/H\(^+\) Exchanger 1 Is Regulated via Its Lipid-Interacting Domain, Which Functions as a Molecular Switch: A Pharmacological Approach Using Indolocarbazole Compounds

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ABSTRACT

The plasma membrane Na\(^+\)/H\(^+\) exchanger 1 (NHE1) is rapidly activated in response to various stimuli. The membrane-proximal cytoplasmic region (~60 residues), termed the lipid-interacting domain (LID), is an important regulatory domain of NHE1. Here, we used a pharmacological approach to further characterize the role of LID in the regulation of NHE1. Pharmacological analysis using staurosporine-like indolocarbazole and bisindolylmaleimide compounds suggested that the phorbol ester- and receptor agonist-induced activation of NHE1 occurs through a protein kinase C-independent mechanism. In particular, only indolocarbazole compounds that inhibited NHE1 activation were able to interact with the LID, suggesting that the inhibition of NHE1 activation is achieved through the direct action of these compounds on the LID. Furthermore, in addition to phorbol esters and a receptor agonist, okadaic acid and hyperosmotic stress, which are known to activate NHE1 through unknown mechanisms, were found to promote membrane association of the LID concomitant with NHE1 activation; these effects were inhibited by staurosporine, as well as by a mutation in the LID. Binding experiments using the fluorescent ATP analog tritrophenyl ATP revealed that ATP and the NHE1 activator phosphtidylinositol 4,5-bisphosphate binds competitively to the LID. These findings suggest that modulation of NHE1 activity by various activators and inhibitors occurs through the direct binding of these molecules to the LID, which alters the association of the LID with the plasma membrane.

Introduction

The ubiquitous Na\(^+\)/H\(^+\) exchanger isoform 1 (NHE1) catalyzes acid extrusion across the cell membrane by electroneutral ion exchange coupled to Na\(^+\) influx and serves as a key regulator of intracellular pH (pHi). Na\(^+\) concentration, and cell volume in virtually all tissues (Wakabayashi et al., 1997; Orłowski and Grinstein, 2004). NHE1 elicits relatively high exchange activity under unstressed physiologic conditions (basal state) and is further activated in response to various stimuli, including hormones, growth factors, and mechanical stress (activated state). This activation is attributable to a change in its affinity for intracellular H\(^+\); thus, it can be easily detected in the neutral pH range as a stimuli-induced cytoplasmic alkalinization in the absence of bicarbonate (Wakabayashi et al., 1997). Although NHE1 activation plays a physiologically important role in optimizing the intracellular ionic environment, it is also thought to promote the pathogenesis of disease, such as heart failure and cancer (Cardone et al., 2005; Karmazyn et al., 2008; Wakabayashi et al., 2013). In fact, treatment with NHE1 inhibitors reduced the pathologic phenotypes of animal models with acute and chronic heart diseases (Karmazyn et al., 2008).

The molecular mechanism of the hormonal activation of NHE1 has been the focus of many investigations. The stimulation of Gq-coupled receptors induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate \[\text{PtdIns(4,5)}^2\] via the activation of phospholipase C, which in turn produces two

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The plasma membrane Na\(^+\)/H\(^+\) exchanger 1 (NHE1) is rapidly activated in response to various stimuli. The membrane-proximal cytoplasmic region (~60 residues), termed the lipid-interacting domain (LID), is an important regulatory domain of NHE1. Here, we used a pharmacological approach to further characterize the role of LID in the regulation of NHE1. Pharmacological analysis using staurosporine-like indolocarbazole and bisindolylmaleimide compounds suggested that the phorbol ester- and receptor agonist-induced activation of NHE1 occurs through a protein kinase C-independent mechanism. In particular, only indolocarbazole compounds that inhibited NHE1 activation were able to interact with the LID, suggesting that the inhibition of NHE1 activation is achieved through the direct action of these compounds on the LID. Furthermore, in addition to phorbol esters and a receptor agonist, okadaic acid and hyperosmotic stress, which are known to activate NHE1 through unknown mechanisms, were found to promote membrane association of the LID concomitant with NHE1 activation; these effects were inhibited by staurosporine, as well as by a mutation in the LID. Binding experiments using the fluorescent ATP analog tritrophenyl ATP revealed that ATP and the NHE1 activator phosphtidylinositol 4,5-bisphosphate binds competitively to the LID. These findings suggest that modulation of NHE1 activity by various activators and inhibitors occurs through the direct binding of these molecules to the LID, which alters the association of the LID with the plasma membrane.

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The molecular mechanism of the hormonal activation of NHE1 has been the focus of many investigations. The stimulation of Gq-coupled receptors induces the hydrolysis of phosphatidylinositol 4,5-bisphosphate \[\text{PtdIns(4,5)}^2\] via the activation of phospholipase C, which in turn produces two
second messengers: diacylglycerol (DAG), which is a protein kinase C (PKC) activator, and inositol 1,4,5-triphosphate, which leads to the release of calcium ($Ca^{2+}$) from the endoplasmic reticulum through the inositol 1,4,5-triphosphate receptor (Taylor, 2002). $Ca^{2+}$ was suggested to activate NHE1 via the direct interaction of $Ca^{2+}$/calmodulin with the cytoplasmic domain of NHE1 during $Ca^{2+}$ mobilization (Wakabayashi et al., 1994a). However, the role of DAG in NHE1 activation is controversial. The potent PKC activators, phorbol esters (PES), activate NHE1; thus, PKC has long been believed to be a key molecule in the hormonal activation of NHE1 (Wakabayashi et al., 1997). However, NHE1 is not phosphorylated by PKC.

We recently hypothesized that NHE1 is activated by the direct binding of DAG and PES to the juxtamembrane cytoplasmic region of NHE1 and a subsequent conformational change of this region upon increased interaction with membrane lipids, rather than PKC (Wakabayashi et al., 2010). This region (aa 542–598) of NHE1 contains a cluster of cationic residues that interact with acidic phospholipids such as PtdIns(4,5)$^\text{P}_2$ (Aharonovitz et al., 2000; Fuster et al., 2004; Abu Jawdeh et al., 2011) and hydrophobic residues, which are predicted to constitute a lipid-binding motif; therefore, we tentatively refer to it as the lipid-interacting domain (LID).

Many studies to date have used protein kinase inhibitors to determine whether NHE1 is regulated by phosphorylation. However, the reported effects of these inhibitors have led to ambiguous conclusions in many cases (i.e., different inhibitors targeting the same kinases produced variable effects). We predicted that some kinase inhibitors may inhibit NHE1 regulation not via inhibition of PKC but through a direct interaction with NHE1. Indeed, we previously reported that one such compound, staurosporine, abolished the PE- and receptor agonist–induced activation of NHE1, probably by interacting directly with the LID (Wakabayashi et al., 2010). Staurosporine belongs to a class of indolocarbazole compounds that are potential anticancer drugs (Sanchez et al., 2006). Various indolocarbazoles developed for pharmaceutical applications work by competing with ATP at ATP-binding sites of protein kinases (Sanchez et al., 2006; Nakano and Omura, 2009). The finding that staurosporine, which structurally resembles ATP, potently inhibits NHE1 regulation (Wakabayashi et al., 2010) led to our recent discovery that NHE1 is an ATP-binding protein and that ATP may directly interact with NHE1 by interacting with the LID (Shimada-Shimizu et al., 2013). These findings raise the interesting possibility that the LID is a critical regulatory region that can interact with many activators and inhibitors; however, how NHE1 is regulated by these interactions remains unknown.

To address this question, we used a series of indolocarbazole and bisindolylmaleimide (BIS) compounds that are potent protein kinase inhibitors. We first screened for drugs that inhibit PE- or receptor agonist–induced activation of NHE1 and found that—with the exception of a few compounds related to staurosporine—NHE1 regulation was not inhibited by most of the tested drugs. Furthermore, a few inhibitors competed with ATP for direct binding to the LID. These results suggest that the LID functions as a molecular switch that dictates the activation state of NHE1 in response to various stimuli.

### Materials and Methods

#### Chemical Inhibitors and Other Reagents.

The template plasmid carrying human NHE1 cDNA cloned into the pEC2 mammalian expression vector has been previously described (Wakabayashi et al., 2000). All constructs were generated via a polymerase chain reaction (PCR)–based strategy as described previously (Wakabayashi et al., 2000). For construction of the green fluorescent protein (GFP)–tagged LID, the cytoplasmic region (aa 542–598) of NHE1 was amplified by PCR using pECE constructs as templates and inserted into the mammalian expression vector pEGFP-C1 (Clontech, Mountain View, CA) (Wakabayashi et al., 2010). For construction of myristoylated alanine-rich C kinase substrate (MARCKS)–GFP, the entire coding region of cloned human MARCKS was inserted into the pEGFP-N1 vector (Wakabayashi et al., 2010). The mouse $\alpha_1$-adrenergic receptor ($\alpha_1$-AR) cDNA was purchased from Invitrogen and cloned into the expression vector pT-REx-DEST30 (Invitrogen). The DNA sequences of the PCR fragments were confirmed using a Model 3130 autosenser (Applied Biosystems, Foster City, CA).

#### Cells and Transfection.

The wild-type and mutant NHE1 constructs were transfected into exchange-deficient PS120 cell lines (Poyseyegur et al., 1984) by Lipofectamine 2000 (Invitrogen), and stable NHE1 transfectants were prepared as described previously (Wakabayashi et al., 2000). NHE1 transfectants were further stably transfected with mouse $\alpha_1$-AR under selection with G418.

#### Measurement of Intracellular $\text{pH}$ Change.

Extracellular stimuli-induced changes in pH were measured using the dual-excitation ratiometric pH indicator BCECF/AM [2’,7’-bis(2-carboxyethyl)-5(6)-carboxyfluorescein/acetoxymethyl], as previously described (Wakabayashi et al., 2010). Briefly, cells expressing the wild-type or mutant NHE1 variants were serum-depleted for more than 2 hours and loaded with 0.3 $\mu$M BCECF/AM (Invitrogen) for 3 minutes at room temperature in Hepes-buffered saline (HBS) [140 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM glucose, and 20 mM Hepes/Tris, pH 7.0]. Cells were then placed in a flow chamber connected to a perfusion system and superfused (0.6 ml/min) with HBS at 35°C. Fluorescence was measured at 510–530 nm with alternating excitations at 440 and 490 nm through a 505-nm dichroic reflector. Images were collected every 10–20 seconds using a cooled CCD camera (ORCA-ER; Hamamatsu Photonics K.K., Hamamatsu, Japan) mounted on an inverted microscope (IX 71; Olympus, Japan).
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Tokyo, Japan) with a 20× objective (UApo/340; Olympus) and processed with AQUACOSMOS software (Hamamatsu Photonics). Changes in pH were monitored by switching perfusions from normal medium to HBS containing various reagents. The resting pH was calibrated as previously reported, using a high [K+] solution (containing 140 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, and 5 μM nigericin) and adjusted to various pH values ranging from 7.0 to 7.5. The change in pH was also measured by the [14C]benzoic acid-equilibration method (L’Allemain et al., 1984; Wakabayashi et al., 2010). In this experiment, serum-depleted cells were preincubated for 30 minutes in bicarbonate-free Hepes-buffered Dulbecco’s modified Eagle’s medium (pH 7.0) and then incubated in the same medium containing [14C]benzoic acid (1 μCi/ml) and various agents for 15 minutes at 37°C. For the experiments measuring drug effect, cells were preincubated for 15 minutes in Hepes-buffered Dulbecco’s modified Eagle’s medium containing each inhibitor and then switched to the radioisotope medium containing various stimuli and each inhibitor. After washing four times with ice-cold phosphate-buffered saline, the cellular uptake of [14C]-radioactivity was measured. The change in pH was calculated according to the following equation:

\[ \Delta pH = \log_{10}(14C_{stim})/14C_{rest} \]  

(1)

where [14C]stim and [14C]rest are the intracellular [14C]-radioactivity in the presence or absence of extracellular stimuli, respectively.

Confocal Microscopy. Exchanger-deficient PS120 cells or PS120 cells stably expressing wild-type NHE1 were transfected with the GFP-labeled LID (aa 542-598) or GFP-labeled MARCKS for 6-8 hours by Lipofectamine 2000. Cells were trypsinized and plated onto 35-mm glass-bottom dishes coated with collagen. Fluorescent signals were observed by confocal microscopy with an Olympus Fluoview FV1000 confocal microscope at 16-24 hours after trypsination. Various agents were added directly to dishes at 22-25°C. Fluorescence intensities in the whole cells and in areas including plasma membrane were measured after selection by eye. For the MARCKS transfection analysis, fluorescence intensities in the plasma membrane (I_mem) and cytosol (I_cyt) were measured using Image-Pro Plus software (Media Cybernetics, Inc., Rockville, MD) and the ratios (I_mem/I_cyt) before and after addition of stimulant were calculated. The dissociation index was calculated as (I_mem/I_cyt) before/(I_mem/I_cyt) after.

Trinitrophenyl-ATP Fluorescence Measurement. TNP-ATP fluorescence was measured at room temperature in a buffer containing 100 mM NaCl, 1 mM MgCl₂, and 10 mM Hepes/Tris (pH 7.4) using a fluorometer (F-7000; Hitachi, Tokyo, Japan) essentially as described previously (Guarnieri et al., 2011). Fluorescence data were obtained with emission and excitation wavelengths of 380 nm and 545 nm, respectively, using a quartz cuvette in a 500-μl reaction volume. To examine the interaction between TNP-ATP and the peptide GP57, we carried out titration experiments in which increasing concentrations of GP57 were added to a constant concentration of TNP-ATP (usually 20 μM). To obtain the value of the Kd for TNP-ATP, we applied the following Langmuir single-site binding equation (Eq. 2) for a curve fit:

\[
\Delta F = (\Delta F_{max}/2/T_1) \left( K_d + [P]_i + [T_1]_i \right) - \sqrt{\left( K_d + [P]_i + [T_1]_i \right)^2 - 4[P]_i [T_1]_i} \right)
\]

(2)

where ΔF is the observed fluorescence increase induced by the peptide, and [P]_i and [T_1]_i are the total peptide and TNP-ATP concentrations, respectively. In the presence of competitors like staurosporine, we applied the following equation (Eq. 3) as described previously:

\[
\Delta F = (\Delta F_{max}/2/T_1) \left( K_d + (K_d/K_d+c) [C]_i + [P]_i + [T_1]_i \right) - \sqrt{\left( K_d + (K_d/K_d+c) [C]_i + [P]_i + [T_1]_i \right)^2 - 4[P]_i [T_1]_i} \right)
\]

(3)

where K_d and [C]_i are the dissociation constant and total concentration of competitor, respectively. Curve-fitting was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA) and the best-fitted values with standard error were obtained.

Statistical Analysis. Values are presented as the mean ± S.D. from three or more determinations. Data were analyzed with the unpaired Student’s t test for comparisons between two mean values. A value of P < 0.05 was considered significant.

Results

Phorbol Ester- or Hormone-Induced Activation of NHE1 Is Inhibited by Only a Few Indolocarbazole Compounds. We first screened for drugs that inhibit the stimulus-induced activation of NHE1. As shown in Fig. 1A, phorbol-12-myristate-13-acetate (PMA) induced a rapid cytoplasmic alkalinization of approximately 0.2 pH units in the NHE1 transfectants, as determined by pH monitoring with BCECF pH indicator dye. Such PMA-induced cytoplasmic alkalinization was also observed when pH was monitored by the [14C]-benzoic acid equilibration method (Fig. 2B). We used these methods to determine whether various chemical compounds inhibit NHE1 activation. For example, consistent with the previous report (Wakabayashi et al., 2010), staurosporine almost completely abolished the PMA-induced activation of NHE1 (Figs. 1A and 2B). We challenged a screening of 70 chemicals (selected by virtual screening as described in Materials and Methods). One such chemical, N, 2-diphenyl-4-propylimidazo[1,2a]benzimidazole-1-carbothioamide (designated drug 1), had no apparent inhibitory effect (Fig. 1A). Surprisingly, no significant inhibitory effects on PMA-induced cytoplasmic alkalinization were detected for any of the 70 chemicals. In addition, none of these compounds alone was able to induce cytoplasmic alkalinization. The chemical structures and effects on cytoplasmic alkalinization of the 18 top-ranking chemicals based on the similarity screening are shown in Supplemental Figs. 1 and 2, respectively.

Fig. 1. Effects of various chemical compounds on the resting pH in the presence or absence of PMA. (A) Changes in pH were measured using the pH indicator dye BCECF-AM. PS120 cells stably transfected with the wild-type NHE1 were stimulated with PMA (1 μM) in the absence or presence of 1 μM of the indicated compounds. St, staurosporine; drug 1, N,2-diphenyl-4-propylimidazo[1,2a]benzimidazole-1-carbothioamide; K252a and K252c, staurosporine derivatives that possess an indolocarbazole ring. (B) Effects of EIPA (50 μM) and staurosporine (St) (1 or 10 μM) on the basal pH. Data are presented as means (n > 20 cells).
Given the results from screening of the initial 70 chemicals, we next focused on indolocarbazoles (e.g., staurosporine) and BIS, which are all commercially available as inhibitors of PKC or other protein kinases (see Supplemental Figs. 3 and 4 for chemical structures). We first assessed the effect of these compounds on the basal exchange activity in the physiologic neutral pHi range. Whereas staurosporine slightly reduced the resting pHi at 1 μM, its higher concentration (10 μM) resulted in a clear cytoplasmic acidification (∼0.2 pH unit) after a short lag phase, comparable to that observed for NHE1 inhibitor EIPA—induced acidification (Fig. 1B). However, staurosporine did not affect the surface expression level of NHE1 (Supplemental Fig. 5). These data are consistent with the previous finding that staurosporine inhibits the basal exchange activity by inducing the acidic shift of pHi dependence, without change in the Vmax (Wakabayashi et al., 2010). When the 30 compounds were assessed using the 14C-benzoic acid equilibration method for 15 minutes at 1 μM, most of these chemicals alone had no effect on basal pHi, although some drugs induced slight cytoplasmic acidification (Fig. 2A).

Next, we examined the effects of these 30 compounds on the activation of NHE1 in response to Gq-coupled receptor agonist. The NHE1 transfectants were further stably transfected with the α1-AR, and the cells were stimulated with the receptor agonist phenylephrine. Stimulation with phenylephrine resulted in a large cytoplasmic alkalinization only in the NHE1 transfectants expressing α1-AR (Fig. 3). Similar to the results for PMA-induced alkalinization, none of the compounds, other than staurosporine, had a strong inhibitory effect on phenylephrine-induced cytoplasmic acidification (Fig. 3). Notably, N-Bz-staurosporine, UCN-01, K252a, and lestaurtinib exerted only a marginal inhibition of phenylephrine-induced cytoplasmic
alkalinization at 1 µM (Fig. 3), although they were more effective when PMA was used (Fig. 2B). However, we observed that each of these four compounds resulted in almost complete inhibition of phenylephrine-induced cytoplasmic alkalinization when used at 10 µM (data not shown, but see Supplemental Fig. 7 for concentration dependence of several drugs). The results of this screen indicated that most protein kinase inhibitors, with a few exceptions, had no effect on PE- or phenylephrine-induced activation of NHE1.

Most Indolocarbazole Compounds Inhibit PKC in Cells. To determine whether the 30 indolocarbazole and BIS compounds could inhibit PKC at the concentration used for pH measurement (1 µM), we evaluated the effects of the compounds by using fluorescently tagged MARCKS, which is a PKC substrate known to dissociate from the plasma membrane in response to PKC-mediated phosphorylation (Ohmori et al., 2000). In fact, PMA resulted in a nearly complete dissociation of MARCKS-GFP from the plasma membrane after 15 minutes' incubation (Fig. 4A, leftmost panels). Staurosporine, UCN-01, and K252a, which inhibited the PMA-induced activation of NHE1, abrogated the PMA-induced MARCKS-GFP translocation at 1 µM (Fig. 4A). However, BIS1, BIS2, KT5823...


((9S,10R,12R)-2,3,9,10,11,12-hexahydro-10-methoxy-2,9-dimethyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid, methyl ester), and K252b ((9S)-2,3,9,10,11,12-hexahydro-10-b-hydroxy-9-methyl-1-oxo-9b,12b-epoxy-1H-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid), which did not affect the PMA-induced activation of NHE1, also almost completely abolished the PMA-induced MARCKS-GFP translocation at 1 μM (Fig. 4A). These data indicate that these compounds can inhibit PKC under the similar conditions used for pH measurement, regardless of their effects on NHE1 activation. Among the 30 compounds tested, all except for drug 1 and 5B218078 [9,10,11,12-tetrahydro-9,12-epoxy-1H-diindolo(1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-i][1,6]benzodiazocine-1,3(2H)dione] significantly inhibited MARCKS-GFP translocation, as shown by a decrease in the dissociation index (Fig. 4B), indicating that these 28 drugs were indeed capable of inhibiting PKC at 1 μM. Taken together, these data suggest that PMA or receptor agonist–induced activation of NHE1 occurs through a PKC-independent mechanism.

### Indolocarbazole Compounds that Block NHE1 Activation Interact with a Peptide Corresponding to the LID of NHE1.

As described, only a few drugs were shown to block the PMA- or agonist-induced activation of NHE1 (Figs. 2B and 3). We predicted that effective compounds such as staurosporine interact directly with the LID, which is the critical regulatory domain of NHE1. Attempts to determine whether staurosporine interacts with the LID were hampered by the nonspecific interaction of this drug with the affinity beads and dialysis bags used to study protein-ligand interaction and also by interference from the strong fluorescence of staurosporine. We assessed the interaction of various compounds with an LID-derived peptide (GP57) by using the fluorescent ATP analog trinitrophenyl ATP (TNP-ATP) (Guarnieri et al., 2011). Based on our previous finding that ATP binds to the LID (Shimada-Shimizu et al., 2013), it was expected that any molecule that can interact with the LID would compete with ATP for binding.

We first determined whether TNP-ATP is a suitable reporter for monitoring the interaction of compounds with the LID. The intensity of TNP-ATP fluorescence increased upon incubation with GP57, with a slight blue shift in the emission spectrum (Fig. 5A), indicating the interaction of TNP-ATP with GP57. TNP-ATP fluorescence reached saturation at higher GP57 concentrations (Fig. 5B); the $K_d$ for TNP-ATP was determined to be $28.0 \pm 1.8 \mu M$. The presence of nonfluorescent ATP (5 mM) reduced the affinity of TNP-ATP for GP57 (i.e., the titration curve did not show saturation at concentrations less than $150 \mu M$ of GP57) (Fig. 5B); the $K_d$ for ATP binding to GP57 was $430 \pm 43 \mu M$. Similarly, the inclusion of water-soluble PtdIns(4,5)P$_2$, a putative activator of NHE1, reduced the affinity of TNP-ATP for GP57 (Fig. 5B), with a $K_d$ of $22.3 \pm 6.9 \mu M$ for PtdIns(4,5)P$_2$ binding to GP57. These data suggest that PtdIns(4,5)P$_2$ and ATP compete with TNP-ATP for binding to the LID. The increase in TNP-ATP fluorescence was not observed upon incubation with a control peptide, IL54 (Fig. 5A). The mutation of two hydrophobic residues of the LID (Leu6573 and Ile6574) to alanine (LI-2A) also abolished the peptide-induced increase in TNP-ATP fluorescence (Fig. 5A), suggesting that these residues are critical for the binding of ATP to the LID.

A similar displacement assay was used to assess the interaction of chemical compounds with GP57. Whereas staurosporine itself emitted fluorescence (Fig. 6A, upper middle trace), it greatly reduced the GP57-induced fluorescence increase of TNP-ATP (Fig. 6A, compare upper left and upper middle traces), suggesting that staurosporine inhibits the interaction of TNP-ATP with GP57. Similarly, in the presence of UCN-01 or K252a, the GP57-induced fluorescence increase was reduced compared with the control (Fig. 6A). In contrast, BIS1 and arcyriaflavin A did not notably affect the TNP-ATP fluorescence (Fig. 6A). The effects of all 30 compounds on the GP57-induced increase in TNP-ATP fluorescence are shown in Fig. 6B. Several of the compounds that inhibited NHE1 activation at a low concentration (staurosporine, N-Bz-staurosporine, UCN-01, UCN-02, and K252a; see Fig. 2B) reduced the GP57-induced increase in TNP-ATP fluorescence (Fig. 6B). Conversely, the compounds that did not affect NHE1 activation did not affect TNP-ATP fluorescence (Fig. 6B). This apparent correlation between drug-induced inhibitions of NHE1 activation and TNP-ATP fluorescence is consistent with the notion that the inhibition of NHE1 activation occurs through a direct interaction of indolocarbazole...
compounds with the LID. Analysis of the dose dependence of GP57 in the presence of competitive drugs indicated that staurosporine, UCN-01, and K252a interact with GP57 with $K_d$ values of $1.70 \pm 0.05 \mu M$, $2.54 \pm 0.13 \mu M$, and $3.35 \pm 0.08 \mu M$, respectively (Fig. 7A). These drugs inhibited the PMA-induced activation of NHE1 within a similar range of concentrations (Fig. 7, B–D). These results provide evidence that these effective compounds inhibit the PMA- or agonist-induced activation of NHE1 through a direct interaction with the LID, and not by inhibiting protein kinase activity.

**Staurosporine Can Inhibit NHE1 Activation Induced by Okadaic Acid and Hyperosmotic Stress.** We next examined whether staurosporine could inhibit NHE1 activation induced by stimuli that are not known to activate PKC. We found that the phosphatase inhibitor okadaic acid, which was previously reported to activate NHE1 (Sardet et al., 1991), indeed induced a large cytoplasmic alkalinization (Fig. 8A). This activation was completely blocked by 1 $\mu M$ of staurosporine (Fig. 8A). At higher concentrations (>10 $\mu M$), staurosporine also inhibited NHE1 activation induced by hyperosmotic stress, which is also reported to activate NHE1 with unknown mechanism (Fig. 8B). Okadaic acid and hyperosmotic stress did not promote MARKCS-GFP dissociation from the plasma membrane in the presence or absence of staurosporine (Fig. 8, C and D), whereas PMA did so even in the presence of okadaic acid (Fig. 8C, right panels). These results indicate that PKC-independent mechanisms underlie the NHE1 activation induced by okadaic acid and hyperosmotic stress, as well as the staurosporine-mediated inhibition of NHE1 activation. Furthermore, in contrast to okadaic acid, all other phosphatase inhibitors did not affect pHi (Fig. 8A); thus, NHE1 activation by okadaic acid occurs through a mechanism, which is not due to the inhibition of protein phosphatase activity. Importantly, the mutation of hydrophobic residues in the LID (LI-2A) abolished or greatly reduced the NHE1 activation induced by okadaic acid and hyperosmotic stress (also observed for PMA and $\alpha_1$-AR agonist) (Fig. 8E), suggesting that the NHE1 activation induced by these stimuli also occurs through the LID.

**Staurosporine Inhibits the Plasma Membrane Translocation of the GFP-Labeled LID Induced by Various Stimuli.** We previously suggested that NHE1 activation occurs via a direct interaction of PEs/DAG and a subsequent conformational change of the LID accompanied by increased affinity...
with membrane lipids (Wakabayashi et al., 2010). To assess the effects of staurosporine on this process, we monitored changes in the membrane association using a GFP-labeled LID probe as described previously (Wakabayashi et al., 2010). Under unstimulated conditions, GFP-LID was localized mainly within the nucleus because of the nuclear localization tendency of GFP itself (Fig. 9, A and B). PMA promoted the plasma membrane translocation of GFP-LID (Fig. 9B), suggesting that PMA increases the affinity of the LID for the membrane lipids. This translocation was inhibited by staurosporine, which can interact with the LID, but not by BIS1 (Fig. 9B). Interestingly, phenylephrine (Fig. 9C), okadaic acid (Fig. 9D), and hyperosmotic stress (Fig. 9E) also promoted the plasma membrane translocation of the LID, which was inhibited by staurosporine in all cases. These results suggest that the extent of membrane association of LID determines the NHE1 activation induced by various stimuli.

The depletion of cellular ATP is known to inhibit drastically the exchange activity of NHE1 and render it inactive in response to various stimuli (Cassel et al., 1986; Little et al., 1988; Wakabayashi et al., 1997) (see Fig. 8E). We found that ATP depletion abrogated the PMA-induced plasma membrane translocation of the LID (Fig. 9F), whereas PMA was able to promote the nuclear export of the LID. Furthermore, in PMA-stimulated cells, incubation for 15 minutes in 5 mM 2-deoxyglucose, which was reported to deplete ATP to less than 2% (Shimada-Shimizu et al., 2013), gradually removed the LID from the plasma membrane (Fig. 9G). These data suggest that the continuous existence of ATP is required for the association of the LID with the plasma membrane.

Discussion

In this study, we addressed the role of the LID in regulation of NHE1 mainly by pharmacological approach as an extension of our previous work (Wakabayashi et al., 2010). Our new findings obtained in this work are summarized as follows: 1) Among diverse array of staurosporine derivatives or other tested compounds, only a few chemicals directly bind to the LID in competition with ATP at low concentration and effectively inhibits NHE1 activation in response to PEs and receptor agonist, providing evidence that inhibition of NHE1 regulation by these chemicals is mediated via direct action on the LID, rather than inhibition of protein kinases. 2) In addition to PEs and receptor agonist, okadaic acid and hyperosmotic stress promoted the translocation of a GFP-tagged LID to the plasma membrane concomitant with NHE1 activation, and these effects were inhibited by staurosporine, as well as the LID mutation. Thus, the LID functions as a key element to receive diverse signals and plasma membrane association of LID determine the activity-state of NHE1. 3) ATP depletion blocks the LID interaction to the plasma membrane concomitant with the drastic inhibition of NHE1 activity, and ATP and PtdIns(4,5)P_2 alternatively interact with the LID, suggesting that the basal exchange activity is preserved by the interaction of ATP and/or acidic membrane lipids to the LID.

Most indolocarbazole or BIS compounds are known to inhibit protein kinases such as PKC at submicromolar concentrations. Importantly, many of these chemicals did not inhibit NHE1 activation induced by PEs or receptor agonists at 1 μM (Figs. 2 and 3), whereas they did inhibit PKC under similar conditions, as shown by the inhibition of MARCKS-GFP removal from the plasma membrane (Fig. 4). One could argue that these compounds may be inert for certain PKC isoforms that are not involved in MARCKS phosphorylation. However, at least some of these drugs can inhibit various PKC isoforms at submicromolar concentrations. For example, BIS1 potently inhibits the PKCa, β1, βII, γ, δ, and ε-isoforms (Toullec et al., 1991; Heikkila et al., 1993) but did not inhibit NHE1 activation in our study. These data strongly suggest that a large part of NHE1 activation occurs through a PKC-independent mechanism. Furthermore, except for okadaic acid, no other potent phosphatase inhibitor induced cytoplasmic alkalinization (Fig. 9A). The inert properties of many protein kinase/phosphatase inhibitors argue against the concept that NHE1 activation in our study. These data strongly suggest that the basal exchange activity is preserved by the interaction of ATP and/or acidic membrane lipids to the LID.

The inhibition of NHE1 activation appears to occur via direct interactions of a few effective drugs with LID. Consistent with the previous 22Na^- uptake data (Wakabayashi et al., 2010), staurosporine reduced the resting pH_i (Fig. 1B), suggesting that staurosporine inhibits the basal exchange activity by directly interacting with NHE1, probably via the LID. Notably, the effective dose of the inhibitors varied with the different stimuli: for example, N-Bz-staurosporine, UCN-01, and lestaurtinib inhibited PMA-induced activation at 1 μM, but not receptor agonist-induced activation. Such a difference may be due to the activating substances that interact with the LID (PMA versus DAG).

In the present study, we found that PtdIns(4,5)P_2 reduces the binding affinity of TNP-ATP with the GP57 LID-derived peptide (Fig. 5B). Additionally, PtdIns(4,5)P_2 inhibited the equilibrium binding of [γ^{32}P]ATP to GP57 (data not shown).
These findings suggest that ATP can be at least partly replaced by PtdIns(4,5)P₂ as a binding partner of the LID. Acidic lipids such as PtdIns(4,5)P₂ and phosphatidylserine are known to interact with the cationic regions of the LID of NHE1, and the mutation of cationic or hydrophobic residues in the LID abrogated the interaction of the LID with acidic lipids and reduced the basal exchange activity (Aharonovitz et al., 2000; Wakabayashi et al., 2010). Furthermore, the present study indicated that one such mutation (LI-2A) blocked the interaction of the LID with TNP-ATP (Fig. 5A). These findings, together with previous data (Shimada-Shimizu et al., 2013), suggest that in addition to acidic phospholipids, ATP also plays an important role in maintaining the basal activity of NHE1. Indeed, a study using whole-cell patch-clamp technique has revealed that NHE1 activity, which had been once abolished by ATP depletion, was able to be restored by perfusion with PtdIns(4,5)P₂ inside the cells (Fuster et al., 2004). However, GFP-LID was localized primarily in the plasma membrane upon stimulation, but not in the resting state (Fig. 9). These findings raise the possibility that ATP, rather than acidic phospholipids, may be a major cofactor for preserving the basal exchange activity, and the exchange from ATP to acidic phospholipids occurs upon activation of NHE1 in response to stimulants, presumably via a conformational change of the LID.

Based on the current and previous data (Wakabayashi et al., 2010), we present a model for NHE1 regulation via the LID (Fig. 10). PEs/DAG directly interacts with the LID and induces the translocation of the LID to the plasma membrane, resulting in NHE1 activation. Staurosporine derivatives or LID mutations abolish these processes and thus inhibit NHE1 regulation. According to this model, movement of the juxtamembrane region of NHE1 (i.e., the LID) toward or away from the lipid bilayer determines the activation state of NHE1, possibly by modulating H⁺ affinity. From the negative results obtained using various protein kinase inhibitors, we propose that NHE1 phosphorylation makes a negligible contribution to PE- or agonist-induced NHE1 activation, consistent with the conclusions of our previous study (Wakabayashi et al., 1994b). In the resting state, ATP or acidic phospholipids would bind to the LID and maintain the basal exchange activity (Fig. 10). In contrast, ATP depletion would promote primarily the dissociation of ATP from NHE1 and simultaneously reduce the level of PtdIns(4,5)P₂ by facilitating its dephosphorylation. In fact, we observed that ATP depletion promoted the removal of the LID from the plasma membrane (Fig. 9, F and G). Thus, ATP depletion drastically inhibits basal exchange activity and renders NHE1 unresponsive for various stimuli. Indolocarbazoles directly interact with the LID and inhibit the interaction

![Fig. 8. Effects of staurosporine and the LID mutation on NHE1 activation induced by okadaic acid or hyperosmotic medium.](image-url)
of ATP or acidic phospholipids with LID. Several pieces of evidence in this study suggest that NHE1 activation in response to okadaic acid and hyperosmolarity may also occur via the LID: 1) staurosporine inhibited the activation of NHE1 induced by these stimuli (Fig. 8); 2) these stimuli facilitated the plasma membrane translocation of the LID, which was inhibited by staurosporine (Fig. 9, C and D); and 3) mutation of hydrophobic residues (LI-2A) in the LID abolished NHE1 activation in response to these stimuli (Fig. 8E). Thus, we propose that the LID is the key regulatory domain of NHE1, and it receives diverse signals that affect the modulation of exchange activity (i.e., the LID functions as a molecular switch). Thus, the pH dependence of exchange activity is controlled by species and effective doses of substances that interact with NHE1 through its LID.

The variable effects of protein kinase inhibitors that have been previously reported, in many cases, led to ambiguous conclusions, likely because the possibility of a direct action of these inhibitors on NHE1 was not considered. Our present results suggest the structural preference of a few chemicals for the LID of NHE1. The effectiveness of the compounds on NHE1 is clearly different from that on protein kinases. Staurosporine derivatives are indolocarbazole alkaloids containing similar structures, including an indole[2,3-a]carbazole core with a C-N linkage to a sugar moiety (see Supplemental Figs. 3 and 4). Since bisindolylmaleimide derivatives did not affect NHE1 at least at low concentration, the presence of at least the g-lactam ring and sugar moiety (see Supplemental Fig. 4) appears to be important for inhibition of NHE1 activation. The lactam ring and sugar moieties of staurosporine are analogous to the adenine ring and ribose moieties of ATP, respectively (Toledo and Lydon, 1997). In fact, these moieties were shown to be important for the interaction of staurosporine with ATP-binding sites in the crystal structures of staurosporine-bound phosphoinositide 3-kinase (Walker et al., 2000), cAMP-dependent kinase, and cyclin-dependent kinase 2 (Toledo and Lydon, 1997). Our present study provides an important step for elucidating the structural basis of NHE1 regulation.
The activation of NHE1 is linked to various diseases, such as heart failure and cancer. We previously reported that the activation of NHE1 is sufficient to induce cardiac hypertrophy and heart failure (Nakamura et al., 2008). Whereas NHE1 inhibitors such as cariporide, which completely shuts off the ion transport, were shown to prevent ischemia-reperfusion injury and chronic heart failure in animal models (Baartscheer et al., 2008; Karmazyn et al., 2008; Huber et al., 2012), they did not clearly show the beneficial effects on patients in several clinical trials (Karmazyn, 2013). A specific drug that selectively inhibits only the regulation of NHE1 while preserving its transport activity may be useful as a new therapeutic approach against heart failure.

Authorship Contributions

**Participated in research design:** Shimada-Shimizu, Wakabayashi.  
**Conducted experiments:** Shimada-Shimizu, Hisamitsu, Wakabayashi.  
**Contributed new reagents or analytic tools:** Hirayama.  
**Performed data analysis:** Shimada-Shimizu, Wakabayashi.  
**Wrote or contributed to the writing of the manuscript:** Nakamura, Wakabayashi.

References


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Na\textsuperscript{+}/H\textsuperscript{+} exchanger 1 is regulated via its lipid-interacting domain which functions as a molecular switch: A pharmacological approach using indolocarbazole compounds

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Department of Molecular Physiology, National Cerebral and Cardiovascular Center Research Institute (N.S.-S., T.H., T.Y.N., S.W.); and Basic Medical Science & Molecular Medicine, Tokai University School of Medicine (N.H.)
Supplemental Fig. S1. Chemical structures of 18 compounds used for the initial screening.

<table>
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<tr>
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<th>1. N-2-diphenyl-4-propylimidazo[1,2-a]benzimidazole-1-carboxyamide</th>
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<tr>
<td>2. 4-amino-2-[2-(2,2-dimethyl-3-oxoperazin-1-yl)-2-oxoethyl]6-(turan-2-yl)pymazin-3-one</td>
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<td>3. 2-[3-(4-methoxy-3-propoxypyrimidinyl)methylidene]-1H-indol-2-one</td>
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<td>4. 2-(2,3-dihydro-1,4-benzodioxin-6-yibopyrrolidin-1-yl)-1-(2-methyl-1H-indol-3-yl)propan-1-one</td>
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<td>5. N-(5-carbamoyl-2-pyridin-1-ylphenyl)-5-cyclopropyl-1,2-oxazole-3-carboxamide</td>
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<td>6. 1-N-(2,3-dimethoxyphenyl)-2-N-ethyl-2,3-dihydroidole-1,2-dicarboxamide</td>
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<td>7. 3aR,3a-[2-(1H-indol-3-yl)-2-oxoethyl]-8-methyl-3,3a,4,5,6,hexahydro-1H-pyrizino[3,2,1-kl]carbazol-3-lum</td>
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<td>8. 2-[2-methoxy-S-(2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-2-yl)-1-phenylmethyl]isodinole-1,3-dione</td>
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<td>9. 5-[2-(2-methylphenyl)amino]-3-[4-methylpiperazin-1-yl]pyrido[1,9-c][1,2]oxazol-6-one</td>
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<td>10. 5-[4-methoxyphenyl]amino]-3-[piperazin-1-yl]-6H-anthra[1,9-c][1,2]oxazol-6-one</td>
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<td>13. 10-[1H-indol-5-yl]-5-(4-methoxyphenyl)-1,3-dimethyl-1,7,8,10-tetrahydro-2H-pyrinoido[4_5;3,4;1]pyrrole[2,1-c][1,4]oxazine-2,4(3H)-dione</td>
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<td>14. 3-[6,7-dimethoxy-1-methyl-3,4-dihydroisoquinolin-2(1H)-yl]-1-(naphthalen-1-yl)pyrrolidine-2,5-dione</td>
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<td>15. 1,4-dimethyl-10-(1-methyl-1H-indol-3-yl)-5-phenyl-1,7,8,10-tetrahydro-2H-pyrinoido[4_5;3,4;1]pyrrole[2,1-c][1,4]oxazine-2,4(3H)-dione</td>
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<td>16. 6-(3,4-dimethoxyphenyl)-N,N,N-trimethylpyrazol-1(5H)-yl)pyrimidin-7-amine</td>
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<td>17. 2,11,17,21-tetrahydro-xephen-4-one-3,20-dione</td>
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<td>18. 7-acetoxyl-6-(chloromethyl)-6,6a-dihydroxy-9-methyl-3-methylidene-2-oxo-3a,4,5,6a,7,7b-hexahydroazuleno[4,5-b]turan-4-yl]2-methylbut-2-encate</td>
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Supplemental Fig. S2. Effects of various chemical compounds on PMA-induced cytoplasmic alkalinization

(A) Changes in pH$_i$ were measured using the [$^{14}$C]benzoic acid equilibration method 15 min after stimulation. NHE1 transfectants were stimulated with 1 μM of PMA or 1 μM of each compound. Drugs 1–18 had no ability to induce alkalinization alone. (B) Effects of drugs 1–18 on PMA-induced cytoplasmic alkalinization. NHE1 transfectants were stimulated for 15 min with 1 μM PMA in the absence or presence of 1 μM of each chemical. These drugs did not significantly inhibit PMA-induced cytoplasmic alkalinization. In these experiments (A and B), PMA-induced cytoplasmic alkalinization was in the range of 0.17–0.23 pH units when several different 24-well plates were used. The data were normalized to the values of PMA alone, and are presented as means ± standard deviation (n = 3).
Supplemental Fig. S3. Chemical structures of bisindolylmaleimide (BIS) and indolocarbazole derivatives
**Supplemental Fig. S3 (continued). Chemical structures of bisindolylmaleimide (BIS) and indolocarbazole derivatives**

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<th>K252b</th>
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- K252c, NGIC-I, SB210078, PKCδ inhibitor, PKCδ/EGFR inhibitor
- arcyriaflavin A, isogranulatimide, nabeccomycin, bacatacarin, lestaurtinib
Supplemental Fig. S4. Functional groups of staurosporine and BIS1
Supplemental Fig. S5. Effect of staurosporine on surface expression of NHE1

NHE1 transfectants were treated with 10 μM staurosporin for 15 or 30 min in phosphate-buffered saline containing 0.1 mM CaCl₂ and 1 mM MgCl₂. During the last 15-min incubation, a long-chain, NHS-ester activated biotinylated reagent (0.5 mM) (NHS-LC-biotin, Pierce Chem.) was added for labeling primary amines of proteins. Cells were lysed with 1% Triton X-100 and biotinylated proteins were collected using 30 μl of streptavidin agarose beads (Invitrogen). Input and biotinylated proteins (ppt) were analyzed using immunoblot with anti-NHE1 antibody and anti-GAPDH antibody (internal control) as described previously (Wakabayashi et al., 2000). In the leftmost lane (control), samples from cells not treated with NHS-LC-biotin were analyzed. The total expression level of NHE1 relative to GAPDH and biotinylated NHE1 (surface expression) was analyzed using imaging software. Data are presented as means ± standard deviation (n = 3). Staurosporine did not significantly affect the surface expression level of NHE1.

Supplemental Fig. S6. Concentration dependence of several chemical compounds on PMA-induced pH_i change

NHE1 transfectants were stimulated for 15 min with 1 μM PMA in the absence or presence of various concentrations of chemicals. PMA-induced cytoplasmic alkalinization without drugs was in the range between 0.17–0.23 pH units for different 24-well plates. Data are presented as means ± standard deviation (n = 3), normalized to the values of PMA alone, which were set to 1. Up to 10 μM concentrations of BIS1, BIS6, BIS9, KT5823, and K252c did not have significant effects on changes in pH_i, while UCN-02 significantly inhibited the alkalinization at more than 1 μM. *p < 0.05 versus phenylephrine alone.
Supplemental Fig. S7. Concentration dependence of several chemical compounds on phenylephrine-induced pH$_i$ change

NHE1 transfectants were stimulated for 15 min with 10 μM phenylephrine in the absence or presence of various concentrations of chemicals. Phenylephrine-induced cytoplasmic alkalinization without drugs was in the range between 0.15–0.20 pH units for different 24-well plates. Data are presented as means ± standard deviation (n = 3), normalized to the values of phenylephrine alone, which were set to 1. Concentrations of up to 10 μM of BIS1, BIS6, BIS9, KT5823, and K252c did not have significant effects on changes in pH$_i$. *p < 0.05 versus phenylephrine alone