NHE3 mobility in brush borders increases upon NHERF2-dependent stimulation by lyosphatidic acid

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Introduction
Lysophosphatidic acid (LPA) is the simplest of the water-soluble phospholipids. Once released as part of the inflammatory response from a cell, it elicits a variety of responses, including stimulation of fibroblast proliferation, suppression of apoptosis, platelet aggregation, cellular motility, tumour cell invasiveness, inhibition of cell-cell communication and neurite retraction (Moolenaar, 1999; Contos et al., 2000; Moolenaar et al., 1990; Moolenaar et al., 1995; Moolenaar et al., 1997; Gennero et al., 1999; Jalink et al., 1995; Retzer and Essler, 2000; Young et al., 2000). LPA levels are increased in the intestine of inflammatory bowel diseases, where it is thought to have reparative functions. LPA also directly exerts intestinal anti-secretory effects, acutely stimulating NHE3 activity and inhibiting cholera-toxin-induced secretory diarrhea (Li et al., 2005).

The BB epithelial Na⁺/H⁺ exchanger NHE3 is responsible for a major part of intestinal and renal Na⁺ absorption. Its activity is determined mostly by the amount of NHE3 on the brush border (BB). Under basal conditions, NHE3 cycles between the plasma membrane and the endosomal recycling compartment (Janecki et al., 2000; Donowitz et al., 2000; Lee-Kwon et al., 2001; Akhter et al., 2002; Kurashima et al., 1998; D’Souza et al., 1998). NHE3 is acutely stimulated and inhibited as part of digestive physiology by changes in trafficking. Given this regulation, it appears paradoxical that NHE3 is anchored to the actin cytoskeleton in two different ways. NHE3 directly interacts with ezrin, which is an actin-membrane-linking protein (Cha et al., 2006; Cha and Donowitz, 2008), as well as interacting indirectly via binding to the PDZ-domain-containing proteins, NHERF1 or NHERF2, which also bind to ezrin via their C-terminal ERM-binding domains (Yun et al., 1998; Donowitz and Li, 2007).

LPA stimulates NHE3 transport activity in polarized OK cells by increasing the surface amount of NHE3 via exocytotic trafficking to the apical surface by a mechanism that requires the PDZ-domain protein NHERF2 (but not NHERF1) and phosphoinositide 3-kinase (PI3K). The agonist studied was lysophosphatidic acid (LPA), an inflammatory mediator, which acutely stimulates NHE3 activity by increasing the amount of NHE3 on the BBs by stimulated exocytosis. LPA acutely stimulated NHE3 activity in OK cells stably expressing NHERF2. Two conditions that totally prevented LPA stimulation of NHE3 activity only partially prevented stimulation of NHE3 mobility: the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, and the NHE3F1 double mutant which has minimal direct binding of NHE3 to ezrin. These results show that LPA stimulation of NHE3 mobility occurs in two parts: (1) PI3K-dependent exocytic trafficking to the BB and (2) an increase in surface mobility of NHE3 in BBs under basal conditions. Moreover, the LPA stimulatory effect on NHE3 mobility required NHERF2. Although NHE3 and NHERF2 co-precipitated under basal conditions, they failed to co-precipitate 30 minutes after addition of LPA, whereas the physical association was re-established by 50-60 minutes. This dynamic interaction between NHERF2 and NHE3 was confirmed by acceptor photobleaching Förster Resonance energy Transfer (FRET). The restricted mobility of NHE3 in BBs under basal conditions as a result of cytoskeleton association is therefore dynamic and is reversed as part of acute LPA stimulation of NHE3. We suggest that this acute but transient increase in NHE3 mobility induced by LPA occurs via two processes: addition of NHE3 to the BB by exocytosis, a process which precedes binding of NHE3 to the actin cytoskeleton via NHERF2-ezrin, and by release of NHERF2 from the NHE3 already localized in the apical membrane, enabling NHE3 to distribute throughout the microvilli. These fractions of NHE3 make up a newly identified pool of NHE3 called the ‘transit pool’. Moreover, our results show that there are two aspects of LPA signaling involved in stimulation of NHE3 activity: PI3K-dependent stimulated NHE3 exocytosis and the newly described, PI3K-independent dissociation of microvillar NHE3 from NHERF2.

Summary
The epithelial brush border (BB) Na⁺/H⁺ exchanger NHE3 is associated with the actin cytoskeleton by binding both directly and indirectly to ezrin; indirect binding is via attachment to NHERF family proteins. NHE3 mobility in polarized epithelial cell BBs is restricted by the actin cytoskeleton and NHERF binding such that only ~30% of NHE3 in the apical domain of an OK cell line stably expressing NHERF2 is mobile, as judged by FRAP analysis. Given that levels of NHE3 are partially regulated by changes in trafficking, we investigated whether the cytoskeleton association of NHE3 was dynamic and changed as part of acute regulation to allow NHE3 trafficking. The agonist studied was lysophosphatidic acid (LPA), an inflammatory mediator, which acutely stimulates NHE3 activity by increasing the amount of NHE3 on the BBs by stimulated exocytosis. LPA acutely stimulated NHE3 activity in OK cells stably expressing NHERF2. Two conditions that totally prevented LPA stimulation of NHE3 activity only partially prevented stimulation of NHE3 mobility: the phosphoinositide 3-kinase (PI3K) inhibitor LY294002, and the NHE3F1 double mutant which has minimal direct binding of NHE3 to ezrin. These results show that LPA stimulation of NHE3 mobility occurs in two parts: (1) PI3K-dependent exocytic trafficking to the BB and (2) an increase in surface mobility of NHE3 in BBs under basal conditions. Moreover, the LPA stimulatory effect on NHE3 mobility required NHERF2. Although NHE3 and NHERF2 co-precipitated under basal conditions, they failed to co-precipitate 30 minutes after addition of LPA, whereas the physical association was re-established by 50-60 minutes. This dynamic interaction between NHERF2 and NHE3 was confirmed by acceptor photobleaching Förster Resonance energy Transfer (FRET). The restricted mobility of NHE3 in BBs under basal conditions as a result of cytoskeleton association is therefore dynamic and is reversed as part of acute LPA stimulation of NHE3. We suggest that this acute but transient increase in NHE3 mobility induced by LPA occurs via two processes: addition of NHE3 to the BB by exocytosis, a process which precedes binding of NHE3 to the actin cytoskeleton via NHERF2-ezrin, and by release of NHERF2 from the NHE3 already localized in the apical membrane, enabling NHE3 to distribute throughout the microvilli. These fractions of NHE3 make up a newly identified pool of NHE3 called the ‘transit pool’. Moreover, our results show that there are two aspects of LPA signaling involved in stimulation of NHE3 activity: PI3K-dependent stimulated NHE3 exocytosis and the newly described, PI3K-independent dissociation of microvillar NHE3 from NHERF2.

Key words: Brush border, NHE3, FRET, FRAP, Exocytosis, Cytoskeleton, NHERF2
(PI3K) (Lee-Kwon et al., 2003a; Choi et al., 2004) and also requires elevation of intracellular Ca²⁺. The presence of NHERF2 increases activation of LPA-induced phospholipase Cβ3 (PLCβ3) and the magnitude of the subsequent elevation of intracellular Ca²⁺ (Choi et al., 2004).

The anchoring of NHE3 to the actin cytoskeleton under basal conditions results in its having a very limited mobile fraction in the apical domain, as previously shown using confocal microscopy combined with FRAP (Cha et al., 2004). The current studies were undertaken to examine how NHE3 could be both fixed to the cytoskeleton and regulated by LPA by increased exocytosis and we investigated (1) whether NHE3 mobility in the BBs was dynamic as a part of acute NHE3 stimulation by LPA and (2) the role of NHERF2 in the LPA-dependent stimulation of NHE3 activity.

Results
LPA transiently stimulates the NHE3 mobile fraction in OK/NHERF2 cells
We previously reported that LPA acutely stimulates NHE3 activity in OK cells in a NHERF2- and PI3K-dependant manner as a result of stimulated exocytic trafficking (Lee-Kwon et al., 2003a). We also previously showed that under basal conditions there are two different pools of apical domain NHE3: a mobile and an immobile pool, the latter limited by binding to NHERF family PDZ-domain-containing proteins (Cha et al., 2004). In this study, FRAP was used to determine whether the LPA stimulation of NHE3 was associated with a dynamic effect on the mobile fraction of apical NHE3. To measure the time-dependent effect of LPA on the NHE3 lateral mobility, FRAP was performed before, and every 10 minutes after, LPA treatment for up to 70 minutes, as shown in Fig. 1A. The mobile fraction of NHE3 under basal conditions was ~30% (obtained by curve-fitting the FRAP data), with a plateau in fluorescence after bleaching, as previously reported, by ~150-200 seconds (Cha et al., 2004). LPA caused a significant stimulation of the NHE3 mobile fraction which first became significant 20 minutes after addition of LPA, peaked at 30 minutes (Fig. 1C), and returned to baseline by 50-60 minutes. Fig. 1B shows a representative experiment with fluorescent intensity determined before and after photobleaching of NHE3 at 30 minutes after LPA treatment (initial fluorescence set at 100). When we estimated the mobile fraction by curve-fitting the FRAP data with the Ellenberg equation (see Materials and Methods), which approximates one-dimensional diffusion, the equation fitted control data very well. By contrast, this equation did not fit the LPA-treated mobile fraction owing to failure of the recovered fluorescence to reach a stable plateau. Specifically, in contrast to the control, which plateaued post-bleach at ~150 seconds, LPA-treated cells exhibited increasing fluorescence intensity, although they demonstrated an intermediate plateau at 300-400 seconds (Fig. 1B). We show below that this is due to the presence of two components in this recovered fluorescence. Therefore, in these studies of the effect of LPA on mobile fraction of NHE3, we analyzed the NHE3 mobile fraction at a fixed time point after the bleach (t=400 seconds). Using this approach, the estimated NHE3 mobile fractions (Mf) were 30.8±3.5% for the control and 74.8±3.7% at 30 minutes after LPA exposure (Fig. 1C).

To show that the stimulatory effect of LPA on NHE3 mobility is specific to NHE3, we also performed FRAP studies in OK-NHERF2 cells with YFP-GL-GPI, a GPI-anchored protein present in the outer leaflet of the plasma membrane that does not associate with either the actin cytoskeleton or with PDZ-domain-containing proteins. LPA treatment for 30 minutes had no effect on the mobile fraction of YFP-GL-GPI (data not shown). These results indicate that the LPA stimulatory effect on NHE3 mobility in BBs is specific.

Because the effect of LPA on the NHE3 mobile fraction is a complicated parameter, which we predicted to contain two components (consisting of free resident apical NHE3 and newly exocytosed NHE3), we attempted to examine each component separately. Initially, the water-soluble crosslinker, Bis(sulfosuccinimidyl) suberate (BS3) was used to fix NHE3 in the apical domain as a way of quantifying the contribution of trafficking in the effects of LPA. The effects of BS³ on NHE3 activity (Fig. 2A), percentage of total NHE3 in the apical domain (Fig. 2B) and the NHE3 mobile fraction in the apical domain (Fig. 3C,D) were determined under basal conditions and 30 minutes after LPA exposure. BS³ treatment increased NHE3 activity, an effect that was similar in magnitude to that of treatment with LPA for 30 minutes; moreover, the effects of BS³ and LPA on NHE3 activity were additive (Fig. 2A). Mechanistically, BS³ treatment caused only a small (non significant) increase in surface NHE3 under basal conditions (Fig. 2B), which contrasted with its effects on the stimulation of NHE3 activity (Fig. 2A). We interpret this to indicate that crosslinking of NHE3 on BBs stimulates NHE3 turnover. The increase in the amount of surface NHE3 upon addition of BS³ plus LPA only minimally exceeded the increase caused by LPA alone. This is interpreted to indicate that BS³ does not alter changes in trafficking occurring with LPA. LPA was shown previously to stimulate NHE3 exocytosis without affecting NHE3 endocytosis, and the current studies indicate persistence in the LPA-induced
increase in balance of exocytosis-endocytosis towards stimulated exocytosis. As shown in Fig. 2C,D, after BS3 treatment for 30 minutes, LPA still caused a similar time-dependent increase in the NHE3 mobile fraction, although to a slightly less extent than with LPA in the absence of BS3. The mobile fraction (at t=400 seconds) of NHE3 in BS3-treated cells increased to 62.6±5% at 30 minutes after initiating LPA exposure (Fig. 2D). BS3 alone decreased the mobile fraction (Mf) of NHE3 from 30.8±3.5% to 16.2±1.8% (Fig. 2D). These results suggest that BS3 fixes NHE3 initially in the apical domain while allowing NHE3 trafficking to continue, albeit with a slightly extended extent of fixation.

Taken together, these results suggest that FRAP shows the dynamic addition of NHE3 to the BB by LPA-induced exocytic trafficking, even when the mobility of NHE3 initially on the BB is fixed by BS3. Since the stimulatory effect of LPA on NHE3 mobility was significantly decreased by pretreatment with BS3 (Fig. 2D), we suggest that the LPA stimulatory effect on NHE3 mobility contains two different components: (1) a contribution of NHE3 added to the BB by changes in its trafficking and (2) a population of NHE3 already present on the apical membrane that is dissociated from the cytoskeleton by LPA signaling. In the absence of BS3 treatment, this latter pool of NHE3 distributes throughout the microvilli before being again fixed to the cytoskeleton.

**Stimulatory effect of LPA on NHE3 mobile fraction is partially dependent on PI3K**

To further demonstrate that there are several components to the LPA-induced increase in the BB NHE3 mobile fraction, we prevented the stimulatory LPA effect on BB NHE3 activity or amount, initially by exposure to a PI3K inhibitor, LY294002, which we previously showed totally prevented the LPA-induced acute stimulation on NHE3 activity and increase in amount (Choi et al., 2004; Lee-Kwon et al., 2003b). LY294002 had no effect on the mobile fraction of BB NHE3 under basal conditions, as previously reported. The basal mobile fraction of NHE3 with LY294002 (Mf=33.6±4.1%) (Fig. 3A) was similar to the control (Mf=30.8±3.5% as shown in Fig. 1C). In spite of totally preventing LPA stimulation of NHE3 activity in OK cells, LY294002 treatment did not prevent the stimulatory effect of LPA on BB NHE3 mobility, although the magnitude of stimulation was decreased (Fig. 3A,B). The mobile fraction of NHE3 with LPA at 30 minutes in the presence of LY294002 still increased to Mf=63.1±5.2% compared with LY294002 treatment alone (Mf=33.6±4.1%). This suggests that LPA stimulation of NHE3 activity by increasing exocytosis is accompanied with an increase in NHE3 mobility related to this delivery. However, there is an additional LPA-induced increase in the NHE3 mobile fraction that occurs via a PI3K-independent mechanism, and thus in the absence of increased trafficking of NHE3 to the apical membrane. These results show that the stimulatory effect of LPA on BB NHE3 mobility is due both to a PI3K-dependent pathway with exocytic insertion of NHE3 into the apical domain and to an increase in the mobile fraction of the NHE3 pool initially on the BB, which is independent of PI3K.

**Stimulatory effect of LPA is significantly decreased in the direct ezrin-binding mutant**

We further demonstrated that LPA increases NHE3 mobility by the two mechanisms by studying the effect of LPA on mobility of an NHE3 mutant that fails to bind ezrin directly. This NHE3 mutant, NHE3F1D double mutant (NHE3FID), minimally binds ezrin, but still has a significant plasma-membrane pool, as we previously reported (Cha et al., 2006). NHE3FID had lowered basal NHE3 activity and lacked any change in NHE3 activity upon LPA exposure (Fig. 4A). NHE3 and NHE3FID were expressed similarly in OK-NHERF2 cells, as was NHERF2 (Fig. 4B). As reported, NHE3FID reduced basal NHE3 mobility (Mf=19.2±1.5%) compared with wild-type NHE3 (Fig. 4D) (Cha et al., 2006). However, LPA still stimulated NHE3 mobility in this mutant, but at a lower level than in the wild type (Fig. 4C,D). The mobile
LPA increases NHE3 brush-border mobility

**Fig. 4.** LPA stimulation of NHE3 mobility was significantly decreased in the ezrin-binding mutant NHE3F1D. (A) NHE3-WT and NHE3F1D were transiently transfected in OK NHERF2 cells. NHE3 transport activity (ΔpHi/minute) was measured with or without LPA (100 μM). Means ± s.e.m. were calculated from n=3 experiments. P values compare effect of LPA with untreated controls. (B) Protein expression of VSV-G tagged NHE3-WT and NHE3F1D detected using anti-VSV-G antibody. 20 μg and 30 μg of total cell lysate was loaded (upper panel). Similar amounts of NHERF2 are expressed in these cells (lower panel). (C) FRAP studies of NHE3F1D with LPA (100 μM) performed on OK NHERF2 cells. LPA was added at the time zero (arrow). A representative experiment is shown. (D) NHE3F1D mobile fraction with or without LPA (100 μM) measured at 30 minutes after addition of LPA. n=5. P represents comparison of LPA effect in NHE3F1D cells.

fraction of NHE3F1D at 30 minutes after LPA treatment was 39.2±4.5% compared with the control value of Mf=19.2±1.5% (Fig. 4D). Note the similar times of the maximum post LPA increase in NHE3 mobile fraction in cells expressing wild-type NHE3 and NHE3F1D. We conclude that in the absence of direct ezrin binding to NHE3, although LPA is not able to stimulate NHE3 activity or to increase the amount of apical membrane NHE3, LPA still increases the NHE3 mobile fraction. The latter process does not require direct ezrin binding to NHE3, although the magnitude of the effect was somewhat dependent on direct ezrin binding.

**NHERF2 is required for the LPA stimulation of NHE3 mobility**

LPA has no effect on NHE3 activity in OK cells which lack endogenous NHERF2 expression, whereas LPA stimulated NHE3 activity in OK cells exogenously expressing NHERF2 (Choi et al., 2004). We next asked whether the LPA stimulatory effect on NHE3 mobility also required NHERF2. FRAP studies were performed on OK cells lacking NHERF2. In the absence of NHERF2, the stimulatory effects of LPA on NHE3 mobility did not occur (Fig. 5A). Endogenous NHERF1 in OK cells without NHERF2 expression failed to support the stimulatory effect of LPA on NHE3 mobility. FRAP studies were also performed on NHE3-585 (residues 1-585) which is a deletion mutant that lacks all NHERF family binding regions (the NHERF proteins bind NHE3 at its C-terminus residues 586-660) (Yun et al., 1998). NHE3-585 also failed to show any stimulatory effect of LPA on the NHE3 mobile fraction as shown in Fig. 5B. The mobile fractions were ~50% in control and ~70% in NHE3-585, which was similar to our previously reported results showing NHERF2 fixed NHE3 to the BB (Cha et al., 2004). These results show that the stimulatory effect of LPA on both the NHE3 mobile fraction and NHE3 activity require NHERF2.

**NHERF2 ERM-binding domain is required for LPA stimulation of both NHE3 mobile fraction and transport activity**

The ERM-binding domain of NHERF1 or NHERF2 has a necessary role in the cAMP inhibition of NHE3 activity (Yun et al., 1998). Expression of NHERF2Δ30, which lacks the NHERF1 ERM-binding domain abolished cAMP regulation of NHE3 (Weinman et al., 2001). Therefore, we studied the role of the NHERF ERM-binding domain on the LPA effects on NHE3 activity and NHE3 mobile fraction. NHERF2Δ30 and wild-type NHERF2 were stably transfected into OK cells and their expression was found to be similar using an anti-NHERF2 antibody (Fig. 6A). We initially determined whether NHERF2Δ30 still supported the LPA effect on the transport activity of NHE3. The basal activity of NHE3 was significantly decreased in NHERF2Δ30 cells compared with cells expressing WT NHERF2. NHE3 activity (ΔpHi/minute) was 0.078±0.007 for NHERF2 cells and 0.045±0.004 for NHERF2Δ30 cells, respectively (n=3; P<0.05) (Fig. 6B). NHERF2Δ30 failed to reconstitute the LPA stimulation of NHE3 activity. Therefore this result shows that LPA stimulation of NHE3 activity requires the ERM-binding domain of NHERF2.

Next, we studied the role of the NHERF2 ERM-binding domain on the NHE3 mobile fraction. As shown previously, NHERF2 overexpression in OK cells limits NHE3 mobility (Cha et al., 2004). In this study, FRAP was determined in OK cells expressing NHERF2Δ30. The mobile fraction of NHE3 in cells expressing NHERF2Δ30 was increased similarly to that of control OK cells not expressing NHERF2 (Fig. 6C). The basal mobile fractions...
of NHE3, NHE3-NHERF2, and NHE3-NHERF2Δ30 were 47.5±2.2%, 29.1±3.1%, and 54.7±2.5%, respectively (Fig. 6C). We then studied the effect of LPA on NHE3 mobility in OK cells stably expressing NHERF2Δ30. The LPA stimulatory effect on NHE3 mobility did not occur in NHERF2Δ30 mutants. We then studied the effect of LPA on NHE3 mobility in OK cells expressing NHERF2Δ30. The low basal NHE3 mobility related to NHERF2 was abolished in NHERF2Δ30 mutant. Results from a single cell are shown with this pattern of mobility was seen in the majority of cells studied 10, 20, 30 and 40 minutes after LPA with return to a normal response by 50 minutes.

Membrane NHERF2 dynamically co-precipitates with NHE3 in a LPA-related time-dependent manner

Since the PI3K inhibitor LY294002 only partially blocked the stimulatory effect of LPA on NHE3 mobility, we investigated the increase in NHE3 mobility by LPA when exocytic insertion was blocked by LY294002 by studying the interaction of NHE3 with NHERF2. LPA was exposed to OK cells stably transfected with NHERF2 and HA-tagged NHE3 for 0, 30 and 60 minutes. NHERF2 was immunoprecipitated and the co-precipitation of HA-NHE3 was confirmed by immunoblotting. Representative results of three experiments are shown.

The dynamic dissociation of NHE3 from NHERF2 by LPA is confirmed by acceptor photobleaching FRET

In this study, we confirmed the temporal dissociation of NHE3 from NHERF2 by LPA treatment using FRET. The most commonly used pair of fluorescent proteins for FRET analysis are CFP and YFP. CFP was tagged to the N-terminus of NHERF2 and YFP was tagged to the C-terminus of NHE3. In supplementary material Figs S1-S4 we show that these NHERF2 and NHE3 constructs function (basal and regulated activity) similarly to the untagged proteins and that they physically associate with each other. FRET between NHE3-YFP and CFP-NHERF2 was demonstrated using a Zeiss 510/META to concentrate on interactions in the apical domain of polarized OK cells. FRET images were acquired before (Fig. 8A) and 30 minutes after (Fig. 8B) LPA exposure. FRET between NHE3 and NHERF2 was present initially but disappeared when studied 30 minutes after addition of LPA (Fig. 8E). To determine whether the FRET interaction between NHERF2 and NHE3 was specific, we examined whether FRET occurred between YFP-GL-GPI and CFP-NHERF2 (Fig. 8C,D). GPI-anchored proteins are located exclusively on the extracellular side of the plasma membrane and thus are not expected to physically associate with NHERF2, which is not an intrinsic membrane protein. Transiently transfected YFP-GL-GPI and CFP-NHERF2 were present in the apical domain of the OK cells and appeared to co-localize strongly with CFP-NHERF2 (Fig. 8C,D). This co-localization does not mean interaction and might be due to the lack of resolution of the confocal microscope. In fact, there was no FRET between YFP-GL-GPI and CFP-NHERF2 and LPA did not change the lack of FRET (Fig. 8E).

Discussion

These studies use assessment of the mobile fraction of NHE3 to newly define a pool of apical membrane NHE3 in polarized epithelial cells, which is part of acute stimulation of NHE3 by increased NHERF2. Note that LPA treatment had no effect on the co-precipitation of NHE3 with NHERF1 (Fig. 7A). Therefore, LPA stimulation of the BB NHE3 mobile fraction specifically depends on NHERF2, but not NHERF1, and the decrease in the physical association of NHE3 with NHERF2 correlates with changes in the NHE3 mobile fraction.
exocytosis. We call this the ‘transit pool’ (Fig. 9). We propose that this is a pool of NHE3 that trafficks to the intervillus cleft way-station in the apical domain and consists of newly exocytosed NHE3 plus NHE3 already localized to microvillar membranes that is transiently released from the cytoskeleton. We speculate that both NHE3 components normally take part in the stimulation of NHE3 activity, but we show here that the latter component of NHE3 occurs even if no further NHE3 is added to the apical domain.

Fig. 8. NHE3 transiently dissociates from NHERF2 after LPA exposure, demonstrated by disappearance of FRET. (A) FRET images in OK cells expressing NHE3-YFP and CFP-NHERF2 acquired before (top panel) and after (bottom panel) photobleaching under basal conditions. Top panel (before photobleaching) shows: CFP-NHERF2 (pseudocolored red), NHE3-YFP (pseudocolored green) and overlay image. Bottom panel (after photobleaching) shows: CFP-NHERF2 (pseudocolored red) and NHE3-YFP (pseudocolored green), demonstrating nearly complete bleaching. (B) As in A, except cells were exposed to LPA for 30 minutes before acquisition of FRET images, before (top panel) and after (bottom panel) the acceptor photobleaching. Top, CFP-NHERF2 (pseudocolored red), NHE3-YFP (pseudocolored green) and overlay image. Bottom (after photobleaching), CFP-NHERF2 (pseudocolored red), NHE3-YFP (pseudocolored green). NHERF2 and NHE3 are highly co-localized on the microvillar region of OK cells, but not in the juxtanuclear region. MetaMorph percentage localization program showed that ~50% of NHE3 co-localized with NHERF2, whereas ~90% of NHERF2 co-localized with NHE3. (C) FRET images in OK cells expressing YFP-GL-GPI and CFP-NHERF2 acquired before (top panel) and after (bottom panel) the photobleaching under basal conditions. Top panel shows FRET images before photobleaching and bottom panel after photobleaching. (D) FRET images as in 8C, except 30 minutes after LPA exposure. (E) FRET efficiencies in the OK cell apical domain calculated with MetaMorph software for NHE3-YFP and CFP-NHERF2 (left pair), and YFP-GL-GPI and CFP-NHERF2 (right pair) with or without LPA (100 µM, 30 minutes). Ten ROIs from the microvillar area were analyzed for each cell and at least seven cells were quantified from three different experiments. Results are means ± s.e.m.
The identity of this newly recognized NHE3 pool was based on the ability to use confocal microscopy to examine the apical domain in a polarized epithelial cell and the use of FRAP to separate NHE3 into a mobile pool that recovers in the apical domain after bleach and a pool that fails to recover. Several previous studies of apical-domain NHE3 mobile fractions have been reported under conditions that limited the contribution of NHERF3 trafficking to the ‘mobile’ fraction (Alexander et al., 2005; Alexander et al., 2007; Cha et al., 2004). Major differences in these approaches were use of an N-terminus extracellular HA-epitope-tagged NHE3 compared with a C-terminally GFP-tagged NHE3. The previous reports suggested how each technique could lead to artifacts, but in fact, the mobile fractions of NHE3 determined by the two methods were very similar: ~25% apical-domain mobile fraction in polarized MDCK cells using extracellular HA-tagged NHE3 and 25-30% apical-domain mobile fraction in polarized OK-NHERF2 cells using NHE3-EGFP.

Identification of the ‘transit pool’ in LPA regulation of NHE3 was made possible by identifying two conditions that prevented LPA stimulation of NHE3 activity and amount at the BB – PI3K inhibition and study of the NHE3F1D mutant, which has minimal direct ezrin binding. Surprisingly, it was initially shown that when NHE3 mobility in the apical domain was studied after LPA exposure, the NHE3 mobile fraction increased, even when PI3K was inhibited with LY294002, as shown here, or with wortmannin (not shown), which totally prevents an increase in NHE3 activity and amount (Lee-Kwon et al., 2003a). Increased apical-domain mobility in the absence of an increase in NHE3 activity or an increase in apical domain NHE3 similarly occurred when the direct ezrin-binding mutant NHE3F1D was studied. These results show that even in the absence of addition of newly trafficked NHE3 to the apical domain, LPA still increased the apical-domain mobile fraction of NHE3 and that this process is not PI3K dependent and occurred even with minimal direct binding of ezrin to NHE3. The similarity in effects on NHE3 mobility in the absence of an increase in PI3K activity and with minimal direct ezrin binding to NHE3 suggests that the same process accounts for the increase in apical mobility in both cases, although the magnitude of increased mobility was less in the NHE3 mutant that only minimally bound ezrin directly. We next attempted to identify this newly defined pool of apical-domain NHE3.

LPA stimulates NHE3 activity in OK cells by causing a NHERF2-dependent increase in BB concentration via PI3K-dependent stimulated exocytic insertion of NHE3 in the plasma membrane. The previously identified role of the NHERF2 requirement demonstrated its involvement in elevation of intracellular Ca\(^{2+}\) by a PLC\(\beta\)-dependent process, which was necessary for the LPA-induced increase in NHE3 activity (Lee-Kwon et al., 2003b). NHERF2 was also shown in the current study to be necessary for the increase in the NHE3 mobile fraction, which occurs within a few minutes of LPA exposure; moreover, it is the cytoskeletal-associated NHERF2 that is linked to actin indirectly via binding to ezrin through its C-terminal ERM-binding domain (deleted in the NHERF2A30 mutant) that is required. Based on its localization in the apical domain, we suggest that the ‘transit pool’ is the NHE3 that associates with NHERF2 in the apical domain. It was previously reported in mouse proximal tubule epithelial cells, that NHERF1 is in the outer part of the microvilli, whereas NHERF2 has a microvillar component that is distributed particularly towards the base of the microvilli. NHERF1 and NHERF2 have a similar distribution to the proximal tubule in Caco-2/bbe microvilli, in which NHERF1 under basal conditions has excellent BB overlap with ezrin and NHE3 above the intervillus clefts, whereas NHERF2 has a similar microvillar component but also occurs below the X-Z plane of the microvilli and therefore below the distribution of NHE3 under basal conditions (B.C. and M.D., unpublished results). The smaller size of the OK apical domain precludes the ability to separate the distribution of NHERF1 and NHERF2 by light microscopy. We hypothesize that NHERF organization is similar in different types of epithelial cells, with the NHERF2 pool localizing to the lower microvillus and below the microvilli in the general area of the intervillus clefts, where it provides a target for trafficking NHE3 in both basal and stimulated exocytosis (the role of apical domain NHERF2 in endocytosis is under study and will be reported separately). We speculate that the NHERF2 pool on the microvilli, which overlaps with NHE3 localization under basal conditions, associates with NHE3 dynamically to allow NHE3 to move over the entire microvillus surface. Although both NHERF1 and NHERF2 associate with NHE3 in the apical domain, NHERF1 did not change its association with NHE3 after LPA treatment, measured under the same experimental conditions used to study NHERF2. This suggests different functional roles of the NHE3 populations that associate with these two NHERF proteins. These results also provide some insights concerning NHE3 activity in this pool. Under conditions where the amount of BB NHE3 was not affected by LPA treatment (i.e. via inhibition of PI3K or by studying NHE3 mutants that fail to directly bind ezrin), the release of microvillar NHE3 from the cytoskeleton was not associated with a change in NHE3 activity. This suggests that this pool of NHE3, whether fixed to the cytoskeleton or free, has similar NHE3 activity.

Relevant to our study is that the NHERF1 dependence of recycling to the plasma membrane of the \(\delta\)-opioid receptor required binding of the receptor to the second PDZ domain of NHERF1 (Lauffer et al., 2009). This role of NHERF1 was replaceable by direct binding of the receptor to ezrin or actin, but there was a requirement for NHERF1 and specifically its PDZ1-binding domain, for regulated exocytosis of this receptor by the hepatocyte-growth-factor-regulated substrate. In this study, the difference in the dynamic aspects of apical-domain binding of NHERF1 and NHERF2 suggests that freeing up of BB NHE3 is not mediated by direct binding to ezrin or to actin, but rather is differentially dependent on specific NHERF proteins.

Several apical-domain pools of NHE3 in epithelial cells have been defined previously. These include NHE3 in lipid rafts and outside lipid rafts; NHE3 bound to megalin (with heavier density, less activity) or not bound to megalin (lighter density, more activity); also, Alexander and Grinstein showed that in renal epithelial apical membrane, NHE3 was both in a fixed, non-cycling pool of functional NHE3 and in a pool that cycles. Intracellular NHE3 also exists in two pools, one of which is rapidly exocytosed, whereas the other moves to the apical membrane with much slower kinetics (Alexander et al., 2005; Alexander et al., 2007; Alexander and Grinstein, 2006). It is not clear whether one of the apical pools described by these authors also represents the ‘transit pool’ characterized in this study as that which increases in the mobile fraction upon LPA treatment and/or which dissociates transiently from NHERF2, but not from NHERF1. Also, there is as yet, no comprehensive study of the functional consequences for NHE3 of being associated or not with NHERF2. Knockdown of NHERF2 in Caco-2/bbe cells did not alter basal NHE3 activity (R.S. and M.D., unpublished results) and there was no effect on NHE3...
activity when transfecting PS120 cells with either NHERF1 or NHERF2 (these cells have a very small amount of endogenous NHERF1 and no NHERF2); by contrast, knockout of NHERF2 in mouse ileum reduces basal NHE3 activity.

The role of NHERF proteins in setting the lateral mobility of NHE3-EGFP on the apical domain of OK cells has been studied in the past using FRAP. NHERF proteins limit the lateral mobility of NHE3 (Cha et al., 2004). This restriction of lateral mobility of NHE3 upon NHERF1 and NHERF2 binding is similar to the effects of PDZ proteins on the lateral mobility of the neural Kv1.4 channel expressed in non-polarized HEK293 cells (Burke et al., 1999) and human CFTR in the plasma membrane of Madin-Darby canine kidney cells (Haggie et al., 2004).

Exocytic trafficking of basal NHE3 depends on direct ezrin binding to NHE3 (Cha et al., 2006). The ezrin-binding mutant NHE3F1D dramatically decreased basal NHE3 activity by decreasing the plasma-membrane expression of NHE3. The current study shows that stimulated exocytosis also depends on direct ezrin binding and leads to the hypothesis that all aspects of regulated NHE3 activity, or at least those occurring upon changes in rates of trafficking, might require direct ezrin binding.

BS3 dramatically increased basal NHE3 activity without causing a comparable increase in surface expression of NHE3, although the effect was associated with a reduced apical membrane fraction of NHE3. This suggests that crosslinking of NHE3 is associated with NHE3 activation, and further shows that NHE3 does not have to be mobile in the apical domain to be active. NHE3 regulation is associated with signaling complexes in which several regulatory proteins bind to its C-terminus. We speculate that BS3 crosslinking affects these complexes to stimulate NHE3 activity.

In conclusion, LPA causes a transient increase in the mobility of apical NHE3 in OK cells. The schematic model for this process is described in Fig. 9. The LPA-induced increase in mobility of NHE3 consists of two components: (1) NHE3 newly delivered to the apical domain by a PI3K-dependent process, which requires direct ezrin binding to NHE3 and which occurs prior to fixing of NHE3 to the microvillar cytoskeleton. This consists of functionally active NHE3 and NHE3 that is newly dissociated from NHERF2 in the intervillus clefts and in the microvillus, which allows the NHE3 to transit over the microvillus prior to cytoskeletal fixation. Definition of the ‘transit pool’ of NHE3 includes NHE3 in the intervillus clefts and lower part of the microvillus, which receives NHE3 from the recycling system and dispenses NHE3 to the microvillus as part of exocytosis. This pool of NHE3 binds NHERF2 dynamically, changing with LPA signaling and associates with the cytoskeleton by ezrin binding to NHERF2. NHERF1 distribution is shown in red and NHERF2 distribution in blue and their locations in the BB are assumed to be static.

The LPA-induced increase of NHE3 mobile fraction consists of two components: NHE3 newly delivered to the apical domain by a PI3K-dependent process, which requires direct ezrin binding to NHE3 and which occurs prior to fixing of NHE3 to the microvillar cytoskeleton. This consists of functionally active NHE3 and NHE3 that is newly dissociated from NHERF2 in the intervillus clefts and in the microvillus, which allows the NHE3 to transit over the microvillus prior to cytoskeletal fixation. Definition of the ‘transit pool’ of NHE3 includes NHE3 in the intervillus clefts and lower part of the microvillus, which receives NHE3 from the recycling system and dispenses NHE3 to the microvillus as part of exocytosis. This pool of NHE3 binds NHERF2 dynamically, changing with LPA signaling and associates with the cytoskeleton by ezrin binding to NHERF2. NHERF1 distribution is shown in red and NHERF2 distribution in blue and their locations in the BB are assumed to be static.

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Materials and Methods

Materials
Monoclonal anti-VSV-G antibodies were derived from the P5D4 hybridoma from Thomas Kreiss and Daniel Loward (Institute Curie, Paris, France). Monoclonal anti-hemagglutinin (HA) antibody was from Sigma. Monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was purchased from US Biological (Swampscott, MA). Polyclonal anti-NHERF1 (S199) and anti-NHERF2 (2570) antibodies were previously described (Yun et al., 1998). BS3 [bis(sulfosuccinimidyl) suberate] was from Thermo Scientific (Rockford, IL). Latrunculin B was from Alexis Thermo Biochemicals (San Diego, CA). Protease inhibitor cocktail (4-[2-aminoethyl] benzenesulfonyl fluoride (AEBSF), trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64), bistratin, leupeptin, aprotinin and sodium EDTA, LPA (Oleoyl-L-a-pyosphosphatic acid sodium salt), PI3K inhibitor LY294002 were from Sigma. Quikchange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). Nigercin and 2',7'-bis (2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) were from Invitrogen. Anti-rabbit Alexa Fluor® 680 dye and anti-mouse IRDye® 800 dye were from LI-COR (Lincoln, NE). DNA primers were from Operon (Huntsville, AL). Peptides were synthesized at the Johns Hopkins Synthesis and Sequencing Facility.

Construction of expression vectors

For measurement of NHE3 transport activity, full-length NHE3 and its ezrin-binding mutant, NHE3F1D were used, as described (Cha et al., 2006). For FRAP measurements, full-length NHE3-EGFP, truncation mutant NHE3585 (residues 1-585), and its direct ezrin-binding double mutant NHE3F1D cDNAs all with VSV-G tag epitope at the C-terminus were assembled using the pEGFP-N3 vector (Clontech, CA), as previously described using Nhel-Xhol restriction sites in-frame with the C-terminal EGFP-coding sequence (Janecki et al., 2000). There was an 84 base pair linker between NHE3 and EGFP. Wild-type full-length rabbit NHE3 cDNA was used as a template for all polymerase chain reactions (PCR) described below. The plasmid containing YFP-GL-GPI (pEGFP-N1, Clontech) was kindly provided by K. Simons (Keller et al., 2001). For FRET, NHE3-YFP or CFP-NHERF2 was assembled using the pcS2-Venus plasmid containing the YFP (Venus) tag or pECFP-C1 vector (Clontech, CA), respectively. Wild-type full-length rabbit NHE3 with C-terminal YSV-G was ligated into the pcS2-Venus with HindIII-BamHI restriction sites in-frame with the C-terminal YFP (Venus), creating NHE3-YFP. Wild-type full-length human NHERF2 was ligated into the pECFP vector with BamHI-EcoRI restriction sites in-frame with the N-terminal ECFP-coding sequence, creating CFP-NHERF2. EGFP was ligated to the N-terminus of NHERF2 because NHERF2 has an ERM-
Phalloidin binds domain at its C-terminal end and removing that domain (Δ30 aa) creates a dominant-negative effect (Weinstein et al., 2001). CDAs were made using the human NHERF2 (residues 1-337) and its ERM-binding domain deletion mutant, NHERF2Δ30 (residues 1-307) were cloned into pcDNA3.1 Hygro− vectors and stably transfected into OK cells using Lipofectam 2000 (Invitrogen), creating OK NHERF2 and OK NHERF2Δ30.

**Cell culture and transfection**

OK cells were cultured on glass-bottom 35 mm plastic culture dishes in D-MEM F-12 medium (GibcoBRL), supplemented with 10% fetal bovine serum, 100 µM penicillin and 100 µg/ml streptomycin at 37°C in a 5% CO2 and 95% air atmosphere. The intrinsic NHE3 activity in OK cells was markedly decreased by repeated applications (once a week) of the ‘acid suicide’ technique (Post et al., 2004). This resulted in OK cells with low NHE3 activity, such that the NHE3 present after transfection was nearly all the transfected NHE3 constructs. These cells were then used without passaging. Post-confluent OK cells with minimal BB NHE3 activity were serum-starved for 2 days and then preincubated with EGTA (3 mM, 30 minutes). The EGTA-treated cells were then transiently transfected with 10 µg NHE3-EGFP or its defective ezrin-binding mutant NHE3F1D-EGFP using 10 µl Lipofectam 2000 (Invitrogen) and following the manufacturer’s instructions.

**Immunoprecipitation and immunoblot analysis**

Co-immunoprecipitation and western blot experiments were performed using lysates from 48 hour serum-starved OK NHERF2 HA-NHE3 cells in the presence (or absence) of LPA (100 µM) for 30 minutes and 60 minutes, as described (Cha et al., 2007). Band visualization and quantification was carried out using the Odyssey System (Li-COR, Lincoln, NE).

**Determination of amount of surface HA-NHE3**

OK cells stably transfected with NHERF2 and HA-NHE3 were plated on 10-cm dishes. When the cells were 100% confluent, they were serum-starved for 2 days before starting the experiments. Cells were treated with DMSO (control), BS1 LPA (100 µM) and BS5 LPA (100 µM) for 30 minutes at 4°C, 95% air atmosphere. Cells were then quickly washed with cold PBS and kept at 4°C for 40 minutes. Anti-HA antibody (1:100 dilution) was added for the surface labeling of NHE3 and cells incubated for 2 hours at 4°C. Cells were then collected and total lysates made using 1 ml lysis buffer (HEPES buffer with 1% Triton X-100, protease inhibitor cocktail (Sigma) and phosphatase inhibitors (Na3VO4, NaF)). 100 µl was used for study of lysate expression and the remaining 900 µl was incubated with Protein-G (Invitrogen) beads (100 µl) for 2 hours to collect surface-labeled HA-NHE3. Beads were then washed five times with lysis buffer and the bound HA-NHE3 eluted with 90 µl SDS sample buffer (10X concentration). SDS-PAGE was performed to detect total HA-NHE3 and surface amount of HA-NHE3 (anti-HA antibody). Antibody against extracellular epitope HA was used to detect surface NHE3 rather than streptavidin-biotin because both BS1 and biotin bound free lys.

**Measurement of Na+/H+ exchange**

Cellular Na+/H+ exchange activity in OK cell lines was determined fluorometrically using the intracellular pH-sensitive dye acetoxymethyl ester of 2′,7′-bis(carboxethyl)5′,6-bis(carboxyfluorescein (BCECF-AM, 5 µM; Invitrogen, Eugene, OR), as described previously (Levine et al., 1995; Cha et al., 2005).

**Fluorescence recovery after photobleaching (FRAP)**

To determine the lateral mobility of NHE3-EFP and the NHE3 F1D mutant-EFP at the apical domain of polarized OK cells, FRAP was used, as previously reported (Cha et al., 2004). OK cells were cultured on glass-bottom 35 mm plastic culture dishes in DMEM, as above. The cells were then transfected using Lipofectam 2000, as described (Cha et al., 2004), with NHE3-EFP, NHE3 585-EFP and NHE3 F1D-EFP and studied ~48 hours later, during which time they were not passaged. FRAP was performed on the stage (heated to 37°C) of a Zeiss LSM 510/Meta confocal microscope (with a Neofluor 40× objective, with signal collected in the OK cell apical domain (0.3 µM). Excitation was at 458 nm for CFP and 514 nm for YFP. Emission was 465–510 nm under conditions of acute NHE3 stimulation and increased apical amount of NHE3, we analyzed the mobile fraction of NHE3 post bleach at 400 seconds. This was a time that the fluorescence had always recovered to plateau level post bleach in untreated control cells. All data are shown as mean ± s.e.m. of the number of cells analyzed, which were obtained in at least two identical experiments, unless stated otherwise. Statistical comparison was performed by unpaired Student's t-test.

**Acceptor photobleaching FRET**

FRET has been used extensively in fluorescence microscopy to quantify protein-protein interactions. To perform acceptor photobleaching FRET, we engineered CFP-NHERF2 and NHE3-YFP constructs, a pair of fluorophores widely used for this approach. A Zeiss 510/Meta confocal microscope (with a Neofluor 10× 1.3 NA oil-immersion objective), with the FRET Plus-Macoro software (Release 4.2; Carl Zeiss Micro Imaging) was used to collect images, which were subsequently used to calculate off-line whether FRET was present between CFP-NHERF2 (donor) and NHE3-YFP (acceptor). OK cells were transfected with CFP-NHERF2 and NHE3-YFP with Lipofectam 2000 with EGTA (3 mM, 30 minutes) pre-incubation as described in the FRAP method. Two days after the transfection, cells were fixed with 4% formaldehyde (cells were incubated at 4°C for 10 minutes, and the inner 10 minutes at room temperature) and then mounted on glass slides (Fluro-Gel, Electron Microscopy Sciences). 40-200 micrometer regions of interest (ROI) were selected manually and images collected before and after bleaching with the 514 nm laser line at maximum intensity for a period that caused almost total acceptor bleaching. Excitation was at 458 nm for CFP and 514 nm for YFP. Emission was 465-510 nm for CFP and 518-561 nm for YFP. The negative control was CFP-GL-GPI, which was studied with CFP-NHERF2. Apparent FRET efficiency, E, was calculated manually with MetaMorph software using the following equation: $F_{E_\text{POST}} - F_{E_\text{PRE}}$. Where $F_{E_\text{POST}}$ and $F_{E_\text{PRE}}$ are the donor fluorescence intensities pre- and post- acceptor photobleaching, respectively (Kenworth and Edidin, 1998) FRET efficiency was calculated by using fluorescence values that had been background-subtracted and corrected for acquisition bleaching, determined with an unbleached ROI.

**Conditions establishing that NHE3 and NHERF2 used as FRET probes behaved similarly to wild-type non-GFP-tagged proteins were determined. We previously showed that NHE3-EFP localized in OK cells similarly to wild-type NHE3, and was distributed in the apical domain and in the juxtanuclear area. NHE3 was functionally inhibited by PI3K inhibitors (wortmannin and LY24003) similarly to wild-type NHE3 (Janecki et al., 2000). We have also reported that NHE3-EFGP stably expressed in PS120 cells was similarly to wild-type NHE3 in terms of the percentage on the surface, basal transport rate and stimulation by growth factors (Janecki et al., 2000). The LPA stimulation of NHE3 in OK cells using endogenous NHE3, the C-terminally GFP (EFGP)-tagged construct and N-terminally HA-epitope-tagged NHE3 were all similar in magnitude (supplementary material Fig. S1). In addition, under stimulatory conditions (dialyzed serum) stimulated NHE3 activity in PS120 cells transiently transfected with NHE3-YFP and CFP-NHERF2 (supplementary material Fig. S2). Moreover, as shown in supplementary material Fig. S3, CFP-NHERF2 co-localized with NHE3-YFP in PS120 cells (transiently co-transfected as in the current studies) and responded to elevation of intracellular Ca2+ with NHE3 inhibition similarly to that reported for wild-type NHEF2/NHE3 in these cells, which is a NHERF2-dependent process. In addition, as shown in supplementary material Fig. S4, CFP-NHERF2 and NHE3-YFP co-precipitated from PS120 cells, as reported for wild-type proteins in these cells.

Supported in part by the National Institutes of Health, NIDDK Grants R01DK26523, R01DK61765, R01 GM 073846, P01DK72084, R24DK64388 (The Hopkins Basic Digestive Diseases Development Core Center), T32DK200763, K01DK080930 and the Hopkins Center for Epithelial Biology. R.L. was supported by an American Gastroenterological Association Jon Isenberg Fellowship. Deposited in PMC for release after 12 months.

**Supplementary material available online at**

http://jcs.biologists.org/cgi/content/full/123/14/2434/DC1

**References**


