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p160ROCK mediates RhoA activation of Na–H exchange

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The ubiquitously expressed Na–H exchanger, NHE1, acts downstream of RhoA in a pathway regulating focal adhesion and actin stress fiber formation. p160ROCK, a serine/threonine protein kinase, is a direct RhoA target mediating RhoA-induced assembly of focal adhesions and stress fibers. Here, stress fiber formation induced by p160ROCK was inhibited by the addition of a specific NHE1 inhibitor, ethylisopropyl-amiloride, in CCL39 fibroblasts, and was absent in PS120 mutant fibroblasts lacking NHE1. In CCL39 cells, NHE1 activity was stimulated by expression of mutationally active p160ROCK, but not by mutationally active protein kinase N, another RhoA target kinase. Expression of a dominant interfering p160-ROCK inhibited RhoA-, but not Cdc42- or Rac-activation of NEH1. In addition, the p160ROCK-specific inhibitor Y-27632 inhibited increases in NHE1 activity in response to RhoA, and to lysophosphatidic acid (LPA), which stimulates RhoA, and it also inhibited LPA-increased phosphorylation of NHE1. A C-terminal truncation of NHE1 abolished both LPA-induced phosphorylation and activation of the exchanger. Furthermore, mutationally active p160-ROCK phosphorylated an NHE1 C-terminal fusion protein *in vitro*, and this was inhibited in the presence of Y-27632. Phosphopeptide maps indicated that identical residues in NHE1 were phosphorylated by p160ROCK *in vivo* and *in vitro*. These findings identify p160ROCK as an upstream, possibly direct, activator of NHE1, and suggest that NHE1 activity and phosphorylation are necessary for actin stress fiber asssembly induced by p160ROCK.

Keywords: cytoskeleton/lysophosphatidic acid/Na–H exchanger/p160ROCK/RhoA

Introduction

NHE1 is a ubiquitously expressed member of the Na–H exchanger family that catalyzes the extrusion of intracellular proton (H^+) ions in exchange for extracellular sodium (Na^+) ions, thereby regulating intracellular pH (pH_i) and cell volume (Noel and Pouyssegur, 1995). This exchanger not only operates under the basal conditions of cells but also is activated by various extracellular stimuli such as hormones, mitogens, oncogenes and extracellular matrix (ECM) proteins. This activation has been shown to be associated with anchorage-dependent growth, cell motility, cell transformation and cell adhesion and spreading (Simchowitz and Cragoe, 1986; Ingber *et al*., 1990; Krump *et al*., 1997; Tominaga and Barber, 1998). Quite recently, a defective mutation of the mouse *Nhe1* gene was found (Cox *et al*., 1997). The mutant mice show selective neuronal death, and a unique epilepsy syndrome, suggesting that NHE1 is required for regulation of neuronal excitability and survival. How NHE1 is activated in a particular signaling pathway and how this activation is linked to cell behavior, however, remain largely unknown.

Low molecular weight GTPases of the Rho family function as control points in regulating the organization of the actin cytoskeleton. In cultured cells, RhoA links mitogen activation to the assembly of focal adhesions and stress fibers (Ridley and Hall, 1992), Rac controls the dynamics of lamellipodia (Ridley and Hall, 1992), and Cdc42 regulates the formation of filopodia (Kozma *et al*., 1995; Nobes and Hall, 1995). RhoA also regulates integrin-induced cell adhesion (Tominaga *et al*., 1993; Laudanna *et al*., 1996) and contractility (Chrzanowska-Wodnicka and Burridge, 1996), cell secretion (Price *et al*., 1995), cytokinesis (Kishi *et al*., 1993; Mabuchi *et al*., 1993), proliferation (Yamamoto *et al*., 1993; Olson *et al*., 1995) and Ras-induced neoplastic transformation (Qiu *et al*., 1995b). We previously found that RhoA stimulates NHE1 activity and mediates NHE1 activation by lysophosphatidic acid (LPA) and the GTPase Gα13, and that activation of NHE1 is necessary for RhoA-induced reorganization of the actin cytoskeleton (Hooley *et al*., 1996; Vexler *et al*., 1996). RhoA-mediated assembly of stress fibers and focal adhesions is inhibited in NHE1-expressing cells treated with NHE1-selective inhibitors. Furthermore, stress fiber induction by RhoA is absent in NHE1-deficient fibroblasts and is restored by expression of NHE1 (Vexler *et al*., 1996; Tominaga and Barber, 1998). Interestingly, NHE1 activity is not required for Rac-induced lamellipodia formation (Vexler *et al*., 1996), although Rac1 also acts upstream of NHE1 to stimulate exchanger activity (Hooley *et al*., 1996). These findings suggest that NHE1 selectively regulates cytoskeletal events induced by RhoA.

Several direct targets of RhoA have been identified recently, including protein kinase N (PKN) (Amano *et al*., 1996b; Watanabe *et al*., 1996), rhophilin (Watanabe *et al*., 1996), rhotekin (Reid *et al*., 1996), p140mDia (Watanabe *et al*., 1997) and a family of Rho-associated kinases, p160ROCK (ROCK-I) (Ishizaki *et al*., 1996) and ROKα/Rho-kinase/ROCK-II (Leung *et al*., 1995; Matsui *et al*., 1996; Nakagawa *et al*., 1996). Although the functions of most RhoA targets remain unknown, p140mDia and Rho-associated kinase isozymes mediate RhoA effects on the actin cytoskeleton. p140mDia, a mammalian homolog of

Drosophila diaphanous, controls actin polymerization by binding and accumulating the actin-binding protein profilin (Watanabe *et al*., 1997). The Rho-associated kinase isozymes, p160ROCK (Ishizaki *et al*., 1997) and ROKα/Rho-kinase/ROCK-II (Leung *et al*., 1996; Amano *et al*., 1997), mediate RhoA-induced assembly of focal adhesions and actin stress fibers. p160ROCK and ROKα/Rho-kinase/ROCK-II are coiled-coil-forming serine/threonine kinases sharing ~90% identity within the kinase domain. These kinases are suggested to regulate cell contractility by indirectly increasing phosphorylation of myosin light chain through the inhibition of myosin phosphatase activity (Kimura *et al*., 1996) or by directly phosphorylating myosin light chain independently of myosin light chain kinase (Amano *et al*., 1996a). The objective of the current study was to determine whether p160ROCK, which is required for RhoA-induced stress fiber and focal adhesion formation, regulates NHE1 activity. We found that p160ROCK specifically mediates activation of NHE1 by LPA, Gα13 and RhoA, and not by Cdc42 and Rac1. p160ROCK mediates LPA-induced phosphorylation of NHE1 *in vivo* and directly phosphorylates NHE1 *in vitro*. Furthermore, we have found that NHE1 phosphorylation was necessary for activation of NHE1 in the LPA–RhoA–p160ROCK pathway. These findings indicate that NHE1 is a downstream, possibly direct, target of p160ROCK and suggest that activation of NHE1 collaborates with increased contractility via myosin to induce p160ROCK-mediated changes in actin cytoskeleton reorganization.

Results

NHE1 activity is required for p160ROCK-induced stress fiber formation

Focal adhesions and stress fibers are clustered structures of, respectively, integrin complexes bound to ECM proteins and actin filaments ligated to the complexes. Such clustering is generated by the force of myosin-based contractility (Chrzanowska-Wodnicka and Burridge, 1996). Because the RhoA-associated kinases such as p160ROCK and ROKα/Rho-kinase have been suggested to enhance phosphorylation of myosin light chain in cells and to work downstream of Rho to induce focal adhesions and stress fibers, it is generally accepted that they induce cytoskeletal effects presumably by regulating myosin contractility through phosphorylation of its light chain. We previously determined that NHE1 activity regulates a RhoA pathway leading to stress fiber formation (Vexler *et al*., 1996). The relationship between Rho-associated kinases and NHE1, however, remains unknown. To determine whether NHE1 activation is also prerequisite for stress fiber induction by RhoA-associated kinases, we transiently expressed a mutationally activated p160ROCKΔ3 in CCL39 fibroblasts. p160ROCKΔ3 is a mutant of p160ROCK truncated at amino acid residue 727, which results in deletion of the Rho-binding domain, the pleckstrin homology domain and the cysteine-rich zinc finger domain. The constitutive activity of the Δ3 mutant was demonstrated previously (Ishizaki *et al*., 1997) and confirmed by its ability to phosphorylate histone *in vitro* (see Figure 6B). As it does in cultured HeLa cells (Ishizaki *et al*., 1997), this mutant induced stress fibers and focal adhesions in CCL39 cells (Figure 1A and B). This induction was abolished by the addition of ethylisopropyl-amiloride (EIPA; 25 μM), an NHE1 inhibitor, suggesting that NHE1 activity was required for p160ROCK-induced stress fiber formation (Figure 1C and D). This suggestion

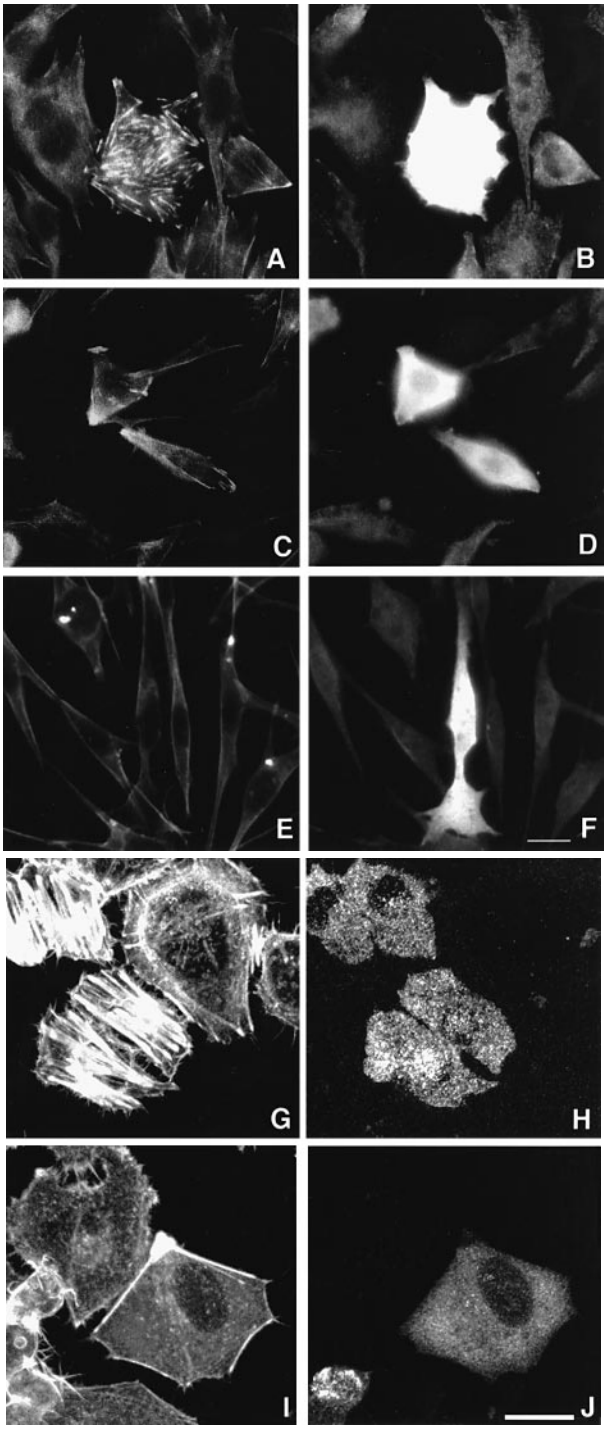

Fig. 1. NHE1 activity is necessary for p160ROCK-induced cell contraction and stress fiber assembly. Cells were transfected with Myc-tagged p160ROCKΔ3 and double-stained with anti-Myc antibodies and with rhodamine–phalloidin after 16 h. (**A** and **B**) CCL39 cells; (**C** and **D**) CCL39 cells treated with 25 μM EIPA for 16 h; (**E** and **F**) PS120 cells; (**G** and **H**) HeLa cells; (**I** and **J**) HeLa cells treated with EIPA for 16 h. F-actin was visualized by phalloidin staining (A, C, E, G and I) and cells expressing p160ROCKΔ3 were visualized by Myc immunostaining (B, D, F, H and J). Bar, 10 μm (F), 20 μm (J).

was supported in experiments using PS120 cells, which are derived from parental CCL39 cells but lack Na–H exchangers (Pouyssegur *et al*., 1984). No stress fibers were induced by the expression of p160ROCKΔ3 in these NHE1-deficient cells (Figure 1E and F). We previously determined that inhibition of NHE1, either in EIPA-treated CCL39 cells or in PS120 cells, selectively inhibits RhoA-mediated cytoskeletal remodeling, as it has no effect on Rac-induced lamellipodia or membrane ruffling, or on Cdc42-induced filopodia formation (Vexler *et al*., 1996; Tominaga and Barber, 1998). To confirm that the effect of NHE1 on stress fiber formation was not specific to CCL39 cells, we demonstrated that induction of stress fibers in HeLa cells expressing p160ROCKΔ3 was also inhibited by EIPA (Figure 1G–J).

p160ROCK stimulates NHE1 activity

The above results demonstrate that NHE1 activity is required for p160ROCK-induced stress fiber formation. They do not, however, necessarily indicate that p160ROCK lies upstream of NHE1 and activates it. To determine whether p160ROCK couples to the regulation of NHE1 activity, we determined the activity of the exchanger in CCL39 cells expressing the Δ3 mutant. The steady-state intracellular pH (pH_i) in a HEPES buffer increased from 7.18 ± 0.03 (mean ± SEM; $n = 4$ transfections) in vector controls to 7.36 ± 0.01 ($n = 4$) in the Δ3-expressing cells. Additionally, NHE1 activity, determined as the rate of pH_i recovery (dpH_i/dt) from an NH_4Cl-induced acid load, was increased in cells transfected with Δ3, compared with vector controls (Figure 2A and B). In two separate cell transfections, expression of p160ROCKΔ1, which is constitutively active due to a truncation at amino acid residue 1080 (Ishizaki *et al*., 1997), also stimulated NHE1 activity (Figure 2B, inset). To determine the specificity of p160ROCK-induced activation of NHE1, we examined the activity of the exchanger in CCL39 cells transfected with a constitutively active PKN. Although PKN also acts directly downstream of RhoA (Amano *et al*., 1996; Watanabe *et al*., 1996) and recently was determined to bind α-actinin (Mukai *et al*., 1997), its functional importance in RhoA-mediated signaling events remains unknown. Myc-PKN, which is constitutively active due to the Myc epitope at the N-terminus (G.Martin, personal communication), was able to phosphorylate histone *in vitro* (see Figure 6B) but had no effect on pH_i (data not shown) or NHE1 activity (Figure 2A and C) compared with vector controls (Figure 2A and C). We also determined that another constitutively active PKN allele, containing only the kinase domain, had no effect on NHE1 activity when transfected into CCL39 cells (data not shown).

p160ROCK selectively mediates activation of NHE1 by RhoA

NHE1 activity is stimulated by activation of RhoA, Cdc42 and Rac1 (Hooley *et al*., 1996). To determine whether p160ROCK selectively mediates NHE1 activity by a specific Rho family GTPase, we co-transfected CCL39 cells with KD-IA, a kinase-inactive p160ROCK. KD-IA, which contains a $K^{105}\rightarrow A$ substitution in the kinase domain and an $I^{1009}\rightarrow A$ substitution in the Rho-binding domain, functions as a dominant-interfering allele to block RhoA-dependent assembly of actin stress fibers (Ishizaki

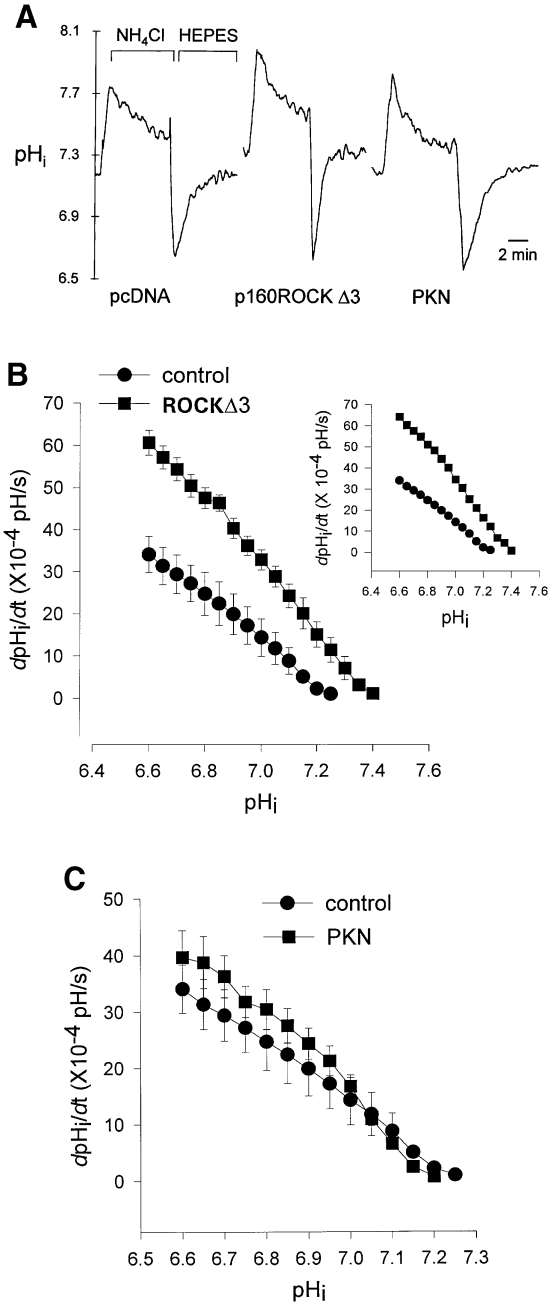

Fig. 2. p160ROCK stimulates NHE1 activity. Intracellular pH recoveries were measured in CCL39 cells transiently transfected with pcDNA3 (2 μg), p160ROCKΔ3 (2 μg), p160ROCKΔ1 (2 μg) or PKN (3 μg). (**A**) Representative tracings of pH_i recoveries from an NH_4Cl-induced acid load, determined in a nominally HCO_3-free HEPES buffer. (**B** and **C**) The rates of pH_i recovery (dpH_i/dt) after an acute acid load at the indicated pH_i values. Data represent the mean ± SEM of four separate transfections for (B) and three separate transfections for (C). (B, inset) dpH_i/dt as a function of pH_i in CCL39 cells transfected with pcDNA vector (●) or p160ROCKΔ1 (■).

et al., 1997). In CCL39 cells transfected with mutationally active RhoAV14, steady-state pH_i increased from 7.23 ± 0.02 in vector controls to 7.44 ± 0.01, and this increase in pH_i was reduced to 7.29 ± 0.03 by co-transfection with KD-IA ($n = 7$ separate matched transfections). Additionally, RhoA-induced increases in NHE1 activity were completely inhibited by co-transfection with KD-IA (Figure 3A). KD-IA had a small inhibitory effect on

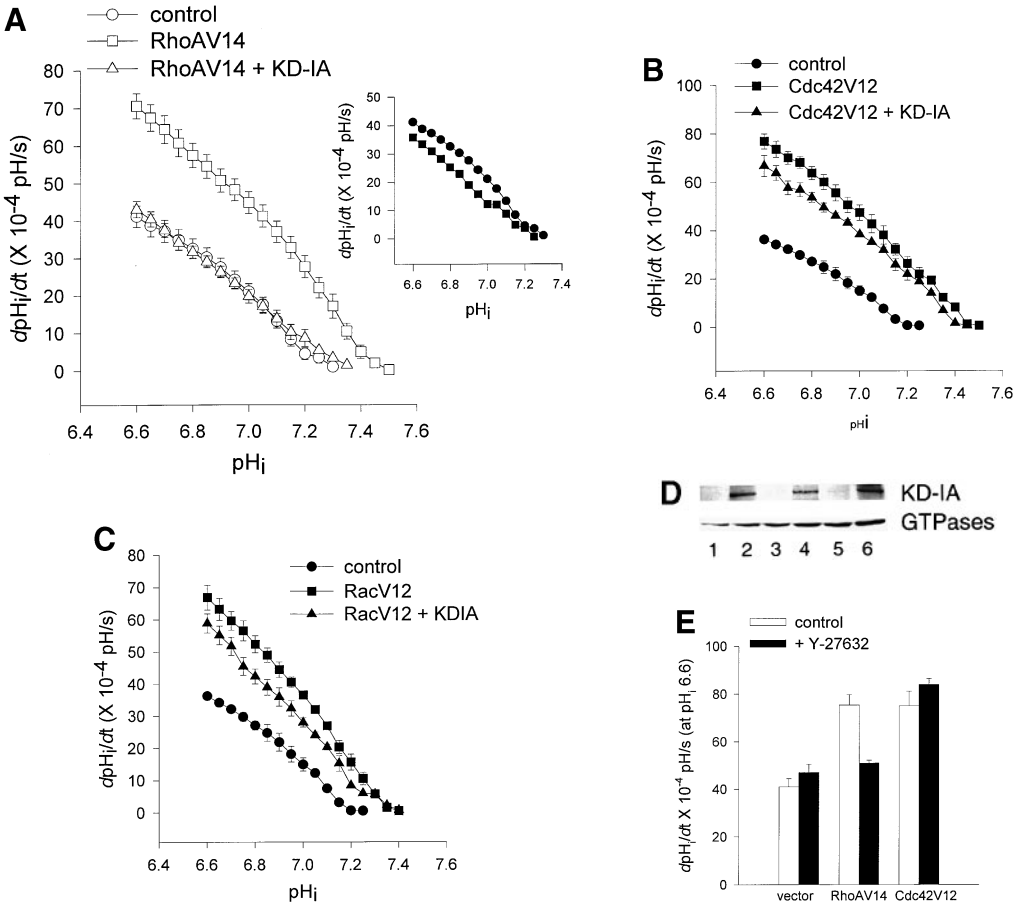

Fig. 3. p160ROCK mediates activation of NHE1 by RhoA. (**A**–**C**) The rates of pH_i recovery from an NH_4Cl-induced acid load were determined at the indicated pH_i values in a HEPES buffer. CCL39 cells were transiently transfected either with 2 μg of pcDNA vector (controls) or with 0.5 μg of RhoAV14 (A), Cdc42V12 (B) or Rac1V12 (C) in the absence and presence of 2 μg of KD-IA. [(A), inset] Vector control in absence (●) and presence (■) of KD-IA. (**D**) Immunoblot of Myc-tagged KD-IA expression and Myc-tagged GTPase expression in the absence and presence of KD-IA. RhoAV14 (lane 1), RhoAV14 + KD-IA (lane 2), Cdc42V12 (lane 3), Cdc42V12 + KD-IA (lane 4), Rac1V12 (lane 5) and Rac1V12 + KD-IA (lane 6). (**E**) The rates of pH_i recovery at pH_i 6.6 in CCL39 cells transfected with 0.5 μg of RhoAV14 or Cdc42V14 measured in the absence and presence of Y-27632 (50 μM, 60 min incubation). Data represent the means ± SEM of 3–7 separate transfections.

NHE1 activity in vector control cells (Figure 3A, inset), suggesting that an upstream regulator of p160ROCK might be constitutively active in serum-starved CCL39 cells. Co-transfection of KD-IA, however, had only a minor inhibitory effect on NHE1 activity stimulated by mutationally active Cdc42V12 (Figure 3B) or mutationally active Rac1V12 (Figure 3C). The magnitude of this inhibition was similar to that observed in vector control cells (Figure 3A, inset). Immunoblot analysis indicated that KD-IA expression was similar in cells transfected with RhoAV14, Cdc42V12 or Rac1V12, and that co-transfection of KD-IA had no effect on the expression of mutationally active GTPases (Figure 3D). The ability of p160ROCK to mediate specifically RhoA activation of NHE1 was confirmed further by treating cells with the p160ROCK-selective inhibitor Y-27632. The compound Y-27632 is a novel pyridine derivative that suppresses RhoA- and p160ROCK-induced stress fiber assembly but has no effect on Rac-induced membrane ruffling or Cdc42-induced filopodia formation (Uehata *et al*., 1997). In four separate cell preparations, Y-27632 significantly inhibited activation of NHE1 in cells transfected with RhoAV14 ($P \leqslant 0.01$), but not in those with Cdc42V12 (Figure 3E). These findings indicate that although three members of the Rho family of GTPases couple to the stimulation of NHE1, p160ROCK selectively mediates only RhoA activation of the exchanger.

p160ROCK mediates activation of NHE1 by Gα13

The α subunit of the heterotrimeric GTPase G13 couples to the stimulation of NHE1 (Dhanasekaran *et al*., 1994; Voyno-Yasenetskaya *et al*., 1994; Kitamura *et al*., 1995). Although previous findings indicated that Gα13 acts downstream of the LPA receptor to activate NHE1 through both Cdc42- and RhoA-dependent signaling pathways (Hooley *et al*., 1996), expression of mutationally active Gα13QL in fibroblasts induces a RhoA-like phenotype of increased stress fiber formation, but not a Cdc42-like phenotype of filopodia extension (Buhl *et al*., 1995; Hooley *et al*., 1996). Hence, RhoA, and not Cdc42, may be the preferred downstream effector of Gα13 *in vivo*. To determine whether p160ROCK mediates Gα13 activation of NHE1, we used co-transfections in CCL39 cells. Transfection of mutationally active Gα13QL alone increased the steady-state pH_i from 7.16 ± 0.04 in vector controls to 7.37 ± 0.03, but co-transfection with KD-IA completely inhibited Gα13QL-induced increases in pH_i, resulting in a value of 7.14 ± 0.03 (n = 3 separate

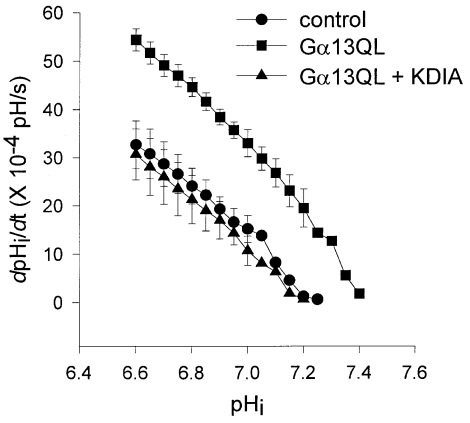

Fig. 4. p160ROCK mediates activation of NHE1 by Gα13. The rates of pH_i recovery (dpH_i/dt) at the indicated pH_i values were determined in CCL39 cells transfected with 2 μg of pcDNA vector (control) or 0.5 μg of α13QL and 0.5 μg of α13QL plus 2 μg of KD-IA. Data represent the means ± SEM of three separate matched transfections.

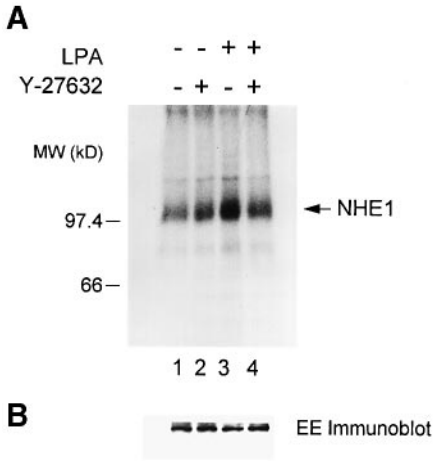

Fig. 5. p160ROCK mediates LPA-induced phosphorylation of NHE1 *in vivo*. Serum-starved, ^{32}P-labeled PS120N cells were dissociated, suspended in serum-free medium for 45 min in the absence or presence of Y-27632, and stimulated by LPA. (**A**) EE-tagged NHE1 was immunoprecipitated and its phosphorylation was analyzed on a 7.5% acrylamide SDS–PAGE gel. Lanes 1 and 2, no stimulation; lanes 3 and 4, stimulation by LPA (10 μM; 10 min); lanes 2 and 4, cells pre-incubated with Y-27632 (30 μM; 30 min). (**B**) Immunoblot analysis of immunoprecipitated NHE1 using EE antibodies.

matched transfections). Co-transfection of KD-IA also completely inhibited the stimulation of NHE1 activity by Gα13QL (Figure 4). Together with our previous findings (Voyno-Yasenetskaya *et al*., 1994; Hooley *et al*., 1996; Vexler *et al*., 1996), these results suggest that NHE1 activity is stimulated by a signaling cascade involving LPA→Gα13→RhoA→p160ROCK.

p160ROCK mediates LPA-induced phosphorylation of NHE1 in vivo

Growth factor activation of NHE1 is associated with increased phosphorylation of the cytoplasmic domain of the exchanger on serine residues (Sardet *et al*., 1991). To determine whether p160ROCK regulates the phosphorylation of NHE1, we stably expressed full-length human NHE1, tagged at the C-terminus with an EE epitope, in NHE-deficient PS120 cells (PS120N cells). The transient transfection efficiency of PS120 cells is <10%, which prevented us from studying the effects of mutationally active or dominant-interfering p160ROCK on NHE1 phosphorylation. We therefore determined whether an LPA pathway involving p160ROCK phosphorylated NHE1. LPA, which activates RhoA (Ridley and Hall, 1992) and stimulates NHE1 activity (Vexler *et al*., 1996), increased the phosphorylation of NHE1 (Figure 5A, lane 3), a result similar to the previously described effect of growth factors (Sardet *et al*., 1991). In PS120N cells pre-treated with the specific p160ROCK inhibitor Y-27632 (30 μM), however, LPA-induced phosphorylation of NHE1 was reduced to basal levels (Figure 5A, lane 4). Y-27632 had no effect on basal NHE1 phosphorylation (Figure 5, lane 2). Immunoblot analysis indicated that the amount of immunoprecipitated NHE1 in the absence and presence of LPA was similar (Figure 5B). These findings suggest that p160ROCK mediates LPA-induced phosphorylation of NHE1 and, as shown below (Figure 8C), LPA-stimulated NHE1 activity.

p160ROCK directly phosphorylates NHE1 in vitro

We next investigated whether p160ROCK directly phosphorylates NHE1 *in vitro*. Mutationally activated Myc-tagged p160ROCKΔ3 and PKN were transiently expressed in CCL39 cells and immunoprecipitated with anti-Myc mAb. Immunoprecipitates were then used for *in vitro* kinase assays with either histone or GST–NHE1 as a substrate. The GST–NHE1 fusion protein included amino acid residues 638–815 of the C-terminal cytoplasmic domain and contained all serine residues that are phosphorylated *in vivo* (Sardet *et al*., 1991; Wakabayashi *et al*., 1992; see Figure 8B). The abundance of kinases in the immunoprecipitates was determined by immunoblotting with anti-Myc antibodies (Figure 6A). As previously reported, both Δ3 (Ishizaki *et al*., 1997) and PKN (Mukai *et al*., 1997) phosphorylated histone (Figure 6B). In contrast, only Δ3 phosphorylated GST–NHE1 (Figure 6B). This finding correlates with the ability of p160ROCK, but not PKN, to stimulate NHE1 activity (Figure 2). GST alone was not phosphorylated by either kinase (data not shown). In the presence of Y-27632 (100 μM) added to the kinase reaction buffer, Δ3-induced phosphorylation of GST–NHE1 was completely inhibited (Figure 6B, lane 3), suggesting that p160ROCK acts directly on the exchanger. We also confirmed that the NHE inhibitor EIPA had no effect on p160ROCK activity. Phosphorylation of histone by immunoprecipitated Δ3 was decreased in the presence of Y-27632 in a dose-dependent manner, but was unchanged in the presence of EIPA (3–300 μM; Figure 6C).

To assess further whether p160ROCK directly phosphorylates NHE1, two-dimensional phosphopeptide mapping was performed to analyze the sites of NHE1 phosphorylated *in vivo* and *in vitro*. EE-tagged NHE1 phosphorylated *in vivo* was isolated by SDS–PAGE, transferred to nitrocellulose membranes and digested with trypsin. Incorporation of ^{32}P into several phosphopeptides increased in response to LPA (Figure 7A and B). Two phosphopeptides from *in vivo*-phosphorylated NHE1 (spots a and b in Figure 7B) appeared also to be generated in GST–NHE1 phosphorylated by p160ROCKΔ3 (Figure 7C). This was confirmed by mixing the two reactions (Figure 7D). These findings suggest that identical residues

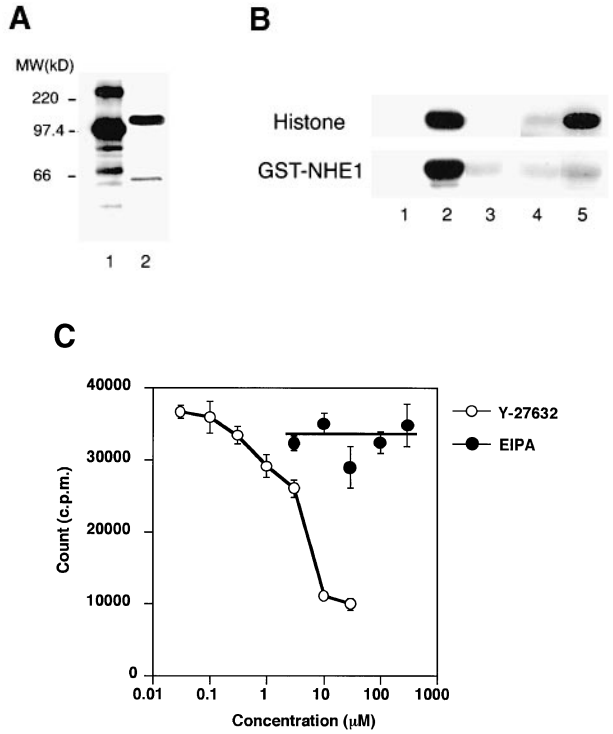

Fig. 6. p160ROCK directly phosphorylates NHE1 *in vitro*. (**A**) Myc-tagged p160ROCKΔ3 (lane 1) and PKN (lane 2), transiently expressed in CCL39 cells, were immunoprecipitated and subjected to immunoblotting with anti-Myc antibodies. (**B**) These same immunoprecipitates were used for *in vitro* kinase assays with histone or GST–NHE1$^{638–815}$ as a substrate. Lanes 1 and 4, vector control; lane 2, p160ROCKΔ3; lane 3, p160ROCKΔ3 in the presence of Y-27632; lane 5, PKN. Exposure time of lanes 1 and 2 is 30 min and that of lanes 3–5 is 4 h. (**C**) Effects of Y-27632 and EIPA on p160ROCKΔ3 kinase activity were determined by phosphorylation of histone in the presence of the indicated concentrations of these inhibitors. The results shown are representative of 2–3 determinations.

are phosphorylated by p160ROCK *in vivo* and *in vitro*. Phosphopeptides not shared by the two reactions may be generated by the different tryptic digestion pattern of full-length NHE1 in the *in vivo* reaction and of the GST–C-terminal tail fusion in the *in vitro* reaction, although we could not exclude the possibility that LPA stimulation also induces phosphorylation of NHE1 by pathways other than p160ROCK.

Increased phosphorylation of NHE1 is necessary for LPA-stimulated exchange activity

Our findings suggest that p160ROCK increases the phosphorylation and activity of NHE1. Previous findings indicate that NHE1 activity is regulated by both phosphorylation-dependent and -independent mechanisms (Grinstein *et al*., 1992; Wakabayashi *et al*., 1992, 1994a; Winkel *et al*., 1993; Goss *et al*., 1994). To determine the functional importance of phosphorylation in p160ROCK-mediated NHE1 activity, we stably expressed NHEΔ635-EE in NHE-deficient PS120 cells (PS120Δ635). All C-terminal serine residues are deleted in this truncated exchanger. The expression of NHEΔ635-EE was confirmed and compared with the expression of full-length NHE1 in PS120N cells (Figure 8A). Lysates from PS120 cells were used to confirm the specific immunoprecipitation of NHE1 (Figure 8A, lane 1 and Figure 8B, lane 1). The phosphorylation of full-length NHE1 in quiescent PS120N cells increased with serum (10% for 10 min; Figure 8B, lanes 1 and 2) or with LPA, as shown in Figure 5, lane 3. In Figure 8B, the relatively high basal phosphorylation of NHE1 is probably due to constitutive integrin activation of NHE1 in adherent cells (Tominaga and Barber, 1998). We were unable to detect basal or stimulated phosphorylation of NHE1 in PS120Δ635 cells (Figure 8B, lanes 3 and 4), confirming the loss of phosphorylation of NHEΔ635.

We next determined whether phosphorylation of NHE1 is important for an increase in NHE1 activity induced by an LPA–p160ROCK pathway. As explained above, the low (<10%) transfection efficiency of PS120 cells prevented us from studying NHE1 activity in response to transient expression of mutationally active or dominant-interfering RhoA or p160ROCK. We therefore used LPA to stimulate the activity of full-length NHE1 expressed in PS120 cells, as previously described (Vexler *et al*., 1996), and found that this increase in activity was blocked by the p160ROCK inhibitor Y-27632 (Figure 8C). In PS120Δ635 cells, quiescent NHE1 activity was greater than in PS120N cells (Figure 8C), possibly due to the loss of an internal calmodulin-binding site at residues 636–656, which has been suggested to function as an autoinhibitory domain in quiescent cells (Wakabayashi *et al*., 1994b). The LPA-induced increase in exchanger activity observed in PS120N cells was reduced by 80% in PS120Δ635 cells (Figure 8C), although the absolute activity of both exchangers in the presence of LPA was similar. In contrast, the magnitude of serum-induced increases in NHE1 activity in PS120Δ635 cells, relative to PS120N cells, was reduced by only 45% (Figure 8C). Hence, NHE1Δ635 almost completely lost the ability to be stimulated by LPA, but only partially lost the ability to be stimulated by serum.

Discussion

In this study, NHE1 activity was stimulated by the dominant active forms of p160ROCK, a RhoA-associated kinase, and RhoA-induced stimulation of NHE1 activity was blocked by the dominant-negative form of this kinase. These findings suggest that p160ROCK acts downstream of RhoA and upstream of NHE1 to mediate RhoA activation of the exchanger. Furthermore, Y-27632, a specific inhibitor of p160ROCK, not only inhibited NHE1 activation by LPA but also suppressed phosphorylation of NHE1 induced by this stimulus. Consistently, no phosphorylation and greatly reduced activation by LPA were found with NHE1Δ635, in which all serine residues in the cytoplasmic domain are deleted. These findings suggest that increased phosphorylation of NHE1 may be necessary for a p160ROCK-mediated pathway to stimulate exchange activity. The importance of NHE1 phosphorylation in regulating exchange activity has been controversial. Deletion of the C-terminal phosphorylation sites in NHE1 inhibits growth factor-induced increases in pH_i by only 50% (Wakabayashi *et al*., 1992, 1994a). However, microinjection of antibodies directed against the C-terminal phosphorylation domain completely inhibits activation of NHE1 by thrombin and endothelin (Winkel *et al*., 1993). On the other hand, activation of NHE1 by osmotic stress (Grinstein *et al*. 1992) and inhibition of NHE1 by ATP

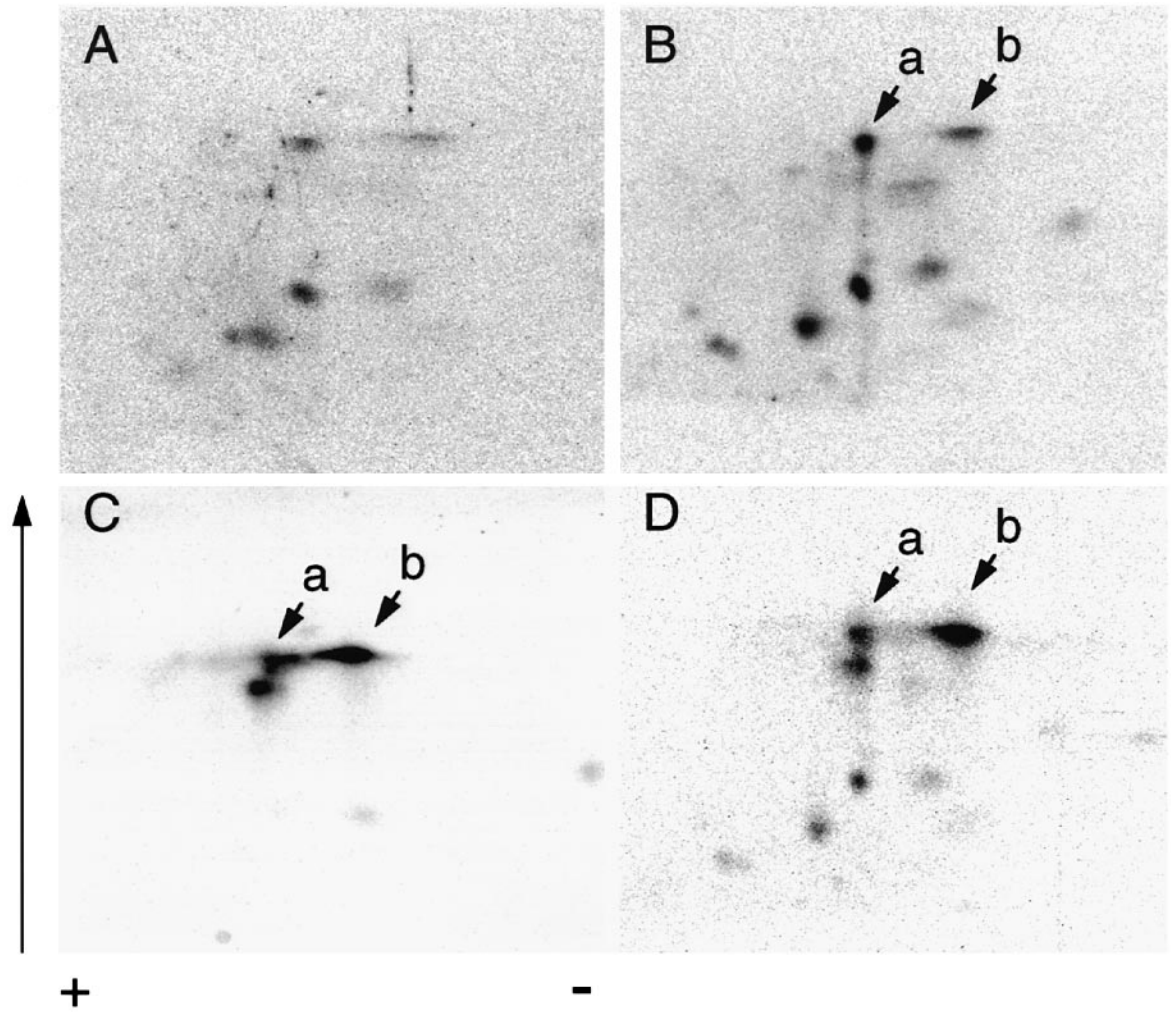

Fig. 7. Phosphopeptide mapping of NHE1 phosphorylated by LPA *in vivo* and by p160ROCKΔ3 *in vitro*. EE-tagged NHE1 immunoprecipitated from ^{32}P-labeled quiescent (**A**) and LPA-stimulated (**B**) PS120N cells, and GST–NHE1 phosphorylated *in vitro* by p160ROCKΔ3 (**C**). Phosphorylated NHE1 was isolated by SDS–PAGE, digested with trypsin and samples were loaded onto thin-layer cellulose plates. Phosphopeptides were separated by electrophoresis (horizontal dimension) and chromatography (vertical dimension), and visualized by Image Analyzer. Phosphopeptides derived *in vivo* (B) and *in vitro* (C) were mixed and analyzed (**D**). Identical radioactive spots observed with *in vivo* and *in vitro* reactions are indicated by arrows (a) and (b).

depletion (Goss *et al*., 1994) occur without detectable changes in phosphorylation of the exchanger. Our current finding that deletion of the C-terminal domain abolished most of the LPA stimulation of NHE1 but suppressed only 50% of serum stimulation indicates that, depending on the extracellular stimulus or intracellular signaling pathway, NHE1 activity is regulated by phosphorylation-dependent and -independent mechanisms, and that the LPA–RhoA pathway utilizes only the phosphorylation-dependent mechanism to stimulate the exchanger. Our findings that the *in vitro* phosphorylation of a GST–NHE1 fusion protein by p160ROCK is inhibited by Y-27632, and that identical sites on NHE1 are phosphorylated *in vivo* and *in vitro* further suggest that p160ROCK may regulate NHE1 by direct phosphorylation.

We previously determined that NHE1 acts downstream of RhoA in a pathway regulating the actin cytoskeleton (Hooley *et al*., 1996; Vexler *et al*., 1996). Activation of NHE1 is necessary for RhoA-induced stress fiber formation but is not sufficient. Increased NHE1 activity in the absence of a RhoA-mediated signal has no effect on stress fiber formation (Vexler *et al*., 1996). A direct RhoA target molecule, p160ROCK, was shown previously to induce stress fiber formation (Ishizaki *et al*., 1996). Our current results show that p160ROCK itself can activate NHE1 and selectively mediates activation of NHE1 by RhoA, but not by Rac1 or Cdc42. We also found that activation of NHE1 is necessary for p160ROCK-induced stress fiber formation. p160ROCK (Uehata *et al*., 1997) and ROKα/Rho-kinase (Kimura *et al*., 1996) previously were shown to increase myosin light chain phosphorylation and to induce cell contractility, and this action on contractility has been suggested as a mechanism for RhoA-induced stress fiber formation (Chrzanowska-Wodnicka and Burridge, 1996). Our current study indicates that activated NHE1 acts cooperatively with this myosin-based mechanism to mediate the RhoA action. The NHE1 action may precede the action on myosin, because without activation of the exchanger, p160ROCK cannot induce the assembly of stress fibers (Figure 1). What is the NHE1 action in this event? Our recent findings suggest that activation of NHE1 is required for cell spreading on a fibronectin-

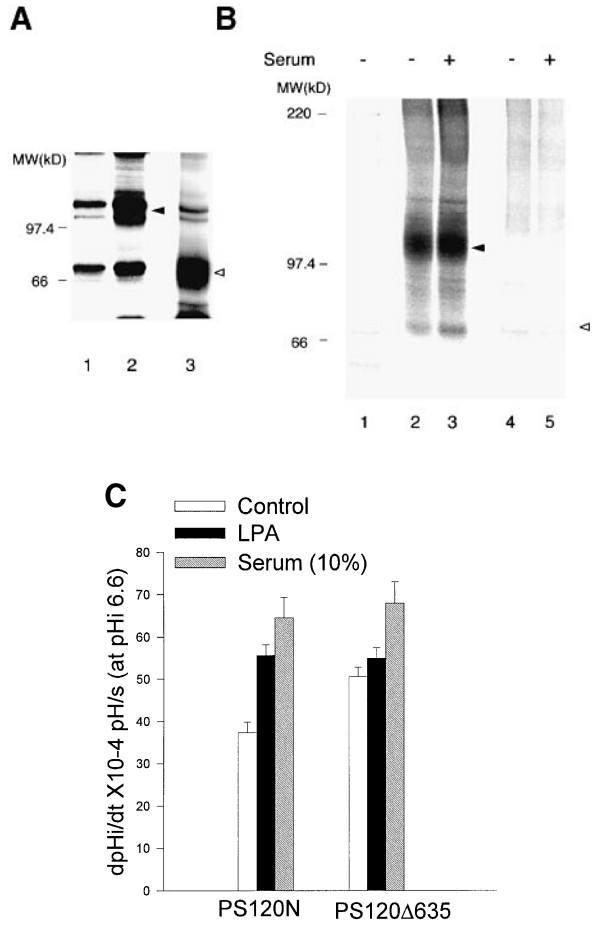

Fig. 8. Increased phosphorylation of NHE1 is necessary for LPA-stimulated exchange activity. (**A**) PS120 cells (lane 1) and PS120 cells stably expressing the EE epitope-tagged full-length NHE1 (PS120N) (lane 2) or the EE epitope-tagged C-terminally truncated NHE1Δ635 (PS120Δ635) (lane 3) were labeled with [^{35}S]methionine. Each exchanger variant was then immunoprecipitated with anti-EE antibodies and analyzed on a 7.5% acrylamide SDS–PAGE gel. (**B**) Serum-starved and ^{32}P-labeled PS120 (lane 1), PS120N (lanes 2 and 3) and PS120Δ635 cells (lanes 4 and 5) were stimulated for 10 min with 10% serum. Phosphorylated NHE1 was immunoprecipitated and analyzed. Black arrows indicate the position of wild-type NHE1, and white arrows indicate the position of truncated NHE1. (**C**) The rate of pH_i recovery from an acid load, determined at pH_i 6.6 in PS120N and PS120Δ635 cells. Data are expressed as the mean ± SEM of 4–6 separate cell preparations.

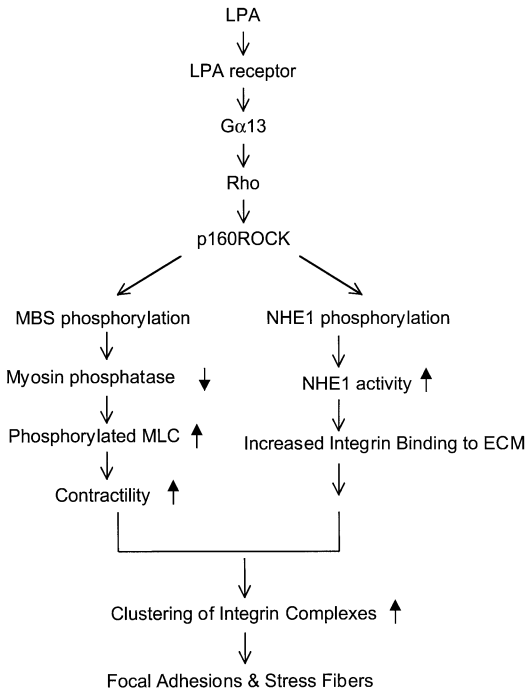

Fig. 9. A proposed pathway of the LPA–RhoA–p160ROCK signaling for induction of focal adhesions and stress fibers. MBS, myosin-binding subunit of myosin phosphatase; MCL, myosin light chain.

coated dish (Tominaga and Barber, 1998). Activation of NHE1 was found previously to be associated with cell spreading, and this activation required integrin ligation of ECM proteins (Ingber *et al*., 1990; Schwartz *et al*., 1991). Probably the initial attachment of integrins to ECM proteins triggers activation and recruitment of p160ROCK and NHE1 to a particular site of the membrane, and the subsequent activation of NHE1 further strengthens the integrin binding to facilitate cell spreading. Consistent with this idea is the report that both LPA activation and attachment to integrins are required for cell spreading and stress fiber formation (Hotchin and Hall, 1995). It is interesting in this respect that p160ROCK is recruited to the cytoskeleton complexes in a manner dependent on integrin ligation of ECM proteins (Fujita *et al*., 1998) and that NHE1 molecules accumulate focally with cytoskeletal proteins such as vinculin, talin and F-actin along the border of lamellipodia of spreading cells (Grinstein *et al*., 1993; Plopper *et al*., 1995). A proposed sequence of events is depicted in Figure 9.

The specific signal that NHE1 is contributing to regulate Rho-mediated cytoskeletal remodeling remains to be determined. The effects of NHE1 are likely to be mediated by changes in intracellular concentrations of H^+ or Na^+, or by changes in cell volume. The predominant localization of NHE1 at sites of focal contact (Grinstein *et al*., 1993; Plopper *et al*., 1995) suggests that if H^+ is an important signal, then perhaps localized pH_i gradients might be critical for the assembly of focal adhesions and the focal attachment of actin stress fibers. If localized pH_i gradients are an important signal, our findings suggest that these are generated primarily by NHE1, as HCO_3-dependent exchangers are unable to compensate for the loss of NHE1 activity. An alternative possibility is that NHE1 is structurally linked to the actin cytoskeleton, analogously to the role of the erythrocyte Cl–HCO_3 exchanger, AE1. AE1 and NHE1 share a similar structural topology of 12 transmembrane domains and a long cytoplasmic domain, and they share a similar function in regulating pH_i. AE1, however, also functions to tether actin to the plasma membrane through the binding of its cytoplasmic domain to the actin-associated proteins ankyrin (Ding *et al*., 1996) and protein 4.1 (An *et al*., 1996).

What are the functional consequences of the p160ROCK-induced activation of NHE1? NHE1 has been proposed to be involved in anchorage-dependent cell proliferation and, paradoxically, cell transformation (Grinstein *et al*., 1989; Maly *et al*., 1989; Ingber *et al*., 1990; Kaplan and Boron, 1994). Similarly, RhoA has been suggested to be involved in the G_1–S progression and cell transformation. In the former process, RhoA is presumed, but has not been proved, to induce focal adhesions and to

transmit the growth signal from these complexes (Yamamoto *et al*., 1993; Schwartz, 1997). Quite recently, RhoA has been shown to induce $p27^{Kip1}$ degradation, which is essential in G_1–S progression (Hirai *et al*., 1997). The p27 degradation is also dependent on cell adhesion, at least in some instances. On the other hand, RhoA in collaboration with Raf induces cell transformation manifested as serum independence and anchorage-independent growth. The transformed cells showed no sign of focal adhesions and stress fibers, although activated RhoA is present (Qiu *et al*., 1995b), indicating that generation of a growth signal is somehow dissociated from cell adhesion. It would be interesting to test whether these processes are also sensitive to an NHE1 inhibitor such as EIPA. NHE1 and RhoA may also be involved in cell motility (Simchowitz and Cragoe, 1986; Stasia *et al*., 1991). Cell migration has been proposed to occur by cycling the extension and adhesion in the front and the de-adhesion and retraction in the rear (Mitchison and Cramer, 1996). RhoA, if involved, is supposed to work in the former process. It would be interesting to know whether NHE1 inhibition and RhoA inactivation induce a similar inhibitory phenotype of migrating cells.

In summary, we determined that NHE1 is a downstream, and possibly direct, target of p160ROCK, and that NHE1 activity and/or phosphorylation is necessary for actin stress fiber assembly induced by p160ROCK. Together with our previous studies (Hooley *et al*., 1996; Vexler *et al*., 1996; Tominaga and Barber, 1998), these findings suggest that NHE1 activity is a critical component of normal cytoskeletal functions regulated by a RhoA–p160ROCK-mediated pathway, including cell adhesion and contractility. Additionally, a functional link between p160ROCK and NHE1 may be an important determinant in pathophysiological conditions associated with abnormal cytoskeletal organization. Recent experiments using Y-27632 implicate a role for RhoA–p160ROCK in augmenting blood pressure in hypertensive rats (Uehata *et al*., 1997). Although the inhibitory effect of the Y-compound on hypertension has been attributed solely to its inhibition of smooth muscle contraction, it is quite likely that Y-27632 corrects high blood pressure also by inhibiting enhanced NHE1 activity. NHE1 activity is increased in blood and vascular smooth muscle cells of hypertensive patients and in animal models of genetic hypertension such as the spontaneously hypertensive rat (Aviv, 1996). Additionally, transgenic mice overexpressing recombinant NHE1 have salt-sensitive hypertension (Kuro-o *et al*., 1995). We expect that similar pathophysiological links between NHE1 and the Rho–p160ROCK pathway will be found in other disease states.

Materials and methods

Expression plasmids

pCAG-myc-p160ROCK and related mutants (Ishizaki *et al*., 1997), pcDNA1-Gα13QL (Voyno-Yasenetskaya *et al*., 1994), pEXV-myc-RhoAV14 (Qiu *et al*., 1995b), pEXV-myc-Rac1V12 (Qiu *et al*., 1995a) and pCMV-myc-Cdc42V12 (Hooley *et al*., 1996) constructs were produced as previously described. pCAN-myc-PKN was provided by Dr G.Martin (ONYX Pharmaceuticals). Human NHE1 cDNA was provided by Dr J.Poussegur (University of Nice, France) and subcloned into pcDNA1 (Invitrogen) with a Glu–Glu epitope (EE) tag at the C-terminus (NHE1-EE). Using this plasmid as a template, a PCR product was generated that contained residues 1545–1905 of the NHE1 cDNA along with an in-frame sequence encoding an EE epitope tag (Grussenmeyer *et al*., 1985) and a *Bam*HI site at the 3′ end. The *Hin*dIII–*Bam*HI fragment from this PCR product was subcloned into pcDNA3 (Invitrogen) and sequenced. A *Hin*dIII–*Bal*I fragment from the full-length NHE1 cDNA was then subcloned between the *Hin*dIII site in this plasmid and the *Bal*I site contained within the NHE1 coding region to restore the 5′ end of the NHE1 coding region (NHE1Δ635-EE). A GST fusion protein comprising the 178 C-terminal amino acids of rabbit NHE1 was obtained from Dr L.Fliegel (University of Alberta, Edmonton) (Silva *et al*., 1995) and purified by glutathione–Sepharose 4B beads (Pharmacia) according to the manufacturer's instructions. EIPA was obtained from Molecular Probes (Eugene, OR).

Cells

CCL39 cells, a Chinese hamster lung fibroblast line; PS120 cells, an NHE-deficient clone derived from parental CCL39 cells (Pouyssegur *et al*., 1984); and PS120 cells stably expressing NHE1-EE (PS120N) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). HeLa cells were maintained in DMEM supplemented with 10% heat-inactivated FBS. Full-length NHE1 and NHE1Δ635 were stably expressed in PS120 cells (PS120N and PS120Δ635, respectively) by a calcium phosphate method (Speciality Media Inc., NJ) with 20 μg of cDNA. Clones were selected by geneticin and by sequential proton suicide incubations as previously described (Pouyssegur *et al*., 1984). This latter selection method eliminates cells not expressing NHE1 because in a HEPES buffer they are unable to extrude protons after an NH_4Cl-induced acid load. Expression of NHE1 was confirmed by measuring the rate of pH_i recovery from an acid load in a HEPES buffer (Voyno-Yasenetskaya *et al*., 1994) and by immunoprecipitating NHE1 from ^{35}S-labeled cells using mouse anti-EE monoclonal antibody (Grussenmeyer *et al*., 1985). For transient expressions, cells were plated at a density of 5×10^5 cells per 6 cm dish. After 24 h, the cells were transfected using lipofectamine (Gibco Life Technologies, Inc.) for 3–4 h with 2–3 μg of DNA in Opti-MEM. pcDNA3 empty vector was used to maintain total transfected DNA constant. Cells were maintained in growth medium for 3–4 h and then in serum-free DMEM for 16–20 h prior to experiments. Transfection efficiencies in CCL39 cells were routinely 30–35%, as determined by staining for β-galactosidase expression. Phalloidin staining and myc immunostaining were performed as previously described (Vexler *et al*., 1996).

NHE1 activity and intracellular pH

To determine NHE1 activity and pH_i, cells plated on glass coverslips were serum-starved for 16–20 h, transferred to a nominally HCO_3-free HEPES buffer, and loaded for 10 min at 37°C with 1 μM of the acetoxy methyl ester of the pH-sensitive fluorescent dye BCECF. Cells were placed in a thermostatically controlled (37°C) cuvette holder in a Shimadzu RF5000 spectrofluorometer, and BCECF fluorescence was measured at 530 nm by alternately exciting the dye at 500 and 440 nm. The emission ratio was calibrated to pH for each determination using 10 μM nigericin as previously described (Thomas *et al*., 1979). To determine NHE1 activity, cells were pulsed for 10 min with 30 mM NH_4Cl and then transferred to a HEPES buffer (Boron and De Weer, 1976). The rate of pH_i recovery (dpH_i/dt) from an acid load induced by the rapid removal of NH_4Cl was calculated at pH_i intervals of 0.05 units and used as an index of NHE1 activity. To study the effects of LPA and serum, these agents were added in the NH_4Cl pulse and in the HEPES recovery buffer. The values for pH_i and dpH_i/dt were expressed as the mean ± SEM of the indicated number of matched separate transfections or of separate cell preparations for LPA and serum treatment.

Phosphorylation of NHE1

Cells grown to sub-confluence in 100 mm dishes were serum deprived for 22 h, then pre-incubated in a nominally phosphate-free, serum-free medium for 2 h and labeled for an additional 5 h at 37°C with [^{32}P]orthophosphate (100–200 μCi/ml). For LPA stimulation, labeled cells were first dissociated by dissociation buffer (Gibco), plated on poly-L-lysine-coated dishes and incubated in serum-free medium for 45 min with or without Y-27632 (Uehata *et al*., 1997). LPA (10 μM) was then added for an additional 10 min. For serum stimulation, serum (10% final) was added directly to adherent labeled cells for 10 min at 37°C. After stimulation, the cells were washed with ice-cold phosphate-buffered saline (PBS), frozen in liquid N_2 and resuspended in buffer A [50 mM HEPES–NaOH (pH 7.4), 150 mM NaCl, 3 mM KCl, 12.5 mM sodium pyrophosphate, 1 mM ATP, 5 mM EDTA supplemented with protease inhibitors]. Samples were centrifuged for 15 min at 100 000 *g*. The pellets were resuspended in 500 μl of ice-cold buffer B [buffer A

containing 1% Brij 96 (Sigma)], sonicated for 40 s, and centrifugated for 30 min at 100 000 *g*. The supernatants were pre-cleared with anti-mouse IgG–agarose (Sigma) and then incubated overnight at 4°C with 5 μg of mouse anti-EE monoclonal antibodies. Anti-mouse IgG–agarose was added for 1 h at 4°C and then washed five times with buffer B. Immunoprecipitated proteins were solubilized by boiling in Laemmli sample buffer and separated by SDS–PAGE (8% polyacrylamide). The gel was dried and subjected to analysis using an Image Analyzer (Molecular Dynamics).

Kinase assays

Cells transiently expressing 2 μg of pCAG-myc-p160ROCKΔ3, 3 μg of pCAN-myc-PKN or 2 μg of pcDNA3 were lysed with lysis buffer [10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin and 2 μg/ml aprotinin], and Myc-tagged kinases were immunoprecipitated with anti-Myc (9E10) antibody as described above. After immunoprecipitation, the beads were suspended with 400 μl of kinase wash buffer (20 mM Tris–HCl, pH 7.4, 1 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM Na_3VO_4, 5 mM β-mercaptoethanol). A 100 μl fraction of the sample was sedimentated and resuspended in 20 μl of kinase buffer containing 25 mM HEPES–NaOH (pH 7.5), 1 mM dithiothreitol, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 μM Na_3VO_4, 10 μM ATP and 0.35 μCi of [^{32}P]ATP. GST–NHE1 (3 μg) or histone H1 (3 μg) was added as a substrate for kinase activity and the kinase mixture was incubated at 30°C for 20 min. The reaction was stopped by adding 5 μl of 5× Laemmli's sample buffer. Samples were resolved by SDS–PAGE (12% acrylamide) and incorporaton of radioactivity was determined by autoradiography (Figure 6B) or scintillation counting of excised radioactive bands (Figure 6C). Equal loading of immunoprecipitated kinases was confirmed by immunoblotting with anti-Myc polyclonal antibody (A-14, Santa Cruz Biotechnology) as described previously (Tominaga and Barber, 1998). The effects of EIPA and Y-27632 on p160ROCK activity were determined by including these inhibitors in the *in vitro* kinase reactions.

Phosphopeptide mapping

NHE1 phosphorylated *in vivo*, and GST–C-terminal NHE1$^{638–815}$ phosphorylated by p160ROCKΔ3 *in vitro* were isolated by SDS–PAGE, transferred to nitrocellulose membranes and identified by autoradiography. The immobilized proteins were digested twice with 20 μg of TPCK-trypsin (Promega) for 10–15 h with shaking at 37°C in 1% ammonium bicarbonate, pH 8.3. The resulting tryptic peptides were lyophilized and separated on thin-layer cellulose plates by electrophoresis in the first dimension and by chromatography in the second dimension, as previously described (Sardet *et al*., 1991). Phosphopeptides were visualized by Image Analyzer.

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