

## Two G protein-coupled receptors activate $\text{Na}^+/\text{H}^+$ exchanger isoform 1 in Chinese hamster lung fibroblasts through an ERK-dependent pathway

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### Abstract

The sodium hydrogen exchanger isoform 1 (NHE1) is present in nearly all cells. Regulation of proton flux via the exchanger is a permissive step in cell growth and tumorigenesis and is vital in control of cell volume. The regulation of NHE1 by growth factors involves the Ras-extracellular signal regulated kinase (ERK) pathway, however, the mechanism for G protein-coupled receptor (GPCR) activation of NHE1 is not well established. In this report, the relationship between GPCRs, ERK, and NHE1 in CCL39 cells is investigated. We give evidence that two agonists, the specific  $\alpha_1$ -adrenergic agonist, phenylephrine and the water-soluble lipid mitogen, lysophosphatidic acid (LPA) activate NHE1 in CCL39 cells. Activation of ERK by phenylephrine and LPA occurs in a dose- and time-dependent manner. Optimal ERK activation was observed at 10 min and displayed a maximum stimulation at 100  $\mu\text{M}$  phenylephrine and 10  $\mu\text{M}$  LPA.  $\alpha_1$ -Adrenergic stimulation also led to a rise in steady-state  $\text{pH}_i$  of  $0.16 \pm 0.02$  pH units, and incubation with LPA induced a  $0.43 \pm 0.06$  pH unit increase in  $\text{pH}_i$ . Phenylephrine-induced activation of NHE1 transport and ERK activity was inhibited by pretreating the cells with the MEK inhibitor PD98059. While only half of the LPA activatable exchange activity was abolished by PD98059 and U0126. To further demonstrate the specificity of the phenylephrine and LPA regulation of NHE1 and ERK, CCL39 cells were transfected with a kinase inactive MEK. The data indicate that ERK activation is essential for phenylephrine stimulation of NHE1, and that ERK and RhoA are involved in LPA stimulation of NHE1 by more than one mechanism. In addition, evidence of the convergence of these two pathways is shown by the loss of NHE1 activity when both pathways are inhibited and by the partial additivity of the two agonists on ERK and NHE1 activity. These studies indicate a direct involvement of ERK in the  $\alpha_1$ -adrenergic activation of NHE1 and a significant role for both ERK and RhoA in LPA stimulation of NHE1 in CCL39 fibroblasts.

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**Keywords:** Sodium hydrogen exchanger; ERK; Lysophosphatidic acid; Alpha adrenergic receptor; Phenylephrine

### 1. Introduction

Regulation of several normal and abnormal cellular processes including maintenance of cell volume, growth

control, intracellular pH ( $\text{pH}_i$ ) homeostasis, tumorigenesis and cellular invasion are each partially or fully under the control of the sodium hydrogen exchanger [1–4]. The sodium hydrogen exchanger isoform 1 (NHE1) is expressed in nearly all mammalian cells and is regulated by a wide variety of signaling molecules [5,6]. This regulation occurs in part due to phosphorylation at the carboxyl terminus of the exchanger. NHE1 catalyzes the electroneutral exchange of 1  $\text{Na}^+$  for 1  $\text{H}^+$  using the  $\text{Na}^+$  electrochemical gradient to extrude protons. The transporter displays a hyperbolic dependence upon extracellular  $\text{Na}^+$  and follows simple Michaelis–Menten kinetics. At resting  $\text{pH}_i$ , the hydrogen ion concentration is at the set point of the exchanger and the

*Abbreviations:* BCECF, 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxy-fluorescein; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptors; KI-MEK1, kinase inactive MAP kinase/ERK kinase 1; LPA, lysophosphatidic acid; NHE1, sodium hydrogen exchanger isoform 1; PE, phenylephrine;  $\text{pH}_i$ , intracellular pH; RSK, p90 ribosomal S6 kinase; ROCK, p160 RhoA associated kinase; TMA, trimethylammonium chloride.

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transport function is inactive. Modification of the carboxyl terminus of the exchanger is proposed to result in an increase in affinity for hydrogen ions at the pH sensor domain, presumably activating the transport function at a lower hydrogen ion concentration.

Several protein kinases such as the growth factor-mediated p90 ribosomal S6 kinase (RSK) [7], protein kinase C (PKC) [8], calcium calmodulin-dependent protein kinase [9] and p160 Rho-associated kinase (ROCK) [10,11] have been demonstrated to phosphorylate NHE1 and increase the rate of ion exchange. Abrogation of extracellular signal-regulated kinase (ERK) has been used to identify the requirement for the Raf/MEK/ERK pathway in growth factor activation of NHE1 in CCL39 cells [6]. The importance of the ERK pathway was further demonstrated when RSK, one of the substrates for ERK, was demonstrated to stoichiometrically phosphorylate serine 703 of NHE1 and increase proton exchange in response to serum and angiotensin [7].

Many agonists that signal through heterotrimeric G proteins also alter  $\text{pH}_i$ , however, this mechanism is much more varied and less understood than the growth factor pathways. Expression of constitutively active  $\text{G}\alpha_q$ ,  $\text{G}\alpha_s$ ,  $\text{G}\alpha_{12}$ , or  $\text{G}\alpha_{13}$  subunits can activate the exchanger through various pathways [4]. Phosphopeptide analysis of the carboxyl terminus of the exchanger after transfection with constitutively active  $\text{G}\alpha_{13}$  or incubation with LPA identified additional phosphorylation sites due to the RhoA directed protein kinase, ROCK [10,11]. In HEK293 and COS-7 cells, expression of active  $\text{G}\alpha_q$  also leads to the activation of the exchanger presumably through a Raf-1-independent, PKC-dependent mechanism [12,13]. Because both growth factors and G protein-coupled receptors that activate NHE1 can also lead to the activation of the ERK pathway, it is highly possible that the growth factor pathway is at least partially responsible for the activation of NHE1 by G protein-coupled receptors.

In this study, we investigate the role of two G protein-coupled receptor agonists, lysophosphatidic acid (LPA) and the  $\alpha_1$ -adrenergic agonist phenylephrine in the ERK-dependent activation of NHE1. Both LPA and phenylephrine induce alkalinization of  $\text{pH}_i$  through NHE1 in CCL39 fibroblasts. Furthermore, we show that both agonists can activate ERK and that this activation is a requirement for full phenylephrine activation and partial LPA activation of NHE1. These data provide a link between G protein-coupled receptors, ERK and NHE1 in CCL39 cells.

## 2. Experimental procedures

### 2.1. Cell culture

Chinese hamster lung fibroblasts (CCL39, ATCC) were maintained in attachment culture with high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma) and

10% heat-inactivated fetal bovine serum (FBS Gibco) at 37 °C in a 5%  $\text{CO}_2$  incubator. Cells were split 1:10 upon reaching 60–70% confluence. A maximum of eight passages were allowed to maintain consistent cell growth and morphology. For experiments to determine NHE1 activity, cells were transferred into T-75 flasks and maintained in complete medium. This transfer was conducted in order to increase cell number without exceeding 70% confluency. Twelve to eighteen hours prior to experiments, the cells were harvested and suspended in spinner culture containing high glucose DMEM with 0.5% heat-inactivated FBS. Maintenance of cells in low serum spinner culture allowed maximal inactivation of signaling pathways and optimal responses to agonists. All culture medium included penicillin, streptomycin, and amphotericin B. For experiments to determine ERK activation, cells were transferred from T25 flasks to 35-mm culture dishes and allowed to grow 24 h. Cells were then placed in high glucose DMEM with 0.5% heat-inactivated FBS for 12 to 18 h prior to experiments. For transfection experiments with kinase inactive HA-tagged MEK1 (KI-MEK1, a gift from Natalie Ahn, University of Colorado at Boulder and Howard Hughes Medical Institute), cells were transferred to 35-mm culture dishes and allowed to grow 12 to 18 h. Cells were then transfected and incubated for 36 h for protein expression prior to experiments.

### 2.2. Transfection

CCL39 cells were transiently transfected with KI-MEK1 upon reaching 60–70% confluency. For transfection, 11.5  $\mu\text{l}$  Fugene-6 (Roche Biochemical) was added to 100  $\mu\text{l}$  of serum- and antibiotic-free OptiMEM 1 (Gibco) and incubated for 10 min. Then, 7.5  $\mu\text{g}$  of endotoxin free KI-MEK1 plasmid (prepared by DragonTech) was added to the Fugene-6. This mixture was allowed to incubate for 30 min to form the Fugene-6/DNA complex. During this incubation, the media was removed from the cells and the dishes were washed three times with phosphate-buffered saline (PBS). OptiMEM 1 (1.4 ml) was then added to each dish and the Fugene-6/KI-MEK1 complex (100  $\mu\text{l}$ ) was added to the dishes dropwise. Cells were then allowed to incubate overnight at 37 °C in a 5%  $\text{CO}_2$  incubator. The next morning, 1.5 ml of high glucose DMEM with 0.5% heat-inactivated FBS was added to each dish and the cells returned to the incubator for 24 h. At this time, all the media was removed from the cells, the wells rinsed with PBS, and incubated with 2.0 ml of high glucose DMEM with 0.5% heat-inactivated FBS. Cells were used for experiments 36 h following transfection.

### 2.3. NHE1 measurements

The pH-sensitive fluorescent dye, 2',7'-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), was used to measure changes in  $\text{pH}_i$ . CCL39 cells were incubated

with 10  $\mu\text{M}$  of the uncharged acetoxymethyl ester form of BCECF (BCECF-AM, Molecular Probes) for 30 min at 37  $^{\circ}\text{C}$  in sodium buffer (Na buffer—20 mM HEPES pH 7.40 at 37  $^{\circ}\text{C}$ , 145 mM NaCl, 3 mM KCl, 1 mM  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , and 5 mM Glucose). Cytoplasmic esterases hydrolyze the esterified dye producing a charged BCECF which is trapped in the cells. A Spex Industries FluoroMax was used to measure changes in fluorescence intensity. The fluorescence intensity of emission was measured at 525 nm while the dye was excited alternately at the pH-sensitive wavelength of 502 nm and the pH-insensitive isoexcitation wavelength of 439 nm.  $\text{pH}_i$  was determined by the fluorescence intensity ratio method with a calibration curve generated using the  $\text{K}^+$ -Nigericin method [14].

Three different protocols were employed to study changes in  $\text{pH}_i$ : (1) steady-state  $\text{pH}_i$  changes upon agonist addition, (2) changes in steady-state  $\text{pH}_i$  upon addition of a weak base to determine buffer capacity ( $\beta$ ), and (3) initial rates of alkalization after an acid load.

In the first method, steady-state  $\text{pH}_i$  was measured prior to the cells being subjected to various concentrations of the agonists LPA or phenylephrine. The fluorescence intensity was then measured for 15–30 min following agonist addition to determine the activation of the NHE1 by measuring the change in  $\text{pH}_i$ .

In the second procedure, the weak base trimethylammonium chloride (TMA) was used to determine buffer capacity. Addition of TMA to a final concentration of 10 mM caused an alkalization by extracting protons from the cell cytoplasm. Cells were acid loaded at various  $\text{NH}_4\text{Cl}$  concentrations and resuspended in  $\text{Na}^+$ -free buffer, as explained below, in order to vary initial  $\text{pH}_i$  values. The fluorescence intensity was measured for 5 min prior to and 10 min following addition of TMA in order to determine buffer capacity.

The third procedure, acid loading, was accomplished using the ammonium chloride prepulse technique [15]. During acid loading, dye-loaded cells were treated with varying concentrations of  $\text{NH}_4\text{Cl}$  and incubated for 10 min in a shaking 37  $^{\circ}\text{C}$  water bath. After incubation, the ammonium chloride solution was removed,  $\text{Na}^+$  buffer was added, and the rate of recovery from the ensuing acid load was measured for 15 min.

Utilizing both the buffer capacity and initial rate data, proton efflux ( $J_H$ ) was determined.  $J_H$  was calculated using the equation  $J_H = \beta * \Delta\text{pH}_i/\text{min}$ . In this equation, the buffer capacity of the cells at the beginning of recovery from an acid load was used.  $\Delta\text{pH}_i/\text{min}$  was determined by calculating the slope of the first 30 to 60 s of the recovery. The units of  $J_H$  are expressed as  $\text{mmol H}^+/\text{l cell H}_2\text{O}/\text{min}$ .

#### 2.4. Agonist treatment and cell harvest

Cells were cultured onto 35-mm tissue culture dishes and allowed to grow to approximately 70–80% confluence. Cells were growth-arrested for 12 h with high glucose

DMEM containing 0.5% serum and 0.5% fatty acid-free BSA with antibiotics and antimycotics. One hour prior to agonist treatment, the cells were rinsed with PBS and incubated in serum-free high glucose DMEM containing 0.5% fatty acid-free BSA without antibiotics and antimycotics. Cells were then treated with specific agonists, inhibitors, or activators as indicated. Following the incubation periods, the cells were washed twice with chilled PBS and lysed in 200  $\mu\text{l}$  Laemmli sample buffer containing 0.1 mM orthovanadate and 1 mM  $\beta$ -glycerophosphate. The samples were boiled and DNA sheered with five passes through a 27-gauge needle. The protein content of lysed samples was analyzed by the Non-Interfering Protein Assay (GenoTechnology).

#### 2.5. SDS-PAGE and Western blot analysis

Lysate was resolved by 10 or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane using a semi-wet electroblotting apparatus. Phosphorylated ERK (p-ERK) was detected by immunoblotting the membrane using a 1:1000 dilution of p-ERK mouse monoclonal IgG (Santa Cruz Biotechnology) in TTBS (10  $\mu\text{M}$  Tris and 150 mM NaCl, pH 8.0 with 0.05% Tween 20) with 5% dry milk overnight at 4  $^{\circ}\text{C}$ . Total ERK was detected by immunoblotting using a 1:1000 dilution of rabbit polyclonal IgG (Santa Cruz Biotechnology). Bound antibodies were detected using a secondary antibody, horseradish peroxidase conjugated IgG (Santa Cruz Biotechnology), diluted at 1:10,000 in TTBS with 5% dry milk. Proteins were detected using an enhanced chemiluminescence detection reagent (Santa Cruz Biotechnology). Densitometric analysis of Western blots were quantified using NIH Image 1.61.

#### 2.6. Immunokinase assay

Specific activation of ERK was performed from whole cell lysates after stimulation with LPA or phenylephrine. Cells were scraped in homogenization buffer (10 mM HEPES pH 7.4, 50 mM  $\beta$  glycerol phosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g}/\text{ml}$  aprotinin, 0.5  $\mu\text{M}$  leupeptin, 0.7  $\mu\text{g}/\text{ml}$  pepstatin A, 1  $\mu\text{g}/\text{ml}$  antipain and 1.0 mM dithiothreitol). The cells were lysed by sonication and equal amounts of lysates were incubated for 1–2 h at 4  $^{\circ}\text{C}$  with 1  $\mu\text{g}$  anti-ERK antibody (Santa Cruz Biotechnology) followed by collection of immune complexes for 2 h at 4  $^{\circ}\text{C}$  using protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). The immunoprecipitates were washed three times with RIPA Buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate) and once with assay dilution buffer (20 mM MOPS, pH 7.2, 25 mM  $\beta$ -glycerol phosphate 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol). Assays for ERK activity were performed for 5 min

at 30 °C using the MAP kinase assay kit (Upstate Biotechnology) with PKC and PKA peptide inhibitors, the calmodulin antagonist calmidazolium, 100  $\mu$ Ci of [ $\gamma$ - $^{32}$ P] ATP and myelin basic protein as a substrate. A 25- $\mu$ l aliquot of each sample was spotted onto phosphocellulose paper (P81, Whatman). The paper squares were washed in 75 mM phosphoric acid, with three changes of solution and a final wash with acetone. The bound radiolabeled peptide was quantified by scintillation counting.

### 3. Results

#### 3.1. Lysophosphatidic acid and phenylephrine activation of NHE1

To evaluate the ability of LPA or phenylephrine to activate NHE1 in CCL39 fibroblasts, changes in  $pH_i$  were measured using BCECF (Fig. 1). CCL39 cells that were serum deprived overnight had a resting  $pH_i$  of  $6.93 \pm 0.09$  ( $n=40$ ). The addition of either LPA or phenylephrine stimulated an increase in the steady-state  $pH_i$  in a dose-dependent fashion (Fig. 2). The addition of 100  $\mu$ M LPA caused an average increase in  $pH_i$  of  $0.43 \pm 0.06$  pH units. When LPA was prepared in the presence of fatty acid-free BSA, a similar but muted increase in  $pH_i$  was observed (data not shown). A typical trace upon addition of 100  $\mu$ M LPA is shown in Fig. 1A. The addition of 100  $\mu$ M phenylephrine stimulated an average increase in  $pH_i$  of  $0.16 \pm 0.02$  pH units. Fig. 1B shows an experiment where the resting  $pH_i$  began at 7.01 and increased to 7.19 following the addition of phenylephrine. This increase in  $pH_i$  occurred over a much slower time course taking over 20 min to complete. In both cases, the alkalinization was contributed to the activation of the NHE1 since the activation did not occur in the absence

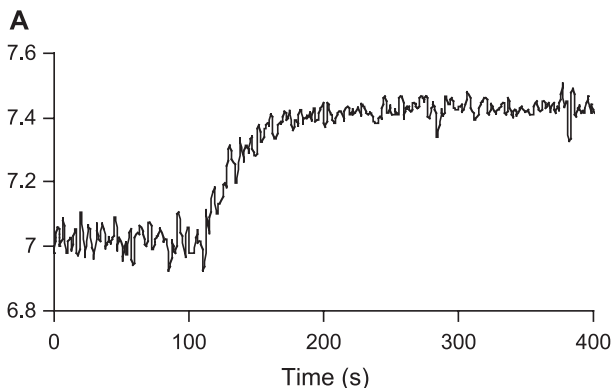


Fig. 1. LPA and phenylephrine activation of NHE1 in CCL39 fibroblasts. Serum-deprived quiescent cells, dye-loaded with BCECF, were suspended in experimental buffer in a quartz cuvette. The average resting  $pH_i$  over the initial 40 experiments was  $6.93 \pm 0.09$ . (A) The initial steady-state  $pH_i$  was measured for 120 s and then cells were exposed with 100  $\mu$ M LPA. The steady-state  $pH_i$  changed from 7.00 to 7.41, an alkalinization of 0.41 pH units. (B) The initial steady-state  $pH_i$  was measured for 200 s and then cells were exposed to 100  $\mu$ M phenylephrine (PE). The steady-state  $pH_i$  changed from 7.01 to 7.19, an alkalinization of 0.18 pH units.

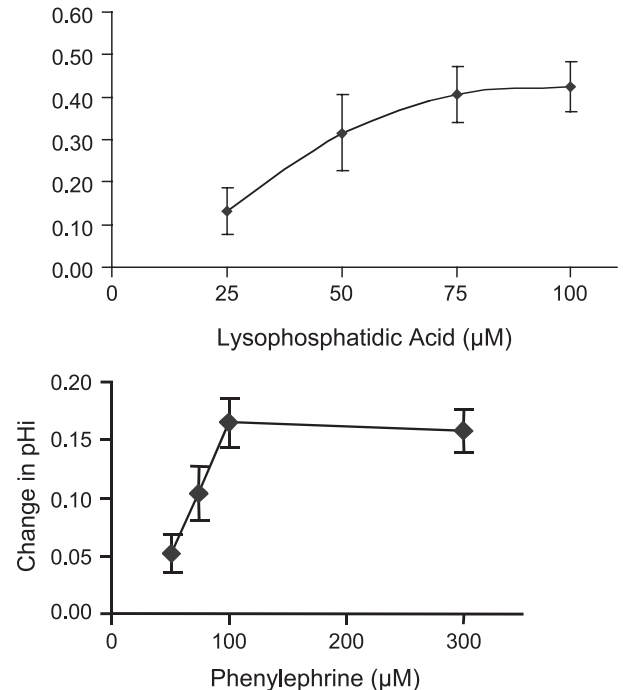


Fig. 2. The dose dependence of NHE1 stimulation by LPA and phenylephrine. Cells were allowed to establish a steady-state  $pH_i$  as seen in Fig. 1. Agonist was then added and a new steady-state  $pH_i$  was established. The change in  $pH_i$  was then calculated. The dose dependence of LPA stimulation of NHE1 was determined by adding 25, 50, 75, or 100  $\mu$ M LPA as shown in A, and plotting against the change in  $pH_i$ . LPA additions caused alkalinizations of  $0.13 \pm 0.05$ ,  $0.32 \pm 0.09$ ,  $0.41 \pm 0.06$ ,  $0.43 \pm 0.06$ , respectively. (B) Phenylephrine (PE) stimulation of NHE1 was determined by adding 50, 75, 100, or 300  $\mu$ M PE as shown in A and plotting against the change in  $pH_i$ . PE additions caused alkalinizations of  $0.051 \pm 0.02$ ,  $0.10 \pm 0.02$ ,  $0.16 \pm 0.02$ ,  $0.16 \pm 0.02$ , respectively.

of extracellular  $Na^+$  or in the presence of ethylisopropyl amiloride (data not shown). At all concentrations of agonist, LPA stimulated a two-to-three fold greater increase in  $pH_i$  than phenylephrine. Maximal activation by LPA occurred at concentrations above 75  $\mu$ M while maximal activation by phenylephrine did not occur until concentrations higher than 100  $\mu$ M (Fig. 2).

#### 3.2. Lysophosphatidic acid and phenylephrine cause an alkaline shift in the $pH_i$ -dependence of NHE1

To better interpret the activation of NHE1 by these agonists, the  $pH_i$ -dependence of transport was determined. These experiments involved the calculation of proton efflux rates ( $J_{H^+}$ ). BCECF-loaded, serum-deprived cells were acidified to different levels using the  $NH_4Cl$  prepulse technique. The acid loading was done either in the presence or absence of agonist. To determine buffer capacity, the cells were exposed to an intracellular acid load and then 10 mM TMA was added. Fig. 3A shows an example of one such experiment where the  $pH_i$  changed from 6.88 to 7.09 an alkalinization of 0.21 pH units, indicating a buffer capacity of 39 mmol  $H^+$ /l cell  $H_2O$ . A series of these experiments were performed to determine

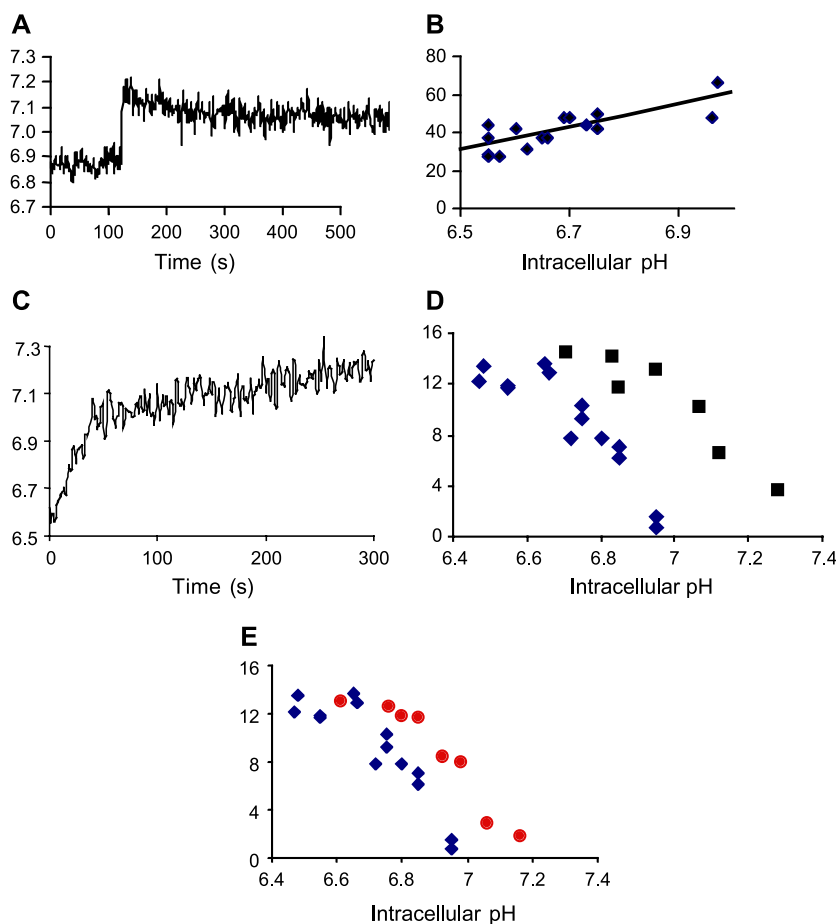


Fig. 3. Determination of  $\text{pH}_i$ -dependence of NHE1 in the presence and absence of LPA and phenylephrine. Serum-deprived quiescent cells, dye-loaded with BCECF, were acid loaded with  $\text{NH}_4\text{Cl}$  for 20 min.  $\text{NH}_4\text{Cl}$  was then removed and the isolated cells were suspended in Na Buffer in a quartz cuvette, while the change in  $\text{pH}_i$  associated with the recovery from an acid load was observed. (A) Isolated cells were suspended in Na buffer in a quartz cuvette and  $\text{pH}_i$  determined for 120 s. Cells were exposed to TMA at a final concentration of 10 mM for 380 s to determine the change in  $\text{pH}_i$ . (B) TMA was added to a final concentration of 10 mM.  $\beta$  was calculated as  $\Delta\text{H}^+/\Delta\text{pH}_i$  upon addition of TMA. (C) Cell recovering from an intracellular acid load. (D) The acid load was conducted in the presence of 100  $\mu\text{M}$  LPA to fully activate NHE.  $\text{H}^+$  efflux was calculated. Diamonds indicate control values determined in the absence of agonist addition. Squares indicate recovery rates with NHE1 stimulated by 100  $\mu\text{M}$  LPA. (E) The acid load was conducted in the presence of 100  $\mu\text{M}$  PE to fully activate NHE1.  $\text{H}^+$  efflux was calculated. Diamonds indicate the control values calculated in the absence of agonist. Squares indicate recovery rates with NHE1 stimulated by 100  $\mu\text{M}$  phenylephrine (PE).

the change in buffer capacity over the physiological  $\text{pH}_i$  range of our studies. A linear regression analysis was then performed giving  $\beta$  values that varied from 30.1  $\text{mmol H}^+/\text{l cell H}_2\text{O}$  at 6.5 to 60.4  $\text{mmol H}^+/\text{l cell H}_2\text{O}$  at 7.0 (Fig. 3B). Following acid loading, the rate of change in  $\text{pH}_i$  over time was determined (Fig. 3C). This rate of change was multiplied by the buffer capacity ( $\beta$ ) at the  $\text{pH}_i$  at which the recovery began. In all cases, maximal recovery rates of 12 to 14  $\text{mmol H}^+/\text{l cell H}_2\text{O}/\text{min}$  were seen at starting  $\text{pH}_i$  values of 6.60 and below. Conducting the acid load in the presence of 100  $\mu\text{M}$  LPA led to an alkaline shift in the  $\text{pH}_i$ -dependence of approximately 0.40 pH units, a value consistent with the LPA-induced changes in steady-state  $\text{pH}_i$  (Fig. 3D). In a similar manner, the addition of 100  $\mu\text{M}$  phenylephrine caused a shift in the  $\text{pH}_i$ -dependence of NHE1 of approximately 0.15 pH units, a value nearly identical to the phenylephrine stimulated alteration in steady-state  $\text{pH}_i$  (Fig. 3E). It appears that both

LPA and phenylephrine induce changes in resting  $\text{pH}_i$  by altering the sensitivity of NHE1 to intracellular  $\text{H}^+$ . It should be noted that the change in steady-state  $\text{pH}_i$  and the shift in the  $\text{pH}_i$ -dependence was substantially larger than those reported in the literature. Vexler et. al. [16] reported that LPA induced a maximal change in steady-state  $\text{pH}_i$  of approximately 0.20 pH units with a similar change in the  $\text{pH}_i$ -dependence of transport activity. One challenge in comparing these data is the substantial difference in LPA concentrations used in the activation experiments. In the earlier work, an LPA concentration of 500 nM was used [16]. This work did not include either a dose–response curve for LPA activation or details of the preparation of LPA for experiments. Additional difficulties are potentially due to the method of LPA preparation. Addition of BSA as a carrier into the stock LPA solution can decrease efficacy of the lipid. These differences make direct comparison particularly difficult.

3.3. Lysophosphatidic acid and phenylephrine activation of ERK in CCL39 cells is both dose and time dependent

Growth factor receptor-mediated activation of ERK is specifically required for the serum-dependent activation of

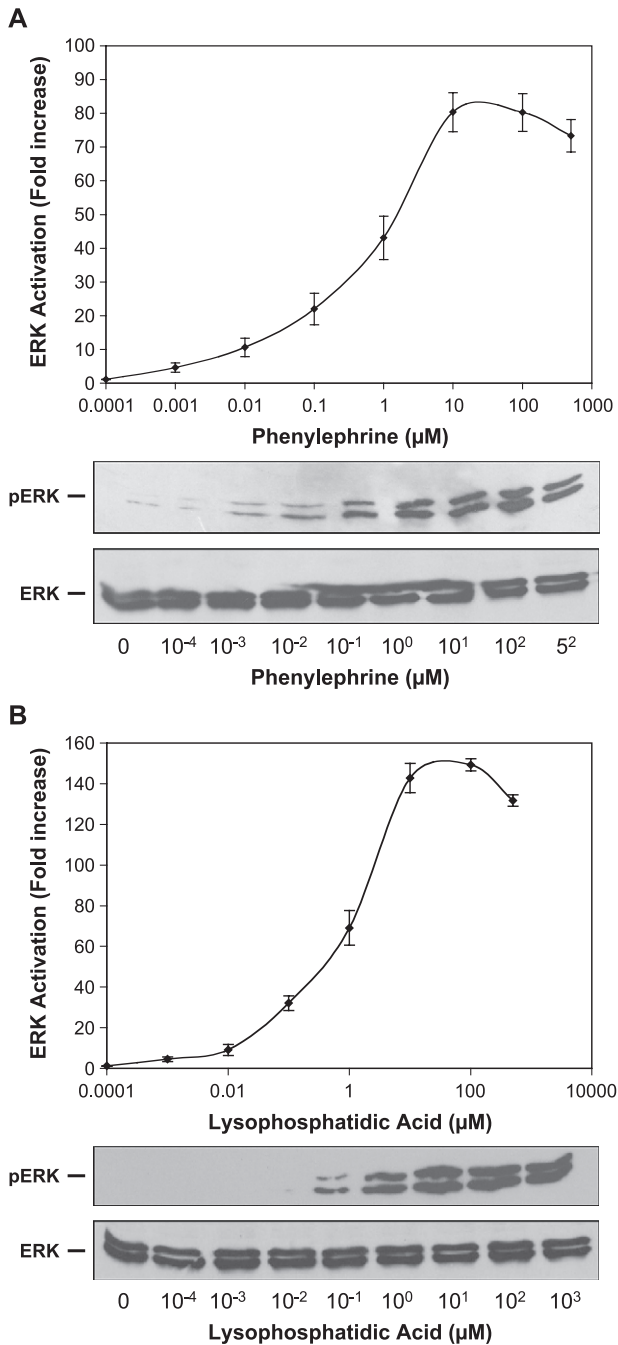


Fig. 4. Dose dependence of ERK stimulation in CCL39 cells. Serum-starved CCL39 cells were stimulated for 5 min with either vehicle (0.1% DMSO) or various concentrations of PE (A) or LPA (B) prior to determination of ERK phosphorylation. Data are expressed as the fold increase of control stimulated responses. Each point is the mean±S.E. of pooled data from at least four independent experiments. The insets in A and B are typical immunoblots representing phospho-ERK (top) and total ERK (bottom).

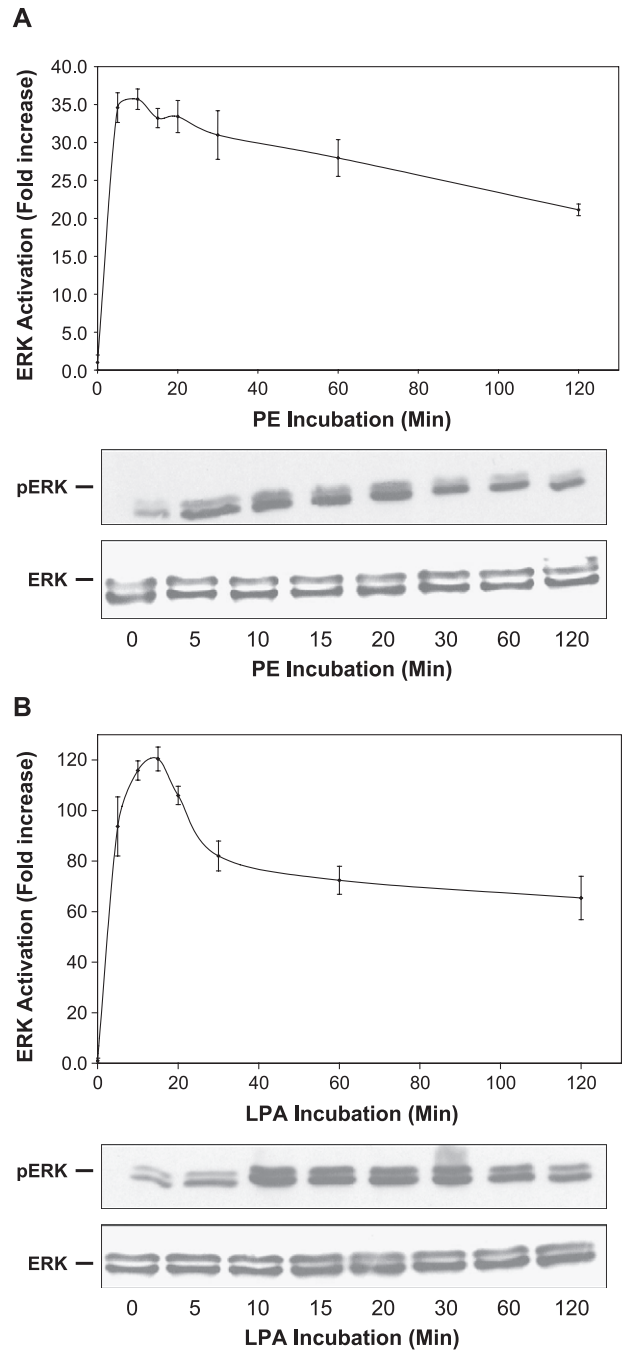


Fig. 5. Time-dependent activation of ERK by phenylephrine and LPA. 70–80% confluent, quiescent cells were treated with agonist for the indicated times. Cells were then washed with PBS and scraped directly into SDS buffer containing orthovanadate and β-glycerophosphate and measured for both total ERK and ERK phosphorylation by Western blot. The fold phosphorylation (ERK Activation) was normalized to the non-stimulated control cells. Serum-starved CCL39 cells were incubated at 37 °C with vehicle (0.1% DMSO), 100 µM phenylephrine (PE) (A) or 10 µM LPA (B) for the indicated times prior to determination of ERK phosphorylation. Data represents means±S.E. (n=4). Lysate (30 and 15 µl) was analyzed for phospho-ERK detection or ERK detection, respectively. The samples were resolved on a 12% SDS-PAGE and assessed by immuno-analysis. Typical blots are shown for phospho-ERK and total ERK.

NHE1 [17]. Both LPA and phenylephrine are capable of inducing ERK activation in a variety of cell lines, however, neither agonist has been demonstrated to activate ERK in CCL39 cells. To assess the ability of these agonists to increase the phosphorylation state of ERK in CCL39 cells, both the dose–response and incubation time for LPA and phenylephrine additions were determined (Figs. 4 and 5). Cells were serum deprived overnight and incubated with the indicated concentration of agonist. ERK activation was determined by Western blotting using the ratio of phosphorylated ERK to total ERK. LPA increased the phosphorylation state of ERK  $142 \pm 7.2$ -fold over basal level with a maximal effect at 10–100  $\mu\text{M}$  (Fig. 4B). Addition of 10 and 100  $\mu\text{M}$  phenylephrine resulted in an  $80.4 \pm 5.79$ - and  $80.3 \pm 5.69$ -fold increase in phosphorylation respectively (Fig. 4A). The kinetics of ERK activation were measured at 10  $\mu\text{M}$  LPA or 100  $\mu\text{M}$  phenylephrine. Maximal phosphor-

ylation occurred at 10 to 15 min for both agonists, and as typical for ERK phosphorylation, decreased with longer incubations (Fig 5). The activation of ERK by LPA while not novel indicates that in CCL39 cells, signaling cascades in addition to RhoA are important for the regulation of NHE1 in these cells. The data also indicate that  $\alpha_1$ -adrenergic receptor regulation of NHE1 potentially involves the ERK pathway.

#### 3.4. ERK activity is required for NHE1 activation by both lysophosphatidic acid and phenylephrine

Because ERK activation is required for serum and growth factor stimulation of NHE1 in CCL39 cells, we investigated the requirement of ERK activation for NHE1 by both G protein-coupled receptor (GPCR) agonists. PD98059 and U0126 are two highly selective MEK

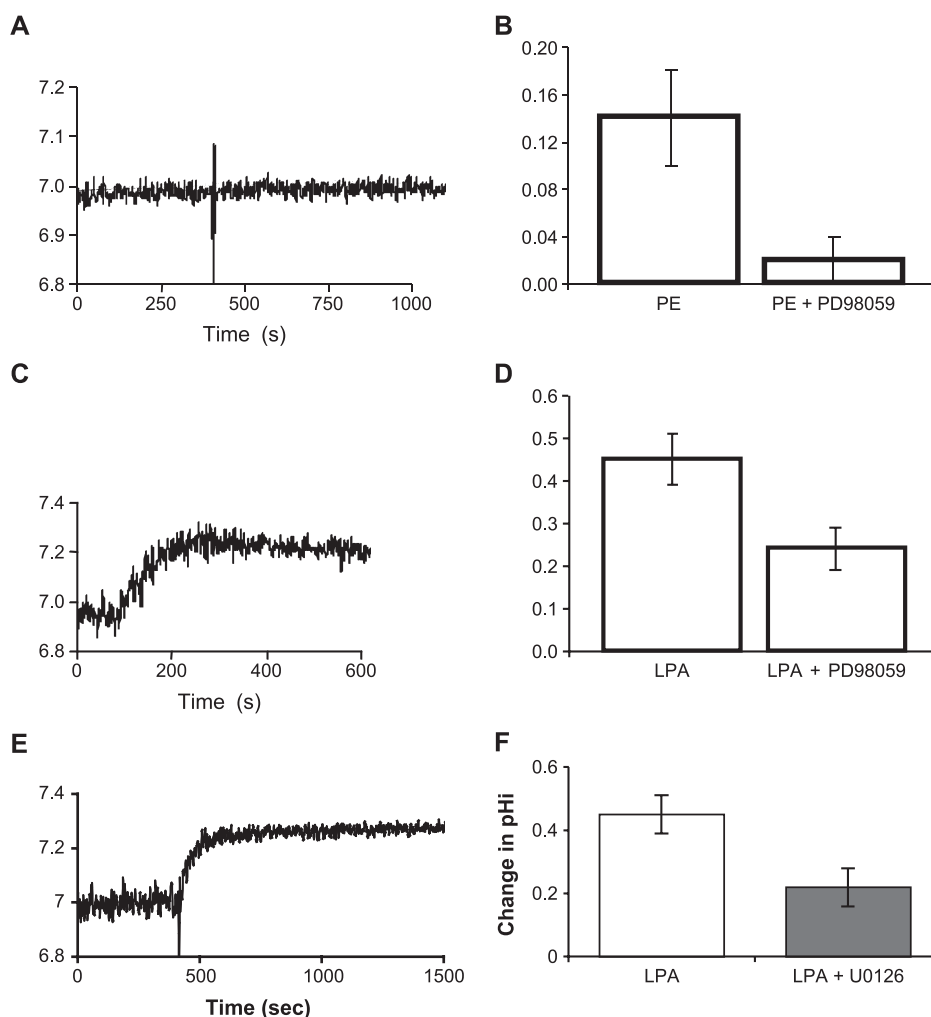


Fig. 6. Pharmacological inhibition of ERK inhibits NHE1 activation by phenylephrine and LPA. (A–F) Quiescent cells were serum deprived for 12 h and then incubated with the indicated MEK inhibitor, dye-loaded with BCECF and acid loaded with  $\text{NH}_4\text{Cl}$  for 30 min.  $\text{NH}_4\text{Cl}$  was then removed and the isolated cells were suspended in Na Buffer containing the inhibitor in a quartz cuvette, while the change in  $\text{pH}_i$  associated with the recovery from an acid load was observed. A, C and E are representative traces of individual experiments. B, D and F represent the variation of  $\text{pH}_i$  with or without agonist. Error bars ( $\pm$  S.E.) are based on an  $n=5$ . (G) Quiescent CCL39 fibroblasts, 70–80% confluent, were treated with 100  $\mu\text{M}$  of either PD98059 or 25  $\mu\text{M}$  U0126 for 30 min. Either 100  $\mu\text{M}$  PE or 50  $\mu\text{M}$  LPA was added for 5 min as indicated. The cells were washed with PBS and lysed in 200  $\mu\text{l}$  Laemmli sample buffer containing phosphatase inhibitors. Twenty micrograms of each sample was resolved on a 10% SDS-PAGE. Phosphorylated ERK and total ERK were determined by Western blot analysis.

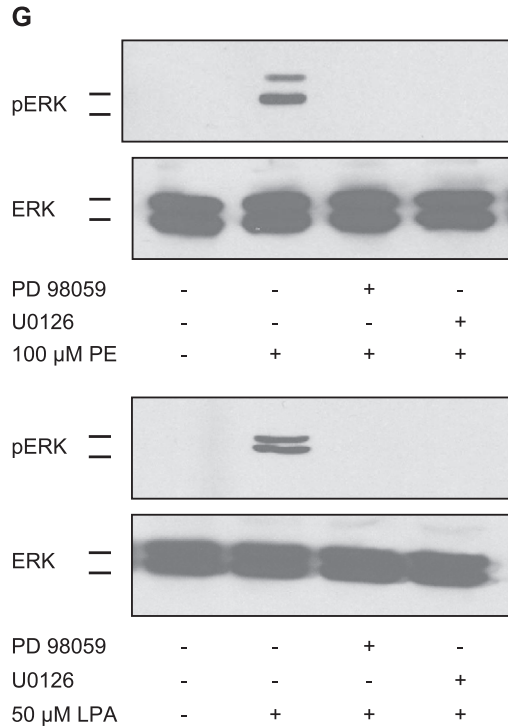


Fig. 6 (continued).

inhibitors. PD98059 binds to and blocks the activation of unphosphorylated MEK [18,19] while U0126 is a non-competitive inhibitor of MEK with respect to ATP and ERK [20,21]. Both drugs were used to evaluate whether ERK activation is required for NHE1 (Fig. 6). CCL39 cells were pretreated with 100 μM PD98059 or 50 μM U0126 for 30 min prior to addition of the indicated agonist. In a series of paired experiments, addition of 100 μM phenylephrine stimulated an increase in steady-state  $pH_i$  of  $0.14 \pm 0.04$  pH units ( $n=5$ ). When this addition was conducted in the presence of 100 μM PD98059, the increase in  $pH_i$  was only  $0.03 \pm 0.02$  ( $n=5$ ; Fig. 6A). Thus, the MEK inhibitor blocked nearly 80% of NHE1 activation by phenylephrine (Fig. 6B). This series of experiments was repeated to observe the impact of two MEK inhibitors on the ability of LPA to activate NHE1. In Fig. 6, C and D show that LPA activation of NHE1 was inhibited by both PD98059 and U0126 but to a lesser extent than phenylephrine. Addition of 100 μM LPA stimulated an increase in  $pH_i$  of  $0.45 \pm 0.06$  ( $n=5$ ). When LPA activation of NHE1 was measured in the presence of 100 μM PD98059, the increase in  $pH_i$  was reduced to  $0.24 \pm 0.05$  ( $n=5$ ). In a nearly identical manner, 50 μM U0126 reduced the LPA-induced alkalinization to  $0.22 \pm 0.06$  pH units ( $n=5$ ; Fig. 6F). Thus, the two MEK inhibitors reduced the LPA triggered activation of NHE1 by 45% to 55%. As expected, both of the MEK inhibitors completely abolished the ability of either LPA or phenylephrine to increase the phosphorylation level of ERK (Fig. 6G). Hence, activation of ERK is required for phenylephrine stimulation of NHE1 exchange activity but is only one component of the LPA stimulation of NHE1.

To more fully determine the role of ERK in the activation of NHE1, we expressed a kinase inactive MEK1 (KI-MEK1) in CCL39 cells prior to NHE1 and ERK activity measurements. Transfection efficiencies of 60–80% were regularly obtained using green fluorescent protein in parallel experiments (data not shown). As judged by the immunoblot (Fig. 7A), KI-MEK1 was expressed at least four times over that of endogenous MEK1. As expected, expression of exogenous interfering KI-MEK1 decreased the LPA and

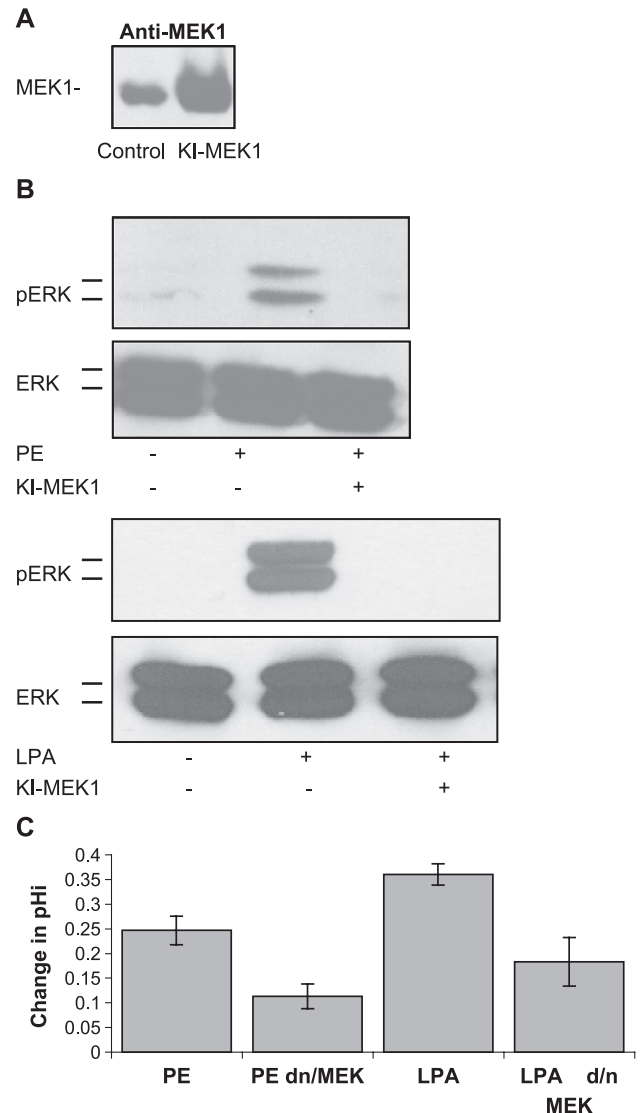


Fig. 7. Kinase-inactive MEK blocks ERK activation of NHE by phenylephrine and LPA. Where indicated CCL39 cells were transfected with 7.5 μg of kinase-inactive MEK (KI-MEK1) or vector only (control). Twenty-four hours after transfection, the cells were serum starved overnight and stimulated with the indicated concentration of LPA, phenylephrine (PE) or vehicle for 10 min. (A) The expression of KI-MEK1 was measured by immunoblotting with an anti MEK antibody. (B) Thirty-six-hour post-transfection, the serum-deprived cells were stimulated with either 50 μM LPA or 50 μM PE for 10 min and phosphorylation determined by Western blot. (C) Cells transiently transfected with KI-MEK1 were made quiescent and serum deprived for 12 h. Cells were then dye-loaded with BCECF and agonist-induced changes in steady-state  $pH_i$  were determined.

phenylephrine-induced phosphorylation of ERK (Fig. 7A). Cells that were transiently transfected with KI-MEK blocked ERK activation at lower LPA concentrations (50–25  $\mu\text{M}$ ). However, at higher concentrations (100  $\mu\text{M}$ ) of LPA, endogenous MEK was still able to phosphorylate ERK1, presumably by the actions of endogenous wild type MEK (data not shown) or by untransfected cells. The addition of phenylephrine to cells transfected with KI-MEK1 also reduced the activation of ERK at 50  $\mu\text{M}$  phenylephrine (Fig. 7B) however as observed with higher concentrations of LPA, cells stimulated with 100  $\mu\text{M}$  v retained a reduced ability to phosphorylate ERK. In a parallel experiment, addition of 50  $\mu\text{M}$  LPA increased  $\text{pH}_i$   $0.36 \pm 0.02$  ( $n=3$ ) and similar to the inhibition with MEK inhibitors, expression of KI-MEK1 inhibited LPA-induced alkalinization with an increase of  $0.18 \pm 0.05$  ( $n=3$ ) (Fig. 7C). Addition of 100  $\mu\text{M}$  phenylephrine increased the intracellular pH by  $0.25 \pm 0.03$  ( $n=3$ ), where KI-MEK1 expressing cells only increased  $\text{pH}_i$   $0.11 \pm 0.02$  ( $n=3$ ) after phenylephrine addition. Together the dominant negative MEK and chemical inhibitor data suggest that ERK is an essential mediator of the phenylephrine cascade and that at least one-half of the LPA pathway in CCL39 cells is due to ERK.

### 3.5. Lysophosphatidic acid stimulation of NHE1 requires both MEK and ROCK activation

Existing studies as well as the information presented here indicate that LPA can signal through both an ERK and a RhoA pathway to regulate NHE1 in CCL39 cells. To investigate the importance of one or both pathways in LPA signaling, we used Y27632, a potent inhibitor of RhoA associated kinases [22] alone or in combination with the MEK inhibitor, PD98059 (Fig. 8). Addition of 50  $\mu\text{M}$  LPA induced a  $\text{pH}_i$  change of  $0.30 \pm 0.03$  ( $n=5$ ). In the presence of 100  $\mu\text{M}$  PD98059, the increase in  $\text{pH}_i$  after 50  $\mu\text{M}$  LPA addition was  $0.13 \pm 0.02$  ( $n=5$ ), a 68% reduction in NHE1 activity. When the 5  $\mu\text{M}$  of the ROCK inhibitor was added to the cells prior to LPA stimulation,

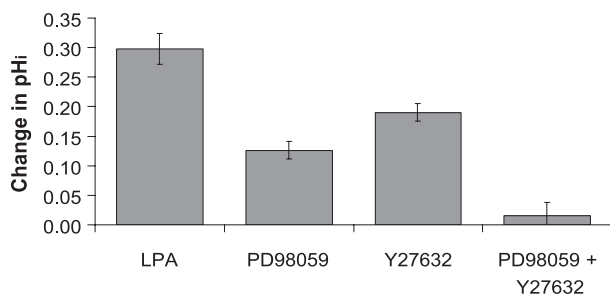


Fig. 8. LPA activation of NHE1 requires both RhoA and ERK signaling pathways. Quiescent cells were serum deprived for 12 h and 15 min prior to 50  $\mu\text{M}$  LPA stimulation, the cells were incubated with 100  $\mu\text{M}$  PD98059 or 5  $\mu\text{M}$  Y27632 as indicated. After dye-loading with BCECF, the cells were acid loaded with  $\text{NH}_4\text{Cl}$  and  $\text{pH}_i$  assessed as described in Experimental procedures. Error bars ( $\pm$ S.E.) are based on an  $n=5$ .

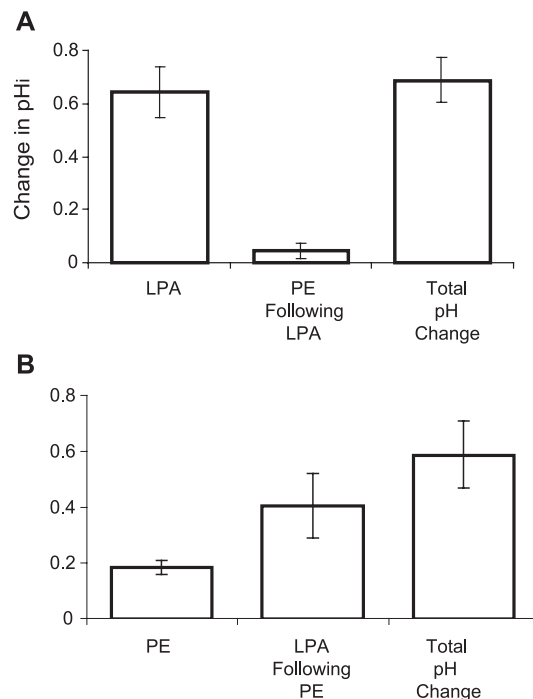


Fig. 9. Summation of LPA and phenylephrine-induced  $\text{pH}_i$  changes. In a series of coupled experiments, the ability of LPA and PE-induced alkalinizations was tested to determine additivity. Addition of 100  $\mu\text{M}$  LPA induced an alkalinization of  $0.64 \pm 0.09$  pH units. When 100  $\mu\text{M}$  PE was added to cells following the LPA-induced  $\text{pH}_i$  increase, phenylephrine (PE) only stimulated an increase in pH of  $0.04 \pm 0.03$  pH units (A). When PE was added first in these experiments, it induced an alkalinization of  $0.18 \pm 0.02$  pH units. The subsequent addition of 100  $\mu\text{M}$  LPA induced an additional  $0.40 \pm 0.11$  alkalinization (B).

only a 36% reduction of  $\text{pH}_i$  ( $0.19 \pm 0.01$  ( $n=5$ )) was observed. In experiments when a higher concentration of LPA (100  $\mu\text{M}$ ) was incubated with Y27632 treated cells, little or no inhibition of NHE1 activity was observed (data not shown). However, when both MEK and ROCK pathways were pharmacologically inhibited, no change in  $\text{pH}_i$  was observed ( $0.02 \pm 0.02$  ( $n=5$ )). Indicating that both pathways are involved in LPA signaling to NHE1 and that for full activation of the exchanger, both pathways need to be stimulated.

### 3.6. Lysophosphatidic acid and phenylephrine activation of NHE1 and ERK display additivity

A separate series of experiments was undertaken to determine whether the phenylephrine and LPA-induced alkalinizations were additive or synergistic (Fig. 9). When both 100  $\mu\text{M}$  phenylephrine and 100  $\mu\text{M}$  LPA were added to CCL39 cells, the resulting change in  $\text{pH}_i$  was  $0.63 \pm 0.11$ . This appears to support the response to the two agonists being additive, though the additivity could only be seen in one set of experiments. Fig. 9A shows the experiment where 100  $\mu\text{M}$  LPA was added to cells and the change in  $\text{pH}_i$  determined. Once the cells had reached a new steady-state, 100  $\mu\text{M}$  phenylephrine was added to the cells. In a series of

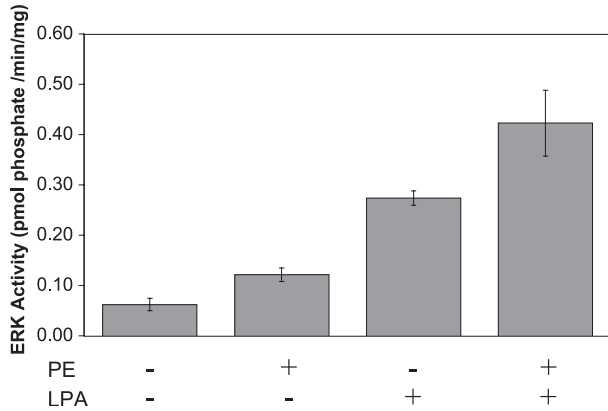


Fig. 10. ERK activation by phenylephrine and LPA is additive. CCL39 Fibroblasts were made quiescent and incubated without agonist or with the indicated combination of 100  $\mu$ M phenylephrine (PE) or 10  $\mu$ M LPA for 10 min. ERK activity was measured after immunoprecipitation of ERK by examining the addition radiolabeled phosphate to exogenously added myelin basic protein, in the presence of inhibitors of PKA and calmodulin. The amount of phosphorylated substrate was determined by filter assay. Data are expressed as a mean  $\pm$  S.E.M. ( $n=4$ ).

five experiments, LPA induced an alkalinization of  $0.64 \pm 0.09$  pH units. The subsequent addition of phenylephrine caused an additional increase in  $pH_i$  of  $0.04 \pm 0.03$ . This appears to suggest that there is no additivity. However, when the experiment is reversed and phenylephrine is added first, the results are strikingly different. The addition of 100  $\mu$ M phenylephrine resulted in an increase in  $pH_i$  of  $0.18 \pm 0.02$  pH units. The subsequent addition of 100  $\mu$ M LPA leads to an additional increase in  $pH_i$  of  $0.40 \pm 0.11$  pH units (Fig. 9B). Thus, it appears that the phenylephrine stimulation cannot add dramatically to the LPA response, but the LPA response can add to the phenylephrine response but not at its normal level. To determine the effect of both agonists on ERK phosphorylation, an immunokinase assay using myelin basic protein was used (Fig. 10). When cells were incubated with 100  $\mu$ M phenylephrine or 10  $\mu$ M LPA, the ERK activity increased 1.95- and 4.38-fold over basal, respectively. When cells were incubated with both agonists simultaneously, the resulting increase in ERK activity was 6.76-fold, a value slightly higher than the anticipated additive value for the two agonists, 6.34. Thus, these data support the hypothesis that two different G protein-coupled receptors signal through a converging pathway that requires ERK to activate NHE1 in CCL39 cells.

#### 4. Discussion

The  $Na^+-H^+$  exchanger isoform 1 is widely involved in the regulation of  $pH_i$  and cell volume. Activation of NHE1 leads to an increase in the rate of proton extrusion by the antiporter and thus an increase in  $pH_i$ . Both the alkalinization of  $pH_i$  and the concomitant extracellular acidification aid in cell growth and tumor invasion [3,23]. While the activation of NHE1 and the kinetic alterations to the

exchanger have been widely studied [1,4], the intricacies of the signaling pathways that regulate NHE1 have not been fully elucidated. To date, activation of NHE1 by growth factor receptors through the MAPK pathway [17] and by G protein-coupled receptors through protein kinase C [24,25], RhoA and its effector ROCK [10,11], and RSK [26] has been described but not fully characterized. While growth factor activation of NHE1 requires ERK activation, the role of ERK in G protein-coupled receptor signaling to NHE1 can vary depending on cell type and the exact mechanism is likely to involve several signaling intermediates. Our results contribute to the characterization of the signaling pathways involved in the G protein-coupled receptor activation of the MAPK pathway and NHE1 in CCL39 cells.

Our initial investigations confirmed the observation of Vexler et al. [16] that LPA activates NHE1 in a dose-dependent fashion. Maximal activation of NHE1 occurred at LPA concentrations greater than 75  $\mu$ M with a maximal change in  $pH_i$   $0.43 \pm 0.06$  pH units occurring at 100  $\mu$ M LPA (Fig. 1). This activation by LPA was due to a shift in the  $pH_i$  dependence of the exchanger, where 100  $\mu$ M LPA induced an alkaline shift in the  $pH_i$ -dependence of approximately 0.4 pH units (Fig. 3). We next demonstrated for the first time in CCL39 cells that the  $\alpha_1$ -adrenergic agonist, phenylephrine activates NHE1. Phenylephrine activated NHE1 in a dose-dependent fashion with maximal activation occurring at 50 to 100  $\mu$ M. The maximal change in steady-state  $pH_i$  was  $0.16 \pm 0.02$  pH units (Fig. 2) with a shift in the  $pH_i$  dependence of 0.15 to 0.20 pH units (Fig. 3). While this is a novel finding in CCL39 cells, it is established that  $\alpha_1$ -adrenergic receptors activate NHE1 in cardiac ventricular muscle cells. Furthermore, the activation of the  $\alpha_1$ -adrenergic receptor in these cells led to nearly the same shift in the  $pH_i$ -dependence and accompanying shift in the steady-state  $pH_i$  of approximately 0.15 pH units in cardiac cells [24,25,27].

G protein-coupled receptor signaling is typically mediated through second messengers leading to metabolic and structural changes. However, it is now clear that G protein-coupled receptors can also play a significant role in the regulation of growth factor pathways as well [28, 29]. Using a platelet-derived growth factor activated ERK, Wang et al. [30] were able to demonstrate a direct phosphorylation of the carboxyl terminus of NHE1. In vascular smooth muscle cells, angiotensin II stimulation leads to the activation of ERK and NHE1 in vivo [31]. One of the most telling indications that ERK is involved in NHE1 activation was demonstrated by Bianchini et al. [17]. Addition of the specific MEK inhibitor PD98059 or the expression of a dominant/negative Raf definitively indicated that the Ras-ERK pathway was important for the activation of the exchanger. More recently, RSK, the downstream effector of ERK, has been shown to specifically phosphorylate the carboxyl terminus of NHE1 in a stoichiometric fashion in vascular smooth muscle cells [26]. Because the regulation of NHE1 by growth factors and thrombin requires the activation of the Ras-ERK pathway and because several GPCR-linked pathways activate ERK, it was

of interest to determine if either LPA or phenylephrine activates ERK as well. Our studies demonstrate that both phenylephrine and LPA activate ERK in a dose- and time-dependent fashion (Fig. 5). Both Western blot analysis of ERK activation and the immunokinase results show that while both agonists are capable of activating ERK, LPA is much more efficacious at inducing ERK activity. This indicates that there is a difference between the mechanisms of the two signaling pathways.

It is important to note that the definitive role for ERK in the regulation of NHE1 is still uncertain. Expression of a constitutively active  $G_{\alpha_q}$  in CCL39 cells did not activate NHE1 [32]. Moreover, CCL39 cells transfected with a dominant negative MEK did not block NHE1 activation by LPA [32]. Because ERK plays such an important role in NHE1 regulation and that phenylephrine and LPA both stimulate ERK, it was interesting to determine if ERK was involved in GPCR signaling in CCL39 cells. In cells pretreated with the MEK inhibitor PD98059 prior to phenylephrine or LPA addition, ERK phosphorylation levels were at or below that of unstimulated cells. However, nearly all of the phenylephrine activation of NHE1 was abrogated while only half of LPA stimulation of NHE1 with PD98059. Because PD98059 inhibits the activation of MEK and does not block phosphorylated MEK activity, we used a second inhibitor, U0126, a compound that can inhibit either phosphorylation state of MEK. When cells were pretreated with 50  $\mu$ M U0126, only half of the LPA stimulated NHE1 activity was inhibited, yet all of the ERK phosphorylation was blocked (Fig. 6). A similar, inhibition of both ERK and NHE1 exchange activity was observed when a kinase-deficient MEK was expressed in the fibroblasts (Fig. 7). Further evidence for a role of ERK in NHE1 regulation was found when cells were incubated with high concentrations (100  $\mu$ M) of the stress kinase regulator anisomycin for 30 min. This resulted in a 2.5-fold increase in ERK phosphorylation and an increase in intracellular pH of 0.2 units. Lower concentrations of anisomycin did not alter either, ERK or NHE1 activation (data not shown). The existence of two pathways converging on NHE1 regulation was shown when both the RhoA kinase ROCK and ERK activation were inhibited (Fig. 8). More of the LPA-induced NHE1 activity was inhibited by the MEK inhibitor than was inhibited by inhibiting ROCK intimates that ERK is as or a more important in NHE1 regulation by LPA in these cells. The fact that NHE1 activity was not inhibited by Y27632 when higher doses of LPA were used indicates that there may be more than one receptor activated in this pathway or that the ERK pathway can somehow compensate for the loss of the second pathway. These data indicate that there are two different pathways involved in the LPA-induced regulation of NHE1 and that the mechanism for this activation needs additional work.

In our studies,  $G_q$  signaling activates NHE1 in an ERK-dependent fashion. However, this observation is not consistent with earlier studies using constitutively activate  $G_{\alpha_q}$  [32]. The difference is likely due to the mechanism of the

signaling pathway. It is possible that the  $\beta\gamma$  subunits are required for activation of the Ras-ERK pathway in a manner already described in other cells involving soluble tyrosine kinases [33–35]. Regardless of the pathway activated by phenylephrine, it is important to note that  $\alpha_1$ -adrenergic receptors are able to stimulate NHE1 in these fibroblasts. Our results also indicate that LPA, at least in part, requires ERK for full stimulation of NHE1 exchange. As mentioned, involvement of ERK was not observed in studies where activated  $G_{\alpha_{13}}$  was co-expressed with either an interfering allele of Raf or MEK [32]. A role for  $G_{\alpha_{13}}$  in regulating the exchanger is believed to take place via the small G protein RhoA and its kinase effector, ROCK. We presented here a significant role for ERK in LPA signaling to NHE1. The observation that only half of the LPA stimulated NHE1 activity was blocked by the use of MEK inhibitors or KI-MEK1 expression the existence of a second, RhoA-mediated pathway. The difference between the two studies may also be explained by the signaling mechanism. Trans-activation of growth factor receptors by  $\beta\gamma$  can involve several tyrosine kinases, protein kinase C or release of epidermal growth factor to create a scaffolding and activation for Ras pathway proteins [34,35]. Use of the constitutively active  $G_{\alpha_{13}}$  may not have been sufficient to stimulate the second growth factor pathway. Also, when CCL39 cells were cultured beyond 8 to 10 passages, the basal level of phosphorylation of ERK was increased by at least three-fold. In these cells, LPA was only able to stimulate a 0.2 pH unit increase in intracellular pH (data not shown). Thus, it is likely that hyperphosphorylated ERK found in cells with several passages could mask the role of ERK in LPA signaling. In this case, a second  $G_{\alpha_{13}}$ -mediated pathway involving RhoA and ROCK would be expected to be the predominant stimulated pathway.

In conclusion, this study presents several important implications to clarify the mechanism for GPCR regulation of NHE1. The first is that phenylephrine can indeed activate NHE1 in CCL39 fibroblasts. Second, our studies indicate that two pathways exist for LPA signaling to NHE1, and that one of the pathways also requires ERK while the other seems to be RhoA dependent. Ongoing studies are investigating the mechanism of ERK and NHE1 activation in these cells. Furthermore, it would be interesting to determine if the two signaling pathways moderated by LPA are due to a single receptor or more than one LPA receptor with different affinity for the lipid ligand.

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## References

- [1] J. Noel, J. Pouyssegur, *Am. J. Physiol.* 268 (2 Pt. 1) (1995) C283–C296.
- [2] J. Orłowski, S. Grinstein, *J. Biol. Chem.* 272 (36) (1997) 22373–22376.
- [3] S.J. Reshkin, A. Bellizzi, V. Albarani, L. Guerra, M. Tommasino, A. Paradiso, V. Casavola, *J. Biol. Chem.* 275 (8) (2000) 5361–5369.
- [4] L.K. Putney, S.P. Denker, D.L. Barber, *Annu. Rev. Pharmacol. Toxicol.* 42 (2002) 527–552.
- [5] J. Orłowski, R.A. Kandasamy, G.E. Shull, *J. Biol. Chem.* 267 (13) (1992) 9331–9339.
- [6] L. Counillon, J. Pouyssegur, *J. Biol. Chem.* 275 (1) (2000) 1–4.
- [7] E. Takahashi, J. Abe, B. Gallis, R. Aebersold, D.J. Spring, E.G. Krebs, B.C. Berk, *J. Biol. Chem.* 274 (29) (1999) 20206–20214.
- [8] P. Fafourmoux, J. Ghysdael, C. Sardet, J. Pouyssegur, *Biochemistry* 30 (39) (1991) 9510–9515.
- [9] L. Fliegel, M.P. Walsh, D. Singh, C. Wong, A. Barr, *Biochem. J.* 282 (Pt. 1) (1992) 139–145.
- [10] T. Tominaga, T. Ishizaki, S. Narumiya, D.L. Barber, *EMBO J.* 17 (16) (1998) 4712–4722.
- [11] T. Tominaga, D.L. Barber, *Mol. Biol. Cell* 9 (8) (1998) 2287–2303.
- [12] T. Voyno-Yasenetskaya, B.R. Conklin, R.L. Gilbert, R. Hooley, H.R. Bourne, D.L. Barber, *J. Biol. Chem.* 269 (7) (1994) 4721–4724.
- [13] S.J. Wadsworth, G. Gebauer, G.D. van Rossum, N. Dhanasekaran, *J. Biol. Chem.* 272 (46) (1997) 28829–28832.
- [14] J.A. Thomas, R.N. Buchsbaum, A. Zimniak, E. Racker, *Biochemistry* 18 (11) (1979) 2210–2218.
- [15] W.F. Boron, A. Roos, P. De Weer, *Nature* 274 (5667) (1978) 190.
- [16] Z.S. Vexler, M. Symons, D.L. Barber, *J. Biol. Chem.* 271 (37) (1996) 22281–22284.
- [17] L. Bianchini, G. L'Allemain, J. Pouyssegur, *J. Biol. Chem.* 272 (1) (1997) 271–279.
- [18] D.T. Dudley, L. Pang, S.J. Decker, A.J. Bridges, A.R. Saltiel, *Proc. Natl. Acad. Sci. U. S. A.* 92 (17) (1995) 7686–7689.
- [19] D.R. Alessi, A. Cuenda, P. Cohen, D.T. Dudley, A.R. Saltiel, *J. Biol. Chem.* 270 (46) (1995) 27489–27494.
- [20] M.F. Favata, K.Y. Horiuchi, E.J. Manos, A.J. Daulerio, D.A. Stradley, W.S. Feesser, D.E. Van Dyk, W.J. Pitts, R.A. Earl, F. Hobbs, R.A. Copeland, R.L. Magolda, P.A. Scherle, J.M. Trzaskos, *J. Biol. Chem.* 273 (29) (1998) 18623–18632.
- [21] S.J. Mansour, J.M. Candia, J.E. Matsuura, M.C. Manning, N.G. Ahn, *Biochemistry* 35 (48) (1996) 15529–15536.
- [22] M. Uehata, *Nature* 389 (1997) 990–994.
- [23] A. Lagana, J. Vadnais, P.U. Le, T.N. Nguyen, R. Laprade, I.R. Nabi, J. Noel, *J. Cell Sci.* 113 (Pt. 20) (2000) 3649–3662.
- [24] M.A. Wallert, O. Frohlich, *Am. J. Physiol.* 263 (5 Pt. 1) (1992) C1096–C1102.
- [25] A.K. Snabaitis, H. Yokoyama, M. Avkiran, *Circ. Res.* 86 (2) (2000) 214–220.
- [26] E. Takahashi, J. Abe, B. Gallis, R. Aebersold, D.J. Spring, E.G. Krebs, B.C. Berk, *J. Biol. Chem.* 274 (29) (1999) 20206–20214.
- [27] M. Puceat, O. Clement-Chomienne, A. Terzic, G. Vassort, *Am. J. Physiol.* 264 (2 Pt. 2) (1993) H310–H319.
- [28] P.H. Sugden, A. Clerk, *Cell. Signal.* 9 (5) (1997) 337–351.
- [29] J.S. Gutkind, *J. Biol. Chem.* 273 (4) (1998) 1839–1842.
- [30] H. Wang, N.L. Silva, P.A. Lucchesi, R. Haworth, K. Wang, M. Michalak, S. Pelech, L. Fliegel, *Biochemistry* 36 (30) (1997) 9151–9158.
- [31] E. Takahashi, J. Abe, B.C. Berk, *Circ. Res.* 81 (2) (1997) 268–273.
- [32] R. Hooley, C.Y. Yu, M. Symons, D.L. Barber, *J. Biol. Chem.* 271 (11) (1996) 6152–6158.
- [33] L.M. Luttrell, T. van Biesen, B.E. Hawes, W.J. Koch, K. Touhara, R.J. Lefkowitz, *J. Biol. Chem.* 270 (28) (1995) 16495–16498.
- [34] L.M. Luttrell, G.J. Della Rocca, T. van Biesen, D.K. Luttrell, R.J. Lefkowitz, *J. Biol. Chem.* 272 (7) (1997) 4637–4644.
- [35] L. Luttrell, Y. Daaka, R. Lefkowitz, *Curr. Opin. Cell Biol.* 11 (1999) 177–183.