Stimulation of the Plasma Membrane Na\(^+\)/H\(^+\) Exchanger NHE1 by Sustained Intracellular Acidosis

EVIDENCE FOR A NOVEL MECHANISM MEDIATED BY THE ERK PATHWAY*

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Activity of the Na\(^+\)/H\(^+\) exchanger (NHE) isoform 1 (NHE1) is increased by intracellular acidosis through the interaction of intracellular H\(^+\) with an allosteric modifier site in the transport domain. Additional regulation is achieved via kinase-mediated modulation of the NHE1 regulatory domain. To determine if intracellular acidosis stimulates NHE1 activity solely by the allosteric mechanism, we subjected cultured neonatal rat ventricular myocytes (NRVM) with native NHE1 expression to intracellular acidosis (pH\(_i\) = 6.6) for up to 6 min by transient exposure to NH\(_4\)Cl and its washout in the presence of NHE inhibition (by zero [Na\(^+\)] or the NHE1 inhibitor cariporide) in HCO\(_3\)\(^-\)-free medium. After the desired duration of acidosis, NHE was reactivated (by reintroduction of [Na\(^+\)] or removal of cariporide), and the rate of recovery of pH\(_i\) (\(dpH_i/dt\)) was measured as the index of NHE activity. Regardless of the method used when intracellular acidosis was sustained for \(\geq 3\) min, subsequent NHE activity was significantly increased (>4-fold). Similar NHE stimulatory effects of sustained acidosis were observed in adult rat ventricular myocytes and COS-7 cells. Sustained (3 min) intracellular acidosis activated several NHE1 kinases in NRVM, in an in-gel kinase assay using as substrate a glutathione S-transferase fusion protein of the NHE1 regulatory domain. Detailed investigation of ERK and its downstream effector p90RSK, two putative NHE1 kinases, revealed time-dependent activation of both by intracellular acidosis in NRVM. Furthermore, inhibition of MEK1/2 by pretreatment of NRVM with two structurally distinct inhibitors, PD98059 (30 \(\mu\)M) or UO126 (3 \(\mu\)M), inhibited the activation of ERK and p90RSK and abolished the stimulation of NHE activity by sustained (3 min) intracellular acidosis. Our data show that not only the duration but also the rate of recovery of pH\(_i\) (\(dpH_i/dt\)) has been shown to produce an allosteric mechanism mediated by the pH\(_i\) sensor (4). Several studies in different cell types indicate a key role for the extracellular signal-regulated kinase (ERK) pathway of the mitogen-activated protein kinase cascade in the stimulation of NHE1 activity by extracellular stimuli, particularly by growth factors and G protein-coupled receptor agonists (7–9). ERK-mediated stimulation of NHE1 activity may occur by direct phosphorylation of the exchanger regulatory domain (10) and/or indirectly through one or more downstream effectors (7). One potential such effector is the 90-kDa ribosomal S6 kinase (p90RSK), which is activated by ERK-mediated phosphorylation (11) and has been shown to phosphorylate Ser-703 within the regulatory domain of NHE1 and thereby increase exchanger activity (12).

In many other cells, NHE1 is the molecular homolog of the plasma membrane (sarcolemmal) NHE in cardiac myocytes (13), a cell type in which the relationship between pH\(_i\) and sarcolemmal NHE activity is particularly steep (14). Thus, in adult rat ventricular myocytes, a reduction in pH\(_i\) from 6.90 to 6.50 (representing a 2.5-fold increase in [H\(^+\)]\(_i\)) has been shown to produce an \(-7\)-fold increase in sarcolemmal NHE activity (14). Although an allosteric mechanism mediated by the pH\(_i\) sensor is widely accepted to underlie this stimulation of sarcolemmal NHE activity by an acute pH\(_i\) reduction, the effect of the duration (cf. the extent) of intracellular acidosis on sarcolemmal NHE activity and the molecular mechanism(s) un-

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The ubiquitously expressed plasma membrane Na\(^+\)/H\(^+\) exchanger (NHE),\(^1\) encoded by the NHE1 gene that was first cloned by Sardet et al. in 1989 (1), consists of a 500-amino acid transport domain that contains 12 putative transmembrane-spanning segments and a 300-amino acid regulatory domain that extends into the cytoplasm (2). NHE1 activity is regulated primarily by pH\(_i\) and increases markedly in response to intracellular acidosis (3), and such regulation has been proposed to occur through the interaction of H\(^+\) with an allosteric modifier site ("pH\(_i\) sensor") within the transport domain (4, 5). Recent evidence suggests critical roles for Gly and Arg residues within the transmembrane segment 11 and the adjacent intracellular loop in pH\(_i\)-mediated regulation of NHE1 activity (6), although the precise molecular mechanism remains unclear. Additional regulation of NHE1 activity in response to various extracellular stimuli, stretch, and altered cell volume is achieved by modification of the regulatory domain, such as phosphorylation and altered interaction with accessory proteins, leading to conformational changes in the NHE1 molecule that are thought to alter the affinity for H\(^+\) of the pH\(_i\) sensor (4). Several studies in different cell types indicate a key role for the extracellular signal-regulated kinase (ERK) pathway of the mitogen-activated protein kinase cascade in the stimulation of NHE1 activity by extracellular stimuli, particularly by growth factors and G protein-coupled receptor agonists (7–9). ERK-mediated stimulation of NHE1 activity may occur by direct phosphorylation of the exchanger regulatory domain (10) and/or indirectly through one or more downstream effectors (7). One potential such effector is the 90-kDa ribosomal S6 kinase (p90RSK), which is activated by ERK-mediated phosphorylation (11) and has been shown to phosphorylate Ser-703 within the regulatory domain of NHE1 and thereby increase exchanger activity (12).

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\(^1\) The abbreviations used are: NHE, Na\(^+\)/H\(^+\) exchanger; NHE1, NHE isoform 1; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase.
derlying any such regulation are unknown. In the present study, we present evidence that a moderate extension of the duration of intracellular acidosis (to \( \geq 3 \) min) produces a marked increase in sarcolemmal NHE activity in both neonatal and adult rat ventricular myocytes. This effect appears to occur through a novel mechanism that is mediated by acidosis-induced activation of the ERK pathway, the inhibition of which allows the sustained stimulation of sarcolemmal NHE activity. Furthermore, our data suggest that this mechanism may have relevance to the regulation of plasma membrane NHE activity in non-myocyte cells also, since similar observations were made in COS-7 cells (African Green monkey kidney cells), which exhibit native NHE1 expression (15).

**EXPERIMENTAL PROCEDURES**

**Materials**—Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were from Amersham Biosciences. Dulbecco’s modified Eagles medium, M199, and fetal bovine serum were from Invitrogen. Antibodies were from Cell Signaling Technology (for phospho-ERK1/2 (Thr(P)-202/Tyr(P)-204) and phospho-p90(RSK) (Ser(P)-381)), Santa Cruz Biotechnology (for ERK2), and Transduction Laboratories (for p90(RSK)). PD98059 and U0126 were from Calbiochem-Novabiochem.

**Cell Culture and Isolation**—Adult and neonatal ventricular myocytes were isolated from Wistar rat hearts by collagenase digestion, as described previously (16, 17). Adult cells were used on the day of preparation, whereas neonatal cells were cultured on glass coverslips for 12-well plates for 1–3 days before use (8, 18). COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (15). Cells were split 1–2 days before pH measurements were made and subsequently cultured on glass coverslips. All cultured cells were serum-starved for 24 h before use for either pH measurement or assessment of kinase activity.

**Induction of Intracellular Acidosis**—In all cases, intracellular acidosis was induced in cells bathed in bicarbonate-free Tyrode solution (137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 10 mM HEPES, pH 7.4, 10 mM glucose) by transient (1–4 min) exposure to 30 mM NH\(_4\)Cl, as in our previous work in various cell types (8, 15–16) rat ventricular myocytes and COS-7 cells (15). Cells were subjected to intracellular acidosis by microepi-ride solutions in the untreated groups.

**Measurement of pH, and NHE Activity**—pH was determined in single cells superfused with bicarbonate-free Tyrode solution by microepi-fluorescence using the fluorescent pH indicator carboxy-seminaphtho
rhodaflo-1, as we have described previously for neonatal (8) and adult (16) rat ventricular myocytes and COS-7 cells (15). Cells were subjected to two consecutive acid pulses, the first of which was transient and the second either transient (in controls) or sustained for 1, 3, or 6 min. As the index of NHE activity, the rate of pH\(_i\) recovery (dpH/dt) was calculated at a fixed pH\(_i\), of 7.0, during recovery from intracellular acidosis.

**In-gel Kinase Assay**—Serum-starved cultured neonatal rat ventricular myocytes were incubated in Tyrode solution at 37 °C for 90 min before experiments. Cells were subjected to intracellular acidosis by exposure to NH\(_4\)Cl (50 mM) for 4 min and its subsequent replacement with sodium-free solution for 0 or 3 min. Cells were then lysed in Laemmli buffer and heated, and protein samples were separated on a 10% polyacrylamide gel containing 0.15 mg/ml GST or GST-NHE178 (prepared from *Escherichia coli* BL21 as described earlier (15)). After electrophoresis, the gel was washed 3 times with wash buffer (50 mM HEPES, pH 7.4, 10 mM MgCl\(_2\), 0.1 mM sodium orthovanadate, 5 mM 2-mercaptoethanol) containing 2% 2-propanol and 20 mM 3-2PATP (0.85 mM). After 60 min, the assay was terminated by transferring the gel to 5% trichloroacetic acid and 10 mM sodium pyrophosphate followed by extensive washing with the same solution to remove unincorporated \(^{32}\)P. The gel was then dried and subjected to autoradiography.

**Western Blotting**—Cells were lysed in Laemmli buffer, and protein samples separated by SDS-PAGE. After transfer to polyvinylidene difluoride membrane, Western analysis was performed using a rabbit polyclonal dual phospho-specific ERK1/2 antibody or a rabbit polyclonal phospho-specific p90(RSK) antibody. To confirm equal protein loading, a mouse monoclonal ERK2 antibody or a mouse monoclonal p90(RSK) antibody was used.

**RESULTS**

**NHE1 Activity Is Increased by Sustained Intracellular Acidosis**—To investigate the effect of the duration of intracellular acidosis on sarcolemmal NHE activity, neonatal rat ventricular myocytes were subjected to two consecutive intracellular acid pulses by transient exposure to NH\(_4\)Cl, as illustrated in Fig. 1. In the control group, Na\(^+\) was present in the superfusion solution throughout NH\(_4\)Cl washout (Fig. 1A). In the sustained acidosis groups, the duration of intracellular acidosis during the second acid pulse was extended to 1, 3, or 6 min by initial washout of NH\(_4\)Cl with Na\(^+\)-free solution (to inhibit the sarcolemmal NHE), and normal extracellular [Na\(^+\)]\(_o\), was subsequently reintroduced (Fig. 1, B–D, respectively). In all groups, sarcolemmal NHE activity was estimated from the rate of change in pH\(_i\) (dpH/dt) in the presence of normal [Na\(^+\)]\(_o\), and at an identical pH\(_i\) of 7.00 (see Fig. 1), the latter to preclude any confounding effects of allosteric regulation of sarcolemmal NHE activity by pH\(_i\). As shown in Table I, there was no significant difference between the four experimental groups in basal pH\(_i\), measured before the first acid pulse (point 1 in Fig. 1A), or the extent of intracellular acidosis attained, as reflected by the maximum pH\(_i\) measured upon NH\(_4\)Cl washout during either the first acid pulse (point 2 in Fig. 1A) or the second acid pulse (point 3 in Fig. 1A). Comparable dpH/dt values were obtained in all groups during recovery from the first acid pulse (Fig. 2A). Furthermore, in the control group there was no significant difference between the dpH/dt values measured during recovery from the consecutive acid pulses, indicating the absence of a temporal increase in NHE activity (Fig. 2A). With increasing duration of intracellular acidosis during the second acid pulse, however, the dpH/dt value was progressively increased such that this was significantly greater than that obtained during the first acid pulse in the cells subjected to 3 or 6 min of sustained acidosis (Fig. 2A). This apparent NHE stimulatory effect of sustained acidosis is more clearly illustrated in Fig. 2B, which shows the change in dpH/dt (\(\Delta dpH/dt\)) during recovery from the second acid pulse relative to that during recovery from the first; \(\Delta dpH/dt\) was close to zero in the control group, again reflecting the absence of a temporal increase in NHE activity, but increased significantly in myocytes exposed to 3 or 6 min of acidosis during the second acid pulse. These data indicate that sarcolemmal NHE activity increases with increasing duration of intracellular acidosis.

To determine whether the observation above reflects a unique property of the sarcolemmal NHE in neonatal rat ventricular myocytes, we carried out similar experiments in adult rat ventricular myocytes and COS-7 cells in which the duration of intracellular acidosis was extended to 3 min during the second acid pulse, again by initial washout of NH\(_4\)Cl with Na\(^+\)-free solution. As in neonatal rat ventricular myocytes, sustained intracellular acidosis significantly increased sarcolemmal NHE activity in adult rat ventricular myocytes (Fig. 3A) and plasma membrane NHE activity in COS-7 cells (Fig. 3B). These findings indicate that the NHE stimulatory effect of sustained intracellular acidosis occurs not only in neonatal ventricular myocytes but also in adult ventricular myocytes.
and in non-myocyte cells with native NHE1 expression.

In the experiments described above, the duration of intracellular acidosis was extended by initial washout of NH₄Cl with Na⁺-free solution; therefore, [Na⁺]ᵢ depletion during this period may underlie the increased NHE activity observed on reintroduction of [Na⁺]ᵢ independently of the extended duration of intracellular acidosis. To address this possibility, we carried out additional experiments in which the duration of intracellular acidosis was extended by initial washout of NH₄Cl in the presence of normal [Na⁺]ᵢ, but with the addition of the NHE1-selective inhibitor HOE-642 (cariporide), whose inhibitory effect is readily reversible upon its removal from the bathing medium (19). As shown in Fig. 4, extending the duration of intracellular acidosis to 3 min by this alternative method also produced a marked increase in sarcolemmal NHE activity in neonatal and adult rat ventricular myocytes and plasma membrane NHE activity in COS-7 cells. These findings indicate that, in a variety of cell types, NHE1 activity is increased markedly by extending the duration of intracellular acidosis and that altered [Na⁺]ᵢ is unlikely to be a significant contributor to this effect.

**Sustained Intracellular Acidosis Activates Cellular ERK and p90RSK**—To explore whether the NHE stimulatory effect of sustained intracellular acidosis might be mediated by activation of NHE1 kinase(s), we carried out an in-gel kinase assay using a GST fusion protein of the NHE1 regulatory domain (GST-NHE1) as the substrate polymerized within the polyacrylamide gel. Analysis of extracts from neonatal rat ventricular myocytes revealed increased activity of several kinases after sustained (3 min) intracellular acidosis, with prominent bands at 44 and 90 kDa (Fig. 5, left panel). These bands reflected the activity of kinases that specifically phosphorylated the NHE1 regulatory domain, since they were absent when GST alone (rather than the GST-NHE1 fusion protein) was used as the in-gel substrate (Fig. 5, right panel). Because previous work by Moor and Fliegel (10) suggests that the 44- and 90-kDa NHE1 kinase activities in rat myocardium represent ERK mitogen-activated protein kinase and its downstream effector p90RSK, we then focused specifically on the potential

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**Table I**

| Experimental group | Basal pHᵢ | Minimum pHᵢ |
|-------------------|-----------|-------------|
|                   | 1st acid pulse | 2nd acid pulse |
| Control           | 7.35 ± 0.04 | 6.70 ± 0.04 |
| Sustained acidosis (1 min) | 7.26 ± 0.02 | 6.72 ± 0.04 |
| Sustained acidosis (3 min) | 7.32 ± 0.03 | 6.68 ± 0.02 |
| Sustained acidosis (6 min) | 7.24 ± 0.06 | 6.71 ± 0.06 |

The duration of intracellular acidosis was extended by initial washout of NH₄Cl with Na⁺-free solution (see “Experimental Procedures” for details). There was no significant difference between groups in each column (n = 6 per group).
activation and NHE regulatory role of the ERK pathway in response to sustained intracellular acidosis.

Fig. 6 illustrates the effects of sustained intracellular acidosis on the activity of the ERK pathway, as reflected by the phosphorylation status of ERK1/2 (Fig. 6A) and p90RSK (Fig. 6B) in neonatal rat ventricular myocytes. Regardless of the method used to regulate the duration of intracellular acidosis (i.e. the washout of NH₄Cl with either Na⁺/H₁⁻ free or cariporide-containing solution), increasing this duration produced a progressive increase in the activity of the ERK pathway, with peak activation achieved after 3 min of intracellular acidosis (Fig. 6). These findings illustrate that sustained intracellular acidosis activates the ERK pathway, with a time course that is consistent with a potential causal role for the activation of this pathway in the observed stimulation of sarcolemmal NHE activity.

PD98059 and UO126 Abolish the Effects of Sustained Intracellular Acidosis on ERK and NHE Activity—To probe the potential functional significance of the activation of the ERK pathway by intracellular acidosis, we investigated the consequences of pharmacological inhibition of MEK1/2, the upstream activators of ERK1/2, using the structurally distinct inhibitors PD98059 (20, 21) and UO126 (22). Once again, exposure of neonatal rat ventricular myocytes to sustained (3 min) intracellular acidosis during the second acid pulse was extended to 3 min by initial washout of NH₄Cl with Na⁺/H⁻ free solution. Pretreatment of the myocytes with PD98059 or UO126 produced a dose-dependent inhibition of the increase
in ERK activity produced by sustained intracellular acidosis (Fig. 7), with complete abolition of such activation by either 30 μM PD98059 or 3 μM UO126 (Fig. 7).

In the light of the above findings, we then used 30 μM PD98059 and 3 μM UO126 as pharmacological tools to explore the contribution of the ERK pathway to the stimulation of sarcolemmal NHE activity by sustained intracellular acidosis in neonatal rat ventricular myocytes. As shown in Fig. 8, after pretreatment with vehicle (Me2SO), sustained (3 min) intracellular acidosis once again significantly stimulated sarcolemmal NHE activity. Pretreatment with the selected concentrations of PD98059 and UO126 did not alter sarcolemmal NHE activity in control cells subjected to transient intracellular acidosis (indicating the absence of an effect of these agents per se on allosteric regulation by pH), but markedly inhibited the stimulation of sarcolemmal NHE activity by sustained intracellular acidosis (Fig. 8). These data indicate that, in neonatal rat ventricular myocytes, the stimulation of sarcolemmal NHE activity by sustained intracellular acidosis is mediated by activation of the ERK pathway.

DISCUSSION

The principal findings of the work described here are as follows: (1). In myocyte and non-myocyte cell types with native expression of NHE1, sustained intracellular acidosis stimulates plasma membrane NHE activity (2). In neonatal ventricular myocytes, intracellular acidosis activates the ERK pathway, with a time course that is consistent with a mechanistic role for this activation in the stimulation of sarcolemmal NHE activity by sustained intracellular acidosis (3). In neonatal ventricular myocytes, pharmacological inhibition of ERK activation during sustained intracellular acidosis also prevents the stimulatory effect of such acidosis on sarcolemmal NHE activity, indicating a causal link between these phenomena.

In considering the effects of sustained intracellular acidosis on NHE1 activity, it is important to emphasize that in the work reported here we manipulated the duration, but not the extent, of intracellular acidosis (e.g. see Table I). Furthermore, NHE1 activity (as reflected by dpH/dt) was determined at an identical pH in each cell. Thus, we can be confident that the observed differences in NHE1 activity arose from the altered duration of intracellular acidosis rather than from allosteric regulation by pH. Indeed, the stimulation of NHE1 activity by sustained...
intracellular acidosis appears to have arisen from an increased affinity for $H^+$, in much the same way that a variety of extracellular stimuli increase NHE1 activity (4). Nevertheless, in the experiments in which the duration of intracellular acidosis was extended by washout of NH$_4$Cl with Na$^+$/H$^+$-free solution, an important contribution from reduced $[Na^+/H^+]_{i}$ cannot be excluded. In sheep Purkinje fibers, Wu and Vaughan-Jones (23) show that pretreatment with Na$^+$/H$^+$-free solution reduces $[Na^+/H^+]_{i}$ and leads to increased sarcolemmal NHE activity after the induction of intracellular acidosis and the reintroduction of extracellular Na$^+$. Such a reduction in $[Na^+/H^+]_{i}$ may stimulate NHE1 activity through an increased thermodynamic driving force (via an increased transmembrane Na$^+$ gradient) (23) or potentially through a Na$^+$/H$^+$-sensitive G protein (24).

How does sustained intracellular acidosis lead to increased NHE1 activity? As noted above, the mode of stimulation of NHE1 activity by sustained intracellular acidosis resembles that achieved by a variety of extracellular stimuli, many of which work through the activation of protein kinases (26). Indeed, we found increased activity of several NHE1 kinases in neonatal ventricular myocytes subjected to sustained intracellular acidosis (Fig. 5). On the basis of previous work by Fliegel and colleagues (10, 27) and others (7, 28, 29) on the NHE1 regulatory role of the ERK pathway in response to a variety of
stimuli in multiple cell types, we focused on the potential activation and NHE1 regulatory role of this pathway in response to sustained intracellular acidosis. Two observations lead us to conclude that activation of the ERK pathway is necessary for the stimulation of NHE1 activity by sustained intracellular acidosis, at least in neonatal rat ventricular myocytes. First, intracellular acidosis activated the ERK pathway in these cells (Fig. 6), with a time course that is consistent with a mechanistic role for this pathway in the stimulation of sarcolemmal NHE activity by sustained (3 min) intracellular acidosis. Second, pretreatment with the structurally distinct MEK inhibitors PD98059 (30 μM) and U0126 (3 μM) abolished both the activation of the ERK pathway (Fig. 7) and the stimulation of sarcolemmal NHE activity (Fig. 8) by sustained intracellular acidosis. PD98059 and U0126 inhibit the ERK pathway by inhibiting the activation and/or activity of MEK1/2, the upstream regulators of ERK1/2 (20–22). Recent work by Davies et al. (30) has called into question the specificity of action of several commercially available protein kinase inhibitors. Nevertheless, the same study has reconfirmed the status of PD98059 and UO126 as highly specific inhibitors of MEK1/2 that remain valuable pharmacological tools in examining the roles of the ERK pathway in cellular processes. Indeed, at a concentration of 50 μM, PD98059 was found not to inhibit a large panel of other protein kinases, including several known regulators of NHE1 activity (such as protein kinase C (31, 32), Rho-dependent protein kinase (33), and p38 mitogen-activated protein kinase (34)) (30). These NHE1 regulatory kinases were also unaffected by UO126 at a concentration of 10 μM (30). On this basis, the common inhibitory effects of both PD98059 and U0126 (each used at the minimum concentration required to abolish ERK activation by the relevant stimulus) on the stimulation of sarcolemmal NHE activity by sustained intracellular acidosis strongly suggests that in neonatal ventricular myocytes this response is mediated by the ERK pathway.

The present study is the first report that illustrates the

**Fig. 7.** PD98059 and U0126 inhibit activation of the ERK pathway by sustained intracellular acidosis in a dose-dependent manner in neonatal rat ventricular myocytes. Activity of the ERK pathway was assessed in control cells (open bars) and those subjected to sustained (3 min) intracellular acidosis (black bars) by determining the phosphorylation status of ERK1/2 (A) and p90RSK (B) by Western blotting with antibodies for phospho-ERK1/2 (Thr(P)-202/Tyr(P)-204) and phospho-p90RSK (Ser(P)-381). Equal protein loading was confirmed by Western blotting with antibodies that detect both phosphorylated and non-phosphorylated forms of ERK2 and p90RSK. Autoradiograms show representative Western blots, and the bar charts show quantitative data. In the sustained acidosis groups the duration of intracellular acidosis was extended to 3 min by wash-out of NH₄Cl with Na⁺-free solution. PD98059, U0126, or vehicle (Me₂SO) was present from 5 min before exposure to NH₄Cl. *, p < 0.05 relative to the control group (n = 6 experiments).
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Fig. 8. PD98059 and U0126 inhibit the NHE stimulatory effect of sustained intracellular acidosis in neonatal rat ventricular myocytes. A, the rate of change in pH (dpH/dt) during recovery from the first (white bars) and second (black bars) acid pulses in the various experimental groups. B, the change in dpH/dt (ΔdpH/dt) during recovery from the second acid pulse relative to that during recovery from the first, in the control groups (white bars) and those subjected to sustained (3 min) intracellular acidosis during the second acid pulse (black bars). In the sustained acidosis groups, the duration of intracellular acidosis during the second acid pulse was regulated by initial washout of NH4Cl with Na+–free solution (see Fig. 1). Vehicle (MeSO4 (DMSO)), PD98059 (30 μM), or U0126 (3 μM) was present from 5 min before exposure to NH4Cl during the second acid pulse. *, p < 0.05, relative to the first acid pulse in A and relative to the control group in B (n = 8–10 per group).

stimulatory effect of sustained intracellular acidosis on NHE1 activity in multiple cell types with native expression of this ubiquitous NHE isoform. Nevertheless, pertinent data have been reported recently with regard to the regulation of NHE3 that is expressed predominantly in epithelial cells of the kidney and the gastrointestinal tract (35), in opioids (36) and porcine (37) cell lines of renal origin. For example, Tsuganezawa et al. (36) show that extended (24 h) incubation of opioid OKP cells in acidic medium increases NHE3 activity after a subsequent acute intracellular acid load, partly through increased NHE3 mRNA and protein abundance. A comparable mechanism is unlikely to be involved in our observations of increased NHE1 activity after intracellular acidosis sustained for a much shorter duration (3 min), although it may contribute to the increased NHE1 activity observed after extended (18 h) incubation of neonatal rat ventricular myocytes in acidic medium (38). Of more direct relevance to the present work is the recent report by Hayashi et al. (37) that exposure of porcine LLC-PK1 cells with native NHE3 expression to sustained (3–5 min) intracellular acidosis by initial washout of NH4Cl with Na+–free solution leads to increased activity of this isoform. Depletion of [Na+]i, and changes in the surface distribution or phosphorylation of NHE3 or in its interaction with the actin cytoskeleton were discounted as mechanisms underlying this stimulatory effect (37), although potential indirect regulation by kinase(s) activated by sustained intracellular acidosis was not addressed. Notably, NHE3 (and to a lesser extent the partially homologous NHE5 isoform) was similarly stimulated by sustained intracellular acidosis when expressed heterologously in AP-1 cells (NHE-deficient Chinese hamster ovary cells). To the contrary, there was no difference in plasma membrane NHE activity between AP-1 cells transfected with the NHE1 isoform and subjected to 1 or 3 min of intracellular acidosis (37). This was interpreted to reflect differential regulation of NHE3/5 versus NHE1 isoforms by the duration of intracellular acidosis (37), although a possible alternative explanation is that in this cell type (unlike in neonatal ventricular myocytes; see Fig. 2) maximal stimulation of NHE1 activity was already achieved after 1 min of intracellular acidosis. It would also be of interest to determine whether intracellular acidosis induces time-dependent activation of the ERK pathway in AP-1 cells with heterologous expression of the relevant NHE isoforms, as it does in the distinct cell types with native NHE1 expression that were studied in the present investigation. Notably, in OKP cells, incubation in acidic medium has been shown to activate the ERK pathway, and this has been implicated mechanistically in the subsequent up-regulation of NHE3 activity (36).

The identity of the “sensor” that activates the ERK pathway and thereby leads to the stimulation of NHE1 activity after sustained intracellular acidosis is not known. It is interesting to note that in MCT cells, a mouse kidney proximal tubular cell line, the tyrosine kinase c-Src is activated rapidly (within 1.5 min) by acid incubation as well as by exposure to intracellular acidosis, with the latter induced by several means, including transient exposure to NH4Cl (39). Because Src family tyrosine kinases are known to regulate ERK activity in response to a variety of stimuli in multiple cell types (40), including cardiac myocytes (41), they represent viable candidates as upstream regulators of ERK activity in response to intracellular acidosis. Contrary to this, however, ERK activation by incubation of OKP cells in acidic medium has been shown to occur through a mechanism that is independent of c-Src activation (36). Future work will focus on identification of the upstream mechanisms that are responsible for ERK activation by intracellular acidosis as well as the determination of the molecular mechanism(s) downstream of ERK activation that is responsible for increased NHE1 activity in response to sustained intracellular acidosis.

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Stimulation of the Plasma Membrane Na\(^+\)/H\(^+\) Exchanger NHE1 by Sustained Intracellular Acidosis: EVIDENCE FOR A NOVEL MECHANISM MEDIATED BY THE ERK PATHWAY

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