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Phosphodiesterase 5A Inhibition Decreases NHE-1 Activity Without Altering Steady State pH_i: Role of Phosphatases

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Key Words

Phosphodiesterase 5A • NHE-1 • Protein kinase G • Protein phosphatase 2A

Abstract

Background/Aims: This study aimed to identify the signaling pathway for the proposed link between phosphodiesterase-5A (PDE5A) inhibition and decreased cardiac Na^+/H^+ exchanger (NHE-1) activity. Methods: NHE-1 activity was assessed in rat isolated papillary muscles by the Na^+-dependent initial pH_i recovery from a sustained acidosis (ammonium prepulse). ERK1/2, p90RSK and NHE-1 phosphorylation state during acidosis was determined. Results: PDE5A inhibition (1 μmol/L sildenafil, SIL) did not modify basal pH_i but significantly blunted pH_i recovery after sustained acidosis. Although preventing ERK1/2- p90RSK signaling pathway (10 μmol/L U0126) mimicked SIL effect, SIL did not blunt the acidosis-mediated increase in kinases activation. SIL+U0126 did not show additive effect on NHE-1 activity. Then, we hypothesized that SIL could be activating phophasatases (PP1 and/or PP2A) to directly dephosphorylate NHE-1 despite preserved ERK1/2-p90RSK activation. Non-specific phosphatases inhibition (1 μmol/L okadaic acid) canceled SIL effect on pH_i recovery from acidosis. Same result was observed by inhibiting PP2A either with a lower dose of okadaic acid (1 nmol/L) or, more specifically, with 100 μmol/L endothall. Consistently, NHE-1 phosphorylation at Ser703 increased after acidosis, SIL prevented this effect and PP2A inhibition (endothall) reverted SIL effect. Conclusion: We suggest that PDE5A inhibitors decrease NHE-1 phosphorylation and activity through a mechanism that involves PP2A activation.

Introduction

The Na^+/H^+ exchanger isoform 1 (NHE-1) is expressed at significant levels in heart tissue [1]. It is the major Na^+ influx pathway in myocardial cells [2] where it also serves as an acid extrusion system. NHE-1 couples H^+ efflux to Na^+ influx in a 1:1 stoichiometry. Enhanced NHE-1 activity leads to an elevated intracellular Na^+ concentration ($[Na^+]_i$) which drives the Na^+/Ca^{2+}

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exchanger (NCX) with a greater sensitivity than Na^+/K^+ ATPase in cardiac myocytes [3-5]. NHE-1 hyperactivity, leading to increased $[Na^+]_i$, has been implicated in myocardial ischemia and reperfusion injury, as well as in cardiac hypertrophy and heart failure [1, 6-14].

There is conclusive evidence that a cause- and-effect relationship exists between many cardiac diseases and exchanger activation; as a result, the NHE-1 has become a potential target for developing new therapeutic strategies against those diseases. In this context, profound knowledge of the mechanisms that regulate NHE-1 activity is necessary. NHE-1 activity is controlled by intracellular pH (pH_i) and other numerous factors, like hormones, catecholamines, and growth factors [1] and also by the time duration of acidosis [15]. One of the main pathways to increase NHE-1 activity is through phosphorylation of specific sites at its cytosolic tail (see review in Reference [16]), resulting in an increased affinity of the H^+ sensor [17]. NHE-1 has consensus sequences for mitogen-activated protein (MAP) kinases, and these enzymes have been implicated in NHE-1 phosphorylation and activation [18-22]. NHE-1 activity can also be regulated by phosphorylation independent mechanisms [16, 17, 23, 24] and even by ATP [25, 26]. We recently proposed that stimulation of the cGMP/ Protein kinase G (PKG) signaling pathway after phosphodiesterase 5A (PDE5A) inhibition depresses NHE-1 activity [27]. This finding coincides with previous reports in tissues other than myocardium [28-31] which may be related to changes in the phosphorylation state of the exchanger. In contrast to the action of specific NHE-1 inhibitors, this novel route to inhibiting NHE-1 only depressed NHE-1 activity after an acidic load, without affecting basal pH_i [27]; therefore, it preserves the important "housekeeping" function of the exchanger. All these properties certainly make the characterization of this unexplored signaling pathway appealing, and suggest that PDE5A inhibitors may be of great therapeutic importance as potential NHE-1 inhibitors. A similar differential effect upon basal and stimulated NHE-1 activity was recently reported after NHE-1 activation by phosphorylation through p90RSK [32]. This report emphasizes the potential therapeutic advantages of using NHE-1 inhibitory compounds that selectively hinder exchanger activity only after an acidic challenge and not under basal conditions. Interestingly, localization of PDE5A at the intercalated disks has been recently reported [33], while same localization had been previously reported for NHE-1 [34, 35]. We may speculate about co-localization of both proteins reinforcing the idea of a possible functional link between PDE5A inhibition and decreased NHE-1 activity.

The present study aimed to identify the underlying mechanism of the inhibitory effect of PDE5A inhibition on NHE-1. We will provide evidence to suggest that PDE5A inhibition decreases NHE-1 phosphorylation and activity by enhancing phosphatase PP2A activity.

Materials and Methods

All procedures followed during this investigation conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and to the guidelines laid down by the Animal Welfare Committee of La Plata School of Medicine.

Measurement of pH_i, determination of NHE-1 activity and intrinsic buffer capacity (β_i)

pH_i was measured in isolated left ventricular papillary muscles from male Wistar rats (n=62) following a previously described BCECF-epifluorescence technique [36]. The muscles were loaded with the ester form of the pH-sensitive fluorescent dye BCECF (Invitrogen, Molecular Probes). The dye was solubilized in dimethyl sulfoxide (Sigma Chemical Co) containing pluronic acid F-127 (20% [wt/vol]) and diluted to a final concentration of 10 µmol/L in HCO_3^-/CO_2-buffered solution. After autofluorescence levels were recorded, the muscles were incubated for 1 hour in the BCECF-AM-containing solution. At the end of the loading period, washout of the extracellular space with dye-free solution was carried out for 30-60 minutes before any pH_i determination was done. At this time, BCECF fluorescence was uniformly distributed throughout the muscle. To measure fluorescence emission from BCECF, excitation light from a 75-W Xenon lamp was band-pass-filtered alternatively at 440 and 495 nm and was then transmitted to the muscles under study by a dichroic mirror (reflecting wavelengths, <510 nm) located beneath the microscope. Fluorescence emission was collected by the microscope objective (x10) and transmitted through a band-pass filter at 535±5 nm to a photomultiplier (model R2693, Hamamatsu). The output of the photomultiplier was collected via an A/D converter (model 2801 A, Data Translation) and stored in a personal computer for later analysis. To limit photobleaching, a neutral density filter (1% transmittance) was placed in the excitation light path, and a manual shutter was used to select sampling intervals (2 seconds every ~10 seconds) during the protocol. The NHE-1 activity after sustained acidosis was assessed by the Na^+-dependent pH_i recovery from an ammonium prepulse-induced acute acid load [37]. The experiments were performed in the nominal absence of bicarbonate (HEPES buffer) to assure that the pH_i recovery after the acidic load was entirely due to NHE-1 activation. The papillary muscles were acid loaded by brief (10 min) exposure to 20.0 mmol/L NH_4Cl followed by washout with Na^+-free HEPES

buffer (NaCl was equimolar replaced with N-methyl-D-glucamine). Recovery of pH_i after acid loading was induced by re-introduction of external Na^+ after 10 minutes of sustained acidosis. For the experiments where the effects of transient (non-sustained) acidosis on NHE-1 activity were studied, ammonium washout was done with Na^+-containing HEPES buffer. In this case, 30 mmol/L NH_4Cl was used to reach a similar degree of acidification after washout to that obtained during sustained acidosis. β_i was determined as previously described [37]. Maximal H^+ efflux ($J_H^+ = {}_{max}dpH_i/dt \cdot \beta_i$) was calculated at the beginning of recovery from acidosis (just after extracellular Na^+ re-addition) and used to compare NHE-1 activity among different groups. Efforts were made to obtain similar degrees of acidification after the 10 minutes of Na^+ deprivation or after transient acidosis, in order to compare the initial J_H^+ at similar pH_i. Table 1 shows that maximal intracellular acidification after the acidic load (measured just before Na^+ re-addition in the case of the experiments of sustained acidosis) was of similar magnitude in all experimental groups.

At the end of each experiment, fluorescence emission was calibrated by exposing the muscles to a high-KCl solution containing 10 µmol/L nigericin, a H^+-K^+ exchanger that equals $[H^+]_o$ to $[H^+]_i$ when extracellular and intracellular K^+ are the same. The calibration solution contained (in mmol/L) KCl 140.0, MgCl2 1.0, CaCl2 1.35, HEPES 5.0, sodium cyanide 4, and BDM 20.0 to prevent muscle contracture. Buffer pH was adjusted with KOH or HCl to four different values ranging from 7.5 to 6.5. Such a calibration revealed a linear relation between pH and the fluorescence ratio (F495/F440) calculated as follows: ratio=(fluorescence at 495 nm-autofluorescence at 495 nm)/(fluorescence at 440 nm-autofluorescence at 450 nm), which was used to correct the experimental fluorescence ratios.

All pharmacological interventions were started 20 minutes before the ammonium prepulse and kept for the entire protocol.

Determination of extracellular signal-regulated protein kinases (ERK1/2), p90 ribosomal S6 kinase (p90RSK) and NHE-1 phosphorylation

Cardiac tissue was homogenized in lysis buffer (300 mmol/l sacarose; 1 mmol/L DTT; 4 mmol/L EGTA, protease inhibitors cocktail (Complete Mini Roche); 20 mmol/L Tris-HCl, pH 7.4). After a brief centrifugation the supernatant was kept and protein concentration determined by the Bradford method. Samples were denatured and equal amounts of protein were subjected to PAGE and electrotransferred to PVDF membranes. Membranes were then blocked with non-fat-dry milk and incubated overnight either with anti-phospho-ERK1/2 (Santa Cruz Biotechnology) or anti-phospho-p90RSK (Cell Signaling). For NHE-1 phosphorylation determination samples were immunoprecipitated using a NHE-1 polyclonal antibody (Chemicon) and then subjected to PAGE, electrotransferred and incubated with an anti-14-3-3 binding motif antibody (Cell Signaling). Previous reports have shown that the regulatory Ser703 of the NHE-1 lies within a sequence which creates upon phosphorylation a binding motif for 14-3-3 proteins [20, 38]. Thus, the anti-14-3-3 binding motif antibody when probed with immunoprecipitated NHE-1 represents a useful tool to estimate NHE-1 phosphorylation at Ser703 [39]. Total p90RSK, ERK-2 and NHE-1 as loading controls were assayed. The signal obtained for the phospho-proteins were each normalized by that obtained with the antibodies that recognized the total amount of the corresponding protein (phosphorylated and non-phosphorylated). Those normalized values were then expressed relative to control. Peroxidase-conjugated anti-rabbit or anti-mouse IgG (Santa Cruz Biotechnology) were used as secondary antibody and bands were visualized using the ECL-Plus chemiluminescence detection system (Amersham). Autoradiograms were analyzed by densitometric analysis (Scion Image).

Experimenta l groups	pH_i	β_i (mmol/L)
Control (n=13)	6.73 ± 0.01	28.4 ± 0.3
SIL (n=4)	6.77 ± 0.03	27.0 ± 1.0
U0126 (n=4)	6.73 ± 0.01	28.4 ± 0.4
SIL+U0126 (n=3)	6.76 ± 0.01	27.5 ± 0.4
SIL+Oka 1 µmol/L (n=5)	6.72 ± 0.03	29.0 ± 1.0
SIL+Oka 1 nmol/L (n=4)	6.72 ± 0.02	29.0 ± 0.9
Oka 1 µmol/L (n=4)	6.74 ± 0.02	28.0 ± 0.7
SIL+End (n=4)	6.72 ± 0.02	29.0 ± 0.7
Transient acidosis (n=5)	6.75 ± 0.02	27.6 ± 0.8
Transient acidosis + SIL (n=4)	6.74 ± 0.04	28.0 ± 1.3
Transient acidosis + U0126 (n=4)	6.75 ± 0.03	27.6 ± 1.0

Table 1. Maximal-induced intracellular acidification after 10 minutes of ammonium washout and intrinsic buffer capacity (β_i) for all experimental groups.

Statistics

Results are presented as mean±SEM. Statistical analysis was performed using one-way ANOVA followed by the Student-Newman-Keuls test. $P<0.05$ was considered significant.

Results

pH_i recovery after sustained acidosis, effect of SIL and ERK1/2 inhibition

The Na^+-dependent initial rate of pH_i recovery from an acute and sustained (10 min) acid load was used to asses NHE-1 activity in isolated papillary muscles. Since the experiments were performed in the nominal absence of bicarbonate, the recovery of pH_i after the acidic load was exclusively due to NHE-1 activation. Maximal-induced acidification and β_i were of similar magnitude in all experimental groups (Table 1). NHE-1 activity was tested before and after PDE5A inhibition by SIL (1 µmol/L). As we previously reported [27], SIL did not affect basal pH_i (7.25 ± 0.02 vs. 7.24 ± 0.02, before and after SIL, respectively, n=8) but significantly inhibited NHE-1 activity after the sustained acidic load (Fig. 1A, B, and E).

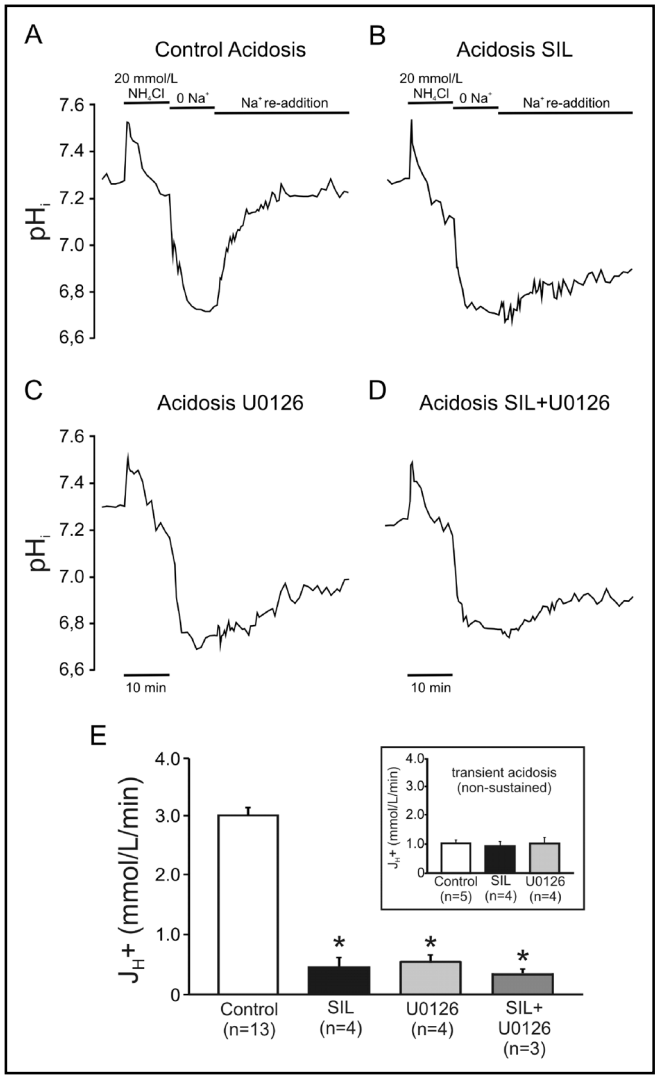

Fig. 1. pH_i recovery after sustained acidosis. Effect of SIL and ERK1/2 inhibition. Panel A: Original raw data from a pH_i recording during and after an ammonium prepulse under control conditions in one papillary muscle. Panel B: Same as "A" but in the presence of the PDE5A inhibitor sildenafil (SIL, 1 μmol/L) which significantly blunted pH_i recovery after the acidic load. Panel C: Same as "A" but in the presence of the MEK inhibitor U0126 (10 μmol/L) to avoid the acidosis-mediated ERK1/2-p90RSK activation. U0126 mimicked the effect of SIL on pH_i recovery. Panel D: Same as "A" but in the presence of SIL+U0126. Combination of compounds did not produce further inhibition of pH_i recovery. Panel E: Averaged initial (maximal) H^+ efflux (J_H^+) obtained from the different protocols shown from A to D. Panel E "inset": Averaged initial J_H^+ obtained after transient (non-sustained) acidosis under control condition, and in the presence of SIL or U0126. Note that neither U0126 nor SIL affected J_H^+ after transient acidosis, suggesting that NHE-1 phosphorylation is not play a significant role under this condition. Maximal intracellular acidification after the ammonium prepulse was similar among groups allowing the comparison of the initial J_H^+ (see averaged results on Table 1). * $P<0.05$ vs. control acidosis.

It was proposed that sustained acidosis promotes an ERK1/2-p90RSK-mediated increase in NHE-1 activity [15]; therefore, we explored the effect of inhibiting this signaling pathway on pH_i recovery under our experimental conditions. Inhibition of MEK, the kinase upstream of ERK1/2-p90RSK, by U0126 (10 μmol/L) during sustained acidosis decreased J_H^+ to values similar to SIL (Fig. 1C and E). A similar decrease in J_H^+ and, therefore, in NHE-1 activity was also detected after inhibition of both PDE5A and MEK (Fig. 1D and E). These results suggest that PDE5A inhibition suppresses the effect of acidosis-mediated ERK1/2-p90RSK activation or that a maximal effect was achieved by the two pharmacological interventions, by mechanisms independent of each other. It is important to highlight that after 30 min of Na^+ re-addition pH_i recovered to similar values in all treated groups (SIL: 6.91±0.03, n=4; U01216: 6.93±0.03, n=4; and SIL+U0126: 6.89±0.02, n=3), suggesting that pH_i would eventually recover to basal values under all experimental conditions.

We particularly focused on the interactions among acidosis, kinases, NHE-1, and PDE5A inhibition under sustained acidosis because of its clinical importance and the already described acidosis-mediated increase in the activity of MAP kinases [15]. However, we also explored the recovery of pH_i after transient (non-sustained) acidosis where MAP kinases were not reported to play a significant role [15]. Under this condition, NHE-1 activity was significantly lower than that observed after sustained acidosis, as it can be appreciated by comparing the corresponding initial J_H^+ during the recovery phase from acidosis on Fig. 1E and 1E "inset" (empty bars). In addition, neither U0126 nor SIL significantly affected J_H^+ after transient acidosis (Fig. 1E "inset"), reinforcing the idea that ERK1/2-p90RSK activation does not play a significant role under this condition. It is important to mention, however, that despite the presumably lack of participation of MAP kinases in determining NHE-1 activity after non-sustained acidosis, its initial J_H^+ was significantly higher than the remaining J_H^+ detected after sustained acidosis plus U0126 or SIL or the combination of both compounds (Fig. 1E, white bar on "inset" vs. black, light-grey and grey bars on main panel). The reason for this unexpected finding is not apparent to us yet but it may be related to the long exposition to Na^+ deprivation used to lengthen acidosis. In any case, the fact that the same protocol to prolong acidosis was repeated under all experimental conditions (with or without interventions) validates comparisons among groups.

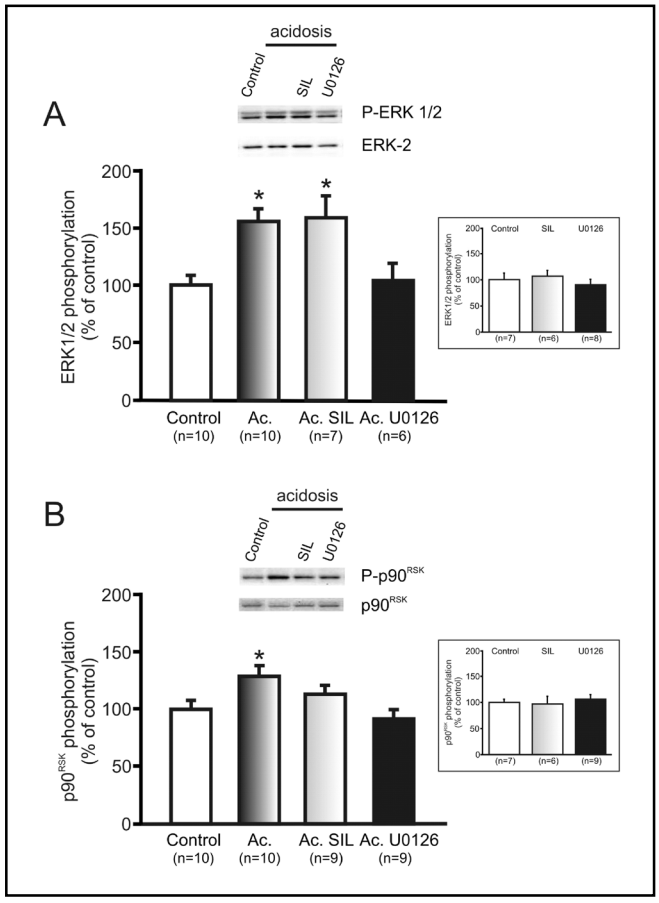

Fig. 2. ERK1/2 and p90RSK phosphorylation after sustained acidosis. As previously shown by Haworth et al. [15], sustained acidosis significantly increased ERK1/2 (panel A) and p90RSK (panel B) phosphorylation. As expected, U0126 canceled the increase in both kinases. Instead, SIL did not affect the acidosis-mediated increase in ERK1/2. SIL slightly decreased p90RSK phosphorylation level although it was not statistically different from acidosis in the absence of SIL. These results demonstrate that these compounds in spite of decreasing NHE-1 activity to a similar extent clearly targeted different intracellular signaling pathways to reach a similar endpoint. Neither SIL nor U0126 modified basal phosphorylation of MAP kinases (insets). "Control": Non-acidotic control; "Ac.": Acidosis. * P<0.05 vs. Control.

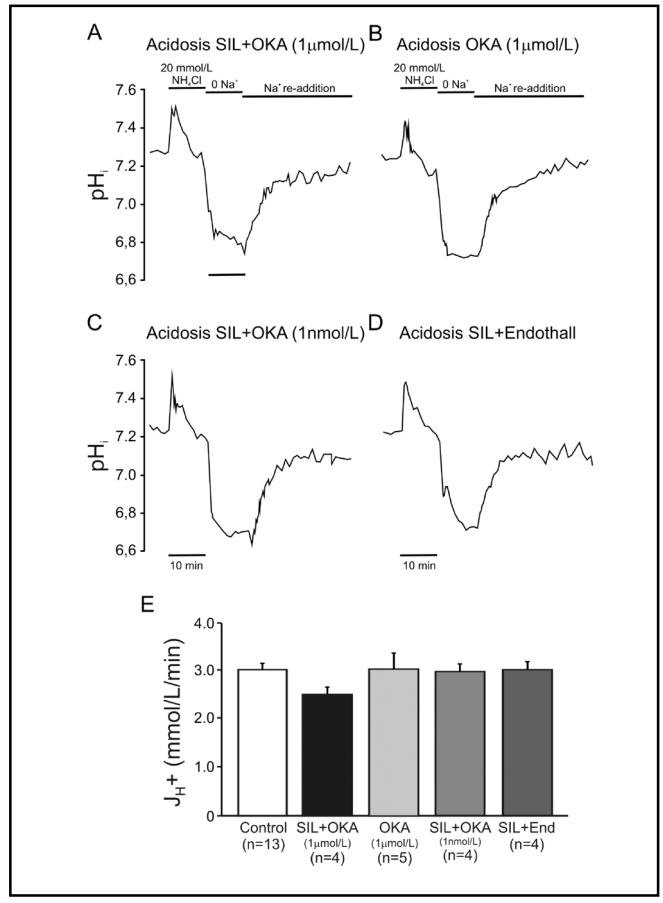

Fig. 3. Role of protein phosphatases PP1 and PP2A in the inhibitory effect of SIL on NHE-1 activity. Non-specific inhibition of PP1 and PP2A with 1 μmol/L okadaic acid on top of SIL restored normal pH_i recovery after the acidic load as shown in the original pH_i recording of panel A and in the averaged results shown in panel E. Okadaic acid alone did not have any stimulatory effect *per se* on NHE-1 activity as shown in panels B (raw data) and E (averaged results). Interestingly, the same result was observed when the dose of okadaic acid was lowered to 1 nmol/L to preferentially inhibit PP2A (Panel C and E) or when PP2A was more specifically inhibited with 100 μmol/L endothall (Panel D and E). For the sake of comparison the averaged initial J_H^+ from control acidosis presented in Figure 1E was also included in the present Figure (panel E).

ERK1/2 and p90RSK phosphorylation after sustained acidosis

After considering the results of the previous section, we examined the phosphorylation state of ERK1/2-p90RSK to determine whether it would be hindered by PDE5A inhibition, thereby preventing the enhanced activity of NHE-1. Sustained acidosis promoted a significant increase in ERK1/2 and p90RSK phosphorylation that was cancelled by MEK inhibition as expected (Fig. 2). Interestingly, SIL did not prevent the acidosis-mediated increase in the phosphorylation state of ERK1/2 (Fig. 2). In the case of p90RSK, SIL slightly decreased its phosphorylation level, though it was not statistically different from acidosis in the absence of SIL. These results provide evidence that the effect of PDE5A inhibition on NHE-1 activity can be mimicked by MEK inhibition (Fig. 1C and E), although both compounds clearly targeted different intracellular signaling pathways to reach a similar endpoint.

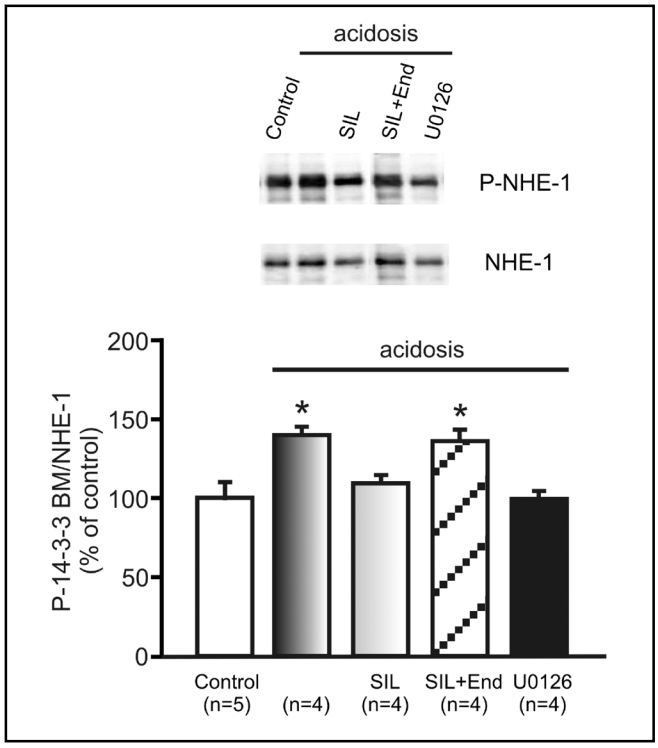

Fig. 4. NHE-1 phosphorylation after sustained acidosis. Consistent with the functional results, NHE-1 phosphorylation at Ser703 estimated by an anti-14-3-3 binding motif (BM) antibody increased after sustained acidosis and SIL blunted this effect. As expected, MEK inhibition with U0126 reproduced SIL effect. PP2A inhibition with endothall ("End") reverted SIL effect on NHE-1 phosphorylation, suggesting that PDE5A inhibition would activate PP2A that in turn would directly dephosphorylate NHE-1 without affecting MAP kinases. "Control": Non-acidotic control. * P<0.05 vs Control.

It is important to stress that neither SIL nor U0126 modified the phosphorylation state of MAP kinases under basal (non-acidotic) conditions (Fig. 2, insets).

Role of protein phosphatases PP1 and PP2A in the inhibitory effect of SIL on NHE-1 activity

Given that SIL did not prevent the increase in ERK1/2-p90RSK phosphorylation after sustained acidosis, we next hypothesized that SIL could be activating phosphatases to directly decrease NHE-1 phosphorylation. Thus, we designed experiments to explore if phosphatase inhibition could revert SIL effect. We planned to target protein phosphatases PP1 and PP2A since they were proven to dephosphorylate NHE-1 under different experimental settings [39, 40]. As an initial approach, we used the phosphatase inhibitor, okadaic acid, at a concentration high enough (micromolar range) to inhibit both PP1 and PP2A [41]. In our experimental conditions, 1 μmol/L okadaic acid added in addition to SIL completely reverted the effect of the PDE5A inhibitor on NHE-1 activity (Fig. 3A and E), suggesting that activation of phosphatases played a crucial role in the inhibitory effect of SIL on NHE-1. Okadaic acid alone did not have any stimulatory effect *per se* on NHE-1 activity since pH_i recovery after the acidic load was not statistically different from that observed in the control condition (absence of drugs), as shown in Fig. 3B and E.

It was previously reported that a lower concentration of okadaic acid (nanomolar range) could preferably inhibit PP2A [41]. To more precisely determine the role of phosphatases on NHE-1 activity after SIL treatment, we evaluated if 1 nmol/L okadaic acid was also able to revert the SIL effect. Figure 3 (C and E) shows that even at this low concentration okadaic acid completely canceled the effect of SIL on NHE-1 activity, suggesting a PP2A-mediated decrease in exchanger phosphorylation after SIL treatment may be responsible for the decreased exchanger activity. To further confirm this, we performed a new series of experiments in which 100 μmol/L endothall, a more specific PP2A inhibitor [39, 42], was added with SIL. Under this condition, the inhibitory effect of SIL on NHE-1 activity was also canceled (Fig. 3D and E).

NHE-1 phosphorylation after sustained acidosis

Taken together, the results presented above suggest a causal link between PDE5A inhibition, PP2A activity, and the profound decrease in NHE-1 activity, which might reflect changes in its phosphorylation state. To provide further evidence in favor of our hypothesis, we analyzed the phosphorylation state of the exchanger at Ser703. We used an anti-phospho-Ser 14-3-3 binding motif antibody in immunoprecipitated NHE-1 to estimate NHE-1 phosphorylation at Ser703 (as explained in the Methods). Sustained acidosis significantly increased NHE-1 phosphorylation and pretreatment with SIL completely cancelled this effect (Fig. 4), in spite of the acidosis-mediated increase in phosphorylation of MAP kinases (Fig. 2). It is important to state that basal NHE-1 phosphorylation was not affected by SIL (111 ± 12 % of control, n=5, P=NS). As expected, NHE-1 phosphorylation at Ser703 decreased when phosphorylation of ERK1/2-p90RSK was prevented by MEK inhibition after the acidic challenge (Fig. 4). Finally, PP2A inhibition fully reverted the inhibitory effect of SIL on NHE-1 phosphorylation (Fig. 4). These results were consistent with the functional results described above, and clearly suggest PP2A

activation after PDE5A inhibition hinders acidosis-mediated increase in NHE-1 phosphorylation, revealing that this phosphatase may directly target NHE-1 without affecting MAP kinases.

Discussion

Sustained acidosis appears to increase NHE-1 activity by two independent mechanisms: 1) the action of H ions upon the allosteric site of the transmembrane domain, which increases the turnover rate of the exchanger, and 2) a time-dependent phosphorylation of the cytosolic tail of the exchanger induced by an acidosis-mediated increase in the activity of MAP kinases, superimposed on the first one. This latter mechanism leads to maximal exchanger activity after 3-5 minutes of acidosis and is inhibited by preventing the activation of kinases [15] and by PDE5A inhibition, as shown in the present work. At a first glance, our findings suggest that PDE5A inhibitors interfere with the acidosis-mediated activation of kinases. However, as shown in Fig. 2, this was not the case; instead, PDE5A inhibition by SIL decreased NHE-1 phosphorylation by a direct action on the cytosolic tail of the exchanger since the effect was observed in spite of increased phosphorylation of MAP kinases. In this context, we suggest that a PKG-mediated PP2A activation would be the signaling pathway by which PDE5A inhibition decreases NHE-1 phosphorylation and, thereby, its activity after sustained acidosis. The exact mechanism, either direct or indirect, by which PKG would activate PP2A is not evident from our experiments and deserves further investigation. Interestingly, a direct PP2A activation by PKG has been previously shown to participate in the mechanism of mucine secretion of human bronchial epithelial cells, [43] and in cellular pathways triggered by atrial natriuretic peptide leading either to stimulation of large conductance calcium and voltage-dependent potassium channels in a rat pituitary tumor cell strain [44] or to inhibition of the carotid body chemoreceptor nerve in rabbits [45].

Our present conclusions were drawn from data obtained after sustained acidosis, which has more important clinical and pathophysiological implications than acute transient acidosis.

If we calculate the increase in NHE-1 activity due to its phosphorylation after sustained acidosis by increased kinase activity or by decreased phosphatase activity, we should be able to discriminate the relative contribution of each mechanism to the total NHE-1 activity. Actually, a decrease in J_H^+ from ~3 to ~0.5 mmol/L/min was detected when the ERK1/2-p90RSK signaling pathway was blocked by U0126. Interestingly, values not statistically different from this were obtained when PDE5A was inhibited by SIL, and the combination of both pharmacological interventions did not induce a further decrease in NHE-1 activity (Fig. 1E). These findings suggest NHE-1 phosphorylation decreased to a maximum after both interventions or that both compounds used the same mechanism to decrease NHE-1 activity. We could speculate about the possibility of reaching the maximal effect that can be obtained by modulating cytosolic tail phosphorylation at Ser703, but remnant NHE-1 activity, through a mechanism other than phosphorylation at Ser703, can be present even after 10 minutes of sustained acidosis. On the other hand, if the assumption that SIL and/or U0126 decreased NHE-1 phosphorylation to a maximum were correct, we should expect to have a similar NHE-1 activity either under these conditions or when kinases are not yet activated like during transient acidosis. However, this was not the case. Without sustained acidosis and Na^+ deprivation, the J_H^+ was higher (Fig. 1E, inset). As mentioned before, the reason for this unexpected finding is not apparent to us yet, but it may reflect an alteration in NHE-1 activity probably promoted by the changes in Na^+ gradient that occurred just after extracellular Na^+ re-addition following a long period of Na^+ deprivation. In any case, this surprising result does not alter the conclusions drawn after Na^+ deprivation, since as stated before all interventions were compared under the same experimental conditions.

Recently, a novel mechanism underlying the protective effects of PDE5A inhibition by SIL upon pathologic cardiac hypertrophy was reported by Koitabashi et al. [46]. The authors showed that transient receptor potential canonical (TRPC) channels were negatively modulated by PKG-dependent phosphorylation. These stretch sensitive non-selective cationic channels can be responsible for increases in Na^+ and/or Ca^{2+} influx mimicking NHE-1 activation. Whether this mechanism is playing a role in our study was not explored.

We are suggesting that the decrease in NHE-1 activity after SIL treatment is due to a direct dephosphorylating action of PP2A on NHE-1. However, the capability of protein phosphatases PP1 and PP2A to target NHE-1 has been a matter of investigation in the last few years with somewhat contradictory results. In 2005, Misik et al. [40] working *in vitro* with AP1 cells, demonstrated that PP1 binds to and dephosphorylates the NHE-1 with consequent modulation of the exchanger's

activity. Although these authors could not exclude the involvement of other protein phosphatases, they clearly stated that PP1 was able to remove virtually all the phosphate from the phosphorylated NHE-1, while PP2A did not completely dephosphorylate the protein.

More recently, Snabaitis et al. [39] working with cultured adult rat ventricular myocytes, demonstrated that PP2A and NHE-1 are co-localized at the level of the intercalated disc regions, and they described an active role for this enzyme with NHE-1 as its target, leading to a decrease in the exchanger activity. The same study demonstrated a dephosphorylating role for PP2A on NHE-1 in intact myocytes after adenosine receptor-mediated Gi protein activation following a phenylephrine-induced increase in NHE-1 phosphorylation, and this dephosphorylation was inhibited by endothall.

Our results appear to support those of Snabaitis et al. [39] in terms of assigning a role to PP2A in targeting NHE-1. As previously stated, we are providing evidence to suggest a novel regulatory mechanism of NHE-1 activity through PDE5A inhibition-induced PP2A activation that dephosphorylates the cytosolic tail of NHE-1 and significantly reduces the exchanger activity during recovery from sustained acidosis.

We estimated the NHE-1 phosphorylation state with a phosphospecific antibody which recognizes the Ser703 phosphorylated 14-3-3 protein binding motif of the immunoprecipitated NHE-1. Previous works have shown that upon ERK/p90RSK phosphorylation, the phospho-Ser 14-3-3 binding motif antibody represents a useful tool for estimating NHE-1 Ser703 phosphorylation [20, 38]. A limitation of our study is that we only assayed NHE-1 phosphorylation at this site and not other identified regulatory sites. In this regard, several different amino acids of the cytosolic domain of the NHE-1 have been reported to be phosphorylated by different protein kinases including Ser693, Ser703, Thr717, Ser722, Ser725, Ser728, Ser766, Thr779, and Ser785 [22]. Furthermore, a recent work by Fliegel et al. reported a novel set of residues, namely Ser770 and Ser771, critical for the sustained acidosis-mediated activation of NHE-1 in neonatal rat cardiomyocytes [47]. Whether phosphorylation/dephosphorylation of residues other than Ser703 plays a role under our experimental conditions requires future investigation. In connection with this, a recent study by Snabaitis et al. [35]. demonstrated for the first time that increased phosphorylation can also be a factor in downregulating the exchanger activity. These authors reported that a protein kinase B-dependent phosphorylation at Ser648 inhibits NHE-1 activity.

Possible reasons for the inconsistencies concerning the capability of PP1 or PP2A to dephosphorylate the NHE-1 are yet unknown. It may be that a type of phosphatase acts on some of the sites and the other on other sites, or that this could be tissue or even species specific. Recent experiments from our own group performed on cat papillary muscles and using similar pharmacological tools to those used herein, suggest that the inhibitory effect of SIL on NHE-1 is also due to dephosphorylation of the exchanger by activation of phosphatase PP1 [48].

It may be argued that our present results in adult rat myocardium, assigning a crucial role for PP2A on NHE-1 dephosphorylation after PDE5A inhibition, are based on pharmacological interventions and this is a limitation of our study. Although the use of a low dose of okadaic acid to preferentially inhibit PP2A activity may conceivably be questioned, the consistency of our functional and biochemical results using another structurally different and reportedly more specific PP2A inhibitor compound, like endothall [39], gives support to our conclusion.

Finally, and from a clinical point of view, we would like to emphasize, as shown previously [27] that in contrast to other NHE-1 inhibitory compounds, SIL inhibited the exchanger only after an acidic load without modifying basal pH_i, thus preserving the important "housekeeping" function of the exchanger. This distinctive property of PDE5A inhibitors confers a potential therapeutic advantage for the treatment of several cardiovascular pathologies characterized by exacerbated NHE-1 activity [22]. This may also be the explanation for the apparent contradiction with previous results by Gergs et al. [49]. showing that overexpression of the catalytic subunit of PP2A in the heart leads to increased baseline necrosis and fibrosis, and decreased cardiac function, suggesting a detrimental role for PP2A activation.

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