Protein Kinase B/Akt Phosphorylates and Inhibits the Cardiac Na\textsuperscript{+}/H\textsuperscript{+} Exchanger NHE1

Andrew K. Snabaitis, Friederike Cuello, Metin Avkiran

Abstract—Sarcolemmal Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) activity is mediated by NHE isoform 1 (NHE1), which is subject to regulation by protein kinases. Our objectives were to determine whether NHE1 is phosphorylated by protein kinase B (PKB), identify any pertinent phosphorylation site(s), and delineate the functional consequences of such phosphorylation. Active PKB\textsubscript{\alpha} phosphorylated in vitro a glutathione S-transferase (GST)-NHE1 fusion protein comprising amino acids 516 to 815 of the NHE1 carboxyl-terminal regulatory domain. PKB\textsubscript{\alpha}-mediated phosphorylation of GST-NHE1 fusion proteins containing overlapping segments of this region localized the targeted residues to the carboxyl-terminal 190 amino acids (625 to 815) of NHE1. Mass spectrometry and phosphorylation analysis of mutated (Ser\rightarrow Ala) GST-NHE1 fusion proteins revealed that PKB\textsubscript{\alpha}-mediated phosphorylation of NHE1 occurred principally at Ser648. Far-Western assays demonstrated that PKB\textsubscript{\alpha}-mediated Ser648 phosphorylation abrogated calcium-activated calmodulin (CaM) binding to the regulatory domain of NHE1. In adult rat ventricular myocytes, adenovirus-mediated expression of myristoylated PKB\textsubscript{\alpha} (myr-PKB\textsubscript{\alpha}) increased cellular PKB activity, as confirmed by increased glycogen synthase kinase 3\beta phosphorylation. Heterologously expressed myr-PKB\textsubscript{\alpha} was present in the sarcolemma, colocalized with NHE1 at the intercalated disc regions, increased NHE1 phosphorylation, and reduced NHE1 activity following intracellular acidosis. Conversely, pharmacological inhibition of endogenous PKB increased NHE1 activity following intracellular acidosis. Our data suggest that NHE1 is a novel PKB substrate and that its PKB-mediated phosphorylation at Ser648 inhibits sarcolemmal NHE activity during intracellular acidosis, most likely by interfering with CaM binding and reducing affinity for intracellular H\textsuperscript{+}.

Key Words: PKB ■ Akt ■ Na\textsuperscript{+}/H\textsuperscript{+} exchanger ■ calmodulin ■ acidosis ■ phosphorylation

The Na\textsuperscript{+}/H\textsuperscript{+} exchanger (NHE) isoform 1 (NHE1) is a membrane glycoprotein encoded by the NHE1/SLC9A1 gene\textsuperscript{1} and is expressed in all tissues. In cardiac myocytes, NHE1 protein contributes significantly to the control of intracellular pH (pHi), particularly in response to intracellular acidosis.\textsuperscript{2} Although basal activity of the sarcolemmal NHE is low under physiological conditions,\textsuperscript{3} increased exchanger activity may mediate inotropic responses to neurohormonal stimuli, such as endothelin-1,\textsuperscript{3} angiotensin II,\textsuperscript{4} and \alpha\textsubscript{1} adrenoceptor agonists,\textsuperscript{5} principally through NHE1-mediated increases in intracellular sodium.\textsuperscript{6,7} The regulation of NHE1 activity in such settings involves modification of the intracellular carboxyl-terminal regulatory domain of the exchanger, either by the binding of accessory proteins (such as calcineurin homologous protein,\textsuperscript{6} carbonic anhydrase II,\textsuperscript{6} and calmodulin [CaM]\textsuperscript{10}) and/or by phosphorylation by protein kinases (such as the extracellular signal-regulated kinases [ERKs],\textsuperscript{11} p90 ribosomal S6 kinase [RSK],\textsuperscript{12,13} Rho-associated kinase [p160-RoCK1],\textsuperscript{14} and p38 mitogen-activated protein kinase [p38–MAPK]).\textsuperscript{15} Although the majority of studies have linked protein kinase–mediated phosphorylation of the NHE1 carboxyl-terminal regulatory domain to the upregulation of NHE1 activity,\textsuperscript{11–15} there are reports that certain protein kinase pathways can inhibit NHE1.\textsuperscript{16,17} Furthermore, although the functionally important phosphorylation sites in NHE1 have been established for some kinases (eg, RSK),\textsuperscript{12,18} they remain to be confirmed for many others.

Interestingly, the carboxyl-terminal regulatory domain of NHE1 contains 3 putative phosphorylation sites that conform to the optimal protein kinase B (PKB) target motif (RxRxxS/T\textsuperscript{\alpha}), which suggests a potential regulatory interaction between PKB and NHE1. In the heart, 3 isoforms of PKB (PKB\textsubscript{\alpha}/Akt1, PKB\textsubscript{beta}/Akt2, and PKB\textgamma/Akt3) are differentially expressed (\alpha=\beta>\gamma), and each constitutes a phosphoprotein of \textasciitextrapolated\textasciitextrapolated\approx 57 kDa that consists of an amino-terminal pleckstrin homology (PH) domain,\textsuperscript{20,21} a central kinase domain,\textsuperscript{22,23} and a carboxyl-terminal hydrophobic motif.\textsuperscript{24} The PH domain is crucial for the activation of PKB as it facilitates the phosphatidylinositol 3,4,5-trisphosphate–dependent translocation of PKB to the inner surface of the cell membrane, where dual phosphorylation at Thr308 and Ser473 by phosphoinositide-dependent kinase 1\textsuperscript{125} and the mTORC2 protein complex,\textsuperscript{25,26} respectively, achieves full activation. In the heart, active PKB plays a role in several physiological and pathological cellular...
processes. For example, increased myocardial PKB activity stimulates glucose uptake, regulates glycogen metabolism through the phosphorylation and inhibition of glycogen synthase kinase (GSK), and is regarded as central to the initiation of cellular survival pathways. PKB activity has also been implicated in the regulation of both exercise-induced physiological cardiac hypertrophy and maladaptive cardiac hypertrophy and its progression to heart failure. Although PKB has been suggested to target multiple substrates in the heart, whether NHE1 is phosphorylated and regulated by PKB is unknown. The objectives of the present study, therefore, were to determine whether NHE1 is a PKB substrate, identify any pertinent phosphorylation site(s), and determine the functional consequences of their PKB-mediated phosphorylation in adult myocardium.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org. Key techniques were adapted from previously published studies, including the isolation, culture and adenoviral infection of adult rat ventricular myocytes (ARVMs), generation of adenoviral vectors, in vitro phosphorylation assays and Western immunoblot analysis, measurement of sarcolemmal NHE activity, determination of cellular NHE1 phosphorylation, and immunocytochemistry and confocal microscopy.

Results
PKB-Mediated Phosphorylation of NHE1
The carboxyl-terminal regulatory domain of NHE1 contains 3 sites that conform to the optimal PKB phosphorylation motif RxRxxS/T, namely Ser648 (RQRLRS), Ser703 (RARIGS), and Ser796 (RIQRCLS), as predicted by a motif scanning algorithm. Therefore, we initially determined whether active PKB would phosphorylate recombinant NHE1, in an in vitro kinase assay using 32P-labeled ATP. Autoradiography revealed that PKB progressively phosphorylated a recombinant GST-NHE1 fusion protein, encompassing the final 300 amino acids of the carboxyl-terminal regulatory domain of human NHE1, in a time-dependent manner (Figure 1A). In parallel, identical in vitro kinase assays were performed in the presence of unlabeled ATP and used a phospho-PKB substrate antibody, which detects phosphorylated Ser or Thr residues within the PKB phosphorylation consensus motif RxRxxS/T, to monitor PKB-mediated phosphorylation of the GST-NHE1 fusion protein. This nonradioactive approach revealed a similar pattern of PKB-mediated phosphorylation of GST-NHE1 fusion protein, encompassing the final 300 amino acids of the carboxyl-terminal regulatory domain of human NHE1, in a time-dependent manner (Figure 1B). Notably, PKB-mediated phosphorylation was absent when either assay used recombinant GST protein as substrate (data not shown). These data suggest that the carboxyl-terminal regulatory domain of NHE1 is a novel substrate for PKB-mediated phosphorylation and that the phospho-PKB substrate antibody is able to detect such phosphorylation. We then used truncated GST-NHE1 fusion proteins, encompassing residues 516 to 630, 625 to 747, and 748 to 815 of the carboxyl-terminal regulatory domain of NHE1, to perform an additional series of in vitro phosphorylation experiments and revealed that PKB-mediated incorporation occurred at residues within 625 to 747 and 748 to 815, but not 516 to 630.

Figure 1. Phosphorylation of GST-NHE1 fusion protein by PKB in vitro (0 to 30 minutes), as detected by 32P incorporation and autoradiography (A) or immunoblotting with a phospho-PKB substrate antibody (B). Equal protein loading was confirmed by Coomassie staining. Data represent means±SEM (n=3). *P<0.05 vs 0 minutes.
all 3 putative PKB phosphorylation sites, was phosphorylated by PKBα in vitro. Interestingly, mutation of Ser648 to Ala (Ser648Ala) markedly reduced PKBα-mediated phosphorylation of GST-NHE1(625–815). In contrast, the Ser703Ala mutation had no discernible effect and the Ser796Ala mutation resulted in only a small reduction in phosphorylation (Figure 3A). The double mutations Ser648/703Ala and Ser648/796Ala also dramatically reduced PKBα-mediated phosphorylation, whereas the double mutation Ser703/796Ala produced only a small reduction (Figure 3A). The triple mutation Ser648/703/796Ala yielded a nonphosphorylatable GST-NHE1(625–815) fusion protein. Parallel immunoblot analysis using the phospho-PKB substrate antibody revealed a similar pattern of phosphorylation (Figure 3B). Taken together, these data suggest that Ser648 is the major PKB phosphorylation site in the NHE1 regulatory domain and that its phosphorylation is detected by the phospho-PKB substrate antibody.

We next performed in vitro kinase assays using the WT and double mutant (Ser648/703Ala, Ser648/796Ala, and Ser703/796Ala) GST-NHE1(625–815) fusion proteins as PKB substrates to study the kinetics of individual phosphorylation of Ser648, Ser703, or Ser796. PKBα-mediated incorporation of 32P into the pertinent GST-NHE1(625–815) fusion protein substrates showed that the phosphorylation of Ser648 proceeded at a rate similar to that of the targeted residues within the WT protein, reaching saturation within 5 to 10 minutes (Figure 4A). In contrast, under the same assay conditions, PKBα-mediated phosphorylation of Ser703 and Ser796 occurred at a much slower rate and did not reach saturation even after 60 minutes (Figure 4A). Again, parallel experiments using the phospho-PKB substrate antibody revealed a similar pattern, with the exception that Western immunoblotting with the phospho-PKB substrate antibody was not as sensitive as 32P autoradiography in detecting Ser703 phosphorylation (Figure 4B). These data provide further support for our finding that Ser648 within the NHE1 carboxyl-terminal regulatory do-
main is a novel substrate for PKBα-mediated phosphorylation. The detection of phosphopeptides containing pSer703 and pSer796 by mass spectrometry analysis likely reflects the fact that, for these studies, the substrate fusion protein was exposed to PKBα for 60 minutes, after which time detectable phosphorylation of these sites also occurs (Figure 4A).

Regulation of CaM Binding by PKBα-Mediated Phosphorylation of Ser648

Ser648 resides within an autoinhibitory CaM-binding region of the NHE1 regulatory domain, with previous evidence indicating that CaM binding to this region relieves the autoinhibitory effect, resulting in increased NHE1 activity. We therefore hypothesized that PKBα-mediated phosphorylation of Ser648 may regulate CaM binding to the NHE1 regulatory domain. To test this hypothesis, we used a far-Western approach and confirmed that CaM did indeed bind to unphosphorylated WT GST-NHE1(625–815) fusion protein, in a calcium-dependent manner (Figure 5A). Such binding was unaffected by Ser→Ala mutations (in the absence of phosphorylation) but was attenuated by the introduction of a phosphomimetic Ser648Asp substitution (Figure 5A). Interestingly, PKBα-mediated phosphorylation abolished CaM binding to WT GST-NHE1(625–815) fusion protein and its mutated variants (Figure 5B), except those carrying a Ser648Ala substitution alone or in combination with other mutations (Figure 5B). These data indicate that PKBα-mediated phosphorylation of Ser648 in the carboxyl-terminal regulatory domain of NHE1 inhibits CaM binding to this domain.

PKB-Mediated Phosphorylation and Regulation of NHE1 in Cardiac Myocytes

To manipulate PKB activity in intact ARVMs, we heterologously expressed constitutively active, hemagglutinin (HA)-tagged myristoylated PKBα (myr-PKBα) protein by adenoviral gene transfer. Western immunoblot analysis using antibodies to the HA-tag or PKBα protein revealed a “dose”-dependent increase in myr-PKBα expression 24 hours after infection (Figure 6A). The constitutively active nature of the heterologously expressed myr-PKBα protein was confirmed by the detection of a parallel increase in glycogen synthase kinase (GSK)3β phosphorylation at Ser9, a known cellular PKB substrate (Figure 6B). Immunocytochemical detection of HA-tag expression confirmed that infection of ARVMs with the AdV:myr-PKBα adenoviral vector at a multiplicity of infection (moi) of 300 (plaque-forming units per cell) was sufficient to achieve 95% infection efficiency (see supplemental Figure III).

Immunocytochemistry and confocal microscopy were also used to determine the cellular localization of heterologously

Figure 4 (Continued). Incorporation and autoradiography (A) or immunoblotting with a phospho-PKB substrate antibody (B). Equal protein loading was confirmed by Coomassie staining. C, Quantitative analysis of PKBα-mediated phosphorylation of WT GST-NHE1(625–815) fusion protein and mutant GST-NHE1 (Ser648/703Ala, Ser648/796Ala, or Ser703/796Ala) fusion proteins in which only Ser648, Ser703, or Ser796 is available for phosphorylation, as detected by immunoblotting with a phospho-PKB substrate antibody. Data represent mean±SEM (n=3).
expression revealed marked colocalization of the 2 proteins, particularly at the intercalated disc regions (Figure 6C).

We then examined whether increased PKBα activity in intact ARVMs, through heterologous expression of myr-PKBα, could increase phosphorylation of the full-length cellular NHE1 protein. ARVMs were coinfected with an adenoviral vector encoding epitope-tagged human NHE1 to amplify the NHE1 signal for phosphoprotein analysis.13 The expression of myr-PKBα was again confirmed by the presence of a HA-tagged protein of appropriate size and increased expression of PKBα protein in the crude lysate of ARVMs infected with the myr-PKBα vector (Figure 7A). Equal amounts of both the ~80-kDa and ~105-kDa moieties of NHE1 protein, representing differentially glycosylated forms,13 were present in crude lysates from all groups (Figure 7B). Following NHE1 immunoprecipitation, equal amounts of heterologously expressed NHE1 protein were present in the immunocomplexes in all groups, as detected by an antibody to the HA-tag (Figure 7C). However, when these immunocomplexes were probed with the phospho-PKB substrate antibody, those from ARVMs infected with the myr-PKBα adenoviral vector were found to contain significantly higher amounts of phosphorylated NHE1, compared to the uninfected control group or a second control group infected with a vector encoding an unrelated protein (β-galactosidase) (Figure 7C). These data show that increased cellular PKB activity in intact ARVMs leads to increased phosphorylation of NHE1; such phosphorylation most likely occurred at Ser648, because PKBα-mediated phosphorylation at this site is readily detected by the phospho-PKB substrate antibody (Figures 3B and 4B).

We then used the interventions described above to determine the effects of increased cellular PKB activity on sarcolemmal NHE activity in intact ARVMs. There were no significant differences between infected groups in cell dimensions or estimated volumes (see supplemental Table I) or the basal intracellular pH (pHi) and the degree of intracellular acidosis achieved after NH4Cl washout (see supplemental Table II). Intrinsic buffering capacity was determined in all 3 groups and used to calculate sarcolemmal NHE activity in the appropriate group (see supplemental Figure IV). Figure 7D shows that sarcolemmal NHE activity, as reflected by the H+ efflux rate across the sarcolemma (JH+), was significantly lower (by 60% to 70%) in ARVMs expressing myr-PKBα, when compared to either control group. These gain-of-function data indicate that, in intact ARVMs, increased PKB activity results in a significant reduction in sarcolemmal NHE activity in response to intracellular acidosis.

Finally, we adopted a complementary loss-of-function pharmacological approach and determined the role of endogenous PKBα/β activity in the regulation of sarcolemmal NHE activity in ARVMs using a recently characterized specific PKB inhibitor [1,3-dihydro-1-((1-((4-((6-phenyl-1H-imidazol-4-5-gquinoxalin-7-yl)phenyl)methyl)-4-piperidinyl)-2H-benzimidazol-2-one], referred to as Akt inhibitor-1/2 (Akti-1/2).38,39 Akti-1/2 interacts with the PH domains of PKBα/β, thereby preventing the conformational change required for their phosphorylation and activation by upstream kinases.40 Consistent with this, pretreatment of ARVMs with 1 μmol/L Akti-1/2 inhibited insulin-induced phosphorylation of endogenous PKB at Ser473 and the PKB substrate GSK3β at Ser9 (Figure 8A). In
subsequent experiments, such pretreatment with Akti-1/2 was found not to alter basal pH, and the degree of intracellular acidosis achieved after NH$_4$Cl washout (see supplemental Table II) but to significantly increase sarcolemmal NHE activity (Figure 8B), through an apparent increase in affinity for intracellular H$^+$(Figure 8C).

Taken together, our complementary gain-of-function and loss-of-function data indicate that PKB activity in cardiac myocytes inhibits sarcolemmal NHE activity during intracellular acidosis, most likely through phosphorylation of Ser648 in the NHE1 regulatory domain and inhibition of CaM binding to this domain.

Discussion

The original findings of the present study are that: (1) NHE1 is a novel PKB substrate; (2) the principal PKB-mediated
phosphorylation site in NHE1 is Ser648, which has not been previously identified as a kinase target; (3) PKB-mediated phosphorylation of Ser648 inhibits binding of Ca\(^{2+}\)-activated CaM to the NHE1 regulatory domain in vitro; (4) PKB-mediated phosphorylation of NHE1 in intact ventricular myocytes markedly depresses H\(^+\) extrusion via sarcolemmal NHE activity, in response to intracellular acidosis.

Several protein kinases including ERK,\(^{11}\) RSK,\(^{12}\) p160-ROCK,\(^{14}\) and p38-MAPK\(^{15}\) have been shown to stimulate NHE1 activity through phosphorylation of its carboxyl-terminal regulatory domain. The targeted amino acid residues have been identified for ERK (Ser770 and Ser771),\(^{11}\) RSK (Ser703),\(^{12}\) and p38-MAPK (Thr717, Ser722, Ser725 and Ser728)\(^{15}\) but remain unidentified for p160-ROCK.\(^{14}\) Furthermore, although the functional importance of ERK-mediated phosphorylation of Ser770 and Ser771 and RSK-mediated phosphorylation of Ser703 has been established,\(^{11,12,18}\) that of p38-MAPK–mediated phosphorylation of the pertinent target sites in NHE1 remains unknown. With specific regard to cardiac physiology, recent studies from our laboratory have confirmed the functional importance of RSK-mediated phosphorylation of NHE1 in \(\alpha_\text{1}-\text{adrenoceptor–induced stimulation of sarcolemmal NHE activity, through the use of a novel RSK inhibitor in ARVMs.}^{41}\) Furthermore, heterologous expression of a dominant negative RSK mutant in neonatal rat ventricular myocytes has been shown to inhibit oxidative stress-induced stimulation of sarcolemmal NHE activity.\(^{42}\) In the same study, transgenic expression of the dominant negative RSK mutant in the mouse heart attenuated oxidative stress-induced stimulation of sarcolemmal NHE activity.\(^{42}\) In this context, the present study is the first to identify PKB as an NHE1 kinase, both in vitro and in intact cells, and also the first to identify Ser648 as a phosphorylation site in NHE1. Furthermore, our data suggest that, unlike most kinases that phosphorylate the NHE1 carboxyl-terminal regulatory domain, PKB inhibits NHE1 activity.

Our findings also provide a potential molecular mechanism through which PKB inhibits NHE1 activity. As noted earlier, Ser648, which we have identified as the principal PKB-mediated phosphorylation site in NHE1, resides in the middle of a high-affinity CaM binding region that comprises amino acids 636 to 656.\(^{10}\) Previous evidence indicates that, in the absence of activated CaM, this region of the NHE1 regulatory domain is not accessible to CaM.

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**Figure 7.** PKB\(\alpha\)-mediated phosphorylation and regulation of NHE1 in intact ARVMs. A, Heterologous PKB\(\alpha\) expression in noninfected cells (CTR) and those infected with AdV:β-gal or AdV:myr-PKB\(\alpha\) (300 moi), detected by immunoblot analysis of Crude Lysate IB: HA tag (myr-PKB\(\alpha\) expression) and Crude Lysate IB: PKB. B, Crude Lysate IB: HA tag (NHE1 expression). C, IP: NHE1, IB: phospho-PKB substrate. D, IP: NHE1, IB: HA tag. Figure 7 (Continued). HA-tag and PKB protein expression. All cells were coinfected with AdV:NHE1 (50 moi). B, Equal expression of heterologous NHE1 in the groups described in A, detected by immunoblot analysis of HA-tag expression. C, Phosphorylation of heterologously expressed NHE1, determined by immunoblot analysis with phospho-PKB substrate antibody. Equal immunoprecipitation of heterologously expressed NHE1 protein was confirmed by HA tag immunoblot analysis. Data represent means±SEM (n=4). *P<0.05 vs CTR or AdV:β-gal. D, Sarcolemmal NHE activity, as reflected by the H\(^+\) efflux rate across the sarcolemma (\(J_h\)) during the initial 60 seconds of recovery from intracellular acidosis, in noninfected ARVMs (CTR) and those infected with AdV:β-gal or AdV:myr-PKB\(\alpha\) (300 moi). Data represent means±SEM (n=36 to 57 cells per group). *P<0.05 vs CTR or AdV:β-gal.
domain is unoccupied and exerts an autoinhibitory effect on the NHE1 transport domain; on its Ca\textsuperscript{2+}-induced activation, CaM binds to this region, abolishing the autoinhibitory interaction between the regulatory and transport domains and increasing NHE1 activity.\textsuperscript{43} Our novel data show that PKB-mediated phosphorylation of Ser648 inhibits the binding of Ca\textsuperscript{2+}/H\textsuperscript{+}-activated CaM to the NHE1 regulatory domain (Figure 5). In the intact cell, this mechanism is likely to sustain the autoinhibitory effect of the CaM binding region on NHE1 activity, even in the presence of activated CaM. Notably, we did not see a significant difference in basal pHi between ARVMs with heterologous expression of myr-PKB and control cells that were either uninfected or infected to heterologously express β-galactosidase in our gain-of-function studies or between ARVMs with or without pretreatment with Akti-1/2 in our loss-of-function experiments (see supplemental Table II). A possible explanation for this observation is the fact that sarcoplasmic NHE activity is very low at physiological pHi in unstimulated cells;\textsuperscript{2} in which the CaM-binding site is likely to be unoccupied.\textsuperscript{43} Under such conditions, PKB-mediated phosphorylation of Ser648 would be expected to have little impact on CaM binding to the NHE1 regulatory domain and, thereby, on NHE1-mediated H\textsuperscript{+} extrusion.

In contrast to the above, we observed a markedly reduced sarcoplasmic NHE activity in response to intracellular acidosis in ARVMs with heterologous expression of myr-PKB\textalpha{}, which occurred concomitantly with increased NHE1 phosphorylation (Figure 7). This increased phosphorylation most likely occurred at Ser648, as determined by the incorporation of \textsuperscript{32}P and the phospho-PKB substrate antibody that we used to detect such phosphorylation (Figures 3 and 4). Furthermore, inhibition of endogenous PKB activity in uninfected ARVMs led to a significantly increased sarcoplasmic NHE activity in response to intracellular acidosis (Figure 8).
together with our finding that PKB-mediated phosphorylation of Ser648 inhibits the binding of Ca\(^{2+}\)-activated CaM to the NHE1 regulatory domain (Figure 5), this observation suggests a new potential mechanism for NHE1 stimulation by acute intracellular acidosis, through enhanced CaM binding. In this regard, intracellular acidosis has long been known to increase free intracellular [Ca\(^{2+}\)]\(_i\) in cardiac cells, and has been proposed to activate another CaM target, Ca\(^{2+}\)/CaM-dependent kinase II. Thus, during intracellular acidosis, Ca\(^{2+}\)/CaM-dependent CaM would be expected to bind to the CaM-binding region of the NHE1 regulatory domain, thereby overcoming its autoinhibitory effect and increasing NHE1 activity. On the basis of our findings, PKB-mediated phosphorylation of Ser648 is likely to inhibit such CaM binding, thereby suppressing NHE1 activity in response to intracellular acidosis. Interestingly, inhibition of phosphodiesterase 5A (which hydrolyzes cGMP) has also been shown recently to inhibit sarcolemmal NHE activity during intracellular acidosis without affecting basal pH, suggesting the possibility that cGMP-responsive pathways, such as the cGMP-dependent protein kinase, may regulate NHE1 activity through an analogous mechanism.

The potential contributions of the other putative PKB phosphorylation sites that we detected by mass spectrometry, namely Ser703 and Ser796, need to be considered. Our studies with site-directed mutagenesis revealed that the NHE1 mutant carrying Ser648/796Ala substitutions, in which only Ser703 was available for phosphorylation, was actually a very poor PKB substrate. Thus, phosphorylation of Ser703 is unlikely to have contributed to PKB-mediated inhibition of sarcolemmal NHE activity. This is consistent with previous data suggesting that Ser703 phosphorylation by RSK stimulates (rather than inhibits) NHE1 activity in response to intracellular acidosis. Relative to the Ser648/796Ala mutant, we detected greater PKBα-mediated phosphorylation of the NHE1 mutant carrying Ser648/796Ala substitutions, in which only Ser796 was available for phosphorylation (Figure 4). Li et al. reported that Ser796 is able to reside within a carbonyl anhydride II–binding region of the NHE1 carbonyl-regulatory domain and to be phosphorylated by unidentified kinase(s) present in a heart cell extract. However, Ser796 phosphorylation had little impact on carbonyl anhydride II binding to NHE1 and phosphorylation of the NHE1 regulatory domain at other, more proximal site(s) was proposed to regulate such binding. Thus, the functional significance of Ser796 phosphorylation, by PKB or other kinase(s), in NHE1 regulation remains unclear. It is noteworthy, however, that Ser796 is a considerably poorer substrate than Ser648 for PKB-mediated phosphorylation in vitro, with detectable Ser796 phosphorylation becoming apparent only after more prolonged exposure to active PKBα (Figure 4).

Sarcolemmal NHE activity increases during myocardial ischemia, largely as a consequence of the intracellular acidosis that develops rapidly on its onset, and is believed to contribute significantly to myocardial injury and dysfunction during ischemia and subsequent reperfusion. Consistent with this, NHE1-selective pharmacological inhibitors have been shown to afford marked cardioprotective benefit during ischemia and reperfusion in both animal models and specific clinical settings. Because increased PKB activity also has a marked protective effect during myocardial ischemia and reperfusion, our findings raise the possibility that reduced NHE1 activity may contribute to the pertinent cardioprotective mechanisms. In this regard, myocardial ischemia is associated with intracellular Ca\(^{2+}\) accumulation, with increased sarcolemmal NHE activity causally implicated in the occurrence of this phenomenon. In such a setting, CaM binding may sustain sarcolemmal NHE activity and contribute to a vicious cycle that further exacerbates intracellular Ca\(^{2+}\) accumulation; to the contrary, PKB-mediated NHE1 phosphorylation would be expected to terminate this cycle by disrupting CaM-mediated stimulation of sarcolemmal NHE activity. In view of the novel findings of the present study, further investigation of the role of CaM binding in the regulation of NHE1 activity and the physiological significance of the inhibition of such binding by PKB-mediated NHE1 phosphorylation appears warranted.

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Disclosures

None.

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1: SUPPLEMENTARY MATERIALS AND METHODS

Materials

Antibodies to phospho-PKB substrates, GSK3β, phospho-GSK3β (Ser9), phospho-PKB (Ser473), PKBα and hemagluttinin (HA) tag were from Cell Signaling Technology. Polyclonal NHE1 antibody was from Santa Cruz Biotechnology. Goat anti-rabbit AlexaFluor® 488 and anti-mouse AlexaFluor® 594 secondary antibodies were from Molecular Probes. Active recombinant PKBα (pleckstrin homology domain deletion mutant) was purchased from Upstate Biotechnology. The bacterial expression vectors (pGEX-KG) encoding glutathione-S-transferase (GST)-NHE1 fusion proteins comprising amino acids 516-815, 516-630, 625-747 and 748-815 of human NHE1 were a kind gift from Dr Bradford Berk (University of Rochester, USA),1 the adenovirus expressing myristoylated PKBα (myr-PKBα) from Dr Kenneth Walsh (University of Boston, USA),2 the HA-tagged human NHE1 construct from Dr Larry Fliegel (University of Alberta, CA) and the AdEasy system components from Dr Bert Vogelstein (Johns Hopkins University, USA). The fluoroprobe 2,7′-bis-(carboxyethyl)-5(6′)-carboxyfluorescein (BCECF), in membrane permeant acetoxymethyl ester form (BCECF-AM), was from Calbiochem. All other chemicals were from Sigma-Aldrich or VWR International, unless otherwise stated.

Analysis of NHE1 Phosphorylation by LC/MS/MS

In vitro phosphorylation of a GST-NHE1 fusion protein (100 pmol) comprising residues 516-815 of human NHE1 by 50 ng of active PKBα (PH domain deletion mutant) was performed for 60 min at 30°C. The phosphorylated fusion protein was then resolved by 12% SDS-PAGE and the gel was fixed and stained with Brilliant Blue G colloidal Coomassie (Sigma-Aldrich, UK). In-gel reduction, alkylation and digestion with trypsin were performed prior to analysis by mass spectrometry. Cysteine residues were reduced with dithiothreitol and derivatized by treatment with iodoacetamide to form stable carbamidomethyl derivatives. Trypsin digestion was carried out overnight at room temperature after an initial 60 min incubation at 37°C. Peptides were extracted from the gel pieces by a series of acetonitrile and aqueous washes. The extract was pooled with the initial supernatant and lyophilised. Each sample was then resuspended in 20 µl of 50 mmol/L ammonium bicarbonate and analysed by LC/MS/MS. Chromatographic separations were performed using an Ultimate LC system (Dionex, UK). Peptides were resolved by reversed-phase chromatography on a 75 µm C18 PepMap column. A gradient of acetonitrile in 0.05% formic acid was delivered to elute the peptides at a flow rate of 200 nL/min. Peptides were ionised by electrospray ionisation using a Z-spray source fitted to a QToF-micro (Micromass, UK). The instrument was set to run in automated switching mode, selecting precursor ions based on their intensity, for sequencing by collision-induced fragmentation. The MS/MS analyses were conducted using collision energy profiles that were chosen based on the m/z and the charge state of the peptide and optimized for phosphorylated peptides; a maximum of nine individual MS/MS spectra were combined for each precursor.

Site-Directed Mutagenesis of NHE1

Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit, according to the manufacturer’s instructions (Stratagene). A new GST-NHE1 fusion protein construct was prepared by subcloning cDNA encoding NHE1 residues 625-815 into the expression vector pGEX-3X. A series of mutant fusion constructs in which Ser648, Ser703 and Ser796 of the NHE1 component were substituted by non-phosphorylatable alanine residues, singly and in all possible combinations, were also prepared.

Preparation of Recombinant NHE1 Fusion Proteins

Recombinant GST-NHE1 fusion proteins were prepared as described earlier.7 Briefly, bacterial expression vectors (pGEX-KG or pGEX-3X) encoding aa 516-815, 516-630, 625-747, 748-815 or 625-815) of human NHE1 (the latter with or without the indicated Ser/Ala mutations), N-
terminally linked to GST, were transformed into BL21 strain of *Escherichia coli*. Cultures were grown to sub-log phase and induced with 0.5 mmol/L isopropyl-β-D-thiogalactopyranoside. Cells were harvested and resuspended in PBS containing 1% v/v Triton X-100 and Mini Complete protease inhibitor cocktail (Roche) and the GST-NHE1 fusion proteins purified at 4°C by affinity chromatography using glutathione-sepharose 4B columns (GE Healthcare, UK).

PKB-Mediated Phosphorylation of NHE1 in vitro

*In vitro* kinase assays were performed as previously described.³ GST-NHE1 fusion proteins (100 pmol) were incubated with active PKBα (PH domain deletion mutant) in kinase reaction buffer containing (in mmol/L) Tris-HCl 20, EGTA 0.04, DTT 6, MgCl₂ 15, pH 7.5 at 30°C for 0-60 min (phosphorylation time course) or 30 min (mutant fusion protein phosphorylation) in the presence of ATP (100 µmol/L), with or without [γ³²P]-ATP. The reaction was terminated by the addition of Laemmli sample buffer. Proteins were separated by 12% SDS-PAGE and phosphorylation assessed by autoradiography (³²P) or western immunoblotting.

Far-Western Analysis of Calmodulin Binding to NHE1

Far-western calmodulin (CaM) binding assays were performed by resolving WT and mutant GST-NHE1(625-815) fusion protein samples (100 pmol) by 10.5% SDS-PAGE and transferring them to polyvinylidene fluoride membranes. Membranes were then incubated with Tris-buffered saline (TBS) supplemented with 1% Tween 20 (TBST) and 10% non-fat milk powder (MTBST), in the absence (+1 mmol/L EGTA) or presence of CaCl₂ (100 µmol/L), overnight at 4°C. Membranes were briefly washed with TBST in the absence or presence of CaCl₂ and then incubated with 0.25 µg/mL biotinylated CaM (Alexis Biochemicals, UK) for 1 h at room temperature (RT). Membranes were then washed with TBST in the absence or presence of CaCl₂ for 1 h at RT before incubation with 1:5000 streptavidin-HRP (GE Healthcare, UK) for 30 min at RT. After washing for 1.5 h with TBST in the absence or presence of CaCl₂, specific protein bands were detected by enhanced chemiluminescence (GE Healthcare, UK). The far-western CaM binding assay was then repeated in the presence of CaCl₂ using WT and mutant GST-NHE1(625-815) fusion protein samples (100 pmol) that had been phosphorylated *in vitro* by PKBα for 30 min at 30°C. Equal protein loading was determined by Coomassie staining of membranes.

Isolation, Culture and Adenoviral Infection of Cardiac Myocytes

Adult rat ventricular myocytes (ARVM) were isolated and maintained in culture for 18-24 h, with or without adenoviral infection shortly after isolation, as described previously.³² Cells to be used for biochemical experiments were maintained in pre-laminated 6-well plastic culture dishes in modified M199 medium (Invitrogen), containing (in mmol/L) creatine 2, carnitine 2 and taurine 5. Cells to be used for pH imaging were plated onto pre-laminated glass coverslips and maintained in an identical medium, in 12-well plastic culture dishes.

Where indicated, adenoviral infection was performed as previously described,⁴ using a vector encoding the C-terminally HA-tagged wild-type human NHE1 (AdV:hNHE1),⁵ β-galactosidase (AdV:β-gal) or myr-PKBα (AdV:myr-PKBα). The recombinant adenoviruses were amplified in HEK-293 cells and purified over CsCl gradients, which produced high-titre viral stocks as determined by serial end-point dilution assay.⁶ After initial plating, ARVM were infected with AdV:hNHE1 (50 MOI), AdV:β-gal (300 MOI) or AdV:myr-PKBα (300 MOI) where appropriate for 60 min and the adenovirus-containing medium was then removed and replaced with fresh modified M199 medium. Infected myocytes were maintained (18-24 h) in an incubator (37 °C, 5% CO₂) until used for experiments.

Analysis of NHE1 Phosphorylation in ARVM

Phosphorylation status of NHE1 was determined using an adaptation of a method that we have described recently.⁷ All ARVM underwent primary infection with AdV:hNHE1 (50 MOI) for 60 min. ARVM were then subdivided into non-infected (no secondary infection) or AdV:β-gal or AdV:myr-PKBα infected groups that underwent secondary infection (300 MOI) for 60 min. After 18-24 h in culture, ARVM were washed with ice-cold PBS and lysed in lysis buffer at pH 7.5 containing (in mmol/L) Tris-HCl 50, EGTA 5, EDTA 2, NaF 100 and Na₃VO₄ 1, as well as 0.05% digitonin and Mini-Complete protease inhibitor cocktail (Roche). The samples were then rapidly frozen by floating the culture plate on a volume of liquid N₂ and thawed at room temperature. Cell lysates were centrifuged at 14000 g for 30 min at 4°C and the supernatant discarded. The pellet was
then solubilized in ice-cold immunoprecipitation lysis buffer at pH 7.5 containing (in mmol/L) Tris-HCl 20, NaCl 150, EDTA 1, EGTA 1, sodium pyrophosphate 2.5, β-glycerophosphate 1, Na3VO4 1 and NaF 100, as well as 1.5 % Triton X-100, 0.1 % SDS and Mini-Complete protease inhibitor cocktail (Roche). The samples were centrifuged at 14000 g for 60 min at 4°C, after which the supernatant containing the solubilized membranes was removed and incubated overnight at 4°C with polyclonal NHE1 antibody. Immune complexes were mixed with protein A magnetic beads (New England Biolabs) for 2 h at 4°C, washed 3 times with ice-cold modified immunoprecipitation lysis buffer (not containing SDS), and separated using a magnetic separation rack (New England Biolabs). The immune complexes were dissociated by the addition of Laemmli sample buffer and heated for 5 min at 75°C. Proteins were resolved on 7.5% SDS-PAGE and analysed by western immunoblotting using rabbit polyclonal phospho-PKB substrate antibody.

**Immunocytochemistry and Confocal Studies**

Immunocytochemistry and confocal microscopy techniques were used to visualize protein localization in ARVM, as described previously. Briefly, ARVM were cultured onto laminin-coated glass coverslips and infected with AdV:myr-PKBα (300 MOI for 60 min). After 18-24 h in culture, HA-tag (myr-PKBα) expression was detected using a mouse anti HA-tag antibody followed by an anti-mouse AlexaFluor® 594 secondary antibody (Molecular Probes). NHE1 protein expression was detected as previously described. Z-stack images (1 μm thick) were acquired on a Zeiss Axiocvert 100M laser scanning confocal microscope equipped with a Plan-NEOFLUAR® x40/1.3 NA objective, using Argon and Helium/Neon lasers to excite AlexaFluor® 488 and 594 at 488 and 543 nm, respectively. Emission from paraformaldehyde-fixed ARVM labelled with AlexaFluor® 488 and 594 were detected at 519 and 617 nm, respectively and processed using Zeiss LSM 510 (v 2.1) software.

**Western Immunoblotting**

Western immunoblotting was carried out as previously described. In brief, protein samples were separated by 7.5-12% SDS-PAGE, transferred to polyvinylidenedifluoride (PVDF) or nitrocellulose membranes and probed with appropriate primary antibodies. Primary antibodies were detected by horseradish peroxidase (HRP)-linked donkey anti-rabbit or sheep anti-mouse secondary antibodies, as appropriate (GE Healthcare, UK). Specific protein bands were detected by enhanced chemiluminescence (GE Healthcare, UK) and phosphorylation status was quantified using a calibrated densitometer (GS-800, Bio-Rad) and Quantity One® software (v 4.5.1).

**Effects of Akt-1/2 on Insulin-Induced PKB Activation**

Cultured ARVM were pre-treated with either vehicle (0.01% DMSO) or PKB inhibitor (Akti-1/2; 1 μmol/L; Calbiochem) for 15 min, and then exposed to either vehicle (PBS) or 1 μU/mL human recombinant insulin (Actrapid; Novo Nordisk) for 3 min (37°C, 5 CO2). Subsequently, ARVM were lysed in Laemmli sample buffer and cellular proteins separated by 9% SDS-PAGE and analysed by western immunoblotting, as described above, using rabbit polyclonal and mouse monoclonal antibodies raised against phospho-PKB (Ser473), phospho-GSK3β (Ser9), GSK3β and PKBα.

**Measurement of Intracellular pH in ARVM**

Intracellular pH (pH$_i$) was measured in quiescent ARVM, as previously described, using the dual excitation/single emission pH-sensitive fluoroprobe BCECF, in conjunction with an imaging system (IonOptix, Milton, MA). Briefly, ARVM were cultured onto laminin-coated glass coverslips and then split into three groups that were uninfected or infected (300 MOI for 60 min) with either AdV:β-gal or AdV:myr-PKBα. For pH$_i$ determination, after 18-24 h in culture, coverslips were placed in a cell chamber (Model RC-25F, Warner Instrument Inc) on the stage of an inverted microepifluorescence microscope (Nikon Eclipse TE300, equipped with a Nikon Plan Fluor 10x/0.3NA objective), loaded with BCECF-AM (2 μmol/L, 10 min) and superfused (3 mL/min) with HCO3-free Tyrode solution at 34°C and pH 7.4 containing (in mmol/L) HEPES 10, NaCl 137, KCl 5.4, MgCl2 0.5 and CaCl2 1.0. Cells were excited with light at 440 nm and 495 nm using a HyperSwitch Light Source (IonOptix), with the BCECF fluorescence emission acquired from multiple cells simultaneously at 535 nm using a Dage MTI CCD integrating video camera and IonWizard v4.4 software (IonOptix). Calibration of the fluorescence signal was carried out, as previously described, using modified calibration solutions at pH 5.8-8.0 containing (in mmol/L)
KCl 140, EGTA 2, MES, PIPES or HEPES 10, MgSO₄ 1.2, FCCP 0.001, valinomycin 0.001, nigericin 0.01 and glucose 10. Cells were exposed transiently (3 min) to 20 mmol/L NH₄Cl to induce intracellular acidosis and the rate of change in pHᵢ (d pHᵢ/dt) was measured during the initial phase (60 s) of or throughout subsequent recovery from intracellular acidosis.

**Determination of Intrinsic Buffering Capacity and Sarcolemmal NHE Activity in ARVM**

The cellular intrinsic buffering capacity (βᵢ) was determined in uninfected (CTR) ARVM and ARVM infected with AdV:β-gal or AdV:myr-PKBα (300 MOI for 60 min), as previously described. In brief, pHᵢ was determined after 18-24 h in culture, as described above, in ARVM sequentially exposed to Tyrode solutions containing 10 μmol/L cariporide, 5 mmol/L BaCl₂ and 20, 10, 5, 2.5, 1 or 0 mmol/L NH₄Cl. βᵢ was calculated as Δ[NH₄Clᵢ]/ΔpHᵢ, sorted into pHᵢ bins 6.40-6.59 (n=16-41 in each group), 6.60-6.79 (n=33-55), 6.80-6.99 (n=45-59), 7.00-7.19 (n=32-40) and 7.20-7.39 (n=23-37), and average βᵢ and pHᵢ values within these bins plotted and subjected to linear least-squares regression (see Online Figure IV). The appropriate equation was then used to estimate βᵢ at various pHᵢ during recovery from intracellular acidosis in each cell. The trans-sarcolemmal H⁺ efflux rate (JᵥH) was calculated as the product of the βᵢ and d pHᵢ/dt, as previously described, and used as the index of sarcolemmal NHE activity.

**Determination of Cell Size**

The dimensions of uninfected (CTR) ARVM and ARVM infected with AdV:β-gal or AdV:myr-PKBα were determined optically. ARVM were viewed with an IonOptix MyoCam™ on the stage of an inverted microepifluorescence microscope (Nikon Eclipse TE300, equipped with a Nikon Plan Fluor 40x/0.6NA objective). Images were displayed and measured on a video screen that had been optically calibrated and the maximum cell length and maximum cell width were determined in a blinded and randomised manner (n=45-60 cells/group). ARVM cell volume (pL) was estimated according to Satoh et al, from the cell area (length x width). The measured cell dimensions and estimated volumes are given in Online Table I.

**Statistical Analysis**

Quantitative data are mean ± SEM. Student’s t test was used to compare two groups. Otherwise, data were subjected to ANOVA, with further analysis by Student-Newman-Keuls test (for multiple comparisons).

**References**


2: ONLINE SUPPLEMENTARY DATA

Online Figure I: LC/MS/MS coverage of NHE1 protein
Peptide fragment coverage (in bold) of the NHE1 domain of PKBα-phosphorylated GST-NHE1(516-815) by mass spectrometry. Putative PKB phosphorylation sites (Ser648, Ser703 and Ser796) are underlined.

516 RSINEEIHTQ FLDHLLTGIE DICGHYGHHH WKDKLNRFNK KYVK KCLIAG 565
566 ERSKEPQLIA FYHKMEMKQA IELVESGGMG KIPSAVSTVS MQNIHPKSLP 615
616 SERILPALK DKEEEIRKIL RNNLQKTRQR LRSYNRHTLV ADPYEEAWNQ 665
666 MLLRQKARQ LEQKINNYLT VPAHKLDSPT MSRARIGSDP LAYEPKEDLP 715
716 VITIDPASPM SPESVDVLLVE ELKGVVELLS RDPKVAEED EDDGGIMMR 765
766 SKETSSPGTD DVFTPAPSIDS PSSRQLQORL SDPGPHPEPG EGEPPFPKQ 815

Online Figure II: LC/MS/MS identification of putative PKB phosphorylation sites

MS/MS Fragmentation spectrum of peptide fragment: 701 IGSDPLAYEPK 711
Monoisotopic mass of neutral peptide (Mr): 1268.57

m/z[635.32]

MS/MS Fragmentation spectrum of peptide fragment: 794 CLSDPGPHPEPGEGEPFK 812
Monoisotopic mass of neutral peptide (Mr): 2272.95

m/z[758.68]
Online Figure III: AdV:myr-PKBα infection efficiency

myr-PKBα  

Online Figure IV: Intrinsic buffering capacity (βᵢ)

CTR  

\[ y = -24.175x + 193.560 \]

AdV:β-gal  

\[ y = -41.390x + 306.646 \]

AdV:myr-PKBα  

\[ y = -40.292x + 296.560 \]

Online Table I. Measured cell dimensions and estimated cell volumes

<table>
<thead>
<tr>
<th></th>
<th>Cell length (µm)</th>
<th>Cell width (µm)</th>
<th>Cell volume (pL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTR (n=45)</td>
<td>130.0 ± 2.4</td>
<td>28.2 ± 0.8</td>
<td>27.8 ± 1.0</td>
</tr>
<tr>
<td>AdV:β-gal (n=45)</td>
<td>126.8 ± 2.4</td>
<td>27.9 ± 0.8</td>
<td>26.8 ± 0.9</td>
</tr>
<tr>
<td>AdV:myr-PKBα (n=60)</td>
<td>127.8 ± 1.9</td>
<td>27.5 ± 0.5</td>
<td>26.6 ± 0.5</td>
</tr>
</tbody>
</table>
Online Table II. Mean basal pH\(_i\) (measured prior to NH\(_4\)Cl exposure) and minimal pH\(_i\) (measured immediately after NH\(_4\)Cl washout) in the study groups

<table>
<thead>
<tr>
<th>Study</th>
<th>Basal pH(_i)</th>
<th>Minimal pH(_i)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gain-of-function study</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR (n=36)</td>
<td>7.14 ± 0.02</td>
<td>6.39 ± 0.03</td>
</tr>
<tr>
<td>AdV:β-gal (n=38)</td>
<td>7.16 ± 0.02</td>
<td>6.42 ± 0.04</td>
</tr>
<tr>
<td>AdV:myr-PKBα (n=57)</td>
<td>7.19 ± 0.01</td>
<td>6.41 ± 0.02</td>
</tr>
<tr>
<td><strong>Loss-of-function study</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CTR (n=39)</td>
<td>7.18 ± 0.02</td>
<td>6.62 ± 0.02</td>
</tr>
<tr>
<td>Akti-1/2 (n=31)</td>
<td>7.14 ± 0.02</td>
<td>6.56 ± 0.03</td>
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</tbody>
</table>