G_{q} protein–coupled receptor stimulation increases sarcolemmal Na\(^+\)/H\(^+\) exchanger (NHE1) activity in cardiac myocytes by an ERK/RSK-dependent mechanism, most likely via RSK-mediated phosphorylation of the NHE1 regulatory domain. Adenosine A\(_{1}\) receptor stimulation inhibits this response through a G\(_{i}\) protein-mediated pathway, but the distal inhibitory signaling mechanisms are unknown. In cultured adult rat ventricular myocytes (ARVM), the A\(_{1}\) receptor agonist cyclopentyladenosine (CPA) inhibited the increase in NHE1 phosphorylation induced by the \(\alpha_{1}\)-adrenoceptor agonist phenylephrine, without affecting activation of the ERK/RSK pathway. CPA also induced significant accumulation of the catalytic subunit of type 2A protein phosphatase (PP2A\(_{C}\)) in the particulate fraction, which contained the cellular NHE1 complement; this effect was abolished by pretreatment with pertussis toxin to inactivate G\(_{i}\) proteins. Confocal immunofluorescence microscopic imaging of CPA-treated ARVM revealed significant co-localization of PP2A\(_{C}\) and NHE1, in intercalated disc regions. In an in vitro assay, purified PP2A\(_{C}\) dephosphorylated a GST-NHE1 fusion protein containing aa 625–747 of the NHE1 regulatory domain, which had been pre-phosphorylated by recombinant RSK; such dephosphorylation was inhibited by the PP2A–selective phosphatase inhibitor endothall. In intact ARVM, the ability of CPA to attenuate the phenylephrine-induced increase in NHE1 phosphorylation and activity was lost in the presence of endothall. These studies reveal a novel role for the PP2A holoenzyme in adenosine A\(_{1}\) receptor-mediated regulation of NHE1 activity in ARVM, the mechanism of which appears to involve G\(_{i}\) protein-mediated translocation of PP2A\(_{C}\) and NHE1 dephosphorylation.

The cardiac sarcolemmal Na\(^+\)/H\(^+\) exchanger (NHE)\(^2\) is a membrane glycoprotein encoded by the NHE1/SLC9A1 gene (1), which is one of nine known members of the solute carrier 9 (SLC9) gene family (2). The sarcolemmal NHE contributes significantly to the control of intracellular pH (pHi) in cardiac myocytes, particularly in response to intracellular acidosis (3). Although basal activity of the sarcolemmal NHE is low under physiological conditions (3), increased exchange activity and resultant increases in intracellular [Na\(^+\)] and/or pH may mediate inotropic responses to neurohormonal stimuli, such as endothelin 1 (4), angiotensin II (5), and \(\alpha_{1}\)-adrenoceptor (\(\alpha_{1}\)-AR) agonists (6). Increased sarcolemmal NHE activity as a result of the release and autocrine/paracrine actions of angiotensin II and endothelin 1 (7) or by an independent mechanism (8), may also underlie the slow increase in force development that occurs in response to myocardial stretch.

With respect to cardiac pathophysiology, there is strong evidence that sarcolemmal NHE activity contributes to myocardial injury and dysfunction during ischemia and reperfusion. Thus, NHE1-selective pharmacological inhibitors, such as cariporide, have been shown to be cardioprotective in this setting in numerous animal studies (see reviews by Avkiran (9) and Karmazyn et al. (10)). Clinical trial data suggest that NHE inhibitors also protect human myocardium in appropriate settings (11), such as in high risk patients who undergo global myocardial ischemia and reperfusion during coronary artery bypass graft surgery (12). Furthermore, sarcolemmal NHE activity may play a causal or permissive role in myocardial hypertrophy and remodeling, as evidenced by studies in animal models of myocardial infarction (13), pressure overload (14), \(\beta\)-adrenergic hyperactivity (15), and rapid ventricular pacing (16), in which adverse changes in cardiac morphology and function have been attenuated by NHE1-selective inhibitors. Despite the apparent potential of the sarcolemmal NHE as a therapeutic target; however, in the recent EXPEDITION trial (17), the significant cardioprotection afforded by cariporide treatment was tempered by serious non-cardiac adverse effects. Until the mechanisms of the adverse effects are delineated, the therapeutic application of agents that directly and globally inhibit the ubiquitously expressed NHE1 protein must remain in abeyance. Nevertheless, better understanding of the molecular mechanisms that regulate the sarcolemmal NHE may pave the way for therapeutic manipulation of exchanger activity in a cardiac- or disease-specific manner.

Previous work in our laboratory (18–21) and by others (22–24) has revealed that activation of the extracellular signal-regulated kinase (ERK) pathway is necessary for the increase in sarcolemmal NHE activity induced by diverse stimuli, includ-
using α1-AR agonists (19). Such stimulation of sarcolemmal NHE activity is thought to occur, at least in part, through activation of the 90 kDa ribosomal S6 kinase (p90RSK or RSK) (19), which lies immediately downstream of ERK (25). Indeed, previous work has shown RSK to increase NHE1 activity in fibroblasts through direct phosphorylation of Ser703 in the exchanger regulatory C-terminal domain (26). Interestingly, in adult rat ventricular myocytes (ARVM), stimulation of adenosine A1 receptors inhibits the increase in sarcolemmal NHE activity induced via Gαi protein-coupled receptors, such as α1-ARs (27). Although this inhibitory effect has been shown to occur through a Gαi protein-mediated pathway (27), the distal signaling mechanism(s) have remained unclear. Therefore, the principal objective of the present study was to identify the pertinent signaling mechanism(s).

EXPERIMENTAL PROCEDURES

This investigation was performed in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act 1986, published by Her Majesty’s Stationery Office, London.

Materials—Antibodies were from the following sources: phospho–Ser 14-3-3 binding motif (polyclonal and monoclonal), phospho-ERK1/2 and phospho-RSK, Cell Signaling Technology; NHE1 (polyclonal), PPIα, RSK (agarose-conjugated) and glutathione S-transferase (GST), Santa Cruz Biotechnology; NHE1 (monoclonal), Chemicon; PP2Ac, Upstate Biotechnology; phospho-Ser16 phospholamban, Badrilla; anti-rabbit AlexaFluor® 488 and anti-mouse AlexaFluor® 594, Molecular Probes. Bacterial expression vector pGEX-KG encoding amino acids 625–747 of human NHE1 linked to glutathione S-transferase (GST) was transformed into the BL21 strain of Escherichia coli. Cells were grown to sublog phase and induced with 0.5 mmol/liter isopropyl-β-D-thiogalactopyranoside. Cells were harvested and resuspended in PBS containing 1% v/v Triton X-100 and the fractionated proteins resolved by 12% SDS-PAGE followed by Western immunoblotting.

Preparation of Recombinant NHE1 Fusion Protein—Recombinant GST–NHE1 fusion protein was prepared as described earlier (31). Briefly, the bacterial expression vector pGEX-KG encoding amino acids 625–747 of human NHE1 linked to glutathione S-transferase (GST) was transformed into the BL21 strain of Escherichia coli. Cultures were grown to sublog phase and induced with 0.5 mmol/liter isopropyl-β-D-thiogalactopyranoside. Cells were harvested and resuspended in PBS containing 1% v/v Triton X-100 and the GST–NHE1 fusion protein purified at 4 °C by affinity chromatography using glutathione–Sepharose 4B columns (Amersham Biosciences).

ARVM Subfractionation—ARVM were subfractionated using a previously described protocol (29), with minor modifications. In brief, ARVM were lysed in ice-cold lysis buffer at pH 7.5 containing (in mmol/liter) Tris–HCl 50, EGTA 5, EDTA 2, DTT 5, as well as 0.05% digitonin and protease inhibitor mixture (Roche, Germany). The samples were then frozen by floating the culture plate on a volume of liquid N2 and thawed at room temperature. Cell lysates were then centrifuged at 14,000 × g for 30 min at 4 °C, and the supernatant, which comprised the cytosolic fraction, was removed. The pellet, which comprised the particulate fraction, was then solubilized in an equal volume of the digitonin-based lysis buffer containing 1% Triton X-100. For PP2A determination, demethylation was performed by adding an equal volume of 0.2 mol/liter NaOH to each cytosolic and particulate fraction, incubation for 30 min at 30 °C, and subsequent pH neutralization by the addition of 1 mol/liter HCl, as previously described (30). Equal volumes of Laemmli sample buffer were added to both fractions, and the fractionated proteins resolved by 12% SDS-PAGE followed by Western immunoblotting.

PP2A Regulation of the Cardiac Na+/H+ Exchanger

ARVM Activity in ARVM—ARVM were lysed by the addition of ice-cold lysis buffer at pH 7.6 containing (in mmol/liter) HEPES 10, NaCl 50, NaF 50, sodium pyrophosphate 50, β-glycerolphosphate 50, EDTA 5, EGTA 5, Na3VO4 2, and AEBSF 1, as well as 0.1% Triton X-100, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. ARVM were scraped off the culture plate on ice and centrifuged at 14,000 × g for 30 min at 4 °C. The lysates were incubated with 10 μg of RSK antibody, purchased as an agarose conjugate (Santa Cruz Biotechnology), for 2 h at 4 °C. The beads were washed twice with lysis buffer, twice with ice-cold wash buffer at pH 7.6 containing (in mmol/liter) Tris–HCl 100, LiCl 500, and DTT 1, as well as 0.1% Triton X-100, and twice with ice-cold wash buffer at pH 7.2 containing (in mmol/liter) HEPES 20, EGTA 2, MgCl2 10, DTT 1, as well as 0.1% Triton X-100. The immune complex was then incubated with 50 μl of reaction mixture containing (in mmol/liter) MgCl2 10, MnCl2 10, unlabeled ATP 0.1, as well as 0.1 μCi of [32P]ATP and 100 pmol of GST–NHE1 fusion protein. This reaction mixture was made up in Tris assay dilution buffer (TADB) at pH 7.5, containing (in mmol/liter) Tris–HCl 50, EGTA 0.1, and DTT 15. The reaction was allowed to proceed for 20 min at 30 °C and terminated by the addition of Laemmli sample buffer. Proteins were resolved by 12% SDS-PAGE and the gels dried by vacuum using Electrophoresis RapiDry (ATTO Corporation, Japan) and subjected to autoradiography.
PP2A Regulation of the Cardiac Na\(^+\)/H\(^+\) Exchanger

RSK2-mediated Phosphorylation of NHE1 in Vitro—The reaction mixture comprised recombinant active RSK2 (100 ng) and GST-NHE1 fusion protein (1 mmol) in TAD buffer (containing 15 mmol/liter MgCl\(_2\)), as well as unlabeled ATP (100 μmol/liter) with or without added [\(^{32}\)P]ATP (1 μCi), and was incubated at 30 °C. Aliquots (50 μl) were removed at 0, 1, 2, 5, 10, 20, and 30 min after the start of the reaction. The reaction was terminated by the addition of these aliquots to an equal volume of Laemmli sample buffer. Proteins were subsequently resolved by 12% SDS-PAGE and analyzed by Western immunoblotting or autoradiography. In the latter case, equal protein loading was determined by staining the gel overnight with colloidal Coomassie stain (containing 1.6% orthophosphoric acid, 0.08% Coomassie Brilliant Blue G250, 8% ammonium sulfate, and 20% methanol); the gel was then destained with 20% methanol and a digital image obtained, prior to drying and autoradiography.

PP2A-mediated Dephosphorylation of NHE1 in Vitro—The GST-NHE1 fusion protein was phosphorylated by recombinant RSK2 as described above, for 30 min at 30 °C. The reaction mixture was then incubated with Ro-318220 (10 μmol/liter) for 10 min at 30 °C, to inhibit RSK2 activity and further GST-NHE1 phosphorylation (31). Subsequently, aliquots of the phosphorylated GST-NHE1 fusion protein were incubated with purified PP2A\(_{\alpha}\) (0.003–0.3 units), in the absence or presence of okadaic acid (10–1000 nmol/liter) or endothall (1–100 μmol/liter), for 60 min at 30 °C. The reaction was terminated by the addition of Laemmli sample buffer, and proteins subsequently resolved by 12% SDS-PAGE and analyzed by Western immunoblotting or autoradiography.

Immunocytochemistry and Confocal Microscopic Imaging—ARVM were cultured on sterile laminin-coated glass coverslips (Warner Instruments Inc.) and exposed to CPA (10 μmol/liter) or vehicle (0.1% Me\(_2\)SO) for 5 min. The following procedures were then performed at room temperature, with all solutions filtered through a 0.2-μm filter unit prior to use. ARVM were washed with PBS and fixed with 4% paraformaldehyde/PBS for 10 min, after which they were washed twice with PBS for 10 min and permabilized with 0.1% Triton X-100/PBS for 20 min. ARVM were then washed twice with PBS for 10 min and incubated with 1% bovine serum albumin/PBS blocking buffer for 60 min. Following removal of blocking buffer, ARVM were incubated with 1% BSA/PBS containing both rabbit NHE1 antibody (1:50) and mouse PP2Ac antibody (1:200) for 60 min. ARVM were then washed twice with PBS for 10 min and incubated in the dark with the secondary antibodies (1:100) Alexa Fluor® 488 (goat anti-rabbit) and Alexa Fluor® 594 (goat antimouse) to detect cellular NHE1 and PP2A\(_{\alpha}\), respectively. ARVM were then washed twice with PBS for 10 min in the dark followed by a brief wash with ddH\(_2\)O and the coverslips mounted on slides with fluorescent mounting medium (Dako-Cytomation). The slides were viewed on an inverted laser scanning microscope (LSM510, Carl Zeiss Inc) equipped with a 40×/1.3NA Plan-Neofluar® oil immersion objective lens (Carl Zeiss, Inc.), and Z-stack images of 1-μm thick were acquired and processed using LSM510 software (v2.01).

Immunoprecipitation of NHE1—ARVMwere washed with ice-cold PBS and lysed in lysis buffer at pH 7.5 containing (in mmol/liter) Tris-HCl 50, EGTA 5, EDTA 2, NaF 100, and Na\(_3\)VO\(_4\) 1, as well as 0.05% digitonin and protease inhibitor mixture (Roche Applied Science). The samples were then frozen by floating the culture plate on a volume of liquid N\(_2\) and thawed at room temperature. Cells lysates were then centrifuged at 14,000 × 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/liter) Tris-HCl 20, NaCl 150, EDTA 1, EGTA 1, sodium pyrophosphate 2.5, β-glycerophosphate 1, Na\(_3\)VO\(_4\) 1, and NaF 100, as well as 1% Triton X-100 and protease inhibitor mixture (Roche). The samples were centrifuged at 14,000 × g for 60 min at 4 °C, after which the supernatant containing the solubilized membranes was removed and incubated overnight at 4 °C with rabbit polyclonal NHE1 antibody or phospho-Ser 14-3-3 protein binding motif antibody. Immune complexes were mixed with protein A magnetic beads (New England Biolabs) for 2 h at 4 °C, washed three times with ice-cold immunoprecipitation lysis buffer, and separated using a magnetic separation rack (New England Biolabs). The immune complexes were dissociated by the addition of Laemmli sample buffer and heating for 5 min at 70 °C. Proteins were resolved on 10.5% SDS-PAGE analyzed by Western immunoblotting using mouse monoclonal NHE1 antibody or phospho-Ser 14-3-3 protein binding motif antibody.

Determination of Sarcolemmal NHE Activity—Sarcolemmal NHE activity was measured, as previously described (19, 27, 32), in quiescent ARVM loaded with the pH-sensitive fluoroprobe carboxySNARF-1. Briefly, ARVM cultured on laminin-coated glass coverslips were placed in a cell chamber (Model RC-25F, Warner Instrument Inc.) on the stage of an inverted microepifluorescence microscope (Nikon) and superfused (3 ml/min) with HCO\(_3\)\(^-\)-free Tyrode solution at pH 7.4 containing (in mmol/liter) HEPES 10, NaCl 137, KCl 5.4, MgCl\(_2\) 0.5, and CaCl\(_2\) 1.0. Cells were excited with light at 540 nm and carboxySNARF-1 fluorescence emission monitored simultaneously at 580 and 640 nm using a Dual Emission Microscope Photometer equipped with photon-counting photomultiplier tubes (Photon Technology International) (19, 27, 32). Calibration of the fluorescence signal was carried out, as previously described (19, 27, 32), using calibration solutions at pH 5.8 – 8.0 containing (in mmol/liter) KCl 140, EGTA 2, MES, PIPES, or HEPES 10, MgSO\(_4\) 1.2, FCCP 0.001, valinomycin 0.001, glucose 10, and nigericin 0.01. The cells were treated with either CPA (10 μmol/liter) or vehicle (0.1% Me\(_2\)SO) for 5 min, prior to treatment with phenylephrine (10 μmol/liter) or vehicle (PBS) during a transient (3 min) exposure to 20 mmol/liter NH\(_4\)Cl to induce intracellular acidosis. The rate of acid efflux (ΔH\(_{\text{efflux}}\)) at a pH\(_i\) of 6.90 (ΔH\(_{\text{efflux}}\)), measured during subsequent recovery from intracellular acidosis, was used as the index of sarcolemmal NHE activity (19, 27, 32). This series of experiments was carried out in a randomized manner in two separate subsets, in the absence or presence of endothall (100 μmol/liter).

Statistical Analysis—Data are mean ± S.E. Student’s t test was used to compare two groups. Otherwise, data were subjected to ANOVA, with further analysis by Dunnett’s test (for comparison of each group with a single control) or Student-Newman-Keuls test (for multiple comparisons).

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RESULTS

Receptor-mediated Regulation of NHE1 Phosphorylation in ARVM—We initially aimed to determine the effects of α₁-AR stimulation, in the absence or presence of adenosine A₁ receptor stimulation, on the phosphorylation status of native NHE1 protein in ARVM. The regulatory Ser⁷⁰³ phosphorylation site in NHE1 that is targeted by RSK lies within the sequence Arg-Ile-Gly-Ser-Asp-Pro (26), which, upon phosphorylation, creates a binding motif for 14-3-3 proteins (33). We therefore speculated that a phospho-Ser 14-3-3 binding motif antibody, which recognizes a motif comprised of phospho-Ser with Pro at the +2 position and Arg at the −3 position, might be useful to detect RSK-phosphorylated NHE1. To test this, we phosphorylated in vitro a GST-NHE1 fusion protein containing amino acids 625–747 of the human NHE1 with recombinant RSK2, and tested whether the phosphorylated protein is recognized by the phospho-Ser 14-3-3 binding motif antibody by immunoblot analysis. As shown in Fig. 1A, the antibody detected a band migrating at ~40 kDa, the molecular mass of the GST-NHE1 fusion protein. There was a progressive increase in the detected signal with increasing duration of the phosphorylation reaction, which reflected a similar progressive increase in GST-NHE1 phosphorylation as detected by autoradiography in parallel radioactive kinase assays utilizing ³²P-labeled ATP (Fig. 1A). Notably, RSK-mediated phosphorylation was not seen by either method when GST was used as substrate (data not shown), confirming that the phosphorylation signal originated within the NHE1 domain of the fusion protein. These data indicated that the phospho-Ser 14-3-3 binding motif antibody might indeed be a useful tool for detecting RSK-mediated NHE1 phosphorylation.

We then exposed intact ARVM to phenylephrine in the absence or presence of the adenosine A₁ receptor agonist CPA and aimed to detect changes in NHE1 phosphorylation, using two complementary protocols. In the first protocol, cellular protein was subjected to immunoprecipitation with NHE1 antibody and subsequent immunoblot analysis with phospho-Ser 14-3-3 binding motif antibody; in the second protocol, a reciprocal approach was used. A protein band migrating at ~100 kDa, the expected molecular mass of the mature NHE1 protein, was detected by both approaches. As illustrated in Fig. 1B, with both protocols, the intensity of precipitation with NHE1 antibody and subsequent immunoblot analysis with phospho-Ser 14-3-3 binding motif antibody; in the second protocol, a reciprocal approach was used. A protein band migrating at ~100 kDa, the expected molecular mass of the mature NHE1 protein, was detected by both approaches. As illustrated in Fig. 1B, with both protocols, the intensity of

![Figure 1A](image1.png)

![Figure 1B](image2.png)
this band was increased in ARVM exposed to phenylephrine, but this increase was abolished by treatment with CPA. These data indicate that α1-AR stimulation induces NHE1 phosphorylation (at site(s) likely to include Ser703) and that this increase in phosphorylation is attenuated by stimulation of adenosine A1 receptors. This led us to hypothesize that adenosine A1 receptor stimulation may inhibit α1-AR-mediated activation of NHE1 kinase(s), such as RSK, which catalyze NHE1 phosphorylation in response to phenylephrine.

Receptor-mediated Regulation of ERK and RSK Activity in ARVM—To test the above hypothesis, we studied the impact of adenosine A1 receptor stimulation on ERK/RSK phosphorylation in ARVM. As shown in Fig. 2A; however, CPA had no effect on the marked increase in ERK1/2 and RSK phosphorylation that arises in response to phenylephrine. Furthermore, CPA treatment did not affect a phenylephrine-induced increase in the catalytic activity of the N-terminal kinase domain of RSK, as reflected by the ability of RSK immunoprecipitated from ARVM to phosphorylate in vitro the GST-NHE1 fusion protein (Fig. 2B). Taken together with the evidence presented above that α1-AR-mediated NHE1 phosphorylation is abolished by A1 receptor stimulation (Fig. 1B), these findings suggest the possible existence of two mechanisms, which are not mutually exclusive. Firstly, RSK-independent mechanisms may contribute to NHE1 phosphorylation in response to α1-AR stimulation and these unknown mechanisms may be subject to inhibition via adenosine A1 receptors. Secondly, adenosine A1 receptor stimulation may up-regulate mechanisms that dephosphorylate the pertinent regulatory phosphorylation sites in NHE1, thus counteracting the effects of α1-AR-mediated activation of NHE1 kinase(s) such as RSK.

**CPA-induced Translocation of PP2Ac in ARVM**—To determine the cellular compartmentation of the principal myocardial protein phosphatases relative to NHE1, we assessed the expression levels of the catalytic subunits of type 1 and type 2A phosphatases (PP1c and PP2Ac, respectively) in cytosolic and particulate fractions from ARVM. As expected, NHE1 protein was detected exclusively in the particulate fraction (Fig. 3A). In contrast, PP1c and PP2Ac were present in both the particulate and the cytosolic fractions (Fig. 3A). Notably, relative to PP1c, a greater proportion of the cellular PP2Ac complement appeared to reside in the cytosolic compartment (Fig. 3A), which is consistent with an earlier report on compartmentation of PP1 and PP2A activities in adult rat myocardial tissue (34). Because Liu and Hofmann (30) have shown previously that adenosine A1 receptor stimulation in ARVM induces PP2Ac carboxymethylation and translocation to the particulate fraction, we then tested whether CPA could induce such translocation of PP1c or PP2Ac under our experimental conditions. As shown in Fig. 3B, CPA had no effect on cellular PP1c compartmentation but induced a significant increase in the PP2Ac content of the particulate fraction. Because the inhibitory effect of A1 receptor stimulation on the α1-AR-mediated increase in sarcolemmal NHE activity is G_{i} protein-mediated (27), we then determined the effect of pertussis...
sis toxin pretreatment (to inactivate G\textsubscript{i} proteins by ADP-ribosylation (28)) on the CPA-induced translocation of PP2A\textsubscript{c}. As illustrated in Fig. 3C, in ARVM pretreated with pertussis toxin, CPA was unable to induce significant enrichment of the PP2A\textsubscript{c} content of the particulate fraction. This finding is consistent with a potential role for PP2A\textsubscript{c} translocation in the G\textsubscript{i} protein-mediated mechanism that underlies A\textsubscript{1} receptor-mediated regulation of sarcolemmal NHE activity (27). It also complements recent data from Liu and Hofmann (35) regarding the proximal signaling mechanisms underlying A\textsubscript{1} receptor-mediated PP2A\textsubscript{c} translocation.  

**Cellular Localization of NHE1 and PP2A\textsubscript{c} in ARVM**—To obtain qualitative visual information on the subcellular localization of PP2A\textsubscript{c} and NHE1, to complement the quantitative data described above, we also performed confocal microscopic imaging of ARVM following dual immunolabeling with specific antibodies for each protein. As illustrated in Fig. 3D, under control conditions, PP2A\textsubscript{c} exhibited diffuse distribution throughout the cell, while NHE1 was concentrated principally in the intercalated disk regions. In response to CPA, however, PP2A\textsubscript{c} appeared to be enriched in the intercalated disk regions, exhibiting marked co-localization with NHE1. Taken together, the data from our fractionation and imaging experiments suggest that stimulation of adenosine A\textsubscript{1} receptors in ARVM gives rise to enhanced co-localization of PP2A\textsubscript{c} and NHE1. This prompted us to determine whether PP2A\textsubscript{c} could dephosphorylate RSK-targeted phosphorylation sites within the NHE1 regulatory domain.

PP2Ac-mediated NHE1 Dephosphorylation in Vitro—In these experiments, the GST-NHE1 fusion protein was first phosphorylated *in vitro* using recombinant active RSK2 and equal aliquots of the phosphorylated protein were then incubated with varying amounts of purified PP2A\textsubscript{c}. Subsequent immunoblot analysis of NHE1 phosphorylation status, using the phospho-Ser 14-3-3 binding motif antibody, revealed reduced phosphorylation of the GST-NHE1 fusion protein with increasing amount of PP2A\textsubscript{c}.

PP2Ac-mediated NHE1 Dephosphorylation in Vitro—Determination of whether PP2A\textsubscript{c} activity is necessary for adenosine A\textsubscript{1} receptor-mediated regulation of the sarcolemmal NHE activity.
PP2A Regulation of the Cardiac Na\(^+\)/H\(^+\) Exchanger

The role of PP2A in cardiac Na\(^+\)/H\(^+\) exchanger (NHE1) regulation is pivotal. Okadaic acid (OA) inhibits both PP2A and PP1, whereas CPA specifically inhibits PP2A. The regulatory effects of these phosphatase inhibitors in cellular contexts were assessed, with the effects on the phosphorylation status of phospholamban, whose phosphorylated Ser\(^{16}\) residue is critical, evaluated. Okadaic acid (1 \(\mu M\)) significantly increased phospholamban phosphorylation at Ser\(^{16}\), whereas endothall (100 \(\mu M\)) was ineffective (Fig. 6A). These data indicate that PP1 inhibition would not contribute to cellular effects of endothall at the relevant concentrations. On the basis of its PP2A-inhibitory efficacy in vitro, lack of effect on PP1 activity in intact cells and favorable cost relative to okadaic acid, we chose to use endothall as a pharmacological tool to determine the role of PP2A in adenosine A\(_1\) receptor-mediated regulation of NHE1 phosphorylation and sarcolemmal NHE activity in ARVM.

Role of PP2A in Receptor-mediated Regulation of NHE1 Phosphorylation in ARVM—To determine NHE1 phosphorylation in ARVM, we used immunoprecipitation with phospho-Ser 14-3-3 binding motif antibody and subsequent immunoblot analysis with NHE1 antibody (Fig. 1B), but this time the cells were pretreated with endothall (100 \(\mu M\)) prior to exposure to phenylephrine and/or CPA. The inhibitory effect of CPA on phenylephrine-induced NHE1 phosphorylation (illustrated in Fig. 1B) was attenuated by endothall pretreatment, such that a significant increase in NHE1 phosphorylation was only observed following \(\alpha_1\)-AR stimulation in both the absence and presence of CPA (Fig. 6B). These data indicate that PP2A activity is necessary for the inhibitory effect of adenosine A\(_1\) receptor stimulation on the \(\alpha_1\)-AR-mediated increase in NHE1 phosphorylation.

Role of PP2A in Receptor-mediated Regulation of Sarcolemmal NHE Activity in ARVM—Finally, we investigated receptor-mediated regulation of sarcolemmal NHE activity in ARVM, in the absence or presence of endothall, using an established microscopy technique. There was no difference between the study groups in basal pH\(_i\) or the minimum pH\(_i\) that was achieved following washout of NH\(_4\)Cl, to induce intracellular acidosis (Table 1). Fig. 7A shows representative recordings of pH\(_i\) in individual cells during recovery from intracellular acidosis. It is evident that, in the absence of endothall, recovery was accelerated by phenylephrine and the effect of phenylephrine was inhibited by treatment with CPA, which is consistent with our previous data (27). In contrast, in the presence of endothall, CPA had no inhibitory effect on the response to phenylephrine. Fig. 7B shows the mean NHE-mediated H\(^+\) efflux rate at pH\(_i\) 6.90 (\(J_{16.9}\)) in each group, illustrating the quantitative effects of the pertinent treatments on sarcolemmal NHE activity. In the absence of endothall, phenylephrine significantly increased sarcolemmal NHE activity and CPA inhibited this response. In the presence of endothall, however, the inhibitory effect of CPA on the phenylephrine response was abolished. These data suggest that PP2A activity plays a critical role in mediating the inhibitory effect of adenosine A\(_1\) receptor stimulation on the \(\alpha_1\)-AR-mediated increase in sarcolemmal NHE activity, most likely by reducing phosphorylation of critical residues, such as Ser\(^{703}\), within the NHE1 regulatory domain.

DISCUSSION

The ability of a variety of protein kinase inhibitors to inhibit the stimulation of sarcolemmal NHE activity by G\(_s\) protein-coupled receptor agonists (18, 19, 23, 32), oxidative stress (20, 22) and intracellular acidosis (21) has implicated protein phosphorylation as an important mechanism in the regulation of the exchanger by multiple stimuli. Furthermore, studies in non-
cardiac cells have identified Ser^{703} of NHE1 (which resides within the sequence Arg-Ile-Gly-Ser-Asp-Pro) as a critical regulatory site, such that RSK-mediated phosphorylation of this residue facilitates serum- and growth factor-induced stimulation of exchanger activity (26, 33). Nevertheless, evidence for direct phosphorylation of the myocardial NHE1 (at Ser^{703} or other identified regulatory sites) by activatory stimuli, such as α-AR agonists, has been lacking. In the present study, we utilized a phosphospecific antibody, which recognizes the Ser-phosphorylated 14-3-3 protein binding motif Arg-Xaa-Xaa-Ser-Xaa-Pro, to show, for the first time, that α-AR stimulation induces phosphorylation of the native NHE1 in ARVM. In view of our data that this antibody detects RSK-phosphorylated residue(s) within the NHE1 regulatory domain (Fig. 1A) and the fact that α-AR stimulation activates the ERK/RSK pathway in ARVM (Fig. 2 and Snabaitis et al. (19)), it is likely that α-AR-mediated NHE1 phosphorylation in ARVM occurs, at least in
part, through the direct action of activated RSK. Furthermore, such RSK-mediated phosphorylation of NHE1 is likely to be of functional significance, particularly since pharmacological inhibition of ERK/RSK activation (by targeting MEK, the upstream activator of this pathway) attenuates the α1-AR-mediated increase in sarcolemmal NHE activity (19).

Whether Ser703, whose RSK-mediated phosphorylation facilitates the stimulation of NHE1 activity in fibroblasts (26), is the sole site that is targeted by RSK (and possibly other α1-AR-responsive NHE1 kinases) in phenylephrine-stimulated ARVM remains to be confirmed. In this context, the NHE1 regulatory domain contains another sequence that conforms to the Arg-Xaa-Xaa-Ser-Xaa-Pro motif, surrounding Ser796 (Arg-Cys-Leu-Ser-Asp-Pro). However, the relative importance of Ser703 versus Ser796 as potential regulatory phosphorylation sites is currently unknown.

Notwithstanding the precise identity of the phosphorylated residues, our data show that phenylephrine-induced NHE1 phosphorylation in ARVM is attenuated by CPA, thereby providing a potential mechanism for the previously reported inhibitory effect of adenosine A1 receptor stimulation on the α1-AR-mediated increase in sarcolemmal NHE activity (27). Importantly, CPA did not affect α1-AR-mediated activation of the ERK/RSK pathway (Fig. 2) but induced significant enrichment of the PP2A, content of the particulate fraction (Fig. 3B). Liu and Hofmann (30) have reported previously that adenosine A1 receptor stimulation in ARVM induces PP2Ac carboxymethylation and translocation to the particulate fraction. In the present work, we demonstrated that the particulate fraction, which was enriched in PP2Ac content following adenosine A1 receptor stimulation, also contained the cellular NHE1 complement (Fig. 3A), raising the possibility that NHE1 might be a target for the translocated phosphatase.

Our confocal immunofluorescence microscopy studies in ARVM revealed that NHE1 protein was not distributed uniformly throughout the sarcolemma, but was enriched in the intercalated disk regions (Fig. 3D). This observation is consistent with earlier data from Petrecca et al. (40), who reported that, in sections from adult rat hearts, NHE1 was localized predominantly to the intercalated disk regions, in close proximity to the gap junctional protein connexin 43. Our present work also suggested that, while PP2A, was distributed diffusely in the resting cell, CPA stimulation induced its accumulation in the intercalated disk regions, where it co-localized with NHE1 (Fig. 3D). There is only limited information available on the subcellular localization of PP2A, in adult mammalian myocardium. However, Ai and Pogwizd (41) have recently reported increased PP2A, co-localization with connexin 43 in the failing rabbit left ventricle, underscoring the dynamic nature of myocardial PP2Ac distribution. The mechanisms that regulate myocardial PP2Ac localization, following adenosine A1 receptor stimulation or in heart failure, are unclear. PP2A is a heterotrimeric holoenzyme and is composed of a core enzyme (which is a high affinity complex of PP2A, and a scaffold protein) that associates with a variety of regulatory subunits (which target PP2A, to specific substrates and intracellular locations) (42). Interestingly, carboxymethylation of PP2Ac regulates the association of the core enzyme with some regulatory subunits, thereby potentially regulating phosphatase targeting to substrates (43). The Gq protein-mediated signaling mechanism through which adenosine A1 receptor stimulation induces PP2Ac carboxymethylation and translocation in ARVM will need to be explored in future work.

An important component of the present work was the demonstration that purified PP2A, could dephosphorylate RSK-phosphorylated NHE1 regulatory domain in vitro (Fig. 4), which suggested that the enhanced proximity of PP2Ac to NHE1 following adenosine A1 receptor stimulation might have a functional impact. Indeed, our subsequent analysis of NHE1 phosphorylation and sarcolemmal NHE activity in intact ARVM substantiated such a functional role. In the pertinent work, the phosphatase inhibitor endothall, at a concentration that inhibited PP2Ac-mediated NHE1 dephosphorylation in vitro (Fig. 5) but did not affect cellular PP1 activity (Fig. 6A), abolished the ability of CPA to inhibit α1-AR-mediated phosphorylation of NHE1 protein (Fig. 6B) and parallel stimulation of sarcolemmal NHE activity (Fig. 7).

Interestingly, endothall did not appear to increase the basal activity of the sarcolemmal NHE, or to potentiate the stimulatory effect of phenylephrine in the absence of CPA (Fig. 7). These data suggest that, in the absence of adenosine A1 receptor stimulation, PP2A, activity does not play an important role in regulating sarcolemmal NHE activity. This is possibly because, under those circumstances, there is little interaction between NHE1 and PP2Ac, as suggested by the absence of significant co-localization of the two proteins (Fig. 3). This situation may be characteristic of ARVM since, in fibroblasts, okadaic acid has been shown to increase basal NHE1 phosphorylation and activity, as well as potentiating responses to α-thrombin (37). Another potential explanation for the divergent findings of the present work with endothall and the earlier work with okadaic acid (37) may be the different phosphatase selectivity of the inhibitors, because okadaic acid potently inhibits both PP2A and PP1 (36), whereas endothall exhibits selectivity for PP2A (38). Indeed, in the present study, we found okadaic acid, but not endothall, to inhibit PP1 activity in

**TABLE 1**

Mean values for resting pHi

|                | Vehicle | CPA |
|----------------|---------|-----|
|                | Cont    | Phen|
| Resting pHi+a  | 7.09 ± 0.02 | 7.09 ± 0.02 |
| Minimal pHi    | 6.78 ± 0.02 | 6.75 ± 0.03 |

**A** Resting pH, measured prior to drug exposure.

**B** All values are mean ± S.E. (n = 12–14).

**C** Minimal pH, measured immediately after NH4Cl washout.

### References

1. Petrecca et al. (40)
2. Liu and Hofmann (30)
3. Ai and Pogwizd (41)
4. Okadaic acid
5. α-thrombin
6. CPA stimulation
7. PP2A regulation
8. NHE1 localization
9. SARCOLEMMA
10. MYOCARDIUM
11. PP1 activity
12. SELECTIVITY

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**Figures and Tables**

- **Fig. 2**: ERK/RSK pathway activation in ARVM.
- **Fig. 3**: Subcellular localization of PP2Ac and NHE1.
- **Fig. 4**: In vitro dephosphorylation of NHE1 by PP2Ac.
- **Fig. 5**: Effect of endothall on NHE1 phosphorylation.
- **Fig. 6**: Cellular PP1 and PP2A activity.
- **Fig. 7**: Basal NHE activity in ARVM.

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ARVM, as reflected by the phospho-lamban phosphorylation status (Fig. 6A).

During the preparation of this article, Misik et al. (44) reported that both PP2A and PP1 could dephosphorylate in vitro a GST-NHE1 fusion protein containing amino acids 637–815 of NHE1, which had been pre-phosphorylated by unidentified NHE1 kinases present in a heart cell extract. In this work, both okadaic acid (in neonatal rat ventricular myocytes) and heterologous expression of a specific PP1-inhibitory peptide (in a Chinese hamster ovary (CHO) cell line) significantly increased NHE1 activity, in the absence of an additional stimulus. Furthermore, through a chemical cross-linking approach in CHO cells expressing an epitope-tagged NHE1 protein, Misik et al. (44) were able to show constitutive PP1 association with NHE1. This is consistent with our observation that a significant proportion of the cellular PP1c complement in ARVM resides in the particulate fraction, which exclusively contains the cellular NHE1 complement (Fig. 3A). Therefore, it appears that PP1 regulates the basal phosphorylation and activity of NHE1 (44), whereas PP2A may participate in a dynamic mechanism of regulation that is responsive to external stimuli.

Fig. 8 illustrates the mechanisms through which α1-AR and adenosine A1 receptors are likely to regulate sarcolemmal NHE activity, on the basis of the novel data reported in the present study and previous pertinent work. Stimulation of α1-AR activates the ERK/RSK pathway in ARVM (19), principally through a Gq protein-mediated mechanism (28). Activated RSK then phosphorylates regulatory site(s), likely to include Ser703 (26), within the NHE1 C-terminal regulatory domain, leading to increased exchanger activity. Stimulation of adenosine A1 receptors, on the other hand, induces the G1 protein-mediated translocation of PP2A to the same cellular compartment as NHE1, leading to dephosphoryl-
PP2A Regulation of the Cardiac Na\(^+\)/H\(^+\) Exchanger

![Diagram of signaling mechanisms](image)

FIGURE 8. Signaling mechanisms through which \(\alpha_1\)-ARs and adenosine \(A_1\) receptors are likely to regulate sarcolemmal NHE activity (see text for details).

...ation of the pertinent regulatory phosphorylation sites and thereby counteracting \(\alpha_1\)-AR-mediated stimulation of exchanger activity. This process represents a novel mechanism in receptor-mediated regulation of the sarcolemmal NHE and is likely to be of (patho)physiological significance. In this context, adenosine \(A_1\) receptor stimulation affords protection against myocardial injury during ischemia and reperfusion (45) and has been shown to attenuate both the positive inotropic (46) and the pro-hypertrophic (47) effects of \(\alpha_1\)-AR stimulation. These actions mimic the reported actions of direct NHE1 inhibitors in similar settings, and it is likely that PP2A\(_{\alpha_2}\)-mediated NHE1 dephosphorylation contributes to the pertinent signaling mechanisms downstream of adenosine \(A_1\) receptor stimulation.

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