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Am J Physiol Heart Circ Physiol 305:H228-H237, 2013. First published 24 May 2013;
doi: 10.1152/ajpheart.00055.2013

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Inhibition of carbonic anhydrase prevents the Na^+/H^+ exchanger 1-dependent slow force response to rat myocardial stretch

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Submitted 18 January 2013; accepted in final form 10 May 2013

Vargas LA, Díaz RG, Swenson ER, Pérez NG, Álvarez BV. Inhibition of carbonic anhydrase prevents the Na^+/H^+ exchanger 1-dependent slow force response to rat myocardial stretch. *Am J Physiol Heart Circ Physiol* 305: H228–H237, 2013. First published May 24, 2013; doi:10.1152/ajpheart.00055.2013.—Myocardial stretch is an established signal that leads to hypertrophy. Myocardial stretch induces a first immediate force increase followed by a slow force response (SFR), which is a consequence of an increased Ca^{2+} transient that follows the NHE1 Na^+/H^+ exchanger activation. Carbonic anhydrase II (CAII) binds to the extreme COOH terminus of NHE1 and regulates its transport activity. We aimed to test the role of CAII bound to NHE1 in the SFR. The SFR and changes in intracellular pH (pH_i) were evaluated in rat papillary muscle bathed with CO_2/HCO_3^- buffer and stretched from 92% to 98% of the muscle maximal force development length for 10 min in the presence of the CA inhibitor 6-ethoxzolamide (ETZ, 100 μM). SFR control was 120 ± 3% (n = 8) of the rapid initial phase and was fully blocked by ETZ (99 ± 4%, n = 6). The SFR corresponded to a maximal increase in pH_i of 0.18 ± 0.02 pH units (n = 4), and pH_i changes were blocked by ETZ (0.04 ± 0.04, n = 6), as monitored by epifluorescence. NHE1/CAII physical association was examined in the SFR by coimmunoprecipitation, using muscle lysates. CAII immunoprecipitated with an anti-NHE1 antibody and the CAII immunoprecipitated protein levels increased 58 ± 9% (n = 6) upon stretch of muscles, assessed by immunoblots. The $p90^{RSK}$ kinase inhibitor SL0101–1 (10 μM) blocked the SFR of heart muscles after stretch 102 ± 2% (n = 4) and reduced the binding of CAII to NHE1, suggesting that the stretch-induced phosphorylation of NHE1 increases its binding to CAII. CAII/NHE1 interaction constitutes a component of the SFR to heart muscle stretch, which potentiates NHE1-mediated H^+ transport in the myocardium.

NHE1 Na^+/H^+ exchanger; carbonic anhydrase II; papillary muscle; slow force response; cardiac hypertrophy

INTRACELLULAR IONIC IMBALANCE is an established contributor to hypertrophic heart growth. Cardiac hypertrophy impairs the ability of the heart to pump efficiently and commonly evolves to heart failure (25, 36). The activity of the cardiac Na^+/H^+ exchanger 1 (NHE1) is central to maintenance of intracellular pH (pH_i) (23, 59); however, experimental and clinical studies demonstrated the pathological implications of increased NHE1 activity during hypertrophy (15, 28, 34, 61). These undesirable effects have been attributed to an increased intracellular Na^+ load (7), leading to elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that triggers widely recognized Ca^{2+}-dependent intracellular signaling pathways leading to cardiac hypertrophy (33, 45, 51).

Mechanical stress triggers immediate intrinsic heart mechanisms to adapt cardiac output to changes in hemodynamic conditions, but it is also the foundation for cardiac hypertrophy if these initial events are sustained (44). When cardiac muscle is stretched there is a rapid augmentation in developed tension due to an increase in myofilament Ca^{2+} sensitivity (the Frank-Starling mechanism), followed by a gradual increase in tension over the next 10–15 min (5, 35, 38). This second phase is called the slow force response (SFR) to stretch and is known to be the result of an increase in Ca^{2+} transient amplitude (3, 6, 27). In the last decade, our laboratory has made efforts to describe the early signals triggered by myocardial stretch that lead to the SFR, which may be the basis for cardiac hypertrophy development. Interestingly, while myocardial stretch-mediated endogenous ANG II release appears to be a key factor for the development of cardiac hypertrophy (17, 32, 45), we have shown that AT1 receptor activation (probably due to endogenous ANG II release) is the initial step of the chain of events leading to the SFR where NHE1 activation is crucial (3, 39). Other hypertrophic responses, which have been mimicked by stretching of isolated cardiomyocytes (45) and ventricular muscle (3, 9, 13, 16, 21, 39, 57, 58), are induced partially through an increase in secretion and synthesis of vasoactive peptides, such as endothelin (ET). In addition, stretch, through AT1 receptor activation, mediates the release of ET-3 in feline papillary muscles, which seems to be responsible for the initial step of the chain of events that underlie the SFR (21).

We have proposed that the chain of events triggered by stretch begins with the release of ANG II and ET and ends with the increase in $[Ca^{2+}]_i$ through a reverse-mode operation of the Na^+/Ca^{2+} exchanger NCX1 (3, 13, 21, 39). More recently, it has been proven that NHE1 activation is a consequence of the activation of redox-sensitive kinases ERK1/2-p90 ribosomal S6 kinase ($p90^{RSK}$) through a mechanism that involves mineralocorticoid receptor activation (10) and epidermal growth factor receptor transactivation (14).

Additionally, maximal activity of NHE1 requires the catalytic activity of the enzyme carbonic anhydrase II (CAII), which provides protons for the Na^+/H^+ exchanger (30). Therefore, NHE1 transport efficiency is influenced by CAII, and this process is mediated through a direct interaction of the enzyme with the NHE1 COOH-terminal region (30), an association that is greatly increased when the NHE1 COOH terminus is phosphorylated (30, 31). Interestingly, a role for CAII in the

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hypertrophy of cultured cardiomyocytes to hypertrophic agents was identified (2) by showing that treatment of the cardiomyocytes with the CA inhibitor, 6-ethoxzolamide (Cardrase, ETZ), prevented the hormonally induced hypertrophy and reversed it even after it was once established (2).

In this context, the present study was designed to examine possible CAII dependence of NHE1 activation to determine SFR development after stretching isolated rat papillary muscles, as well as the binding of CAII to NHE1 after stretch. We provide evidence that *1*) CAII inhibition prevents the SFR to cardiac muscle stretch, an effect that may conceivably be assigned to the limitation of the available proton substrate to maximize the NHE1-mediated H^+ extrusion; and *2*) myocardial stretch increases the interaction of CAII and NHE1 due to $p90^{RSK}$-dependent NHE1 phosphorylation, the prevention of which also blunts the SFR development in rat cardiac muscle. Therefore, CAII induction may be a prognostic molecular marker of stretched muscle that precedes cardiac hypertrophy, and CA inhibition may be effective in preventing the early hypertrophic signals triggered by myocardial stretch.

MATERIALS AND METHODS

Animals. Four-month-old male Wistar rats, originally derived from Charles River Breeding Farms (Wilmington, MA), were used in these studies. All animals were housed under identical conditions and had free access to standard dry meal and water. Protocols that involved rats were reviewed and approved by the Animal Welfare Committee of La Plata School of Medicine and performed in accordance with the Guide for the Care and Use of Laboratory Animals (Argentine Republic Law No. 14346) concerning animal protection.

Isolation of papillary muscles. Rats were anesthetized by an intraperitoneal injection of Euthanyl (pentobarbital sodium, 35 mg/kg). Hearts were rapidly removed, and papillary muscles from the left ventricle were isolated and used to assess the SFR to stretch as previously described (39). The picture was captured with a Panasonic Lumix digital camera, without zoom magnification. Briefly, the muscles were mounted in a perfusion chamber and superfused at a constant rate (5 ml/min) with a CO_2/HCO_3^--buffered solution containing (in mmol/l) 128.3 NaCl, 4.5 KCl, 1.35 $CaCl_2$, 20.23 $NaHCO_3$, 1.05 $MgSO_4$, and 11.0 glucose and equilibrated with 5% CO_2-95% O_2 (pH ~7.40). The muscles were maintained at 30°C, paced at 0.2 Hz at a voltage 10% over threshold, and isometric contractions were recorded. Cross-sectional area (calculated as 0.75 of the product of thickness times width) was used to normalize force records obtained with a silicon strain gauge (model AEM 801, SensoNor). The slack length of each muscle was determined after mounting, and then the muscles were progressively stretched to the length at which they developed maximal twitch force (L_{max}). After a few minutes at L_{max}, they were shortened to obtain 95% of the maximal twitch force (length ~98% of L_{max} and referred to as L_{98}). Muscles were then shortened to 92% of L_{max} (L_{92}) and maintained at this length until the beginning of the experimental protocol, when they were abruptly stretched from L_{92} to L_{98}. Experimental protocols were repeated in the presence or absence of the CA inhibitors, 6-ethoxzolamide ETZ (100 μM, Sigma-Aldrich, St. Louis, MO) or *N*-methyl-acetazolamide (*N*-methyl-AZ, 100 μM), a synthesized compound lacking the unsubstituted sulfonamide group required for CA inhibition (48), or benzolamide (BZ, 10 μM), a hydrophilic potent CA inhibitor with very limited cell penetrance, or in the presence or absence of the $p90^{RSK}$ inhibitor, SL-0101–1 (10 μM, Santa Cruz). The drugs were added to the perfusate 20 min before stretch, and during this period none of them changed developed force by more than 2–4%.

Determination of pH_i in rat papillary muscles. For pH_i measurements the muscles were mounted in a perfusion chamber and loaded with 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF, AM form), for 30 min (final concentration 5 μmol/l) at room temperature. To measure fluorescence emission from BCECF, excitation light from a 75-W Xe 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 (×10) and transmitted through a band-pass filter at 535 ± 5 nm to a photomultiplier (model R2693, Hamamatsu). The photomultiplier output was then collected via an analog-to-digital converter (model 2801 A, Data Translation) and stored in a personal computer. To avoid 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 s every ~10 s) during the protocol. To assess the effect of stretch on pH_i under HCO_3^--free conditions muscles were superfused with a HEPES-buffered solution containing (in mmol/l) 146.2 NaCl, 4.5 KCl, 1.35 $CaCl_2$, 1.05 $MgSO_4$, 11.0 glucose, and 10.0 HEPES, titrated to pH 7.40 with NaOH and equilibrated with 100% O_2. Alternatively, muscles were superfused with CO_2/HCO_3^--buffered solution as above. Exposure to CO_2/HCO_3^--buffered Ringer solution would presumably allow any HCO_3^- transport mechanism not operational in CO_2-free HEPES-buffered solution to assist pH_i regulation. Therefore, in the presence of the HCO_3^-/CO_2 buffer solution, anion transport mechanisms were inhibited by the 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid disodium salt, SITS (Sigma Chemical), added to the superfusate to a final concentration of 2 mmol/l. Under HCO_3^-/CO_2 conditions, experimental protocols were performed in the presence or absence of the CA inhibitors, 6-ethoxzolamide ETZ (100 μM, Sigma-Aldrich) or *N*-methyl-AZ (100 μM). At the end of each experiment, BCECF emitted fluorescence was calibrated in vivo by the high K^+-nigericin method (3, 13).

Preparation of rat papillary muscle lysates. Isolated papillary muscles subjected to different SFR protocols were frozen in liquid nitrogen and stored at −80°C. Muscles were then homogenized with a PRO250 Homogenizer (PRO Scientific, Oxford, CT) in ~300 μl ice-cold IPB buffer (1% Igepal, 5 mM EDTA, 0.15 M NaCl, 0.5% deoxycholate, 10 mM Tris·HCl, pH 7.5), containing 2 mg/ml BSA and protease inhibitors (PI, MiniComplete Tablet, Roche).

Determination of extracellular signal-regulated protein kinase ERK1/2 phosphorylation after muscle stretch. At the end of the protocols, the superfusion solution was quickly removed, and non-stretched papillary muscles, or muscles stretched in the absence or presence of the CA inhibitor ETZ (100 μM), were homogenized in lysis buffer: 300 mmol/l sucrose, 1 mmol/l DTT, 4 mmol/l EGTA, protease inhibitors cocktail (Complete Mini Roche), and 20 mmol/l Tris·HCl, pH 7.4. After a brief centrifugation the supernatant was kept and protein concentration determined. Samples were denatured and equal amounts of protein (100 μg) subjected to PAGE and electrotransferred to PVDF membranes. After blocking with nonfat dry milk, membranes were incubated overnight with anti-p-ERK1/2 polyclonal antibodies (1:1,000 dilution, Santa Cruz) followed by incubation with a donkey anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (1:1,000 dilution, Santa Cruz). Membranes were stripped and incubated with rabbit anti-ERK2 antibody (1:1,000 dilution, Santa Cruz) followed by incubation with donkey anti-rabbit IgG HRP-conjugated antibodies.

Coimmunoprecipitation. Homogenates of papillary muscles were centrifuged at 1,440 *g* for 5 min in a Beckman G5–6K centrifuge. Supernatants were obtained and the Quant-iT protein assay kit (Molecular Probes/Invitrogen Labeling and Detection, Eugene, OR) was used to determine protein concentration on a Qubit fluorometer (Invitrogen), according to the manufacturer's instructions. Supernatants (500 μg) from control stretched muscles, or muscles stretched in the presence of the CA inhibitor ETZ or muscles stretched in the presence of the $p90^{RSK}$ inhibitor SL-0101–1 were applied to 50 μl protein A-Sepharose (50% slurry) for 3 h at 4°C. After centrifugation

(5 min, 8,000 *g*), muscle lysates were incubated overnight with rabbit polyclonal anti-NHE1 antibody (H-160, Santa Cruz) (6 μl, 1.2 μg IgG) or nonimmune serum (6 μl), and 100 μl protein A-Sepharose (at 4°C, overnight). Resin was washed and resuspended in an equal volume of 2X SDS/PAGE sample buffer.

Immunodetection. Papillary muscle immunoprecipitated lysates (25 μl) or total lysates (60 μg protein) were resolved by SDS-PAGE on 8–10% acrylamide gels, as indicated. Proteins were transferred to PVDF membranes and then incubated with rabbit anti-NHE1 (H-160, Santa Cruz, 1:1,000 dilution,), or rabbit anti-CAII antibody (H-70, Santa Cruz, 1:1,000 dilution), or mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1,000 dilution). Immunoblots were then incubated with 1:1,000 dilutions of donkey anti-rabbit IgG, or donkey anti-mouse IgG (Santa Cruz, CA) conjugated to HRP. Blots were visualized and quantified using ECL reagent and a Bio-Rad Image Station.

Statistics. Data are expressed as means ± SE. Student's *t*-test or one-way ANOVA followed by Neuman-Keuls multiple comparison posttest analysis, when appropriate, was used to compare data. $P < 0.05$ was considered a threshold for statistical significance.

RESULTS

Effect of CA inhibition on the SFR to papillary muscle stretch. Figure 1 shows the schematic representation of the rat heart with the papillary muscles used in our studies (Fig. 1*A*). Freshly isolated papillary muscle from rat left ventricles were located in a perfusion chamber and subjected to the stretch protocols (Fig. 1*B*).

Since CAII binds NHE1 to activate NHE1-mediated H^+ efflux rate (30), we reasoned that inhibition of CAII could indirectly inhibit NHE1 activity and thus reduce the SFR to myocardial stretch. The mechanical response to stretch of rat papillary muscles was studied in the presence of the lipophilic cell membrane-permeant CA-inhibitor 6-ethoxyzolamide (ETZ, 100 μM). Cross-sectional area was calculated for control muscles subjected to stretch (0.50 ± 0.10 mm^2), and muscles stretched in the presence of CA inhibitors (0.60 ± 0.06 mm^2) (Fig. 1*C*). Previously, ETZ was found to have a slight effect on isometric force development after 20–30 min in rabbit papillary muscle maintained in a Krebs-Henseleit solution at 20°C (24). Under our experimental conditions, ETZ did not change the isometric force development of rat papillary muscle after 20 min of incubation at a more physiological temperature of 30°C, 100 ± 5% vs. 97 ± 4% (n = 6), before and after treatment, respectively (data not shown). Stretching of the rat papillary muscles promoted the distinctive biphasic mechanical response, an initial abrupt force increase followed by the SFR, which fully developed after ~10 min (Fig. 2*A*, *top*). In contrast, ETZ prevented the SFR development of rat papillary muscles (Fig. 2*A*, *middle*). To prove that prevention of SFR after rat muscle stretch is dependent on CA enzymatic activity, experiments were repeated in the presence of *N*-methyl-AZ, an acetazolamide analog devoid of inhibiting effects on CA (48). *N*-methyl-AZ did not change the developed force of rat heart muscle after 20 min of incubation, 100 ± 5% vs. 104 ± 4% (n = 4), before and after treatment, respectively (data not shown). In addition, as predicted the non-CA-inhibiting analog of acetazolamide, *N*-methyl-AZ, failed to block the SFR after stretch of the cardiac muscle (Fig. 2*B*).

In addition, in another series of experiments we used benzolamide (BZ, 10 μM), a hydrophilic CA inhibitor with reduced permeability, to evaluate the effect on the SFR to heart muscle stretch (Fig. 2*A*, *bottom*). We presume that BZ would primarily inhibit the extracellular luminal membrane-bound CAIV, and transmembrane CAIX and CAXIV in the myocardium, yielding extracellular enzyme inhibition and having a less prominent effect on the SFR development. Prior to the muscle stretching, incubation with BZ (10 μM, 20 min) did not affect the maximal muscle developed force, 100 ± 7% vs. 104 ± 4% (n = 7), before and after treatment, respectively (data not shown). Conversely, in the presence of BZ the SFR to muscle stretch was only modestly developed (Fig. 2, *A* and *B*), suggesting that the contribution of another CA-associated bicarbonate metabolon-system, such as the sodium bicarbonate cotransporter (NBC), may have additional effect on the SFR to rat myocardial stretch.

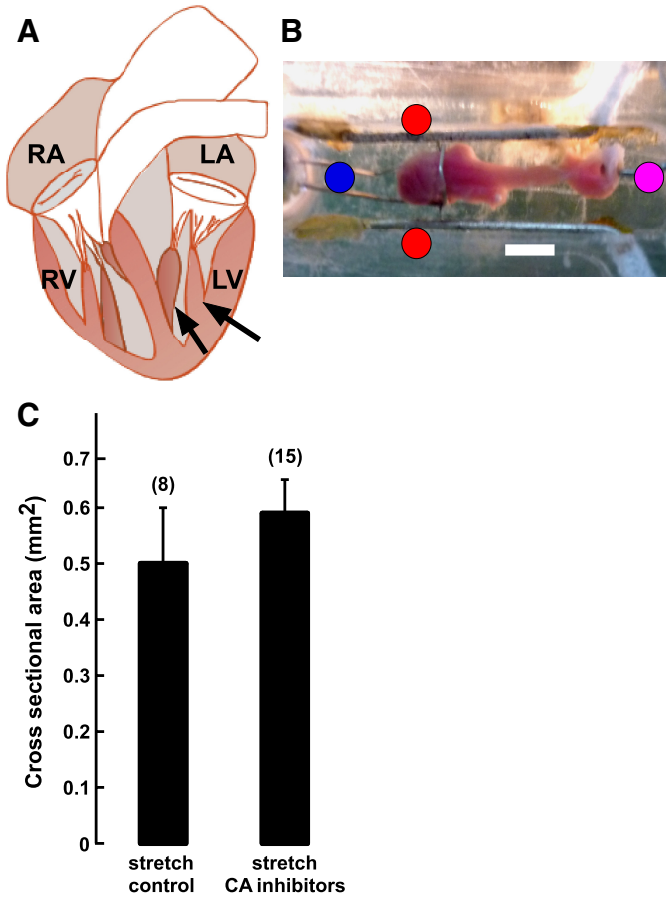

Fig. 1. Papillary muscle morphology. *A*: schematic representation of the rat heart and left ventricle papillary muscles (black arrows) used for muscle contractility recordings. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. *B*: picture of rat ventricular papillary muscle located in a perfusion chamber. Bar = 2 mm; red circle, wired electrodes; blue circle, force transducer; pink circle, fixed support. Picture was captured with a Panasonic Lumix camera without zoom magnification. *C*: summary of cross-sectional area (mm^2) corresponding to control stretched papillary muscles, or papillary muscles stretched in the presence of carbonic anhydrase (CA) inhibitors. Values are means ± SE. Parenthetical values on top of bar indicate number of muscle analyzed.

Previously, we showed that the specific NHE1 inhibitor, HOE642, blocked the SFR to feline papillary muscle stretch (11). In addition, the SFR was absent in rat papillary muscles isolated from NHE1-silenced hearts (40). Thus we suggest that the observed SFR inhibition after blockade of CAII is the result of NHE1 inhibition.

Effect of CA inhibition on pH_i in the SFR to rat papillary muscle stretch. NHE1 transport activity and the SFR. Under nominally HCO_3^--free conditions (HEPES buffer), the stretch

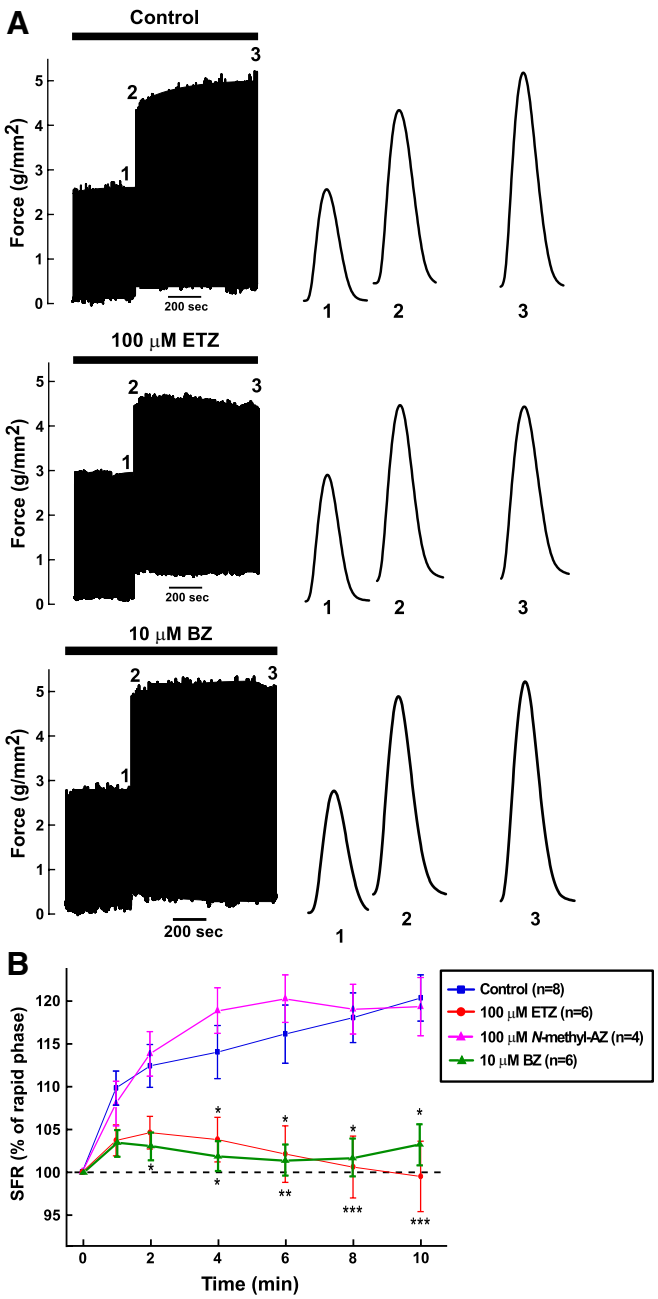

Fig. 2. Slow force response (SFR) and CA inhibition. *A*: typical force recording of rat papillary muscles superfused with CO_2/HCO_3^--buffered solution and subjected to an increase in length from 92 to 98% of maximal force, in the absence (*top*) or presence (*middle*) of the potent membrane-permeant CA-inhibitor 6-ethoxzolamide (ETZ, 100 μM), or in the presence of the potent hydrophilic CA-inhibitor benzolamide (BZ, 10 μM) (*bottom*). After stretching a papillary muscle from 92 to 98% of length at L_{max}, a sudden increase in force immediately occurs (from *1* to *2*; *top, middle,* and *bottom*). After that, under control conditions, a progressive increase in force develops during the next 10 min, the SFR (*top*), which was canceled by ETZ (*middle*), or partially canceled by BZ (*bottom*). Right panels show isolated twitch records corresponding to the full contractile response on the left (*1, 2*, and *3* correspond to twitch force recordings before, immediately after, and 10 min after stretch, respectively), in the absence (*top*) or presence of the CA inhibitors ETZ (*middle*) or BZ (*bottom*). *B*: time course of the SFR expressed as percentage of the initial rapid phase in the absence (control) or presence of ETZ or the acetazolamide analog *N*-methyl-AZ (100 μM), or the presence of BZ. *n* represents the number of animals and muscles analyzed. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ vs. control.

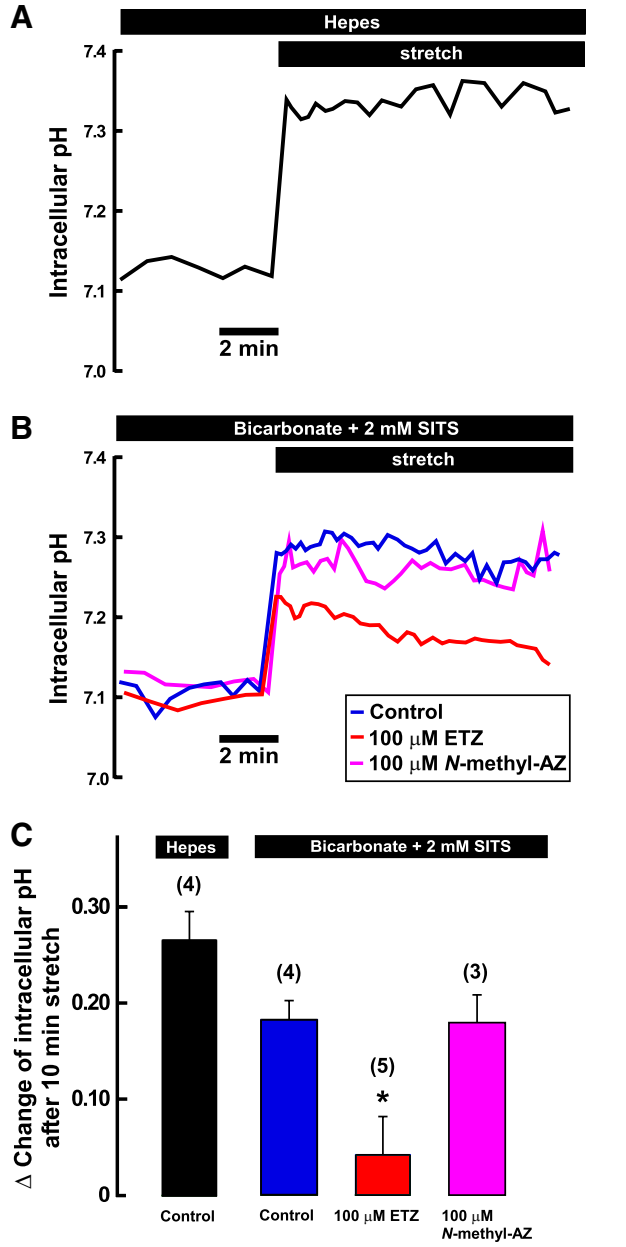

Fig. 3. Effect of CA inhibition on NHE1 transport activity in stretched rat myocardium. Heart muscles were loaded with the BCECF pH-sensitive fluorescent dye to measure intracellular pH (pH_i) and subjected to the stretch protocol (see METHODS). *A*: example of single rat papillary muscles used for pH_i measurement experiments under nominally HCO_3^--free conditions (HEPES buffer). *B*: pH_i recording of a rat heart muscle superfused with a CO_2/HCO_3^- buffer containing SITS in the absence (control, blue trace) or presence of the CA inhibitor ETZ (red trace), or presence of *N*-methyl acetazolamide, an acetazolamide analog devoid of inhibiting effects on CA (*N*-methyl-AZ, purple trace). *C*: summary of ΔpH_i obtained after 10 min of papillary muscles stretched under HEPES buffer (black) or CO_2/HCO_3^- buffer + SITS (blue) conditions, in the presence or absence of ETZ (red) or *N*-methyl-AZ (purple). Parenthetical values on top of bar indicate number of experiment. $*P < 0.05$ vs. control group.

of rat and cat papillary muscles elicits an increase in pH_i which is fully dependent on NHE1 activity (3, 13). Herein, rat papillary muscles were loaded with the pH-sensitive dye BCECF-AM and subjected to the stretch protocol in the absence of HCO_3^- (HEPES, Fig. 3*A*). Stretch of rat heart muscles

causes a rapid and sustained increase in pH_i, which reaches a maximal value after 10 min ($\Delta pH_i = 0.27 \pm 0.03$) (Fig. 3*C*), in accordance with previous results (13). NHE1 binds the cytosolic enzyme CAII (30, 31), which produces the H^+ substrate to maximize NHE1 transport activity. Since the development of the SFR seems to depend primarily on the activation of the NHE1 transport (40), we reasoned that inhibition of CAII could limit NHE1 activity by reducing substrate availability, and therefore affect the SFR to muscle stretch. Given that CA catalytic activity requires CO_2 substrate for HCO_3^- formation, and that under nominally HCO_3^--free conditions the enzyme's activity will be negligible we conducted experiments in HCO_3^--buffered media. Changes in myocardial pH_i elicited by stretching rat papillary muscles were then evaluated in muscles bathed in CO_2/HCO_3^--buffered medium after the addition of 2 mmol/l of the disulfonic stilbene derivative SITS to inhibit the HCO_3^--dependent membrane transporters (Fig. 3*B*). Under these conditions, the only pH_i regulatory mechanism operational in the myocardium is the NHE1 (12). In the presence of HCO_3^- and SITS, stretch of rat heart muscles also produced a rapid and sustained increase in pH_i (Fig. 3*B*), reaching a ΔpH_i of 0.18 ± 0.02 after 10 min (Fig. 3*C*) proving NHE1 activation. In contrast, the potent CA inhibitor, ETZ, prevented the increase in pH_i after the muscle stretch with a maximal change of pH_i of only 0.04 ± 0.03 (Fig. 3*C*). We further investigated the possibility that the effects of ETZ on the SFR-induced changes in pH_i were independent of CA inhibition using *N*-methyl-acetazolamide (*N*-methyl-AZ, 100 μM). Interestingly, *N*-methyl-AZ did not block the NHE1-mediated pH_i increase induced by stretch of rat papillary muscles ($\Delta pH_i = 0.18 \pm 0.03$) (Fig. 3, *B* and *C*).

From these experiments we conclude that activation of NHE1 after stretch is dependent on intracellular CA activity and reinforce the idea that abrogation of the SFR with CAII inhibition may be due to NHE1 blockade.

In summary, to evaluate the effects of CA inhibition on the SFR of rat papillary muscle stretch, pH_i measurement were performed in the present of SITS to eliminate the activity of all other pH_i-regulating processes except NHE1. In addition to these measurements, contractile performance during the SFR developments was determined in the absence of SITS. Therefore, on the basis of previous observations, we can assume that in the absence of SITS, changes in pH_i should be minimal (3, 39) and hence should not affect the myofilament Ca^{2+} responsiveness and the magnitude of SFR development, since the $Na^+{}_i$ increase which follows NHE1 activation is the only factor determining the magnitude of the contractile response.

Binding of CAII to NHE1 in stretched rat papillary muscle. CAII binds to the penultimate group of thirteen amino acids of the NHE1 (30, 31). Within the NHE1 COOH-terminal region amino acids S796 and D797 form part of a novel CAII binding site, and phosphorylation of the NHE1 COOH terminus increases the binding of CAII (30, 31). We studied the physical interaction of NHE1 and CAII in rat papillary muscle subjected to stretch by coimmunoprecipitation experiments. Lysates prepared from stretched papillary muscle were used to examine the SFR of rat heart (Fig. 4). Lysates (500 μg protein) were immunoprecipitated with rabbit anti-NHE1 antibody, resolved by SDS/PAGE in a 10% acrylamide gel, and probed with a rabbit anti-CAII antibody (Fig. 4*A*, *top*). In a parallel blot, immunoprecipitates were resolved by SDS/PAGE in an 8% acrylamide gel and probed with a rabbit anti-NHE1 antibody (Fig. 4*A*, *bottom*). As a control, nonstretched rat papillary muscles were immunoprecipitated with anti-NHE1 antibody, and the immunoblots resolved by anti-CAII and anti-NHE1 antibodies, as above (Fig. 4*A*, *top* and *bottom*). The amount of CAII bound to NHE1 was normalized to the NHE1 expression level (Fig. 4*B*). NHE1-associated CAII increased by ~60% in the stretched compared with nonstretched papillary muscles ($163 \pm 9\%$ $n = 10$ vs. $100 \pm 12\%$, $n = 13$, respectively, $P < 0.05$).

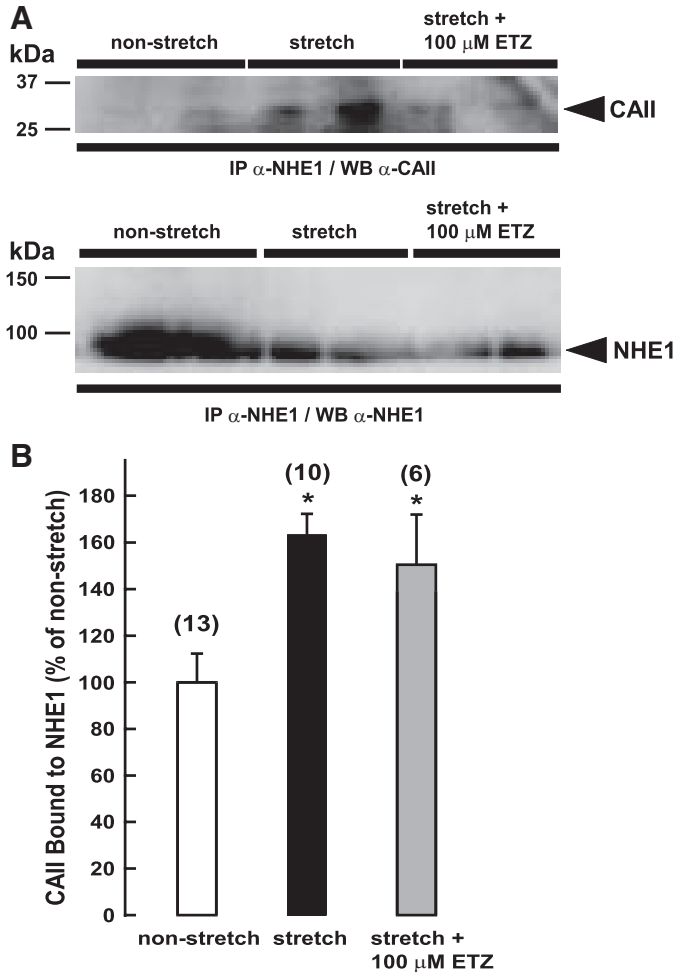

Fig. 4. Coimmunoprecipitation of NHE1/CAII complex from rat heart ventricular muscle. *A*: a total of 500 μg protein of rat papillary muscle lysates (nonstretched control muscles or muscles used for SFR to myocardial stretch measurements in the presence or absence of ETZ) were immunoprecipitated (IP) with rabbit polyclonal antibody directed against NHE1 (H-160, Santa Cruz) (6 μl = 1.2 μg IgG) and resuspended in a final volume of 75 μl. The gels were loaded with 100 μg of immunoprecipitated protein (25 μl). Heart muscle lysates were electrophoresed on 10% acrylamide gels, transferred to a PVDF membrane, and probed with anti-CAII rabbit polyclonal antibody (H-70, Santa Cruz) (*top*). In a parallel blot, immunoprecipitated lysates of nonstretched muscles, and muscles stretched in the presence or absence of ETZ (100 μg protei$n$ = 25 μl), were analyzed on 8% acrylamide gels, transferred to PVDF membranes, and probed with anti-NHE1 antibody (*bottom*) to indicate total amount of NHE1 in each sample. Position of NHE1 and CAII is shown (filled arrow). WB, Western blot. *B*: bar graph quantifies the amount of bound CAII normalized to the expressed NHE1 protein in each muscle sample (n = number of animals and heart muscles analyzed). *Significant difference ($P < 0.05$) compared with nonstretched muscles.

Additional coimmunoprecipitation experiments were repeated in the presence of the CA inhibitor ETZ, to verify the functional

role of the NHE1/CAII interaction in the stretched myocardium (Fig. 4, *A* and *B*). Rat papillary muscle was incubated for 20 min in the presence of 100 μM ETZ and subsequently stretched in the presence of the CA inhibitor. At the end of the experiment, papillary muscle lysates were prepared, the samples were immunoprecipitated with anti-NHE1 antibody, and the immunoblots probed with an anti-CAII antibody (Fig. 4*A*). CAII bound to NHE1, and this interaction was increased 50% upon stretch of the muscles (Fig. 4B, $n = 6$). This confirms that ETZ did not disturb the NHE1/CAII interaction, and that the effect of ETZ on the SFR of stretched papillary muscle could not be attributed to disruption of an effective NHE1/CAII metabolon.

Cardiac muscle releases ANG II and ET after stretch, as seen in cultured myocytes (32, 43, 45, 54, 60) and papillary muscles (21). Moreover, an increase in the prepro-ET3 mRNA was observed after a 15-min stretch in feline papillary muscles (21). To discard changes in CAII and NHE1 expression that could affect interpretation of the coimmunoprecipitation data, CAII and NHE1 expression were measured in lysates of muscles subjected to stretch. Lysates of control rat papillary muscles, or muscles subjected to the stretch protocol in the presence or absence of the CA inhibitor, ETZ, were studied by SDS-PAGE-electrophoresis (Fig. 5*A*), and the amount of CAII and NHE1 on blots was quantified by densitometry and normalized to the amount of GAPDH in the sample. Expression of CAII, normalized to GAPDH, was similar in nonstretched muscles compared with muscles stretched in the presence or absence of ETZ (Fig. 5*B*). In addition, expression of NHE1 normalized to GAPDH did not change in nonstretched muscles, or muscles stretched in the presence or absence of ETZ (Fig. 5*B*). Therefore, while stretch of rat papillary muscle did not affect total CAII or total NHE1 protein expression, it altered the amount of CAII bound to NHE1.

Modulation of the NHE1/CAII complex assembly after myocardial stretch by redox-sensitive p90^{RSK}. The SFR depends on the activation of NHE1 (3, 13, 40), which is a target of redox-sensitive kinases such as p90^{RSK}. Herein, we explored the role played by the p90^{RSK} protein kinase in the SFR to myocardial stretch and on the interaction between CAII and NHE1 after stretch. Pretreatment of rat heart muscles with the specific p90^{RSK} inhibitor, SL0101–1 (10 μM), did not affect developed force under basal conditions (20 min incubation, not shown) or the initial fast response after muscle stretching (Fig. 6*A*). However, SL0101–1 completely prevented the SFR after papillary muscle stretch (Fig. 6*B*). Muscles were frozen immediately after the stretching protocol and processed for coimmunoprecipitation. Coimmunoprecipitation experiments were conducted in the presence or absence of SL0101-1. Anti-NHE1 antibody was able to precipitate CAII from stretched rat heart muscles, either in the presence or absence of SL0101–1 (Fig. 6*C*). Nonetheless, SL0101–1 reduced the amount of CAII bound to NHE1 by 26 ± 7% compared with stretch (control) (Fig. 6*D*).

These results indicate that the phosphorylation of NHE1 by the redox-sensitive p90^{RSK} kinase after stretch increases the NHE1/CAII complex abundance.

Effect of CA inhibition on extracellular signal-regulated protein kinase (ERK1/2) phosphorylation after stretch. Myocardial stretch activates NHE1 through posttranslational modifications, mainly by phosphorylation of its cytosolic regulatory domain (22). Myocardial stretch induces NHE1 phosphorylation at Ser703 through a mechanism that appears to involve an

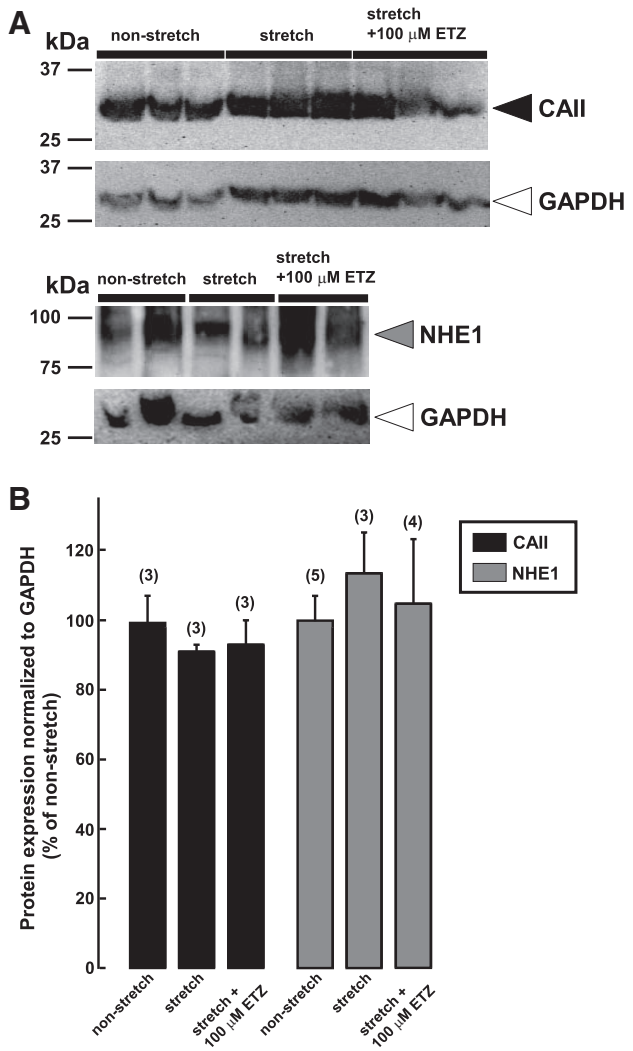

Fig. 5. Effect of ETZ on CAII and NHE protein level in stretched rat papillary muscles. *A*: total ventricular muscle lysates were prepared from isolated rat papillary muscles, which were nonstretched, or stretched in the absence or presence of 100 μM ETZ. Protein (60 μg) was loaded and immunoblots of the muscle lysates were probed with antibody against CAII or antibody against NHE1, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as indicated. *B*: expression levels of CAII, NHE1, and GAPDH were quantified by densitometry. CAII and NHE1 expression was normalized to GAPDH in each muscle sample and expressed as a percentage of nonstretched muscle. Values are means ± SE. Parenthetical values on top of bar indicate number of muscle analyzed.

increase in redox-sensitive ERK1/2 and p90^{RSK} kinases activity (11, 56). Nonstretched rat papillary muscle or muscles subjected to stretch were homogenized in lysis buffer, and the relative amounts of phosphorylated ERK1/2 were determined by immunoblot analysis (Fig. 7*A*, *top*). ERK1/2 phosphorylation was quantified and normalized to ERK2 expression (Fig. 7*B*, *bottom*). We confirmed that myocardial stretch significantly increased phosphorylation of ERK1/2, a kinase upstream of p90^{RSK}, compared with nonstretched cardiac muscle, as previously described (40). We reasoned that if ERK1/2 activation is upstream of NHE1 phosphorylation, myocardial stretch through an autocrine/paracrine mechanism should increase ERK1/2 activity even when NHE1-bound CAII was inhibited. Interestingly, in the presence of the CA inhibitor

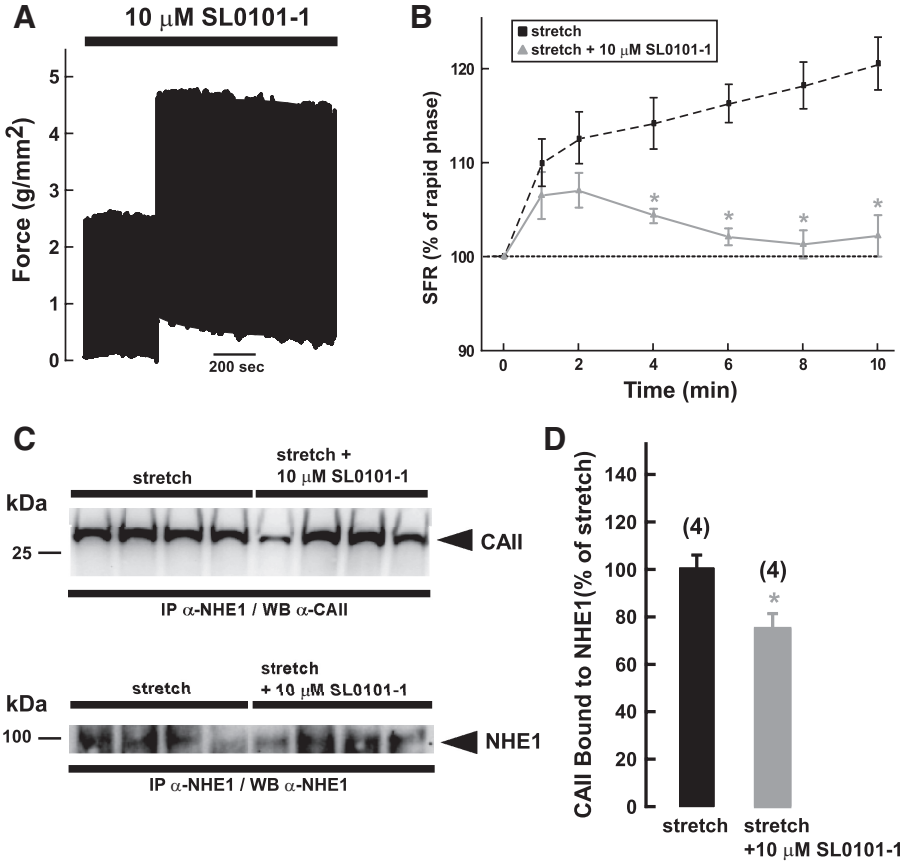

Fig. 6. Effect of p90 ribosomal S6 kinase ($p90^{RSK}$) inhibition on the SFR of rat myocardial stretch and on NHE1/CAII complex formation after stretch of heart muscles. *A*: typical force recording of a rat papillary muscle subjected to an increase in length from 92 to 98% of maximal force, in the presence of the specific $p90^{RSK}$ inhibitor (SL0101–1, 10 μM). *B*: time course of the SFR expressed as percentage of the initial rapid phase in the presence of SL0101–1 ($n = 4$). Dotted line represents the time course of the SFR as percentage of the initial rapid phase in control muscles (see Fig. 1*B*). *$P < 0.05$ compared with stretched muscles. *C*: coimmunoprecipitation experiments on control stretched muscles or stretched muscles in the presence of SL0101-1 were performed and analyzed as described in Fig. 4. Positions of NHE1 and CAII are shown (filled arrow). *D*: The bar graph quantifies the amount of bound CAII normalized to the expressed NHE1 protein in each muscle sample. Parenthetical values at the top of the bar indicates the number of animals and heart muscles analyzed. *Significant difference ($P < 0.05$) compared with stretched muscles.

ETZ, stretch of the rat cardiac muscle increased ERK1/2 phosphorylation to approximately the same extent as control stretch muscles (Fig. 7, *A* and *B*).

This confirms that ETZ did not prevent ERK1/2 phosphorylation that occurred at the end of the measured SFR after myocardial stretch (~10 min), and consequently according to our hypothesis ETZ did not prevent the NHE1-CAII increased interaction mediated by phosphorylation of NHE1 through ERK1/2-$p90^{RSK}$ signaling. However, we cannot exclude the possibility that phosphorylation of other sites on NHE1 that may affect NHE1-CAII interaction could have taken place during the course of the first minutes of SFR development after muscle stretch.

DISCUSSION

Activation of the NHE1 by hormonal and other pathways induces myocardial hypertrophy (22, 26). Conversely, treatment with specific NHE1 inhibitors prevents hypertrophy and blocks heart failure in genetic models or animal models of infarction (19, 20, 28, 61) and improves myocardial function in patients with acute anterior myocardial infarction (42). The present report focused on the availability of substrate for NHE1 activity provided by CAII, as a target to inhibit NHE1 and prevent the predicted initial steps of cardiac hypertrophy development induced by stretch of the mammalian myocardium.

Cardiac muscle expresses membrane-bound CAIV, CAIX, and CAXIV enzymes (46). In addition, cytosolic CAII is present in rat cardiomyocytes and papillary muscles (2, 55). NHE1 is functionally activated by a physical interaction with the cytosolic enzyme CAII (30), and the activation of NHE1 through this association is explained by the ability of CAII to produce the NHE1 substrate H^+ by catalysis of CO_2 hydration. We thus reasoned that inhibition of CAII could limit NHE1 activity through reduced substrate availability, preventing NHE1-mediated SFR to myocardial stretch, a myocardial response with the proclivity to induce early stages of cardiac hypertrophy. Herein, we proved that following stretch of cardiac muscle and during the development of the SFR to myocardial stretch, there is an increase in the CAII binding to NHE1 (Fig. 4), which is dependent on the activation of the redox-sensitive $p90^{RSK}$ kinases (Fig. 6), a kinase known to phosphorylate and regulate NHE1 activity. Furthermore, we demonstrated that ETZ, a sulfonamide with CA inhibitory properties, did not affect the binding interaction of CAII and NHE1 (Fig. 4). Rather we suggest that ETZ inhibits CAII enzymatic activity, preventing formation of the NHE substrate H^+, limiting H^+ availability for NHE1 transport, and consequently reducing NHE1-mediated SFR to cardiac muscle stretch.

Different proteins of cardiac myocytes are preprogrammed at a very early stage of heart development, but in addition, other exogenous factors, such as functional load (stretch, pressure), play an important role in their expression under both physiological and pathological circumstances. A role for CA in the hypertrophic response of cardiomyocytes to hypertrophic agents has been identified (2). The linked activity of AE3 and NHE1 promotes hypertrophy and the hypertrophy-program increases expression of several CA enzymes in cultured rat

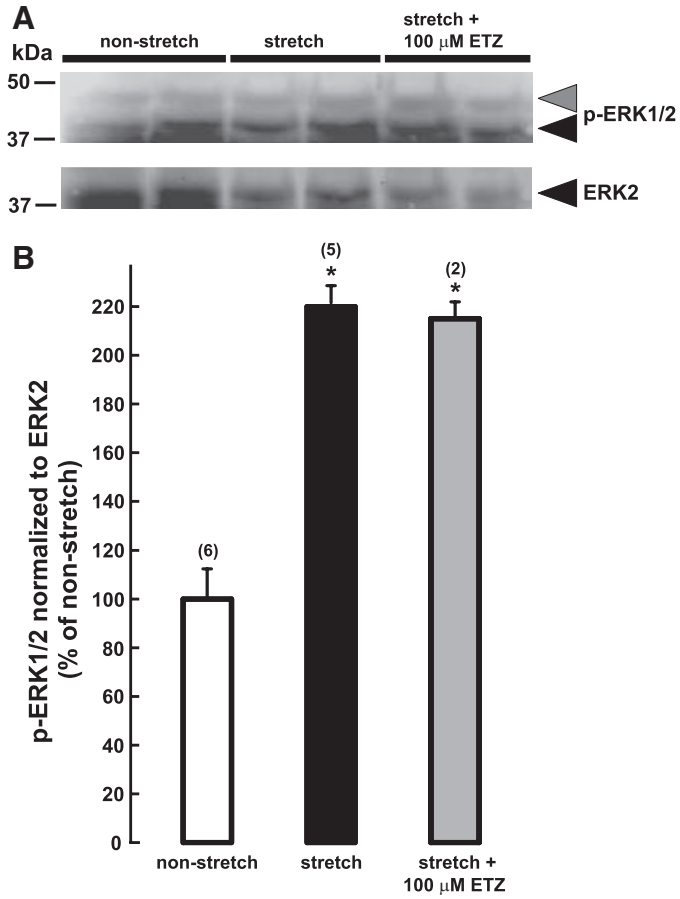

Fig. 7. Effect of stretch on ERK1/2 protein kinase phosphorylation in rat papillary muscles. *A*: rat ventricular lysates were prepared from isolated rat papillary muscles, which were nonstretched, or stretched in the absence or presence of 100 μM ETZ. Protein (60 μg) was loaded and immunoblot of the muscle lysates was probed with p-ERK1/2 antibody or antibody against ERK-2 as indicated. Gray and black arrows indicate position of ERK1 and ERK2, respectively. *B*: expression levels of p-ERK1/2 and ERK2 were quantified by densitometry. p-ERK1/2 expression was normalized to ERK2 in each muscle sample and expressed as a percentage of nonstretched muscle. Values are means ± SE. Parenthetical values on top of bar indicate number of muscles analyzed. *$P < 0.05$ vs. nonstretched.

cardiomyocytes subjected to stimulation with ANG II and the α-adrenergic agonist, phenylephrine (2). Moreover, cardiomyocytes from CAII-deficient mice do not respond to prohypertrophic stimulation, supporting a role of CAII in promoting cardiac hypertrophy (8). Treatment of both neonatal and adult cultured rat cardiomyocytes with the CA inhibitor, ETZ (Cardrase), prevents hormonally induced hypertrophy and reverses it once established (2). In addition, induction of the CAII gene has been reported in rats with spontaneous hypertension and failing heart (47), and in humans with cardiac hypertrophy and heart failure (4). Recently, we found that CAXIV maximizes the transmembrane HCO_3^- gradient for AE3, thereby activating its transport rate (2, 55). In addition, CAXIV expression is upregulated in the hypertrophic myocardium of spontaneously hypertensive rats (SHR), and this increased CAXIV expression enhances AE3-mediated HCO_3^- transport, thus creating a feed-forward cascade that generates a change in the intracellular milieu conducive to greater myocardial hypertrophy (2, 55). As mentioned above, coactivation of NHE1 and AE3 in the SHR loads cardiomyocytes with Na^+. The CAXIV-activated AE3 anion exchange activity in the SHR compensates for the enhanced NHE1 activity and blunts changes in the pH_i. However, the increased $Na^+{}_i$ detected in the hypertrophic myocardium of the SHR may still be present even in the absence of changes in myocardial pH_i. Thus increased $Na^+{}_i$ itself leads to a secondary increase in $[Ca^{2+}]_i$ through augmented NCX activity and so triggers a hypertrophic response. Prevention of SFR development after stretch of cardiac muscle in the presence of the membrane-impermeable CA-inhibitor BZ (Fig. 1) could be putative evidence for the activation of a CAXIV-AE3-NHE1 complex under these conditions. Furthermore, the NBCe1 Na^+/HCO_3^- cotransporter, which is a major contributor to intracellular alkalinization of the heart, has been recently shown to bind cytoplasmic CAII, GPI-anchored membrane CAIV, and transmembrane CAIX (37). Association of NBCe1 and any of these CAs could maximize the HCO_3^- flux through the transporter. Therefore, we cannot exclude the possibility that a fraction of the observed effects of BZ on the SFR to heart muscle stretch does not arise from inhibition of NBCe1. However, this hypothesis should be examined in a future work.

The SFR depends on activation of NHE1 (3, 13, 40), which is a target of redox-sensitive kinases such as the extracellular signal-regulated kinase (ERK), ERK1/2, and $p90^{RSK}$. Previous reports have shown that Ser703 in the regulatory COOH-terminal domain of NHE1 creates a binding motif for 14-3-3 proteins upon phosphorylation by the ERK1/2-$p90^{RSK}$ cascade (18, 29, 49, 53). Phosphorylation in the COOH-terminal NHE1 14-3-3 binding motif increased after feline myocardial stretch (56). In addition, a significant increase in ERK1/2 and $p90^{RSK}$ phosphorylation after stretch of feline myocardium has been reported (11), and inhibition of MEK, a kinase upstream of ERK1/2 and $p90^{RSK}$, abolished the SFR of feline heart muscles (11).

A recent study reports that reactive oxygen species (ROS) can mediate the upregulation of NHE1 gene expression and thus increase cell resistance to death, thus connecting the intracellular redox status with the cell sensitivity to death triggers (1). However, although ROS produce some beneficial effects in cells, it seems possible that ROS may also participate in several steps of the intracellular prohypertrophic signaling elicited by release of ANG II and/or ET1 in the myocardium (50), as well as the stretch of the cardiac muscle (41). In connection with these findings, myocardial stretch triggered the release/formation of ANG II/ET, which in an autocrine/paracrine manner leads to NOX activation and ROS-$O_2^{\cdot -}$ production with concomitant opening of the mitochondrial K_{ATP} channels and increased mitochondrial ROS-$O_2^{\cdot -}$ formation (11). In this context, mitochondrial $O_2^{\cdot -}$ or H_2O_2 (after dismutation) may activate the ERK1/2-$p90^{RSK}$ pathway, leading to phosphorylation and activation of the NHE1, thereby increasing $[Na^+]_i$ and generating the SFR through the NCX. Therefore, increased phosphorylation of kinases known to be activated by ANG II/ET and that target NHE1 occurs after myocardial stretch through a mechanism that appears to involve mitochondrial ROS. Thus we could speculate that after myocardial stretch, enhanced ROS production would phosphorylate and activate NHE1, increasing the binding of CAII to NHE1. However, we have not examined this possibility under our experimental conditions.

On the other hand, in vitro experiments showed that the phosphorylation of a site distant from the ultimate 26 COOH-

terminal amino acids of the NHE1 protein, increases the binding of CAII to NHE1 (30, 31). Previously, stretch of cardiac muscle has been shown to increase ERK1/2 and $p90^{RSK}$ kinase phosphorylation, an effect that is blocked by the AT1 ANG II receptor antagonist, losartan (11). In addition, the SFR after myocardial stretch was blunted by inhibiting the ERK1/2 signaling pathway (11). We found that the specific $p90^{RSK}$ inhibitor SL0101–1 did not affect the twitch force of isometric papillary muscle contraction nor the immediate response, after muscle stretching (Fig. 6*A*). However, SL0101-1 blocked the SFR after myocardial stretch in rat ventricular muscles (Fig. 6*B*) and prevented phosphorylation of NHE1 Ser703 after stretch of the rat cardiac muscle (data not shown). From our experiments, however, we cannot exclude that other kinases acting upstream or downstream of $p90^{RSK}$ phosphorylate NHE1 following cardiac muscle stretch. Inhibition of MEK (a kinase that is upstream of ERK1/2 and downstream of RAS) also abolished the SFR of rat feline muscles (11). Nevertheless, kinase inhibitors, PD98059 inhibiting MEK, and SL0101-1 inhibiting $p90^{RSK}$, both prevented the SFR to papillary muscle stretch, indicating that phosphorylation is a requirement for the mechanical counterpart that occurs after the autocrine/paracrine loop triggered by myocardial stretch.

Carbonic anhydrase II binding occurs on the group of amino acids comprising the length between 790 and 802 of the NHE1 COOH-terminus, with the S796 and D797 amino acids being most critical to the attachment (30, 31). Phosphorylation of the COOH-terminal 182 amino acids of NHE1 resulted in increased CAII binding (30). Although both ERK2 ($p42^{mapk}$) and CaM kinase II phosphorylate the last 182 amino acids of the NHE1 COOH-terminal tail, they did not affect the CAII/NHE1 complex formation, and mutations of both S796A and D797N NHE1 eliminate most of the in vitro phosphorylation of the last 26 amino acids of NHE1 (NHE1-L26) (30, 31). Hence, more proximal phosphorylation sites outside of the NHE1-L26 region have been proposed to be responsible for initiating CAII binding. Herein, we found that following stretch of the ventricular muscle, the binding of CAII to NHE1 increased and that stimulation of the NHE1/CAII complex arrangement triggered by stretch was affected when $p90^{RSK}$ phosphorylation was prevented (Fig. 6). Furthermore, stretch of the rat cardiac muscle increased phosphorylation of ERK1/2 kinase, and this effect was not prevented by CA inhibition (Fig. 7).

Localization of CAII to the cytosolic surface of NHE1 maximizes the local concentration of H^+ at the surface of NHE1, thereby activating the transport flux and regulating the NHE1-dependent SFR of the cardiac muscle after myocardial stretch. Therefore, CA inhibition may be an effective intervention to prevent the early signals following mechanical stretch that lead to pathological cardiac growth, in addition to other possible benefits to cardiac function arising from altering intra- and extracellular pH (52).

GRANTS

This work was supported by the Agencia Nacional de Promoción Científica Grant (PICT 2007 No. 01011, Res. No. 320/08) to B. V. Álvarez. B. V. Álvarez and N. G. Pérez are Established Investigators of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, Argentina). L. A. Vargas and R. G. Díaz are Fellows of CONICET.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.A.V., R.G.D., N.G.P., and B.V.A. performed experiments; L.A.V., R.G.D., N.G.P., and B.V.A. analyzed data; L.A.V., R.G.D., E.R.S., N.G.P., and B.V.A. interpreted results of experiments; L.A.V., R.G.D., and B.V.A. prepared figures; E.R.S., N.G.P., and B.V.A. conception and design of research; E.R.S., N.G.P., and B.V.A. edited and revised manuscript; N.G.P. and B.V.A. approved final version of manuscript; B.V.A. drafted manuscript.

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