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Splice Cassette II of Na^+,HCO_3^- Cotransporter NBCn1 (slc4a7) Interacts with Calcineurin A

IMPLICATIONS FOR TRANSPORTER ACTIVITY AND INTRACELLULAR pH CONTROL DURING RAT ARTERY CONTRACTIONS[*][S]

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Andreas A. Danielsen[‡§1], **Mark D. Parker**[¶1], **Soojung Lee**[‡§], **Walter F. Boron**[¶], **Christian Aalkjaer**[‡§], **and Ebbe Boedtkjer**[‡§2]

From the [‡]*Department of Biomedicine, Aarhus University, DK-8000 Aarhus C, Denmark, the* [§]*Water and Salt Research Center, Aarhus University, DK-8000 Aarhus C, Denmark, and the* [¶]*Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio 44106*

Background: Signaling pathways linking artery contraction to increased acid extrusion and intracellular pH control have been unknown.
Results: Calcineurin A interacts physically with Na^+,HCO_3^- cotransporter NBCn1 and is required for activation of Na^+,HCO_3^- cotransport during artery contraction.
Conclusion: Ca^{2+}-dependent activation of NBCn1 is mediated by calcineurin.
Significance: Calcineurin-dependent activation of NBCn1 is critical for intracellular pH control during artery contraction.

Activation of Na^+,HCO_3^- cotransport in vascular smooth muscle cells (VSMCs) contributes to intracellular pH (pH_i) control during artery contraction, but the signaling pathways involved have been unknown. We investigated whether physical and functional interactions between the Na^+,HCO_3^- cotransporter NBCn1 (slc4a7) and the Ca^{2+}/calmodulin-activated serine/threonine phosphatase calcineurin exist and play a role for pH_i control in VSMCs. Using a yeast two-hybrid screen, we found that splice cassette II from the N terminus of NBCn1 interacts with calcineurin Aβ. When cassette II was truncated or mutated to disrupt the putative calcineurin binding motif PTVVIH, the interaction was abolished. Native NBCn1 and calcineurin Aβ co-immunoprecipitated from A7r5 rat VSMCs. A peptide (acetyl-DDIPTVVIH-amide), which mimics the putative calcineurin binding motif, inhibited the co-immunoprecipitation whereas a mutated peptide (acetyl-DDIATAVAA-amide) did not. Na^+,HCO_3^- cotransport activity was investigated in VSMCs of mesenteric arteries after an NH_4^+ prepulse. During depolarization with 50 mM extracellular K^+ to raise intracellular $[Ca^{2+}]$, Na^+,HCO_3^- cotransport activity was inhibited 20–30% by calcineurin inhibitors (FK506 and cyclosporine A). FK506 did not affect Na^+,HCO_3^- cotransport activity in VSMCs when cytosolic $[Ca^{2+}]$ was lowered by buffering, nor did it disrupt binding between NBCn1 and calcineurin Aβ. FK506 augmented the intracellular acidification of VSMCs during norepinephrine-induced artery contractions. No physical or functional interactions between calcineurin Aβ and the Na^+/H^+ exchanger NHE1 were observed in VSMCs. In conclusion, we demonstrate a physical interaction between calcineurin Aβ and cassette II of NBCn1. Intracellular Ca^{2+} activates Na^+,HCO_3^- cotransport activity in VSMCs in a calcineurin-dependent manner which is important for protection against intracellular acidification.

Acid-base transport across the plasma membrane is crucial for intracellular pH (pH_i) control and hence for a multitude of cellular functions (1, 2). The need for cellular acid extrusion varies greatly under different physiological and pathological conditions, but in many cases the signaling pathways involved in regulating transporter activity are unknown. Functional consequences of disturbed pH_i include changes in ion channel function (3, 4) and intracellular enzyme activity (5, 6).

In vascular smooth muscle cells (VSMCs)[3] of resistance arteries, acid extrusion during intracellular acidification is mostly Na^+-dependent and mediated by the Na^+,HCO_3^- cotransporter NBCn1 (slc4a7) and the Na^+/H^+ exchanger NHE1 (slc9a1) (5, 7–10). In the near physiological pH_i range, NBCn1 is much more active than NHE1, and consequently, NBCn1 plays an important role for control of steady-state pH_i (5, 8, 10). In congruence, knock-out of NBCn1 inhibits vasocontractile responses through a pH_i-mediated decrease in rho-kinase-dependent VSMC Ca^{2+} sensitivity (5). Knock-out of NHE1 affects VSMC steady-state pH_i and rho-kinase-dependent signaling only in the absence of CO_2/HCO_3^- (10).

During artery contractions, VSMCs are exposed to an increased intracellular acid load independent of whether the

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[1] Both authors contributed equally to this work.
[2] To whom correspondence should be addressed: Dept. of Biomedicine, Aarhus University, Ole Worms Allé 6, Bldg. 1180, DK-8000 Aarhus C, Denmark. Tel.: 45-87167716; E-mail: eb@fi.au.dk.

[3] The abbreviations used are: VSMC, vascular smooth muscle cell; ANOVA, analysis of variance; BCECF, 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein; EIPA, 5-(*N*-ethyl-*N*-isopropyl) amiloride; IP, immunoprecipitation; PSS, physiological salt solution.

contraction is induced by norepinephrine (8), angiotensin II (11), Ca^{2+} ionophores (8, 11), or membrane depolarization with elevated extracellular $[K^+]$ (12). The mechanism for the increased acid load has not been determined conclusively but has been shown to depend on the increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (11). Enhanced metabolic acid production may play a role, but countertransport of H^+ by the plasma membrane Ca^{2+}-ATPase also appears to be important (13). Interestingly, previous studies have shown that Na^+,HCO_3^- cotransport in VSMCs is activated during artery contractions (12) but the signaling pathways responsible have not previously been defined.

Modulation of ion channel function by $[Ca^{2+}]_i$ is a widespread mechanism of control with relevance among others to Cl^-, K^+, Na^+, and Ca^{2+}channels (14–17). The mechanistic background varies but may involve direct interaction between Ca^{2+}-calmodulin and the respective ion channel or altered signaling through intracellular kinases or phosphatases (16). The Ca^{2+}/calmodulin-dependent serine-threonine phosphatase calcineurin is involved in regulation of ion transport activity; and in VSMCs, calcineurin modulates for instance the activity of L-type Ca^{2+}-channels (17) and ATP-sensitive K^+-channels (18). Calcineurin consists of a catalytic (calcineurin A) and a Ca^{2+}-binding (calcineurin B) subunit and contributes considerably to intracellular Ca^{2+}-dependent signal transduction (19).

Electroneutral Na^+,HCO_3^- cotransport independent of net Cl^- transport was first described in VSMCs of rat mesenteric arteries (20). Subsequently, NBCn1 was cloned from rat aorta and human skeletal muscle and characterized as an electroneutral, Cl^--independent Na^+,HCO_3^- cotransporter (21, 22). It consists of a large intracellular N terminus, 10–14 transmembrane segments and a smaller intracellular C terminus (23). A number of splice variants have been described with two variable splice regions (cassette I and II) in the N terminus and one (cassette III) in the C terminus; however, the functional consequences of these splice variations remain largely unknown (2, 23). Rat VSMCs express a variant of NBCn1 (rNBCn1-B) which contains the 123 amino acids long splice cassette II (21). A homologous sequence is expressed in humans where it comprises 124 amino acids (23).

In the present study, we tested the hypothesis that calcineurin interacts with binding motifs in cassette II of NBCn1, and that activation of calcineurin by intracellular Ca^{2+} provides a link between artery contraction and activation of Na^+,HCO_3^- cotransport in rat mesenteric artery VSMCs.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Assay—The yeast two-hybrid screen was performed using the MATCHMAKER Two-Hybrid System 3 (Clontech) according to the manufacturer's recommendations. Briefly, our "bait" construct, human NBCn1 cassette II cDNA subcloned into pGBKT7, was transformed by electroporation into the AH109 yeast strain. The transformed AH109 cells were mated with a culture of the Y187 yeast strain pretransformed with a library of human skeletal muscle MATCHMAKER cDNAs in pACT2 (purchased pretransformed from Clontech). Mated yeast that expressed a putative cassette II-interacting partner were selected on agar plates of minimal dropout medium that lacked adenine (complemented by interaction reporter gene 1), histidine (complemented by interaction reporter gene 2), leucine (complemented by pACT2), and tryptophan (complemented by pGBKT7). The medium also contained X-α-Gal to allow blue-white screening for cells expressing a putative cassette II-binding partner (by virtue of interaction reporter gene 3 that produced α-galactosidase). Plates were incubated at 30 °C for 3 days.

Identifying pACT2 Constructs from Positive Yeast Two-hybrid Colonies—Blue colonies were grown in liquid culture overnight. From each culture, the pACT2 cDNA construct encoding a putative cassette II-binding partner was isolated using a QIAprep Miniprep Kit (Qiagen). A single modification to the manufacturer's protocol for bacterial minipreparations was necessary to digest the yeast cell wall and allow for efficient cell lysis: instead of resuspending the cell pellet in their P1 buffer, we resuspended the cell pellet in phosphate-buffered saline (PBS) that contained 5 mg/ml lyticase (L2524; Sigma-Aldrich) and incubated at 37 °C for 30 min. Because the DNA yield was typically poor, the open reading frame of interest was amplified from pACT2 using vector-specific primers that flanked the open reading frame. Each PCR product was purified using a QIAquick PCR Purification Kit (Qiagen) and was sent for automated DNA sequencing (Keck DNA Sequencing Facility, Yale University, New Haven, CT) with those same two primers to confirm the open reading frame. Clones of interest were maintained by transformation into *Escherichia coli*.

One-on-one Yeast Two-hybrid Assay—The secretion of α-galactosidase by yeast that express interacting protein pairs can be quantified by a colorimetric assay and used as a proxy for the strength of interaction between the protein partners. The assay was performed according to the instructions in the Clontech Yeast Protocols Handbook that was supplied with the Yeast Two-Hybrid Kit. Briefly, individual pACT2 constructs were retransformed into Y187 yeast that were subsequently mated with AH109 yeast transformed with a pGBKT7-based bait construct. Mated yeast were selected on agar plates of minimal medium that lacked leucine and tryptophan.

Individual colonies were grown in liquid culture to mid-logarithmic phase (0.4–0.6 OD/ml) and the cells harvested by centrifugation. The supernatant was assayed for α-galactosidase activity by measuring the formation of colored product from colorless PNP-α-Gal in acetate buffer and normalized for the original culture density.

RT-PCR Analyses—Expression of NBCn1 and calcineurin Aβ at mRNA level was investigated in A7r5 rat aortic VSMCs using two-step RT-PCR analyses, as previously described (8). The primers for calcineurin Aβ were: forward, 5′-ACC ATG ATA GAA GTA GAA GCT CC-3′ and reverse, 5′-TAA CCT CTG TCC ACA TAA TCA C-3′. For NBCn1, the primers were: forward, 5′-AAG TTC CTC GGA ATT CGT GAA CAG-3′ and reverse, 5′-CTG GCA TGA GGT CAT CAA GCC AAC-3′.

Co-immunoprecipitation—Binding between native full-length NBCn1 and calcineurin Aβ was investigated using co-immunoprecipitation assays based on homogenates from A7r5 VSMCs. A previously characterized (24) anti-N-terminal antibody against NBCn1 (generously provided by Dr. Jeppe Praeto-

rius, Aarhus University, Denmark) was used. Additionally, commercially available antibodies (Santa Cruz Biotechnology) against calcineurin Aβ (sc-6124) and NHE1 (sc-136239) were employed in conjunction with species-matched horseradish peroxidase-coupled secondary antibodies (anti-rabbit, 7074P2, Cell Signaling; anti-goat, sc-2020, Santa Cruz Biotechnology; anti-mouse, 7076P2, Cell Signaling). Specificity of the bands obtained for calcineurin Aβ was confirmed by preincubating the primary antibody overnight at 5 °C with a five times greater concentration (w/v) of the immunizing peptide (sc-6124P; Santa Cruz Biotechnology). A7r5 VSMCs were grown in 25- or 75-cm^2 culture flasks with Dulbecco's modified Eagle's medium (DMEM, 01-055-1; Biological Industries, Kibbutz Beit-Haemek, Israel) added 2 mM L-glutamine, 50 international units/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum. For protein isolation, the cells were washed twice with cold Dulbecco's PBS (02-023-1; Biological Industries), immersed in 500 μl of lysis buffer, scraped mechanically, and transferred to Eppendorf tubes. Following gentle rotation at 5 °C for 1 h, samples were centrifuged at 16,000 × *g* for 15 min. The supernatant was mixed with the anti-NBCn1 or anti-NHE1 antibody and left at 5 °C overnight. Then, 50 μl of resin (protein A/G-agarose, 20421; Pierce) was washed three times in IP buffer and added to the samples (containing ~500 μg of total protein) and left under gentle rotation for 3 h at room temperature. After centrifugation at 16,000 × *g* for 1 min, the supernatant was removed and kept for immunoblotting. The resin was washed five times in washing buffer before loading buffer was added to give a total of 100 μl. Finally, samples were heated to 95 °C for 10 min and centrifuged at 16,000 × *g* for 1 min. 10 μl of the supernatant was loaded for immunoblotting, which was performed as described previously (5). The IP buffer contained 150 mM NaCl and 25 mM Tris, pH 7.2. The washing buffer was prepared by supplementing the IP buffer with 5 mM EDTA, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1× Halt protease inhibitor mixture (Thermo Scientific). The lysis buffer was obtained by adding 1% Triton X-100 to the washing buffer. The loading buffer contained 1 mM DTT, 58 mM Tris-HCl, 1.6% (w/v) SDS, 5% (v/v) glycerol, and 0.02% bromophenol blue.

To investigate the role of the putative calcineurin binding motif PTVVIH in cassette II of NBCn1, 150 μg of A7r5 cell lysate was incubated with 50 μg of a peptide (acetyl-DDIPTVVIH-amide; 100% purity; Caslo ApS, Lyngby, Denmark), which mimics the calcineurin motif, or with 50 μg of a mutated peptide (acetyl-DDIATAVAA-amide; 99% purity, Caslo ApS). The cell lysates were incubated with the peptides for 3 h before addition of the anti-NBCn1 antibody. The peptide sequences were extended with three N-terminal amino acids (DDI) beyond the putative calcineurin binding motif to increase the hydrophilicity of the peptides. N-terminal acetylation and C-terminal amidation were performed to eliminate the terminal charges and make the peptides more representable of internal protein sequences. Short peptides, which mimic calcineurin binding motifs, have previously been shown to inhibit calcineurin-dependent signaling in other cell types (25). Densitometric analyses were performed using ImageJ software (Rasband; National Institutes of Health).

Artery Tension, pH_i, and $[Ca^{2+}]_i$ Measurements—Mesenteric arteries with an internal diameter of ~250 μm were isolated from 10–12-week-old male Wistar rats euthanized by CO_2 inhalation followed by decapitation. All animal procedures were performed in accordance with Danish national legislation and guidelines from the Danish Animal Care and Use Committee. The isolated arteries were mounted in wire myographs (DMT, Aarhus, Denmark) and normalized to 90% of the internal diameter corresponding to a transmural pressure of 100 mmHg (26). VSMC pH_i and $[Ca^{2+}]_i$ were investigated using wide-field fluorescence microscopy of BCECF- and fura-2-loaded arteries, respectively. These procedures have previously been described in detail (8) except that experiments in the present study were performed on an Olympus IX70 microscope equipped with an Olympus LUCPlanFL N 20× objective (NA 0.45) and an EasyRatioPro fluorescence imaging system (Photon Technology International). Calibration of the BCECF fluorescence ratio was performed with the high-K^+ nigericin method (20) and further confirmed by the null-point technique (27); no discrepancies between results obtained with the two techniques were observed (supplemental Fig. 1). Intracellular buffering capacity was calculated from the change in pH_i following washout of 20 mM NH_4Cl as previously described (8, 28). Buffering capacity in the presence of CO_2/HCO_3^- at pH_i 6.9 was 30 ± 5 mM under control conditions compared with 30 ± 3 mM in the presence of 10 μM cyclosporine A ($n = 6$; $p = 0.96$, paired Student's *t* test). In another series of paired experiments, buffering capacity at pH_i 7.05 in the presence of CO_2/HCO_3^- was 22 ± 4 mM under control conditions compared with 22 ± 3 mM in the presence of 10 μM FK506 ($n = 8$; $p = 0.89$, paired Student's *t* test). Because no effect of the calcineurin inhibitors was seen on intracellular buffering capacity, pH_i recovery rates were calculated at similar pH_i levels and compared directly. NH_4Cl was initially washed out into a Na^+-free solution to measure the Na^+-independent pH_i recovery rate. Subsequently, Na^+ was returned to the bath solution, and the Na^+-dependent pH_i recovery rate measured.

The physiological salt solution (PSS) used for the functional experiments had the following composition: 114 mM NaCl, 10 mM HEPES, 24 mM $NaHCO_3$, 1.20 mM $MgSO_4$, 4.70 mM KCl, 5.50 mM glucose, 0.026 mM EDTA, 1.18 mM KH_2PO_4, and 1.60 mM $CaCl_2$. Solutions containing 50 mM extracellular K^+ (K-PSS) were obtained by substituting part of the NaCl with KCl. In HCO_3^--free solutions, NaCl replaced $NaHCO_3$. In Na^+-free solutions, Na^+ was replaced by *N*-methyl-D-glucammonium, except for $NaHCO_3$, which was replaced by choline-HCO_3. HCO_3^--containing solutions were aerated with 5% CO_2 balance air, HCO_3^--free solutions with air (nominally CO_2-free); pH was adjusted to 7.40 at 37 °C. Phentholamine (1 μM) was added to solutions containing 50 mM K^+ to inhibit α-adrenoreceptor stimulation by norepinephrine released from nerve terminals upon K^+-induced depolarization.

Statistics—Data are expressed as mean ± S.E. Paired two-tailed Student's *t*-tests were used for comparison of matched measurements obtained under two different conditions. The order in which matched measurements under different conditions were performed was alternated to eliminate potential effects of time. If more than two groups were compared, a one-

way ANOVA was employed followed by Bonferroni or Tukey post-tests. To evaluate the effects of two variables on the measured variable, we used two-way ANOVA followed by Bonferroni post-tests. When the variable was measured multiple times for each subject, repeated measures one-way or two-way ANOVA were employed. $p < 0.05$ was considered statistically significant; *n* equals the number of rats except in the yeast two-hybrid assays where *n* equals number of experiments and in the co-immunoprecipitation experiments where *n* equals number of preparations obtained from different culture flasks. Statistical analyses were performed using GraphPad Prism 5.04 software.

RESULTS

NBCn1 Interacts with Calcineurin A in a Yeast Two-hybrid Assay—Using cassette II of NBCn1 as bait to screen products of a human skeletal muscle cDNA library, the yeast two-hybrid assay produced 14 large colonies and 42 small colonies that were potentially expressing cassette II-interacting partners. DNA isolated from 3 of the large colonies and 20 of the small colonies were concluded to be false positives because they did not include useful protein coding sequences (*e.g.* sequence was out of frame) or included DNA sequences of nuclear or mitochondrial proteins. Three of the remaining 9 large colonies produced DNA that encoded calcineurin Aβ (encoded by the *PPP3CB* gene), and 3 of the remaining 22 small colonies produced DNA that encoded calcineurin Aγ (encoded by the *PPP3CC* gene). Other potential binding partners revealed by the assay are shown in supplemental Table 1. Only calcineurin Aβ was investigated further in the present study. We confirmed the interaction between cassette II of NBCn1 and full-length calcineurin Aβ using a one-on-one yeast two-hybrid assay, in which we crossed cassette II-expressing yeast with calcineurin Aβ-expressing yeast (Fig. 1*A*).

Interaction between Cassette II of NBCn1 and Calcineurin Involves Consensus Binding Motifs—In one-on-one α-galactosidase assay-based interaction screens with truncated cassette II baits, we showed that the interaction of NBCn1 with calcineurin Aβ was lost when cassette II was truncated by 57 but not by 14 amino acid residues, suggesting that residues 68–110 are necessary for the interaction (Fig. 1*A*). Truncation of cassette II by 14 amino acids in fact seemed to enhance the interaction with calcineurin Aβ compared with wild type cassette II (Fig. 1*A*); however, this most likely reflects a higher expression of the shorter bait. Removal of amino acid residues 68–110 of cassette II would disrupt or remove three motifs (PSISRLT, PELLVS, and PTVVIH in Fig. 1*B*) that are similar to the optimal consensus calcineurin binding motif (P*X*L*X*IT; for review see Refs. 29, 30). Additional analyses showed that mutation of the most C-terminal of the three putative calcineurin binding motifs PTVVIH (residues 94–99 of cassette II were mutated to ATAVAA) was sufficient to completely abolish the signs of interaction between cassette II and calcineurin Aβ (Fig. 1*B*). Mutation of either of the other two putative calcineurin binding motifs (residues 64–70 and 82–87 of cassette II, respectively) reduced but did not abolish the signs of interaction (Fig. 1*B*). Taken together, the yeast two-hybrid analyses suggest that calcineurin Aβ binds to splice cassette II of NBCn1 in a manner that minimally requires the putative calcineurin binding motif PTVVIH.

Calcineurin Aβ and NBCn1 Co-immunoprecipitate from VSMCs—The isoform of NBCn1 (rNBCn1-B) expressed in VSMCs contains splice cassette II (21, 23); because Ca^{2+}-mediated activation of NBCn1 would provide a relevant mechanism of activation during artery contraction, we investigated the hypothesis that NBCn1 and calcineurin interact physically and functionally in VSMCs. RT-PCR analyses confirmed the expression of both NBCn1 and calcineurin Aβ in A7r5 rat VSMCs at the mRNA level (supplemental Fig. 2).

Using a co-immunoprecipitation assay, we investigated whether calcineurin Aβ interacts with native full-length NBCn1 and/or NHE1 in A7r5 VSMCs (Fig. 2). Both NBCn1 and NHE1 were successfully immunoprecipitated (Fig. 2, *A* and *B*). Additionally, calcineurin Aβ was detected in the eluate from the resin following immunoprecipitation of NBCn1 (Fig. 2*C*) but not after immunoprecipitation of NHE1 (Fig. 2*E*). The band for calcineurin Aβ obtained after immunoprecipitation with the anti-NBCn1 antibody disappeared after preincubation of the anti-calcineurin Aβ antibody with its immunizing peptide (Fig. 2*D*), confirming the specificity. Although calcineurin Aβ did not co-immunoprecipitate with NHE1, it was detected in the cell lysates (Fig. 2*E*).

To further explore the role of the putative calcineurin binding motif PTVVIH for the interaction between native full-length NBCn1 and calcineurin Aβ, we next investigated whether the co-immunoprecipitation between NBCn1 and calcineurin Aβ was affected by a peptide that mimics this motif. We found that incubation of the A7r5 cell lysates with the peptide acetyl-DDIPTVVIH-amide abolished the interaction between NBCn1 and calcineurin Aβ whereas incubation with a mutated peptide (acetyl-DDIATAVAA-amide) did not (Fig. 2*F*). The level of co-immunoprecipitation in the presence of the acetyl-DDIPTVVIH-amide peptide was reduced to ~10% of that seen in the presence of the mutated peptide (Fig. 2*G*). These findings support that in VSMCs native full-length NBCn1 and calcineurin Aβ interact physically through the PTVVIH binding motif in cassette II of NBCn1.

Calcineurin Regulates Na^+,HCO_3^- Cotransport Activity When $[Ca^{2+}]_i$ Is Increased— Na^+,HCO_3^- cotransport activity was investigated in VSMCs of rat mesenteric isolated arteries using the NH_4^+ prepulse technique (an original trace is shown in supplemental Fig. 3). Na^+,HCO_3^- cotransport was measured as the Na^+-dependent pH_i recovery rate in the presence of 10 μM Na^+/H^+ exchange inhibitor EIPA. To evaluate the effect of Ca^{2+}-activated intracellular signaling pathways, experiments were performed in the presence of 50 mM extracellular K^+ to depolarize the VSMCs and activate Ca^{2+} influx through voltage-gated Ca^{2+}-channels. Indeed, under these conditions, VSMC $[Ca^{2+}]_i$ was increased (Fig. 3*A*), and 10 μM calcineurin inhibitor FK506 (also known as tacrolimus) reduced Na^+,HCO_3^- cotransport activity ~30% (Fig. 3, *B* and *C*). No effect of 10 μM FK506 on the Na^+-independent pH_i recovery rate was seen (Fig. 3*B*). When arteries were loaded with 30 μM BAPTA-AM, the increase in VSMC $[Ca^{2+}]_i$ following stimulation with 50 mM extracellular K^+ was greatly attenuated (Fig. 3*A*), and Na^+,HCO_3^- cotransport activity was reduced (Fig.

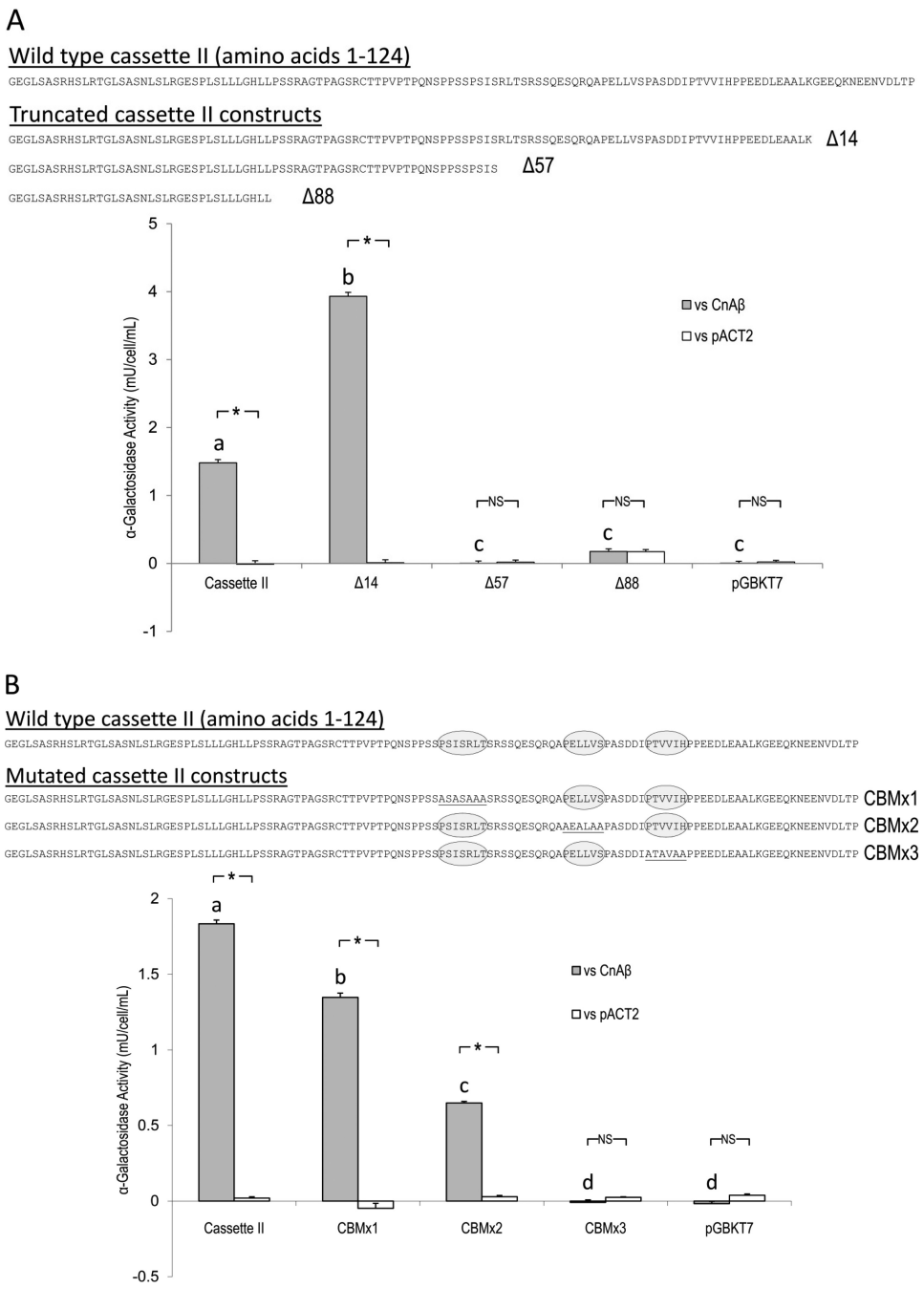

FIGURE 1. **Interaction between splice cassette II of NBCn1 and calcineurin Aβ requires specific binding motifs.** *A*, colorimetric assay ($n = 3$) of reporter gene activity to determine the calcineurin Aβ interaction site of cassette II using truncated cassette II baits. Data from parallel experiments performed without the calcineurin Aβ construct (*versus* pACT2) never elicited a signal >0.21 milliunit/cell per ml. *B*, colorimetric assay ($n = 3$) of reporter gene activity to determine the calcineurin Aβ interaction site of cassette II using mutated cassette II baits. Data from parallel experiments performed without the calcineurin Aβ construct (*versus* pACT2) never elicited a signal >0.04 milliunit/cell per ml. *Columns* marked by *different letters* were significantly different from each other when compared by one-way ANOVA followed by Tukey's post-tests. Reporter gene activity following co-expression of cassette II constructs and calcineurin Aβ was compared with experiments in which the cassette II constructs were co-expressed with pACT2 by a paired two-tailed Student's *t* test. *, $p < 0.05$; *NS*, not significantly different. *Error bars*, S.E.

3*D*). At these reduced levels of VSMC $[Ca^{2+}]_i$, 10 μM FK506 had no effect on the Na^+,HCO_3^- cotransport activity (Fig. 3*D*). The calcineurin inhibitor cyclosporine A (10 μM) inhibited Na^+,HCO_3^- cotransport activity to a similar extent as FK506 under conditions of increased VSMC $[Ca^{2+}]_i$ (Fig. 3*E*).

Binding between NBCn1 and Calcineurin Is Unaffected by $[Ca^{2+}]_i$ and Calcineurin Inhibition—Next, we investigated whether the stimulatory effect of increased $[Ca^{2+}]_i$ and inhibitory effect of FK506 on Na^+,HCO_3^- cotransport activity were due to an altered binding between NBCn1 and calcineurin Aβ. We found (Fig. 4) that the level of co-immunoprecipitation of calcineurin Aβ with NBCn1 was similar from A7r5 cells under control conditions (PSS) and A7r5 cells with an increased $[Ca^{2+}]_i$ induced by exposure to 50 mM extracellular K^+ (K-PSS). The level of co-immunoprecipitation was also unaffected by 10 μM FK506 (Fig. 4). These findings suggest that

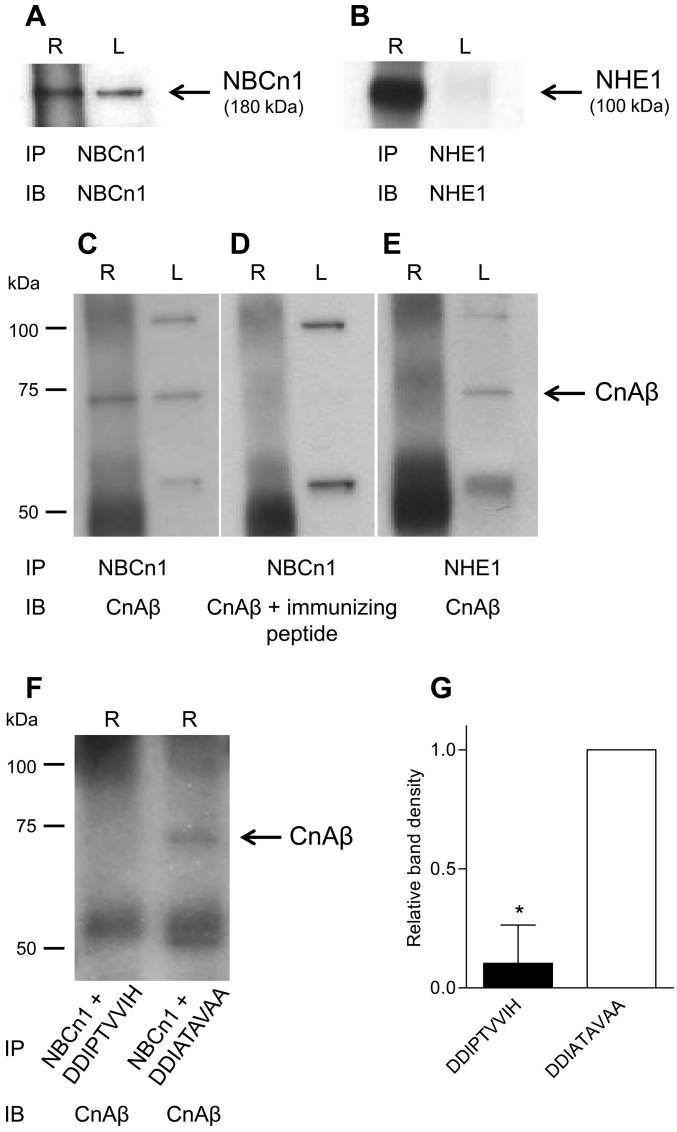

FIGURE 2. **NBCn1 and calcineurin Aβ (CnAβ) co-immunoprecipitate from A7r5 rat VSMCs, and the interaction can be blocked by a peptide that mimics the PTVVIH calcineurin binding motif.** *A*, NBCn1 was detected in the eluate from the resin (*R*) after immunoprecipitation with an anti N-terminal NBCn1 antibody. NBCn1 was also detected in the lysate (*L*). *IB*, immunoblotting. *B*, NHE1 was detected in the eluate from the resin after immunoprecipitation with the NHE1 antibody. A faint band was seen when a sample of the lysate was loaded. *C*, calcineurin Aβ was detected in the eluate from the resin following immunoprecipitation with an anti-N-terminal NBCn1 antibody. Calcineurin Aβ was also detected in the lysate. *D*, the ~70-kDa immunoreactive band for calcineurin Aβ was absent when the anti-calcineurin Aβ antibody was preincubated with its immunizing peptide. *E*, calcineurin Aβ could not be detected in the eluate from the resin following immunoprecipitation with an anti-NHE1 antibody although it was detected in the lysate. *F*, calcineurin Aβ was detected following immunoprecipitation of NBCn1 from A7r5 cell lysates incubated with an acetyl-DDIATAVAA-amide peptide but not when A7r5 cell lysates were incubated with an acetyl-DDIPTVVIH-amide peptide, which mimics the putative calcineurin binding motif in cassette II of NBCn1. *G*, densitometric analyses ($n = 3$) show the level of co-immunoprecipitation of calcineurin Aβ with NBCn1 in the presence of an acetyl-DDIPTVVIH-amide peptide or a mutated acetyl-DDIATAVAA-amide peptide. The comparison was performed using a paired two-tailed Student's *t* test. In lanes loaded with lysate, 10 μg of protein was added to each well. In lanes loaded with eluate from the resin, a 10-μl sample was added representing 10% of the total eluate obtained after addition of ~500 (*A–E*) or 150 (*F*) μg of total protein to the resin. *IB*, immunoblotting. *Error bar*, S.E.

calcineurin Aβ is continuously docked in a complex with NBCn1 and that increased calcineurin activity rather than a change in binding is responsible for the higher Na^+,HCO_3^- cotransport activity observed during conditions of increased $[Ca^{2+}]_i$. Similarly, the inhibitory effect of FK506 is most likely explained by reduced calcineurin activity rather than interference with the binding between NBCn1 and calcineurin Aβ.

Calcineurin Has No Effect on Na^+/H^+ Exchange Activity—We have previously shown that the Na^+/H^+ exchanger NHE1 is responsible for the Na^+-dependent pH_i recovery observed in VSMCs of resistance arteries in the absence of CO_2/HCO_3^- (7, 10). Following an intracellular acid load, no effect of 10 μM FK506 on Na^+/H^+ exchange activity in VSMCs was seen (Fig. 5). Taken together, the pH_i measurements support that calcineurin acts specifically to increase Na^+,HCO_3^- cotransport activity in VSMCs of resistance arteries when $[Ca^{2+}]_i$ is increased.

Calcineurin Is Important for pH_i Control in VSMCs during Artery Contractions—Na^+,HCO_3^- cotransport mediated by NBCn1 has been shown to be important for steady-state pH_i control and is purportedly activated during artery contractions (12). We therefore investigated whether inhibition of calcineurin affects pH_i regulation in contracting rat mesenteric arteries. Representative traces of the pH_i changes seen during norepinephrine-induced artery contraction are shown in Fig. 6*A*. Active wall tension upon norepinephrine stimulation was not significantly affected by 10 μM FK506 (Fig. 6*B*); however, the consequent pH_i decrease was greatly augmented (Fig. 6*C*). These findings support the hypothesis that the interaction between NBCn1 and calcineurin is important for activation of acid extrusion from VSMCs and hence for pH_i control during artery contraction.

DISCUSSION

Control of pH_i in VSMCs during artery contraction depends on activation of Na^+,HCO_3^- cotransport (12), but the signaling pathways responsible have until now been unknown. We have previously shown that NBCn1 is responsible for the Na^+,HCO_3^- cotransport in VSMCs (5, 8). Here, we show that NBCn1 interacts physically with the Ca^{2+}/calmodulin-dependent serine/threonine phosphatase calcineurin and that inhibition of calcineurin decreases Na^+,HCO_3^- cotransport activity in VSMCs during conditions of high $[Ca^{2+}]_i$. We furthermore provide evidence that calcineurin-dependent activation of Na^+,HCO_3^- cotransport plays a substantial role for control of VSMC pH_i during norepinephrine-induced vasoconstriction.

A binding site for calcineurin Aβ was found to reside within splice cassette II of NBCn1. Splice variants containing cassette II are expressed in VSMCs (21), heart (31), and skeletal muscle (22) but absent from most epithelia (24) and adult neuronal tissue (24, 32). The location of a calcineurin binding site within cassette II provides a novel mechanism of tissue-specific regulation of NBCn1 activity and represents an important first step toward understanding the functional importance of the multiple splice variants described for NBCn1 (23). Cassette II contains three putative calcineurin binding motifs. The most C-terminal of these (PTVVIH, amino acid residues 94–99 of cassette II) appears to be essential for the interaction between NBCn1 and calcineurin because mutation of this site completely abolished the interaction with calcineurin Aβ. The importance of the PTVVIH binding motif, which is very similar

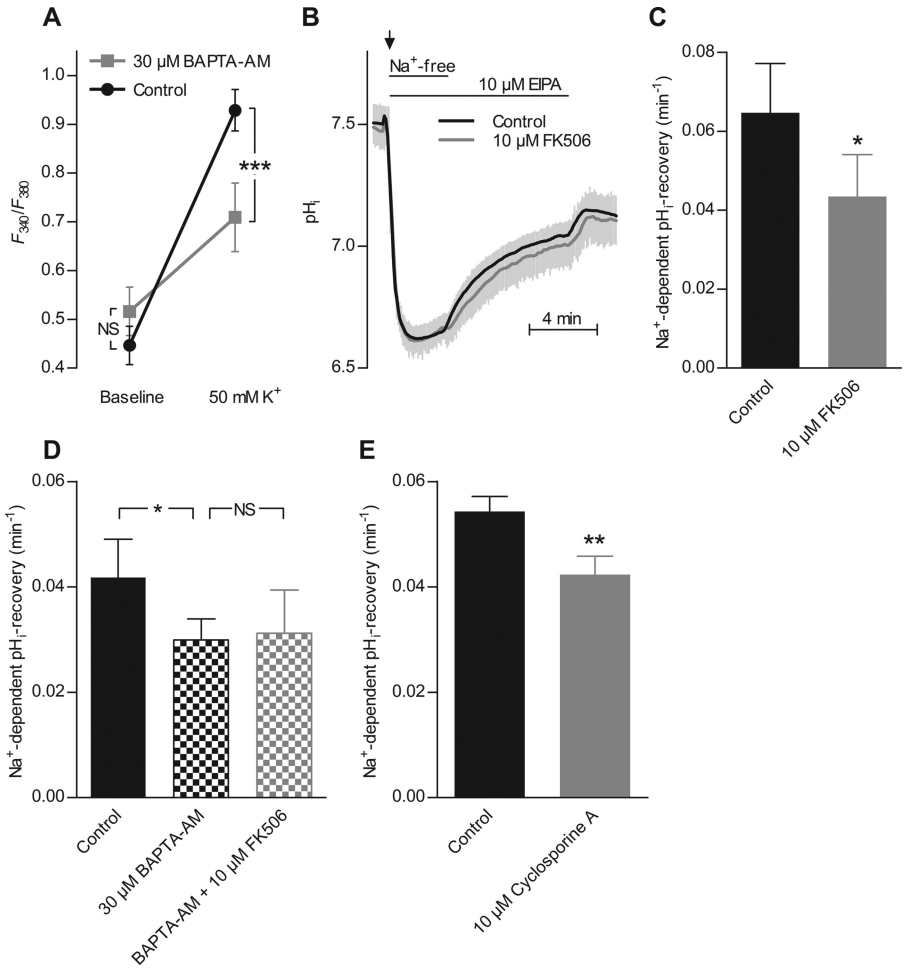

FIGURE 3. **Calcineurin-inhibition with 10 μM FK506 (also known as tacrolimus) or 10 μM cyclosporine A attenuates Na^+,HCO_3^- cotransport activity in VSMCs of rat mesenteric isolated arteries when intracellular [Ca^{2+}] is elevated.** *A*, fura-2-based measurements ($n = 6$) of $[Ca^{2+}]_i$ in VSMCs of mesenteric arteries at rest (with ~5 mM extracellular K^+) and during recovery from an intracellular acidification in the presence of 50 mM extracellular K^+. Comparisons were made with two-way repeated-measures ANOVA followed by Bonferroni post-tests. *B*, average traces ($n = 8$) of the pH_i recovery following an NH_4^+ prepulse in the presence of 50 mM extracellular K^+. The *arrow* indicates the time point at which 20 mM NH_4Cl was washed out. The experiments were performed in the presence of CO_2/HCO_3^-. *C*, net Na^+-dependent, EIPA-insensitive pH_i recovery rate ($n = 8$) measured after an NH_4^+ prepulse in the presence of 50 mM extracellular K^+ was attenuated by 10 μM FK506. The comparison was made by a paired two-tailed Student's *t* test. *D*, net Na^+-dependent, EIPA-insensitive pH_i recovery rates ($n = 6$) after an NH_4^+ prepulse in the presence of 50 mM extracellular K^+. Buffering of intracellular Ca^{2+} using BAPTA-AM significantly slowed down the rate of pH_i recovery. No further effect of adding 10 μM FK506 was seen. Comparisons were made with one-way repeated-measures ANOVA followed by Bonferroni post-tests. *E*, calcineurin inhibition with 10 μM cyclosporine A attenuated Na^+,HCO_3^- cotransport activity in VSMCs of rat mesenteric arteries following an intracellular acid load. The *columns* represent the net Na^+-dependent pH_i recovery rate ($n = 6$) in the presence of CO_2/HCO_3^- and 10 μM EIPA and with 50 mM extracellular K^+ to increase intracellular [Ca^{2+}]. The comparison was made with a paired two-tailed Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *NS*, not significantly different *versus* control or as specified. *Error bars*, S.E.

to the calcineurin docking site PSVVVH within the calcineurin regulatory protein calcipressin/RCAN1 (33, 34), was further confirmed by the ability of an acetyl-DDIPTVVIH-amide peptide to interfere with the interaction between native full-length NBCn1 and calcineurin Aβ in a co-immunoprecipitation assay. Mutation of either of the two more N-terminal calcineurin binding motifs reduced but did not abolish the interaction between NBCn1 and calcineurin, which may suggest (i) that these sites are able to bind calcineurin Aβ but only in the presence of the most C-terminal binding motif or (ii) that the strong binding of calcineurin Aβ to cassette II of NBCn1 is at the C-terminal binding motif whereas the two more N-terminal sites operate through an avidity effect by attracting and retaining calcineurin Aβ close to the C-terminal site.

Our data show that changes in $[Ca^{2+}]_i$ and addition of FK506 modulate NBCn1 activity but do not alter the binding between calcineurin and NBCn1. These findings imply that the regulation of NBCn1 by calcineurin is not due to a conformational change or steric effect induced by the binding of calcineurin *per se* but rather is dependent on its phosphatase activity. We propose that the physical interaction between NBCn1 and calcineurin serves to maintain calcineurin at the appropriate location to efficiently alter the phosphorylation state of NBCn1 or an interacting partner and thereby regulate NBCn1 transport activity. In support of this finding, both NBCn1 (35–37) and known NBCn1-interacting partners (*i.e.* cystic fibrosis transmembrane conductance regulator, CFTR (38)) have previously been shown to be phosphorylated although the functional consequences of the phosphorylations require further investigations. For a more elaborate discussion, see Ref. 2.

Calcineurin inhibitors have a multitude of cellular effects which both in the short and the long term interfere with artery

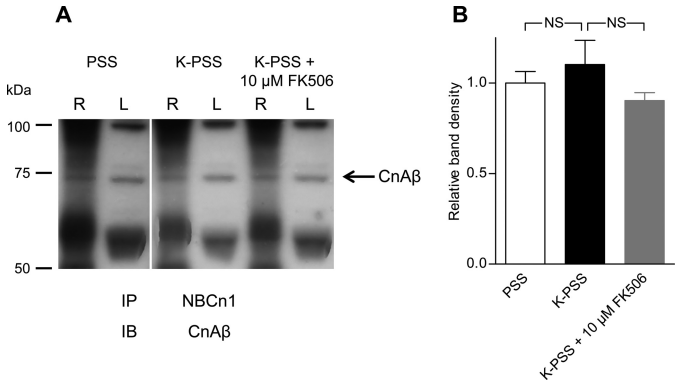

FIGURE 4. **Co-immunoprecipitation of NBCn1 and calcineurin Aβ is unaffected by $[Ca^{2+}]_i$ and calcineurin inhibition.** *A*, representative bands for calcineurin Aβ (*CnAβ*) detected in the eluate from the resin (*R*) following immunoprecipitation with an anti N-terminal NBCn1 antibody. A7r5 cells were incubated in PSS, K-PSS, or K-PSS with 10 μM FK506 (as indicated) prior to cell lysis. Calcineurin Aβ was also detected in the lysate (*L*) under all three conditions. *B*, average results of densitometric analyses ($n = 3–4$). The level of co-immunoprecipitation of calcineurin Aβ with NBCn1 was expressed normalized to the level of co-immunoprecipitation from A7r5 cells in PSS run in parallel experiments on the same gel. The comparison was made using one-way repeated-measures ANOVA followed by Bonferroni post-tests. *NS*, not significantly different. In each *lane*, a 10-μl sample was added representing 10% of the total eluate obtained after addition of ~500 μg of total protein to the resin. *IB*, immunoblotting. *Error bars*, S.E.

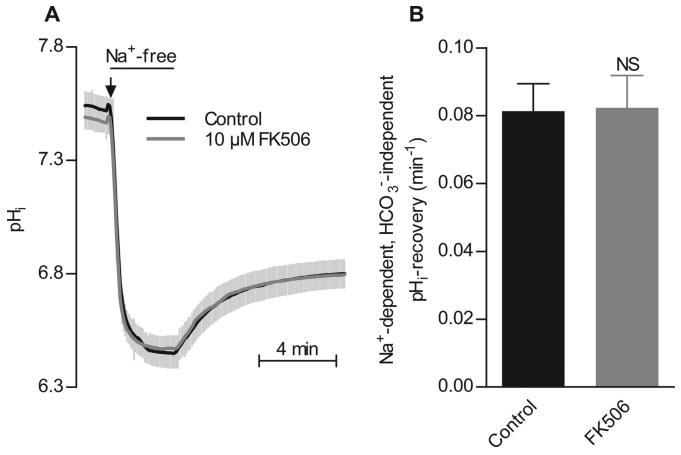

FIGURE 5. **Calcineurin-inhibition with 10 μM FK506 does not affect Na^+/H^+ exchange activity in VSMCs of rat mesenteric arteries.** *A*, average traces ($n = 6$) of the pH_i recovery in VSMCs following an NH_4^+ prepulse. The *arrow* indicates the time point at which 20 mM NH_4Cl was washed out. The experiments were performed in the absence of CO_2/HCO_3^- with 50 mM extracellular K^+ to increase intracellular $[Ca^{2+}]$. *B*, net Na^+-dependent pH_i recovery rate ($n = 6$) measured after an NH_4^+ prepulse in the absence of CO_2/HCO_3^- was unaffected by 10 μM FK506. Experiments were performed in the presence of 50 mM extracellular K^+. The comparison was made by paired two-tailed Student's *t* test. *NS*, not significantly different *versus* control. *Error bars*, S.E.

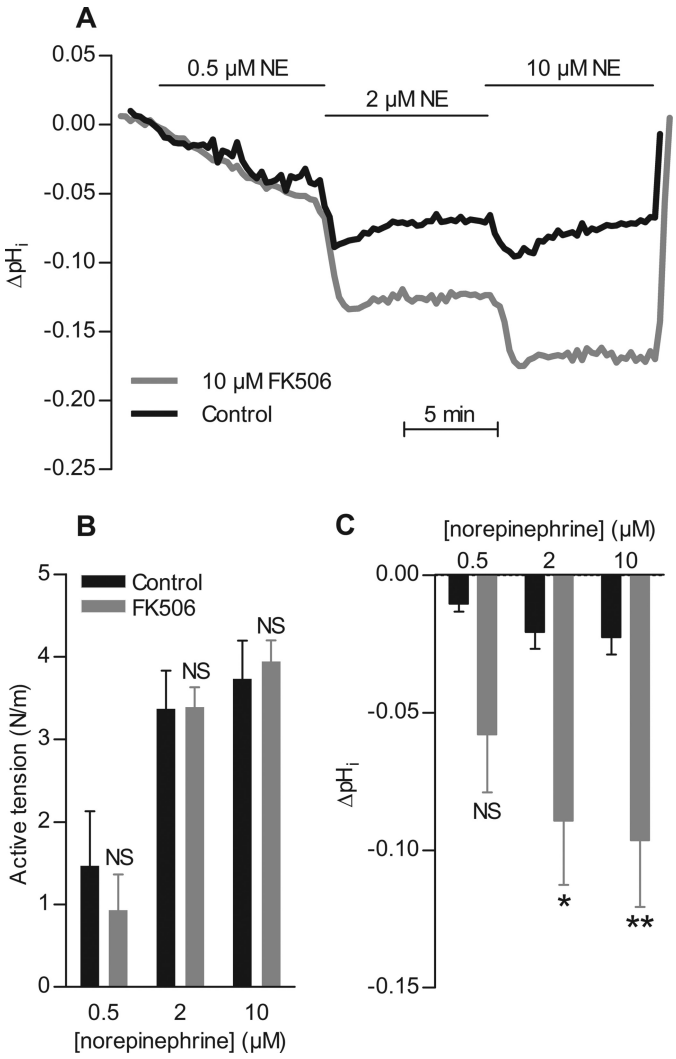

FIGURE 6. **Calcineurin inhibition results in larger contraction-induced intracellular acidifications in VSMCs of rat mesenteric arteries despite unchanged active tension production.** *A*, traces of the VSMC pH_i changes observed in response to increasing concentrations of norepinephrine (*NE*) in rat mesenteric arteries under control conditions and in the presence of 10 μM FK506. *B*, active tension production in rat mesenteric arteries exposed to increasing concentrations of norepinephrine in the presence or absence of 10 μM FK506. Experiments ($n = 8$) were performed with CO_2/HCO_3^- present. No significant net effect of FK506 on active tension development was seen. *C*, average changes ($n = 8$) in pH_i upon exposure to increasing concentrations of norepinephrine in the presence or absence of 10 μM FK506. Experiments were performed in the presence of CO_2/HCO_3^-. Contraction-induced acidifications were enhanced in the presence of FK506. Average values for tension and pH_i during the last 2 min of the 10-min long norepinephrine stimulations are shown. Comparisons were made by two-way repeated-measures ANOVA followed by Bonferroni post-tests. *, $p < 0.05$; **, $p < 0.01$; *NS*, not significantly different *versus* control. *Error bars*, S.E.

constriction and dilation (39, 40). It is likely that effects of VSMC acidification secondary to reduced NBCn1 activity play a mechanistic role although a number of other pathways (*e.g.* signaling through nuclear factor of activated T cells (NFAT)) are also of major importance (29). At this point, it is not possible to directly evaluate the consequences of the interaction between NBCn1 and calcineurin Aβ for vasomotor function and integrated artery responses due to the existence of multiple calcineurin-dependent signaling pathways and the lack of experimental tools to specifically interfere with the interaction in intact arteries. The magnitude of change in VSMC pH_i observed following calcineurin inhibition during artery contraction is, however, predicted to have substantial effects on vasomotor function (5, 10). As recently reviewed in detail (41), both acute and sustained disturbances in VSMC pH_i have pronounced effects on vasomotor function. Acute changes in pH_i interfere with Ca^{2+} homeostasis in VSMCs (42), which can likely be attributed to competition between the intracellular H^+ and Ca^{2+} for buffer binding (43, 44) and possibly to altered ion channel function (3, 4). Sustained changes in pH_i affect VSMC contractions primarily by altering the Ca^{2+} sensitivity of the contractile machinery (5, 8, 10). A number of enzymes critical

for vascular function (*e.g.* rho-kinase (5), NO-synthase (5, 6), endothelin-converting enzyme (45)) show pronounced intrinsic pH sensitivities. Even changes of 0.1 unit magnitude are sufficient to change enzymatic activities to a functionally relevant extent (5) and are expected to be of consequence for normal artery function and cardiovascular disease development.

In the present study, we found no effect of calcineurin inhibition on Na^+/H^+ exchange activity. In contrast, calcineurin homologous proteins, which show protein sequence homology to calcineurin B, have previously been shown to interact with NHE1 and increase transporter activity (46, 47). Considering the strict sequence requirements for many protein-protein interactions and the stringent substrate specificity of most catalytic processes, the ability of calcineurin homologous proteins but not calcineurin to regulate Na^+/H^+ exchange activity is not unexpected and provides a possible mechanism for independent control of NBCn1 and NHE1 activity.

It was recently reported that artery remodeling induced by angiotensin II infusion is prevented by inhibitors of calcineurin (48). A potential role for calcineurin in artery remodeling is interesting in the context of acid-base transport because we and others have reported that NHE1 is important for artery structure development and remodeling (10, 49, 50). Further investigations are required to determine whether changes in NBCn1 activity induced by calcineurin inhibitors contribute to the observed effects on artery remodeling.

In conclusion, we demonstrate a novel physical interaction between calcineurin Aβ and cassette II of NBCn1. We furthermore show that intracellular Ca^{2+} activates Na^+,HCO_3^- cotransport activity in rat VSMCs in a calcineurin-dependent manner and provide a novel mechanism for protection of VSMCs against intracellular acidification during artery contraction.

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