Calcium-Induced Activation of the Rat Vascular Myocyte Na^+/H^+ Exchanger Isoform-1

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An established intermediate phenotype of human hypertension and diabetic nephropathy is an elevation of Na^+/H^+ exchanger (NHE) activity, but the mechanism for this is unclear. This phenotype is maintained in vascular myocytes from the spontaneously hypertensive rat (SHR) compared with the normotensive Wistar Kyoto rat (WKY). Since intracellular calcium levels ($[Ca^{2+}]_i$) following agonist stimulation were elevated in cells from both hypertensive humans and SHR, we have examined the role of calcium-calmodulin (CaM) in the mechanism of increased NHE activity in vascular myocytes of SHR by determining the activity and phosphorylation state of NHE isoform-1 (NHE-1) in cells from SHR and WKY when $[Ca^{2+}]_i$ was elevated by the ionophores A23187 or ionomycin. NHE activity was measured using fluorometry and NHE-1 phosphorylation by immunoprecipitating the exchanger from ^{32}P-orthophosphate–labeled cells with a polyclonal NHE-1–specific antibody. The ionophore A23187 increased $[Ca^{2+}]_i$ in both cell types to approximately 700 to 800 nmol · L^{-1}, and led to stimulation of NHE-1 activity only in WKY myocytes, with no effect on SHR cells. An inhibitor of CaM kinase II (KN-62) failed to abolish stimulation of NHE-1 by A23187 in WKY cells, and had no effect on unstimulated NHE-1 activity in both cell types. Ionomycin also elevated $[Ca^{2+}]_i$ in both cell types to approximately 1,000 nmol · L^{-1} and activated NHE-1 activity in only WKY cells. Activation of NHE-1 in WKY cells by an increased $[Ca^{2+}]_i$ was not mediated by an increase in NHE-1 phosphorylation, whether in the presence or absence of KN-62. The elevated NHE-1 phosphorylation in SHR cells was not affected by elevated $[Ca^{2+}]_i$ or KN-62. Calmodulin-agarose beads bound NHE-1 extracted from SHR cells to a lesser extent than that from WKY cells. We conclude that calcium-induced NHE-1 activation in WKY cells was not mediated by CaM kinase II. The elevated NHE-1 activity and phosphorylation of SHR cells was not further modulated by increased $[Ca^{2+}]_i$, and was also independent of CaM kinase II. Non–phosphorylation-dependent mechanisms of activation of NHE-1 may therefore be responsible for alterations of NHE-1 activity in these cells, such as the direct binding of CaM to NHE-1. This direct binding of CaM to NHE-1 may be impaired in SHR compared with WKY cells.

THE SODIUM/HYDROGEN (Na^+/H^+) exchanger (NHE) is a ubiquitous plasma membrane glycoprotein that is important for regulation of cellular pH and volume and may also be activated during stimulation of growth responses by agonists. Increased NHE activity is a consistent membrane-transport intermediate phenotype described in a variety of cell types from patients with essential hypertension and diabetic nephropathy, and also from the spontaneously hypertensive rat (SHR) compared with the Wistar Kyoto rat (WKY).[1] This enhanced activity is preserved in cultured vascular myocytes from SHR[2-4] and may depend on genetic rather than humoral factors. Further analysis of this exchanger anomaly has indicated that only mRNA of NHE isoform-1 (NHE-1) is present in vascular myocytes and that there is no increased expression of this mRNA species in SHR cells.[5] We have also previously demonstrated that a NHE-1–specific polyclonal antibody could detect the presence of this isoform as a 95-kd protein from cultured vascular myocytes[6] and that there was no increased NHE-1 protein content in cultured cells or tissue extracts from SHR, indicating that increased NHE activity was not due to elevated protein content.

The importance of posttranslational processes such as phosphorylation of the NHE-1 protein has been proposed as a possible mechanism for its activation.[7-9] Agonists such as α-thrombin, growth factors, okadaic acid, and phorbol esters have been demonstrated to cause a dramatic increase in NHE-1 phosphorylation together with enhancement of exchanger activity. We also demonstrated previously that NHE-1 phosphorylation of quiescent SHR vascular myocytes was elevated approximately twofold compared with that of WKY cells,[10] although stimulation of NHE activity by serum did not lead to further enhancement of phosphorylation.[10] Similar experiments confirmed the elevated NHE activity and enhanced NHE-1 phosphorylation in lymphoblasts from hypertensive patients compared with cells from normotensive patients.[11] Thus, cells from hypertensive individuals and SHR may exhibit increased NHE activity not associated with an increased cellular NHE-1 protein content, but with an increase in NHE-1 phosphorylation, although the role that this plays in the activation of NHE-1 remains to be determined.

Recent evidence has pointed to other mechanisms that could activate NHE-1 in addition to phosphorylation. For example, based on deletion mutant studies, unknown regulatory proteins have been postulated to bind to certain domains of the NHE-1 cytoplasmic C-terminal that may affect its agonist-stimulated activity.[12] Furthermore, calcium-calmodulin (CaM) has been demonstrated to bind directly to an autoinhibitory domain on the C-terminal of NHE-1 and may be involved in stimulation of its activity.[13] In addition, previous study has also suggested that there are consensus sites for CaM kinase II on the C-terminal of NHE-1, and direct phosphorylation of the C-terminal of a fusion protein by purified CaM kinase II was demonstrated.[14] Activation of NHE-1 by CaM therefore may involve a kinase-independent mechanism involving direct binding to NHE-1 or may be mediated via CaM kinase II.

Since unstimulated SHR vascular myocytes may exhibit elevated cytosolic calcium levels ($[Ca^{2+}]_i$)[15-17] and also show

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enhanced phospholipase C and $[Ca^{2+}]_i$ responses to agonists,[18-20] we have further examined the role of cytosolic calcium in the activation of NHE-1 in SHR vascular myocytes. $[Ca^{2+}]_i$ was elevated by the calcium ionophore A23187, and NHE activity and NHE-1 phosphorylation were then determined. The dependence of this NHE activity on CaM kinase II was examined using a specific inhibitor of CaM kinase II (KN-62: 1-[*N,O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyrosyl]-4-phenyl-piperazine), as originally described by Tokumitsu et al.[21] Our studies demonstrate that NHE-1 in WKY cells could be activated by an increased $[Ca^{2+}]_i$, but that the activity in SHR cells (which was already elevated) could not be thus modulated. Furthermore, activation in WKY cells was not associated with any increase in NHE-1 phosphorylation and was not inhibited by KN-62. Elevated NHE-1 activity and phosphorylation in SHR cells also was not susceptible to KN-62. The importance of other non–phosphorylation-dependent mechanisms of activation of NHE-1 is thus indicated, with one possibility being direct binding of CaM to NHE-1.[12] At calcium concentrations achieved by ionophore treatment, calmodulin bound NHE-1 extracted from SHR cells to a lesser extent than that from WKY, a finding that may explain the ability of the calcium ionophore to enhance NHE-1 activity in WKY cells with little effect on that in SHR cells.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium, Ham's F12 medium, chick embryo extract, and ^{32}P-orthophosphate were from ICN Flow (High Wycombe, Bucks, UK). Fetal calf serum (FCS) was from Advanced Protein Products (Birmingham, UK). Ham's F12 growth medium was buffered with 14 mmol · L^{-1} $NaHCO_3$ (pH 7.1 with 5% CO_2 in air) and contained 15% FCS, 0.5% (wt/vol) chick embryo extract, 2 mmol · L^{-1} glutamine, 10^5 IU · L^{-1} penicillin, and 100 mg Z L^{-1} streptomycin. ^{14}C molecular-weight markers, the enhanced chemiluminescence kit, and Aurodye were obtained from Amersham International (Amersham, UK). Protein A Sepharose CL4B was purchased from Pharmacia (Uppsala, Sweden). 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) was from Cambridge Bioscience (Cambridge, UK). The CaM kinase II inhibitor KN-62 (1-[*N,O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-ty rosyl]-4-phenyl-piperazine), ionomycin, and the CaM kinase II substrate MHRQETVDCLK (corresponding to residues 281 to 291 of the α-subunit of CaM kinase II) were from Calbiochem (Nottingham, UK). All other chemicals, including the ionophore A23187, 3-[*N*-morpholino]propanesulfonic acid (MOPS), and calmodulin-agarose, were from Sigma Chemical (Poole, UK). The NHE-1–specific inhibitor HOE-694 (3-methylsulfonyl-4-piperidinobenzoyl guanidine)[22] was obtained from Dr W. Scholz (Hoechst, Frankfurt, Germany). P81 phosphocellulose paper was from Whatman (Maidstone, UK).

Culture of Vascular Myocytes and Measurement of NHE Activity

Vascular myocytes were obtained from thoracic aortae of WKY and SHR maintained at a colony established at the Biomedical Services Unit of Leicester University. Blood pressures of 12-week-old rats by the tail-cuff method confirmed values in SHR of greater than 170 mm Hg, with values of less than 120 mm Hg in WKY. Vascular myocytes were obtained by enzymatic digestion with collagenase as previously described[3,6,10,20] and were cultured in Ham's F12 growth medium. All experiments reported were performed on cultures between passage three and eight.

Measurements of NHE activity were performed on confluent vascular myocytes on cover slips.[3,20] Serum was withdrawn for 24 hours before study, and cytosolic pH (pH_i) measurements were performed after loading the cells with the fluorophore BCECF.[3,10] pH_i and Na^+–dependent H^+ fluxes were measured in HEPES-buffered saline (HBSS) composed of NaCl 130 mmol · L^{-1}, KCl 5 mmol · L^{-1}, $CaCl_2$ 1.8 mmol · L^{-1}, $MgSO_4$ 1 mmol · L^{-1}, glucose 5 mmol · L^{-1}, HEPES 20 mmol · L^{-1} and BSA 1 g · L^{-1}, pH 7.4, after clamping pH_i to 6.0.[3,10] Buffering capacity at pH_i 6.0 was also measured using a NH_4Cl pulse. Calibration was achieved with isotonic KCl buffers of different pH, containing nigericin and monensin (5 μmol · L^{-1} of each).

To determine the effects of increased $[Ca^{2+}]_i$, cells were incubated with the calcium ionophore A23187 (10 μmol · L^{-1}) in HBSS for 20 minutes at 37°C. After scavenging the ionophore with BSA and clamping pH_i at 6.0, Na^+-dependent H^+ fluxes were measured, together with buffering capacities. Another calcium ionophore, ionomycin (1 μmol · L^{-1}), was also used to confirm the findings obtained with A23187. $[Ca^{2+}]_i$ was measured using similarly treated fura-2–loaded myocytes,[23] and $[Ca^{2+}]_i$ was calculated as previously described.[23] The dependence of fluxes on CaM kinase II was assessed by preincubation of cells with the inhibitor KN-62 (10 μmol · L^{-1}) in HBSS for 30 minutes at 37°C. $[Ca^{2+}]_i$ was then increased in these cells using A23187 as described earlier.

Assay of CaM Kinase II Activity in Cell Lysates

CaM kinase II was activated in WKY cells by adding 10 μmol · L^{-1} A23187 to quiescent monolayers and incubating for 10 minutes at 37°C. Identical cultures were pretreated with 10 μmol · L^{-1} KN-62 for 30 minutes before stimulation with A23187. Cells were then quickly rinsed twice with ice-cold HBSS and scraped into lysis buffer consisting of MOPS 50 mmol · L^{-1}, pH 7.4, sodium fluoride 100 mmol · L^{-1}, sodium pyrophosphate 100 mmol · L^{-1}, dithiothreitol 2 mmol · L^{-1}, EGTA 2 mmol · L^{-1}, phenylmethylsulfonyl fluoride 0.2 mmol · L^{-1}, aprotinin 0.4 U/mL, and Nonidet P-40 1%. Following sonication for 15 minutes in a bath, extracts were centrifuged at 14,000 × *g* for 10 minutes. Ten microliters of the supernatant was added to 25 μL assay buffer composed of MOPS 10 mmol · L^{-1}, pH 7.4, $MgCl_2$ 10 mmol · L^{-1}, EGTA 3 mmol · L^{-1}, and ATP 0.2 mmol · L^{-1}. To each tube was added 0.2 MBq ^{32}P-γ-ATP and 5 μg CaM kinase II substrate MHRQETVDCLK. Duplicate samples without the substrate were also treated with ^{32}P-γ-ATP (0.2 MBq). Following an incubation of 15 minutes at 30°C, reactions were terminated by addition of 0.5 mol/L trichloroacetic acid. Supernatants were spotted onto P81 phosphocellulose paper. The pieces of phosphocellulose paper were washed four times in 1% orthophosphoric acid and once with acetone before determination of counts by Cerenkov counting. Proteins in the extracts were determined using a BioRad (Hertfordshire, UK) detergent-compatible protein assay kit.

Determination of NHE-1 Phosphorylation

A detailed description of the phosphorylated NHE-1 immunoprecipitation protocol has previously been published.[10,11] Briefly, the quiescent vascular myocyte cultures were incubated with ^{32}P-orthophosphate (50 μCi · mL^{-1}) for 3 hours in phosphate-free HBSS. Following rapid washes with cold HBSS, liquid nitrogen was poured onto the monolayers. One milliliter of cold (4°C) extraction buffer (buffer E) containing 10 g · L^{-1} polyoxyethylene-8-lauryl ether, Tris 30 mmol · L^{-1}, NaCl 130 mmol · L^{-1}, EDTA 5 mmol · L^{-1}, phenylmethylsulfonyl fluoride 1 mmol · L^{-1}, *o*-phenanthroline 1 mmol · L^{-1}, iodoacetamide 1 mmol · L^{-1}, Na fluoride 100 mmol · L^{-1}, Na orthovanadate 5 mmol · L^{-1}, ATP 10 mmol · L^{-1}, Na pyrophosphate 10 mmol · L^{-1}, pepstatin A 1 mg · L^{-1} and leupeptin 2 mg · L^{-1} was poured over the frozen cells. The monolayers were then scraped off using cell scrapers. After sonicating these extracts, they were centrifuged at 14,000 × *g*. The supernatant was then preabsorbed with protein A–Sepharose CL4B beads. NHE-1–

specific antibody G253 (final concentration, 100 μg · mL^{-1}) was added to the supernatants. After 2 hours, immunoprecipitates were recovered following an additional 1-hour incubation with protein A–Sepharose CL4B beads. The beads were washed extensively, Laemmli sample buffer was added, and the extracts were boiled. Phosphoproteins were resolved on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and quantified by autoradiography on preflashed x-ray films. NHE-1 can be identified as a 95-kd phosphoprotein.[10] All values have been normalized to an arbitrary value of 1 for the quiescent WKY cell extract.

The effect of increased $[Ca^{2+}]_i$ was investigated using a protocol similar to that devised for fluorometric studies. Cells preloaded with ^{32}P-orthophosphate were treated with 10 μmol · L^{-1} A23187 for 20 minutes to increase $[Ca^{2+}]_i$. At the end of this period, the fluid was aspirated and the cells were snap-frozen in liquid nitrogen following two brief washes with HBSS. Incubations with KN-62 (10 μmol · L^{-1}) were performed over 30 minutes at 37°C before addition of A23187 for a further 20 minutes. Incubations were then terminated by freezing in liquid nitrogen.

Determination of NHE-1 Binding to Calmodulin-Agarose Beads

Quiescent SHR and WKY myocytes were rinsed in phosphate-buffered saline and then snap-frozen in liquid nitrogen. The cells were scraped into buffer E (which lacked the detergent polyoxyethylene-8-lauryl ether). The suspension of cells was then further disrupted by nitrogen cavitation[24] using a pressure of 10 bars for 15 minutes at 4°C. The homogenate was then centrifuged at 600 × *g* for 10 minutes to obtain a postnuclear supernatant. The supernatant was centrifuged at 100,000 × *g* for 30 minutes to recover a crude membrane pellet. This was washed once in buffer F consisting of NaCl 140 mmol · L^{-1} and HEPES 20 mmol · L^{-1}, pH 7.4, containing ovalbumin 1 g · L^{-1} as carrier, to remove the EDTA and protease inhibitors in buffer E. The pellet was resuspended in buffer F containing the detergent polyoxyethylene-8-lauryl ether (10 g · L^{-1}) and sonicated for 15 minutes at 4°C to extract NHE-1. This suspension was then centrifuged at 50,000 × *g* for 30 minutes, and the supernatant containing solubilized proteins (including NHE-1) was retained.

Calmodulin-agarose beads were washed in buffer F containing varying amounts of EGTA and calcium to obtain calculated concentrations of 0, 50, 100, 200, 400, and 800 nmol · L^{-1} free calcium. However, the precise free-calcium concentrations of these buffers were determined using fura-2 as detailed by Grynkiewicz et al,[23] and these were subsequently used for the plots. After multiple washes of the calmodulin-agarose beads in these buffers of differing calcium concentration, similar amounts of NHE-1 extracted from crude SHR and WKY membranes (as determined by Western blots detailed previously[6,10,11]) were added to the beads. The beads were rotated end-on-end for 2 hours at 4°C. They were then recovered by gentle centrifugation on a benchtop microfuge, and were washed five times in buffer F containing the same free-calcium concentrations as used in the 2-hour incubations. Following the final wash, beads were boiled in Laemmli gel sample buffer and extracts were resolved on 7.5% SDS-PAGE gels. After blotting onto supported nitrocellulose,[6,10,11] NHE-1 was detected by the specific polyclonal antibody G252 as previously described. NHE-1 bound to the calmodulin-agarose beads was expressed as a percent of total NHE-1 protein added to each tube.

Statistics

Results are expressed as the mean ± SEM, and comparisons were made by ANOVA and Student's *t* test, performed on an Oxstat statistics package (Microsoft, Reading, UK). Two-tailed *P* values less than .05 were considered significant.

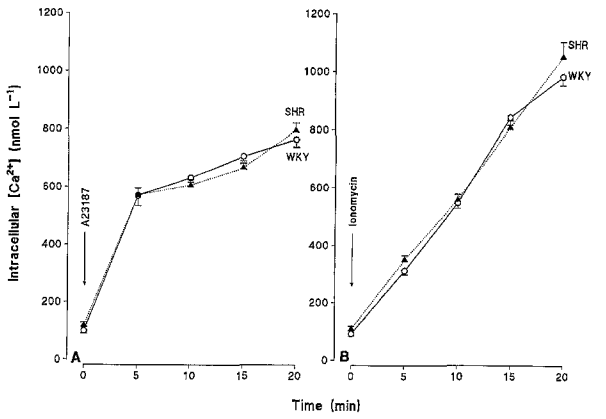

Fig 1. $[Ca^{2+}]_i$ in WKY and SHR vascular myocytes before (A) and after (B) addition of the ionophores A23187 (10 μmol · L^{-1}) or ionomycin (1 μmol · L^{-1}). The mean ± SEM is plotted (n = 6 experiments).

RESULTS

Incubation of the quiescent SHR and WKY vascular myocytes with A23187 at 37°C for 20 minutes effectively increased $[Ca^{2+}]_i$ to 700 to 800 nmol · L^{-1}, which was sustained over the duration of the determination of H^+ fluxes (Fig 1). There were no significant differences in $[Ca^{2+}]_i$ of quiescent SHR and WKY cells, and A23187 increased $[Ca^{2+}]_i$ to approximately similar levels. Another calcium ionophore, ionomycin (1 μmol · L^{-1}), also increased vascular myocyte $[Ca^{2+}]_i$ to approximately 1,000 nmol · L^{-1}, with no differences between the strains.

Figure 2 shows typical traces from SHR and WKY cells incubated in the presence or absence of A23187 and then clamped to pH_i 6.0 before studying H^+ efflux into HBSS. A23187 had no overall effect on H^+ efflux in SHR cells, but preincubation with the ionophore markedly activated H^+ efflux in WKY cells. In the absence of extracellular Na^+ (where Na^+ was replaced with *N*-methyl-D-glucamine), H^+ efflux was much

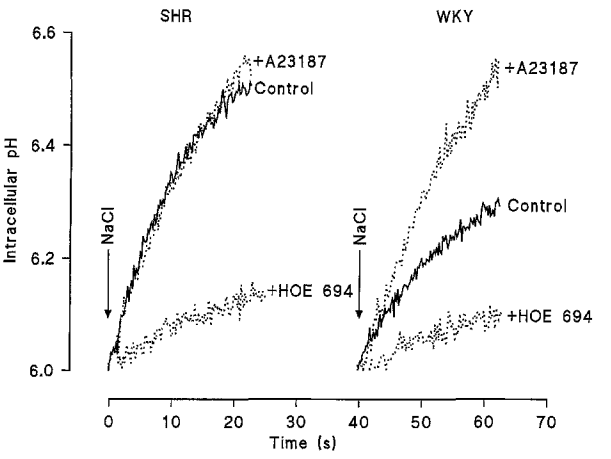

Fig 2. Typical pH_i recordings from WKY and SHR vascular myocytes with and without preincubation with A23187 (10 μmol · L^{-1}). pH_i was clamped to 6.0, and H^+ efflux was determined in HBSS. In Na^+-free buffers (replacing Na^+ with *N*-methyl-D-glucamine) or HBSS containing the NHE-1–specific inhibitor HOE-694 (10 μmol · L^{-1}), H^+ efflux was substantially reduced. For clarity, only traces with HBSS + HOE-694 are shown, but fluxes in Na^+-free buffers resembled these traces.

slower. In the presence of the NHE-1–specific inhibitor HOE-694 (10 μmol · L^{-1}), H^+ efflux in Na^+-containing buffers was reduced to that observed in Na^+-free buffers, indicating that the majority of Na^+-dependent H^+ efflux was mediated by NHE-1.

Figure 3 illustrates the effect on pH_i and NHE activity of incubating these serum-deprived SHR and WKY cells with A23187, and data averaged over 12 experiments are presented. ANOVA showed differences between pH_i- ($P < .005$) and NHE-1–mediated H^+ efflux ($P < .005$) in WKY cells incubated with A23187, KN-62, or both agents, but for experiments on SHR vascular myocytes, only pH_i was different between the different incubation conditions ($P < .005$). pH_i of quiescent SHR cells exceeded that of WKY cells ($P < .005$), confirming our previous experiments on these cells.[10,16] NHE-1–mediated H^+ efflux was also higher in these quiescent SHR cells compared with WKY controls ($P < .001$). pH_i of both types of cells was elevated by the ionophore, which is not surprising since A23187 may mediate the exchange of extracellular Ca^{2+} for intracellular H^+. When NHE-1–mediated H^+ effluxes were measured after A23187 incubation, WKY cells showed a marked enhancement of NHE activity ($P < .001$) to approximately the levels measured in quiescent SHR cells (Fig 3). In contrast, NHE activity of SHR cells was not significantly altered by incubation with A23187. This phenomenon was not unique to A23187, since another calcium ionophore, ionomycin, was also able to elevate pH_i of both cell types ($P < .001$) but NHE-1 activity was elevated only in WKY cells ($P < .001$).

Since elevations of $[Ca^{2+}]_i$ may enhance NHE activity by routes dependent on phosphorylation (eg, by stimulation of CaM kinase II[14]) or by direct binding of CaM to NHE-1,[13] we investigated the susceptibility of the phenomenon of activation of NHE to inhibition by the specific CaM kinase II inhibitor KN-62.[21] This inhibitor had no significant effect on pH_i or NHE activity of quiescent SHR and WKY cells, indicating that the elevated NHE activity in SHR cells was independent of this kinase. After a preincubation of these cells with KN-62, treatment with A23187 led to an elevation of pH_i and Ca^{2+}, as in cells without the inhibitor (Fig 3), indicating that Ca^{2+}/H^+ exchange mediated by the ionophore was unaffected. Despite this, NHE activity of WKY cells treated with KN-62 was still enhanced by A23187 incubation and SHR cells showed no response, as described previously. The apparent failure of KN-62 to inhibit the stimulatory effect of A23187 on NHE-1 in WKY cells was not due to a lack of effect of KN-62 on cellular CaM kinase II, since WKY cells activated by A23187 (following preincubation with KN-62) showed significant inhibition of CaM kinase II (81.3% ± 1.8%, n = 3) as assayed by the peptide phosphorylation kinase. Thus, these positive controls exclude the possibility that KN-62 was inactive in these cells.

NHE-1 phosphorylation was then investigated in quiescent ^{32}P-loaded cells, using immunoprecipitation with the NHE-1–specific antibody G253.[10] In all experiments, we ensured that equivalent amounts of NHE-1 protein were immunoprecipitated from the cell extracts, by determining NHE-1 content of extracts using Western blotting with the NHE-1–specific antibody G252 (as previously described.[6,10,11]). NHE-1 phosphorylation of quiescent SHR cells exceeded that of WKY cells by about twofold, confirming previous findings[10] (Table 1). However, incubation of either cell type with KN-62 did not affect

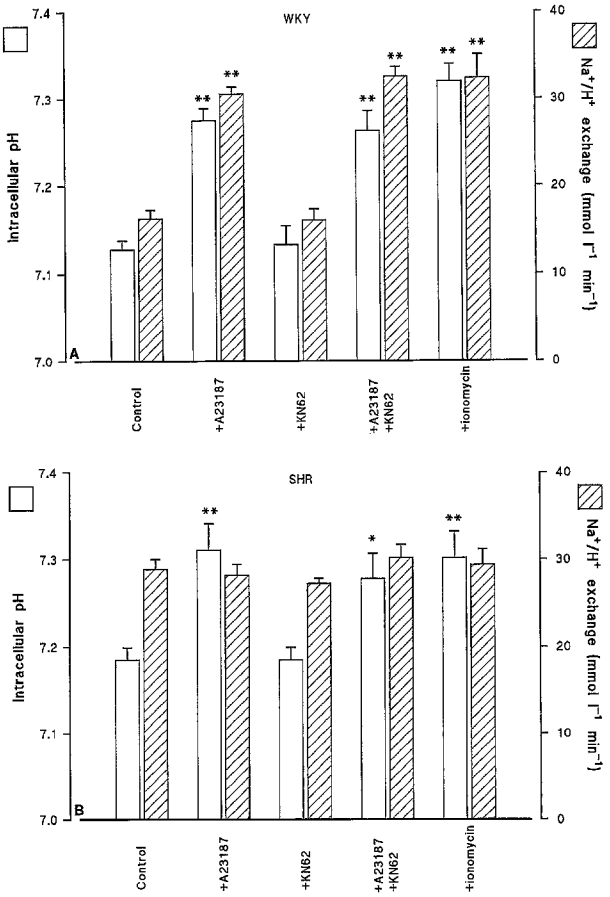

Fig 3. pH_i (□, left axis) and NHE activity at pH_i 6.0 (▨, right axis) of WKY (A) and SHR (B) vascular myocytes in the quiescent state and after incubation with A23187 (10 μmol · L^{-1}) or ionomycin (1 μmol · L^{-1}) for 20 minutes at 37°C. Cells were also preincubated with the CaM kinase II inhibitor KN-62 (10 μmol · L^{-1}) and subsequently incubated with or without A23187 before determination of pH_i and NHE-dependent H^+ fluxes. A23187 or ionomycin led to elevations of pH_i in both cell types in the presence or absence of KN-62 (n = 12 experiments, $^{**}P < .001$ and $^{*}P$ M .01 *v* incubation without ionophores). NHE activity was only enhanced in A23187- or ionomycin-treated WKY cells, and A23187-enhanced NHE activity was not affected by KN-62 (n = 12 experiments, $^{**}P < .001$ *v* control cells).

NHE-1 phosphorylation in the quiescent state, indicating the independence of this phosphorylation from the activity of CaM kinase II. Furthermore, activation of NHE activity in WKY cells with A23187 did not lead to any enhancement of its phosphorylation, and KN-62 had no significant effect on NHE-1 phosphorylation in A23187-treated cells. Similarly, NHE-1 phosphorylation of SHR cells was not affected by A23187 in either the presence or absence of KN-62 (Fig 4 and Table 1).

Table 1. Phosphorylation of Immunoprecipitated NHE-1 in Cell Extracts From Equal Numbers of Quiescent Serum-Free Cells (n = 3 experiments) Treated With A23187, KN-62, or Both

Cell Type	Control	A23187	KN-62	KN-62 + A23187
WKY	1.00	1.17 ± 0.09	1.07 ± 0.09	1.11 ± 0.13
SHR	2.20 ± 0.16	2.22 ± 0.16	2.47 ± 0.24	2.13 ± 0.16

NOTE. Density of NHE-1 phosphoprotein was normalized to a value of 1 for quiescent WKY extracts. Results are the mean ± SEM.

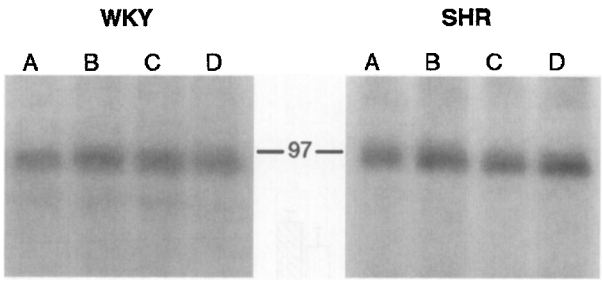

Fig 4. Autoradiographs of phosphorylated NHE-1 from WKY and SHR vascular myocytes, specifically immunoprecipitated from extracts of equal cell numbers with the polyclonal antibody G253. (A) Immunoprecipitates from control quiescent cells. Phosphoprotein immunoprecipitates loaded onto track (B) were from cells incubated with 10 μmol · L^{-1} KN-62, (C) from cells stimulated with 10 μmol · L^{-1} A23187 for 20 minutes, and (D) from cells pretreated with KN-62 and then stimulated with A23187. No significant differences of NHE-1 phosphorylation were apparent in these different incubation conditions in either cell type. Although the immunoprecipitates were derived from cell extracts containing equal amounts of NHE-1 protein (as determined by Western blotting), the autoradiograph from WKY cells was exposed for twice the duration of that from SHR cells, so that the latter autoradiograph was not overexposed for quantitation purposes. When duration of exposure is similar, NHE-1 phosphorylation of quiescent SHR cells was approximately double that of WKY cells.

To investigate whether NHE-1 extracted from WKY and SHR cells bound to calmodulin beads to different extents, we incubated detergent-extracted NHE-1 with calmodulin-agarose at different free-calcium concentrations (as determined by fura-2 fluorescence[23]). With increasing calcium concentrations, a higher proportion of the added NHE-1 from both cell types was recovered (Fig 5). However, for calcium concentrations of about 200 nmol · L^{-1} to 1 μmol · L^{-1}, recoveries of NHE-1 extracted from WKY cells were higher than those from SHR cells ($P < .005$, n = 4; Fig 5). At concentrations less than 200 nmol · L^{-1}, differences in recovery of NHE-1 between cell types were smaller but significant ($P < .05$).

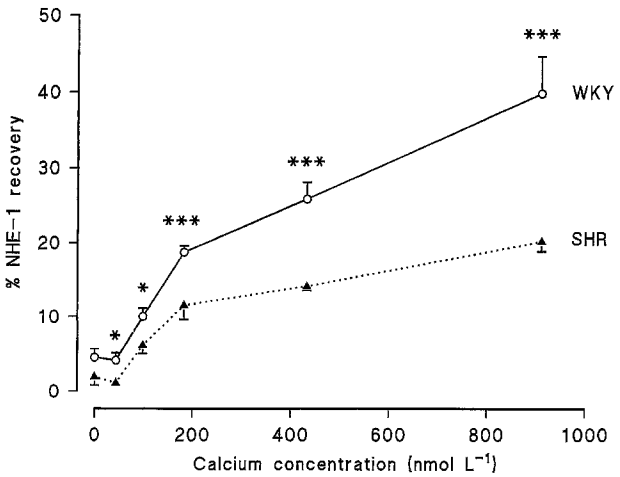

Fig 5. Recoveries of detergent-extracted NHE-1 from SHR or WKY cells with calmodulin-agarose incubated with different free-calcium concentrations, expressed as a percentage of NHE-1 protein added. ***$P < .005$, *$P < .05$ (n = 4).

DISCUSSION

Several studies have demonstrated elevated basal $[Ca^{2+}]_i$ levels in SHR vascular myocytes,[15-17] and these differences in $[Ca^{2+}]_i$ between SHR and WKY vascular myocytes are exaggerated especially following activation by a variety of agonists.[18-20] In the present study, there was no significant difference in resting $[Ca^{2+}]_i$ levels between SHR and WKY cells. One possible interpretation is that SHR cells may display an altered sensitivity to agonist-induced $[Ca^{2+}]_i$ transients without any elevation in basal $[Ca^{2+}]_i$. We have therefore proceeded to investigate the role of elevated $[Ca^{2+}]_i$ in modulating the activity of NHE-1, an exchanger that has been demonstrated to exhibit increased activity in SHR cells. NHE activity in vascular myocytes is predominantly mediated by NHE-1 rather than the other epithelial-specific isoforms,[5] and the enhanced NHE activity in SHR cells was independent of NHE-1 protein content.[6] NHE-1 phosphorylation was previously demonstrated to be one mechanism associated with its activation,[7-9] and we previously showed that quiescent SHR vascular myocytes exhibited enhanced NHE-1 phosphorylation compared with WKY cells.[10] We therefore investigated whether this altered NHE-1 activity and phosphorylation in SHR cells was dependent on the activity of an important Ca^{2+}-dependent signaling kinase, CaM kinase II.

The present studies confirm the enhanced NHE-1 activity and phosphorylation of SHR cells compared with WKY myocytes. However, when $[Ca^{2+}]_i$ was elevated using the Ca^{2+} ionophore A23187, only WKY cells showed marked enhancement of NHE activity to levels measured in quiescent SHR cells. In contrast, the elevated NHE activity of SHR cells was not further enhanced by this procedure. These effects of elevating $[Ca^{2+}]_i$ are not unique to A23187, since ionomycin produced similar results of activating NHE-1 in WKY cells and not in SHR cells. Furthermore, the effects of A23187 are unlikely to be due to its activation of protein kinase C, since stimulation of this kinase with a phorbol ester results in an elevated maximal transport capacity (V_{max}) of NHE-1 for both WKY and SHR vascular myocytes.[25] Activation of NHE-1 in WKY cells was independent of the activity of CaM kinase II, and NHE-1 immunoprecipitation experiments confirmed that there was no enhancement of this phosphorylation by elevated $[Ca^{2+}]_i$. This is in contrast to previous suggestions that NHE-1 may be a substrate for CaM kinase II from in vitro studies, in which the C-terminal of NHE-1 possesses three putative consensus sites for this kinase[14] and was phosphorylated by CaM kinase II.[14] Extrapolation of such in vitro experiments to cellular physiology may not be appropriate. However, the recent evidence that NHE-1 is a CaM binding protein with domains in the C-terminal with high and low affinities for calmodulin[13] is an interesting observation. The calmodulin binding domain has an autoinhibitory effect on NHE activity,[13] and interaction with CaM releases the exchanger mechanism from this inhibition. Our data are compatible with one possible interpretation that the elevation of NHE activity induced by $[Ca^{2+}]_i$ in WKY cells was mediated by direct binding of CaM to the C-terminal of NHE-1, releasing the exchanger from autoinhibition. The lack of stimulation of NHE-1 activity by elevated $[Ca^{2+}]_i$ in SHR cells may also suggest that the increased NHE activity of these quiescent cells could result from release of the exchanger from autoinhibition

by the calmodulin binding domain, resembling the behavior of mutants that do not possess this particular domain on the C-terminal cytoplasmic tail of NHE-1.[13]

The present experiments also demonstrate that the elevated NHE activity and NHE-1 phosphorylation of SHR vascular myocytes are not affected by inhibition of CaM kinase II, so this particular kinase may not be responsible for the altered NHE activity in cells from hypertensive animals. We could not demonstrate further enhancement of NHE activity or NHE-1 phosphorylation in SHR cells in which $[Ca^{2+}]_i$ was elevated by A23187. Whether the elevated NHE activity in quiescent SHR cells uses pathways similar to the mechanism of $[Ca^{2+}]_i$-induced activation remains to be demonstrated.

A further finding was that NHE-1 extracted from WKY cells was bound by calmodulin-agarose beads to a greater extent than NHE-1 extracted from SHR cells. This difference existed over the concentration of calcium expected in cells treated with the calcium ionophore A23187. Thus, one possible explanation for the stimulation of NHE activity by the ionophore in WKY cells but not in SHR cells may be this reduced binding of CaM to the autoinhibitory domain on the regulatory C-terminal of NHE-1 in SHR cells compared with WKY cells. However, the precise cause of this reduced binding of calmodulin to NHE-1 of SHR cells remains to be explored. Figure 6 illustrates one possible interpretation of these phenomena, where NHE-1 of quiescent WKY cells is subjected to autoinhibition from its calmodulin binding domain. Elevation of $[Ca^{2+}]_i$ leads to a release of NHE-1 from autoinhibition. In quiescent SHR cells, the reduced binding of calmodulin to this domain may be due to the presence of a putative accessory protein or to the hyperphosphorylation of this domain. These mechanisms may then release NHE-1 from autoinhibition and also reduce its further stimulation by CaM.

Our finding of a dissociation of stimulation of NHE activity from an increase in its phosphorylation contrasts with previous descriptions of enhancement of NHE-1 phosphorylation concomitant with agonist stimulation of NHE activity.[7-9] Some of these experiments were performed on cells possessing an excess of EGF receptors,[7] which may have altered the balance of intracellular signaling mechanisms between tyrosine kinase–mediated and G-protein–mediated pathways. Furthermore, Rao et al[26] also demonstrated that stimulation of NHE-1 activity in

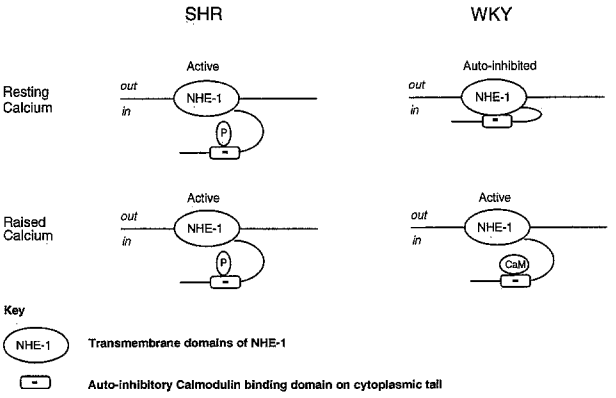

Fig 6. Schematic diagram showing NHE-1 in quiescent WKY and SHR cells and following stimulation with A23187. The autoinhibitory calmodulin binding domain on the cytoplasmic tail is illustrated. In SHR cells, the presence of a putative accessory regulatory protein or hyperphosphorylation of the calmodulin binding domain (P) may impede calmodulin binding, thereby releasing NHE-1 from autoinhibition.

HL-60 cells was not accompanied by an increase but instead a decrease in NHE-1 phosphorylation. Another example of dissociation between NHE activation and its phosphorylation is the increase in NHE activity with a hyperosmotic stimulus.[27] These reports, together with our present study on vascular myocytes, confirm the presence of non–phosphorylation-dependent mechanisms for activation of NHE-1, either as putative regulatory proteins that bind to domains on the C-terminal of NHE-1 as suggested by Wakabayashi et al,[12] or as the direct interaction of CaM with NHE-1.[13] However, phosphorylation of NHE-1 at some crucial sites may still have a role to play in agonist stimulation of NHE activity, since deletion mutants with some sections of the regulatory C-terminal removed had a reduced but unabolished response to agonists.[12] In conclusion, one possible reason for the differences in response of SHR and WKY cells to increased $[Ca^{2+}]_i$ may be an altered interaction between such putative regulatory proteins and the regulatory C-terminal of NHE-1 (in addition to the altered interaction of CaM with the autoinhibitory domain of NHE-1), since this important region of the exchanger is phosphorylated to a higher degree in SHR cells. Confirmation of this hypothesis must await identification of these NHE-1 C-terminal regulatory proteins.

REFERENCES

1. Rosskopf D, Dusing R, Siffert W: Membrane sodium-proton exchange and primary hypertension. Hypertension 21:607-617, 1993

2. Berk BC, Vallega G, Muslin AJ, et al: Spontaneously hypertensive rat vascular smooth muscle cells in culture exhibit increased growth and Na^+/H^+ exchange. J Clin Invest 83:822-829, 1989

3. Davies JE, Ng LL, Ameen M, et al: Evidence for altered Na^+/H^+ antiport activity in cultured skeletal muscle cells and vascular smooth muscle cells from the spontaneously hypertensive rat. Clin Sci 80:509-516, 1991

4. LaPointe MS, Ye M-H, Moe OW, et al: Na^+/H^+ antiporter (NHE-1 isoform) in cultured vascular smooth muscle from the spontaneously hypertensive rat. Kidney Int 47:78-87, 1995

5. Lucchesi PA, Deroux N, Berk BC: Na^+-H^+ exchanger expression in vascular smooth muscle of spontaneously hypertensive and Wistar-Kyoto rats. Hypertension 24:734-738, 1994

6. Siczkowski M, Davies JE, Ng LL: Na^+/H^+ antiporter protein in normal Wistar-Kyoto and spontaneously hypertensive rat. J Hypertens 12:775-781, 1994

7. Sardet C, Counillon L, Franchi A, et al: Growth factors induce phosphorylation of the Na^+/H^+ antiporter, a glycoprotein of 110 kD. Science 247:723-726, 1990

8. Sardet C, Fafournoux P, Pouyssegur J: α-Thrombin, epidermal growth factor, and okadaic acid activate the Na^+/H^+ exchanger, NHE-1, by phosphorylating a set of common sites. J Biol Chem 266:19166-19171, 1991

9. Bianchini L, Woodside M, Sardet C, et al: Okadaic acid, a phosphatase inhibitor, induces activation and phosphorylation of the Na^+/H^+ antiport. J Biol Chem 266:15406-15413, 1991

10. Siczkowski M, Davies JE, Ng LL: Na^+/H^+ exchanger isoform 1 phosphorylation in normal Wistar-Kyoto and spontaneously hypertensive rats. Circ Res 76:825-831, 1995

11. Ng LL, Sweeney FP, Siczkowski M, et al: Na^+/H^+ antiporter

phenotype, abundance and phosphorylation of immortalized lymphoblasts from humans with hypertension. Hypertension 25:971-977, 1995

12. Wakabayashi S, Bertrand B, Shigekawa M, et al: Growth factor activation and "H^+-sensing" of the Na^+/H^+ exchanger isoform 1 (NHE1). J Biol Chem 269:5583-5588, 1994

13. Bertrand B, Wakabayashi S, Ikeda T, et al: The Na^+/H^+ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins. Identification and characterization of calmodulin-binding sites. J Biol Chem 269:13703-13709, 1994

14. Fliegel L, Walsh MP, Singh D, et al: Phosphorylation of the C-terminal domain of the Na^+/H^+ exchanger by Ca^{2+}/calmodulin-dependent protein kinase II. Biochem J 282:139-145, 1992

15. Erne P, Hermsmeyer K: Intracellular vascular muscle Ca^{2+} modulation in genetic hypertension. Hypertension 14:145-151, 1989

16. Ng LL, Davies JE, Ameen M: Intracellular free magnesium levels in vascular smooth muscle and striated muscle cells of the spontaneously hypertensive rat. Metabolism 41:772-777, 1992

17. Bukoski RD: Intracellular Ca^{2+} metabolism of isolated resistance arteries and cultured vascular myocytes of spontaneously hypertensive rats and Wistar-Kyoto normotensive rats. J Hypertens 8:37-43, 1990

18. Nabika T, Velletri PA, Lovenburg W, et al: Increase in cytosolic Ca^{2+} and phosphoinositide turnover metabolism induced by angiotensin II and [arg]vasopressin in vascular smooth muscle cells. J Biol Chem 260:4661-4670, 1985

19. Osani J, Dunn MJ: Phospholipase C responses in cells from spontaneously hypertensive rats. Hypertension 19:446-455, 1992

20. Morton C, Baines R, Masood I, et al: Stimulation of two vascular smooth muscle–derived cell lines by angiotensin II: Differential second messenger responses leading to mitogenesis. Br J Pharmacol 115:361-367, 1995

21. Tokumitsu H, Chijiwa T, Hagiwara M, et al: KN-62, 1-[*N,O*-bis-(5-isoquinolinesulfonyl)-*N*-methyl-L-tyr osyl]-4-phenyl-piperazine, a specific inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II. J Biol Chem 265:4315-4320, 1990

22. Scholz W, Albus U, Lang HJ, et al: HOE 694, a new Na^+/H^+ exchange inhibitor and its direct effect in cardiac ischaemia. Br J Pharmacol 109:562-568, 1993

23. Grynkiewicz G, Poenie M, Tsien RY: A new generation of calcium indicators with greatly improved fluroescence properties. J Biol Chem 260:3440-3450, 1985

24. Ng LL, Davies JE, Siczkowski M, et al: Abnormal Na^+/H^+ antiporter phenotype and turnover of immortalized lymphoblasts from type I diabetic patients with nephropathy. J Clin Invest 93:2750-2757, 1994

25. Siczkowski M, Ng LL: Phorbol ester activation of the rat vascular myocyte Na^+/H^+ exchanger isoform 1. Hypertension 27:859-866, 1996

26. Rao GN, Sardet C, Pouyssegur J, et al: Phosphorylation of Na^+/H^+ antiporter is not stimulated by phorbol ester and acidification in granulocytic HL-60 cells. Am J Physiol 264:C1278-C1284, 1993

27. Grinstein S, Woodside M, Sardet C, et al: Activation of the Na^+/H^+ antiporter during cell volume regulation. J Biol Chem 267: 23823-23828, 1992