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Calmodulin-dependent binding to the NHE1 cytosolic tail mediates activation of the Na^+/H^+ exchanger by Ca^{2+} and endothelin

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Li X, Prins D, Michalak M, Fliegel L. Calmodulin-dependent binding to the NHE1 cytosolic tail mediates activation of the Na^+/H^+ exchanger by Ca^{2+} and endothelin. *Am J Physiol Cell Physiol* 305: C1161–C1169, 2013. First published October 2, 2013; doi:10.1152/ajpcell.00208.2013.—The mammalian Na^+/H^+ exchanger isoform 1 (NHE1) is a ubiquitous plasma membrane protein that regulates intracellular pH by removing a single proton (H^+) in exchange for one extracellular Na^+. The human protein contains a ~500-amino acid membrane domain and a regulatory, ~315-amino acid cytosolic domain. NHE1 is activated by a number of hormones including endothelin (ET) and by Ca^{2+}. The regulatory tail possesses an inhibitory calmodulin (CaM)-binding domain, and inhibition of NHE1 is relieved by binding of a Ca^{2+}-CaM complex. We examined the dynamics of ET-1 and Ca^{2+} regulation of binding to NHE1 in vivo. CFP was linked to the NHE1 protein cytoplasmic COOH terminus. This was stably transfected into AP-1 cells that are devoid of their own NHE1 protein. The protein was expressed and targeted properly and retained NHE1 activity comparable to the wild-type protein. We examined the in vivo coupling of NHE1 to CaM by Förster resonance energy transfer using CaM linked to the fluorescent protein Venus. CaM interaction with NHE1 was dynamic. Removal of serum reduced CaM interaction with NHE1. Addition of the Ca^{2+} ionophore ionomycin increased the interaction between CaM and NHE1. We expressed an ET receptor in AP-1 cells and also found a time-dependent association of NHE1 with CaM in vivo that was dependent on ET treatment. The results are the first demonstration of the in vivo association of NHE1 and CaM through ET-dependent signaling pathways.

calmodulin; endothelin; sodium/hydrogen exchanger; pH regulation

THE SODIUM/HYDROGEN EXCHANGER isoform-1 (NHE1) is a plasma membrane glycoprotein that protects cells from intracellular acidification by extruding a single intracellular proton in exchange for a single extracellular Na^+ (12). Ten Na^+/H^+ exchanger isoforms (NHE1–NHE10) are known. NHE1 was the first isoform discovered and is ubiquitously distributed (13). Other isoforms are restricted to specific tissues, and some have predominantly intracellular localization. In mammals, aside from its pH regulatory role, NHE1 is also important in regulation of cell volume, proliferation, and differentiation and in metastasis of some types of tumor cells (4, 12, 24, 45).

NHE1 has two major domains. The ~500-amino acid NH_2-terminal membrane domain transports ions. This is followed by a hydrophilic 315-amino acid COOH-terminal cytosolic domain in human NHE1. The cytosolic domain regulates the membrane domain. Regulation occurs by protein-protein interaction on the cytosolic tail and phosphorylation of the distal region of the tail (29). Calmodulin (CaM) is a Ca^{2+}-binding protein that plays an important role in regulating NHE1 function in response to Ca^{2+} signaling. In the presence of elevated Ca^{2+}, CaM can bind to NHE1 at two sites in the cytoplasmic tail. Residues 636–656 constitute a high-affinity site, and residues 657–700 constitute a low-affinity site (2). When CaM is bound to the high-affinity site, it blocks an autoinhibitory interaction of the tail with the membrane domain, thereby activating the Na^+/H^+ exchanger (2, 20, 44). There are also seven conserved acidic amino acids, ^{753}EEDEDDD^{759}, in the distal region of the COOH-terminal tail that play a role in CaM binding (23), possibly maintaining a proper conformation of the cytosolic tail that is required for CaM binding. The physiological relevance of the CaM-binding domain was demonstrated in transgenic mice. When the autoinhibitory site was mutated to cause a hyperactive NHE1 protein, expression of this protein in the myocardium promoted heart hypertrophy much more than overexpression of the wild-type NHE1 protein (30, 31, 49).

Activation of the NHE1 protein by a variety of hormones, including activation by epidermal growth factor and angiotensin II, has been suggested to be coupled through CaM (7, 10, 14). Endothelin-1 (ET-1) is another hormone that activates the Na^+/H^+ exchanger in a number of cell types (6, 15, 19, 38, 43), and stimulation of NHE1 activity is coupled with increasing intracellular Ca^{2+} (16, 17, 47). However, the precise mechanism by which ET activates NHE1 activity is unclear, and, in particular, whether it has an effect on CaM binding and regulation of NHE1 is not known. In this study we develop an in vivo system for the detection of CaM binding to the NHE1 protein in single cells. Using this system, we show that Ca^{2+} and ET-1 stimulate the interaction of CaM with NHE1 in intact cells. The results represent the first demonstration of the association of CaM with NHE1 in intact cells with ET and Ca^{2+} regulation.

EXPERIMENTAL PROCEDURES

Materials. BCECF-AM was purchased from Molecular Probes (Eugene, OR), W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride] and trifluoperazine (TFP) from Sigma, anti-MAPK (ERK1/2), anti-phosphorylated ERK1/2, anti-phosphorylated p38, and anti-p38 from Cell Signaling Technology (Beverly, MA), anti-hemagglutinin (HA) antibody (Y-11) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-NHE1 antibody from BD Biosciences, Lipofectamine 2000 reagent from Invitrogen (Carlsbad, CA), anti-DDK (DYKDDDDK) monoclonal antibody, human ET receptor cDNA (catalog no. 4812050, type A), EDNRA (NM_001957), and anti-DDK antibody from OriGene Technologies (Rockville, MD), sulfo-NHS-SS-biotin from Pierce Chemical (Rockford, IL), and synthetic DNA from IDT (Coralville, IA). The expression plasmid pmCerulean3-Dectin1An-10 [which is derived from pEGFP-N1 (GenBank accession no. U55762)] was a generous gift of Dr. N. Touret (Dept. of Biochemistry, University of Alberta). All other chemicals were of analytical grade and purchased from Fisher Scientific (Ottawa, ON, Canada), Sigma (St. Louis, MO), or BDH (Toronto, ON, Canada). A plasmid containing rat CaM linked to yellow fluorescent protein (YFP) was a generous gift of Dr. Walter Stühmer (Max-Planck

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Institute of Experimental Medicine, Göttingen, Germany). BAPTA-AM was obtained from Sigma.

Construction of expression vectors. The plasmid pmCerulean3-Dectin1An-10 was used to construct an expression vector for the human NHE1 protein. pmCerulean3-Dectin1An-10 contains dectin fused in-frame to the cyan fluorescent protein (CFP) cerulean, separated by a short flexible linker. The human NHE1 protein was amplified from the plasmid pYN4$^+$ (25) using the primers 5′-AAGAATTCGCTAGCGCCACCATGGTTCTGCGGTCTGGC-ATC-3′ and 5′-CCGAATTCCCCGGGGGAACGTCATATG-GATAGGATCCTGC-3′, which flanked the cDNA with a 5′ *Nhe*I site and an in-frame 3′ *Xma*I site. The plasmid pmCerulean3-Dectin1An-10 was digested with *Nhe*I and *Age*I to remove dectin cDNA, and the amplified NHE1 gene was inserted. This resulted in a construct with a 5′ cDNA for NHE1 with cerulean fused in-frame to the NHE1 cytosolic tail, separated by a flexible polylinker.

For expression of the human ET type A (ET_A) receptor, cDNA (pCMV6-ETaR) transfection-ready DNA was purchased from OriGene Technologies.

Cell culture and stable transfection. AP-1 is a mutant cell line derived from Chinese hamster ovary (CHO) cells that do not express endogenous active NHE1 (37). Stably transfected cells were established using Lipofectamine 2000 reagent (Invitrogen Life Technologies, Carlsbad, CA) essentially as described elsewhere (40). Plasmids carrying a neomycin resistance gene allowed the selection of transfected cells using Geneticin (G418) antibiotics. pYN4$^+$ plasmid, which expresses the human NHE1 cDNA, HA-tagged, was used as a source of NHE1 (25). Where indicated, cells were cotransfected with the plasmids described. Stable cell lines for experiments were regularly reestablished from frozen stocks at *passages 5–11*.

For some experiments, cells were incubated overnight in low serum (0.5%) and then treated with ET-1 (1 nM). In some cases, cells were treated with BAPTA-AM (50 μM) to chelate intracellular Ca^{2+} (46) in the presence of 10% serum medium.

Cell surface expression. Cell surface expression was measured as we have described elsewhere (39). The cell surface was labeled with sulfo-NHS-SS-biotin (Pierce Chemical), and plasma membrane Na^+/H^+ exchanger was removed with immobilized streptavidin resin. Equivalent amounts of total and unbound proteins were separated using SDS-PAGE followed by Western blotting and densitometry measuring immunoreactive (HA-tagged) NHE1 protein. It was not possible to efficiently and reproducibly elute proteins bound to immobilized streptavidin resin; therefore, total and unbound fractions were used. The amounts of NHE1 on the cell surface were measured by comparison of both HA-immunoreactive species (upper and lower) in Western blots of the fractions.

SDS-PAGE and immunoblotting. Expression of NHE1 was confirmed by immunoblotting using anti-HA antibody. Samples were resolved on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membrane. After incubation with anti-HA monoclonal antibody, peroxidase-conjugated goat anti-mouse antibody (Bio/Can, Mississauga, ON, Canada) was used for detection. Immunoreactive proteins were detected on film using the Amersham enhanced chemiluminescence Western blotting and detection system.

For detection of ERK1/2, phosphorylated ERK1/2, p38, and phosphorylated p38, antibodies to these proteins were used according to the manufacturer's instruction and visualized with the appropriate secondary antibodies as described above. For some experiments, immunoreactive proteins were detected on X-ray film by using secondary antibodies conjugated to horseradish peroxidase.

Densitometric analysis was carried out using ImageJ 1.35s software (National Institutes of Health, Bethesda, MD).

Intracellular pH measurement. BCECF was used to measure the rate of intracellular pH (pH_i) recovery after acute acid load by addition of NH_4Cl (50 mM for 3 min). NHE1 activity was measured as the slope of the first 20 s of recovery from acidification and expressed as ΔpH/s, as described elsewhere (40). Cells grown to ~90% confluence on coverslips were incubated overnight in reduced (0.5%) serum where indicated.

For pH_i measurement, BCECF-loaded cells were subjected to a two-pulse acidification assay using a PTI Deltascan spectrofluorometer for measurement, as previously described (8). NH_4Cl prepulse was used to acidify the cells for both pulses. In the first pulse, acidification of cells by NH_4Cl removal was followed by ~20 s of incubation in a Na^+-free buffer; then the cells were allowed to recover in a normal-Na^+ buffer. The second pulse was the same as the first, except 1 nM ET-1 was added where indicated in the recovery in normal-Na^+ buffer. In some experiments we tested the effect of CaM inhibitors on the activity of the Na^+/H^+ exchanger. In these cases, a two-pulse assay was used, and the effect of W7 (60 μM) or TFP (60 μM) was examined. W7 or TFP was present throughout the second pulse. Controls included vehicle (DMSO) for this same time period.

Values are means ± SE, and statistical significance was determined using Wilcoxon's signed-rank test.

Fura 2 Ca^{2+} measurements. For measurements of cytoplasmic Ca^{2+} concentrations, a Ca^{2+} buffer (143 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 20 mM HEPES, pH 7.4, 0.1% glucose, 1 mM $CaCl_2$, and 100 μg/ml sulfinpyrazone) was used. Cells were incubated with 2 μM fura 2-AM in serum-free medium for 30 min at room temperature and then with serum-free medium alone for 15 min at room temperature to allow for deesterification of the dye. Cells were washed with phosphate-buffered saline, trypsinized, washed with Ca^{2+} buffer, and resuspended in Ca^{2+} buffer for analysis. Fura 2 fluorescence was measured on a fluorometer (Photon Technology International) with excitation wavelengths of 340 nm (Ca^{2+}-bound) and 380 nm (Ca^{2+}-free) and an emission wavelength of 510 nm, as described elsewhere (35). Cells were treated with ET-1. To convert the ratio of signal at 340 nm to signal at 380 nm into cytoplasmic Ca^{2+} concentrations, two calibration steps were performed: *1*) a maximum ratio from treatment with 1 μM ionomycin and 4 mM $CaCl_2$ and *2*) a minimum ratio from treatment with 32 mM EGTA, 24 mM Tris, and 0.4% Triton X-100.

Acceptor photobleaching by Förster resonance energy transfer. Protein-protein interactions were studied using the acceptor photobleaching method Förster resonance energy transfer (FRET) following a procedure similar to that described by Zhu et al. (52). To perform FRET, we used NHE1-CFP (cerulean) and CaM-YFP (Venus) constructs. The CFP and YFP variants of green fluorescent protein (GFP) are a useful donor and acceptor pair, respectively, for assessing the presence of FRET. Their spectroscopic characteristics consist of well enough separated spectra to allow for efficient transfer between donor and acceptor. After excitation of CFP at 458 nm, the emission fluorescence was collected from 464 to 505 nm. No YFP signal was detected under this condition. YFP excitation was at 514 nm, and emission was collected from 522 to 590 nm. No CFP signal was detected under this condition. A Leica TCS SP5 confocal microscope was used to assess the presence of FRET between CFP-NHE1 (donor) and CaM-YFP (acceptor) (18, 36, 52). CFP-dectin (donor) expressed in AP-1 cells was used as the control cell line. For some experiments, a stable cell line was made with AP-1 cells expressing CFP-NHE1 plated on the round coverslips, and this was transiently transfected with CaM-YFP with Lipofectamine 2000, as described above. At ~36–48 h after the transfection, the cells were kept at 4°C for 20 min while they were fixed with 4% formaldehyde. For some experiments, the cells were serum-starved overnight and then treated with 1 nM ET-1 for 5 min before they were fixed with 4% formaldehyde. For other experiments, the cells were treated with 50 μM BAPTA-AM for 30 min at 37°C before they were fixed. The cells were mounted with Dako fluorescent mounting medium. For FRET detection, a ×60/1.2 water immersion objective was used. The particular regions of interest were selected, and the images were collected before and after bleaching with the 514-nm laser light at a maximum intensity for 10 frames.

The apparent FRET efficiency (E) was calculated using the following equation: $E = (I_{D\text{-}post} - I_{D\text{-}pre})/I_{D\text{-}post}$, where $I_{D\text{-}pre}$ and $I_{D\text{-}post}$ represent the donor fluorescence intensities before and after acceptor photobleaching, respectively (18, 36, 52).

FRET efficiency was calculated using fluorescence values that had been background-subtracted and corrected for bleaching during acquisition, as determined from an unbleached region of interest.

Immunoprecipitation of the NHE1-CaM complex. NHE1-CaM complexes were immunoprecipitated using anti-CaM antibody and the cleavable cross-linker dithiobis(succinimidylpropionate) (Pierce Chemical), essentially as described elsewhere (22). Anti-CaM antibody was obtained from Santa Cruz Biotechnology.

RESULTS

Expression and characterization of NHE1-cerulean. Initial experiments verified the correct expression, targeting, and activity of the various constructs used in the experiments. NHE1 was constructed with a cerulean tag at the COOH terminus of the protein and stably transfected into AP-1 cells that are devoid of their own Na^+/H^+ exchanger protein. We used Western blotting to determine if the protein was expressed. The results are shown in Fig. 1, *A* and *B*. Initially, we used anti-GFP antibodies that were cross-reactive with the cerulean fusion protein added to the COOH terminus of NHE1. They detected an immunoreactive ~140-kDa protein and a second smaller ~110-kDa band. The larger protein is the correct size of the full-length NHE1 with cerulean. We previously noted this characteristic pattern of NHE1, with a second smaller band that corresponds to the deglycosylated or partially glycosylated NHE1 protein (40). There was no immunoreactive protein in the lysate from mock-transfected AP-1 cells or in cells transfected with HA-tagged

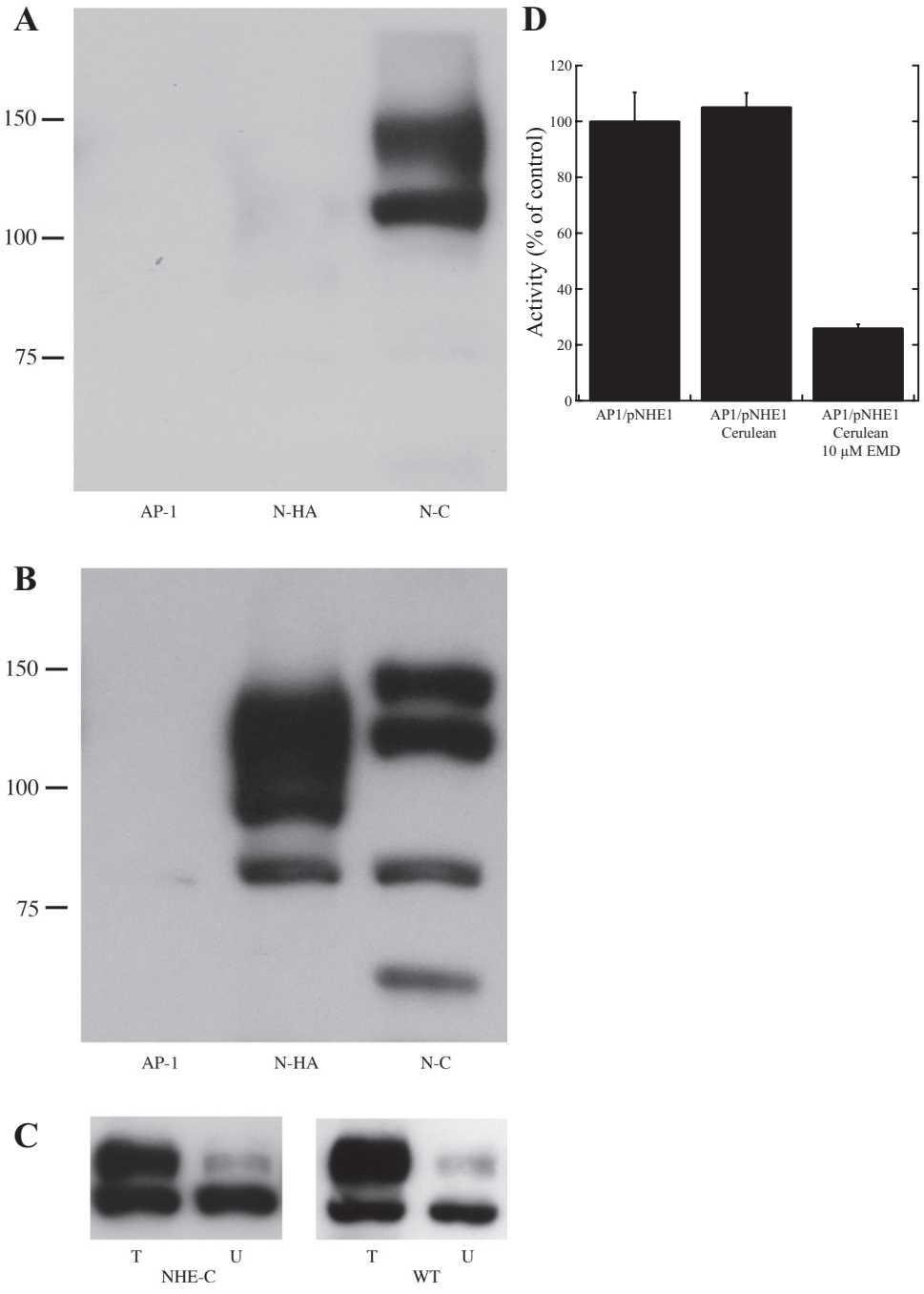

Fig. 1. Characterization of Na^+/H^+ exchanger isoform 1 (NHE1)-cerulean expression in AP-1 cells. *A* and *B*: Western blots contained cell lysates from controls and cells stably expressing wild-type $pYN4^+$ plasmid [containing hemagglutinin (HA)-tagged NHE1 (N-HA)] or cells containing NHE1-cerulean (N-C). *A*: Western blot with anti-green fluorescent protein (GFP) antibodies. *B*: Western blot with anti-HA antibodies. *C*: subcellular localization of NHE1-cerulean (NHE-C) and NHE1 wild-type (WT, HA-tagged) in AP-1 cells. Cells treated with sulfo-NHS-SS-biotin were lysed, and streptavidin-agarose beads were used to bind labeled proteins. Equivalent amounts of total cell lysate (T) and unbound intracellular lysate (U) were examined by Western blotting, with anti-HA antibody used to identify NHE1 protein. One typical experiment is shown. Results are typical of ≥4 determinations. *D*: comparison of Na^+/H^+ exchanger activity of AP-1 cells transfected with wild-type NHE1 (pNHE1) or NHE1 tagged with cerulean (pNHE1-cerulean). Effect of 10 μM EMD-87580 (EMD) on NHE1 activity is also illustrated. Values are means ± SE of ≥8 determinations.

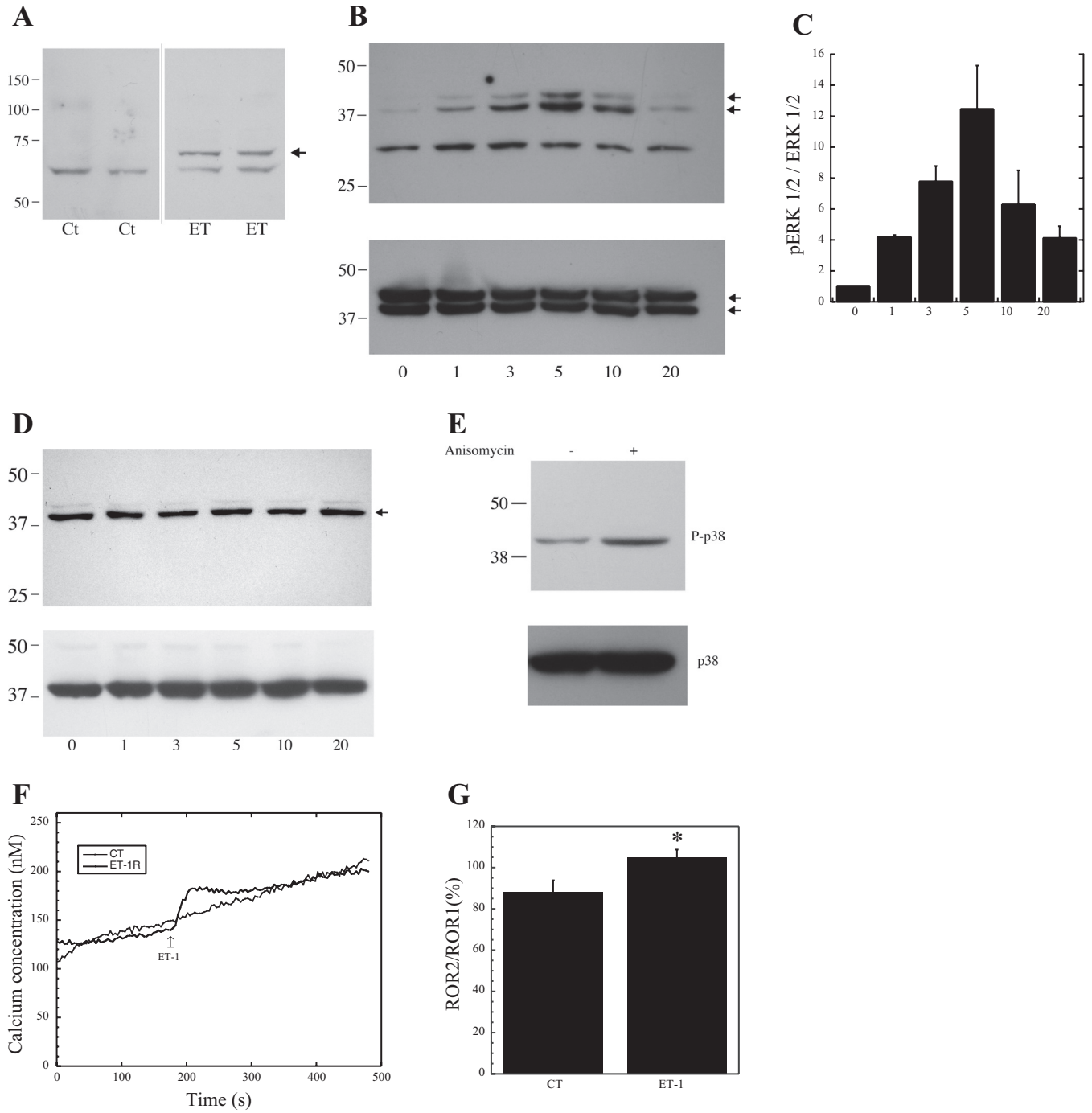

Fig. 2. Characterization of cells stably transfected with NHE1 and endothelin (ET) receptor. *A*: Western blot of cells probed with anti-DDK antibody. Arrow indicates immunoreactive protein found in cells transfected with ET receptor but absent in control (Ct) cells. *B*: Western blot of activation of AP-1 cells stably transfected with ET receptor. Cells were treated with 1 nM ET-1 for 0–20 min at 37°C. *Top*: immunoblot of cell lysates with anti-phosphorylated ERK1/2 antibodies. *Bottom*: immunoblot of cell lysates with anti-ERK1/2 antibodies. Arrow denotes immunoreactive ERK1/2. *C*: summary of results of activation of ERK1/2. Level of phosphorylated ERK1/2 (pERK1/2) was normalized by the level of ERK protein. Values are means ± SE of ≥3 experiments. *D*: Western blot of activation of AP-1 cells stably transfected with ET receptor. Cells were treated with 1 nM ET-1 for 0–20 min at 37°C. *Top*: immunoblot of cell lysates with anti-phosphorylated p38 antibodies. *Bottom*: immunoblot of cell lysates with anti-p38 antibodies. Arrow denotes immunoreactive p38 protein. *E*: effect of 25 μg/ml anisomycin on phosphorylated p38 (P-p38) levels. Serum-starved cells were treated with anisomycin for 30 min, and cell lysates were immunoblotted with anti-phosphorylated p38 antibodies (*top*) or anti-p38 antibodies (*bottom*). Results are typical of 7 experiments. *F*: effect of 1 nM ET-1 on Ca^{2+} levels in NHE1-containing AP-1 cells in the presence or absence of ET receptor. CT, cells without the ET receptor; ET-1R, cells stably transfected with the ET-1 receptor. Arrow indicates addition of 1 nM ET-1. Results are typical of 4 experiments with each cell type. *G*: effect of 1 nM ET-1 on NHE1 activity of Chinese hamster ovary cells containing the ET receptor and NHE1. Serum-deprived cells were subjected to a 2-pulse assay in the presence and absence of 1 nM ET-1 for 5 min. Rate of recovery (ROR) of the 2nd pulse (ROR2) was compared with that of the 1st pulse (ROR1). Values are means ± SE of 7 determinations. *$P < 0.01$, significantly different from the 1st pulse.

NHE1 protein. Western blotting with anti-HA antibody (Fig. 1*B*) again showed the same characteristic two bands in cells stably transfected with NHE1 linked to cerulean. The NHE1-cerulean expression plasmid was constructed from the $pYN4^+$ plasmid and included the HA tag as a spacer accounting for the immunoreactivity. Cells stably transfected with $pYN4^+$ plasmid (containing NHE1, HA-tagged and without cerulean) showed the characteristic immunoreactive pattern described previously (40). The major immunoreactive bands were smaller than the band observed in cells with cerulean-tagged NHE1, which is accounted for by the size of the additional cerulean protein. AP-1 cells showed no immunoreactivity with anti-HA antibodies.

We confirmed that the NHE1 protein was properly targeted to the plasma membrane by examining its surface processing using cell surface biotinylation, as we described previously (40). Approximately 50% of the NHE1 protein (Fig. 1*C*) was targeted to the plasma membrane, whether it contained the cerulean tag or not. This amount is similar to earlier results with wild-type NHE1 with a HA tag (Fig. 1*C*), as reported elsewhere (40).

We also examined the ability of NHE1-cerulean to respond to an acute acid load induced by NH_4Cl. The results (Fig. 1*D*) show that NHE1 linked to cerulean had activity similar to NHE1 that was not linked to cerulean. Additionally, most NHE1 activity was inhibited by addition of the NHE1 inhibitor EMD-87580 (10 μM), which is typical of this protein.

ET receptor expression. NHE1 has been reported to be stimulated by elevation of intracellular Ca^{2+} and by treatment with ET (1, 33). To study NHE1 in AP-1 cells, which are a derivative of CHO cells, we introduced the ET receptor. The receptor contained a tag that was used for Western blot analysis. When pCMV6-ETaR plasmid was transfected into the AP-1 cells, Western blot analysis (Fig. 2*A*) demonstrated the expression of a 60- to 70-kDa immunoreactive band that was not present in cells that were not transfected with the receptor. The identity of a smaller cross-reactive band was not known.

ET receptor coupling. Binding of ET to the ET receptor has been shown to result in activation of a cascade pathway that includes activation of ERK (41). To examine whether AP-1 cells stably transfected with ET_A receptor were appropriately coupled to signaling pathways, we serum-starved the cells overnight, treated them with 1 nM ET-1 for 0–20 min, and examined levels of activated phosphorylated ERK1/2 by Western blotting. The results are shown in Fig. 2, *B* and *C*. Treatment of the cells with ET-1 resulted in a rapid increase in phosphorylated ERK1/2 levels that peaked at 5 min after stimulation. Similar experiments examined whether p38 was activated by ET-1 in the cells stably transfected with the ET receptor. The results are shown in Fig. 2*D*. There was no increase in the level of p38 phosphorylation with ET-1 treatment. Figure 2*E* demonstrates that treatment of cells with another stimulus, anisomycin, can cause an increase in phosphorylated p38 levels. Treatment with 25 μg/ml anisomycin resulted in an average increase of 73% of phosphorylated p38 levels when normalized to p38 protein levels ($n = 7$). Figure 2*F* demonstrates that the ET_A receptor-containing cells respond to treatment with an elevation of intracellular Ca^{2+}. Application of 1 nM ET-1 to cells with ET receptor always resulted in a transient increase in Ca^{2+} levels. Cells without ET receptor never showed this response.

Activation of NHE1 by ET-1. We examined whether treatment with ET-1 activated NHE1 activity in the CHO cells with ET receptor. We used a two-pulse assay similar to that described above. Cells were subjected to two 3-min treatments with 5 mM NH_4Cl and then allowed to recover in NaCl-containing medium essentially as described elsewhere (8, 26). For the second recovery, cells were treated with 1 nM ET-1 or left untreated for 5 min immediately prior to recovery from acid load. We compared the second rate of recovery (with or without ET-1) with the first rate of recovery. There was no difference in pH_i at the start of recovery between the treated and untreated groups. The results are shown in Fig. 2*G*. In a control two-pulse assay, the rate of recovery of the second pulse declined slightly from that of the first pulse. In ET-1-treated cells, the rate of recovery of the second pulse increased and was elevated significantly, ~20%, compared with the control experiment. Longer (10 min) treatment with ET-1

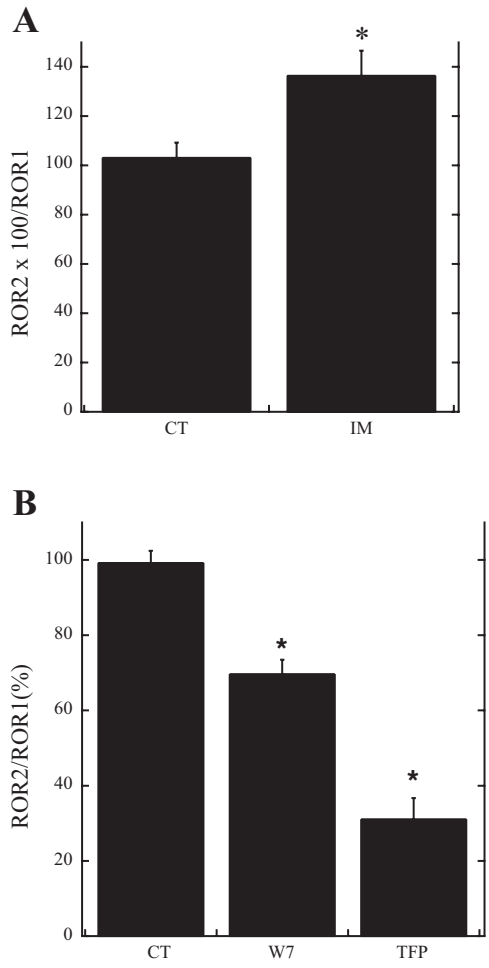

Fig. 3. Effect of ionomycin and calmodulin (CaM) antagonists on NHE1-cerulean activity. Cells expressing NHE1 linked to cerulean were treated twice with NH_4Cl (5 mM) as described in Fig. 2*E* legend. For control (CT) experiments, the 2nd pulse was sham (vehicle) treatment. *A*: cells were subjected to the 2nd pulse for 50 s with 0.5 μM ionomycin (IM), and rate of recovery after the 2nd NH_4Cl pulse is compared with that of the 1st pulse. Values are means ± SE of 10 experiments. $*P < 0.01$, significantly different from the 1st pulse. *B*: effect of W7 and trifluoperazine (TFP, 60 μM) on rate of recovery after the 2nd NH_4Cl pulse compared with the 1st pulse. Values are means ± SE of 8 experiments. $*P < 0.001$, significantly different from the 1st pulse.

resulted in less effect (not shown), consistent with the decreased activation of ERK at this time point (Fig. 2*C*).

Ca^{2+} activation of NHE1. Several physiological stimuli are known to affect activity of the NHE1 protein. We also examined the effect of ionomycin on activity of NHE1. The results are shown in Fig. 3*A*. Similar to the protocol described above, cells were mildly acidified by two exposures to 5 mM NH_4Cl and treated with and without 0.5 μM ionomycin for 50 s prior to the second pulse. There was no difference in pH_i at the start of recovery between the treated and untreated groups. When cells recovered in the presence of ionomycin, the rate of recovery was significantly increased. The effect was apparent

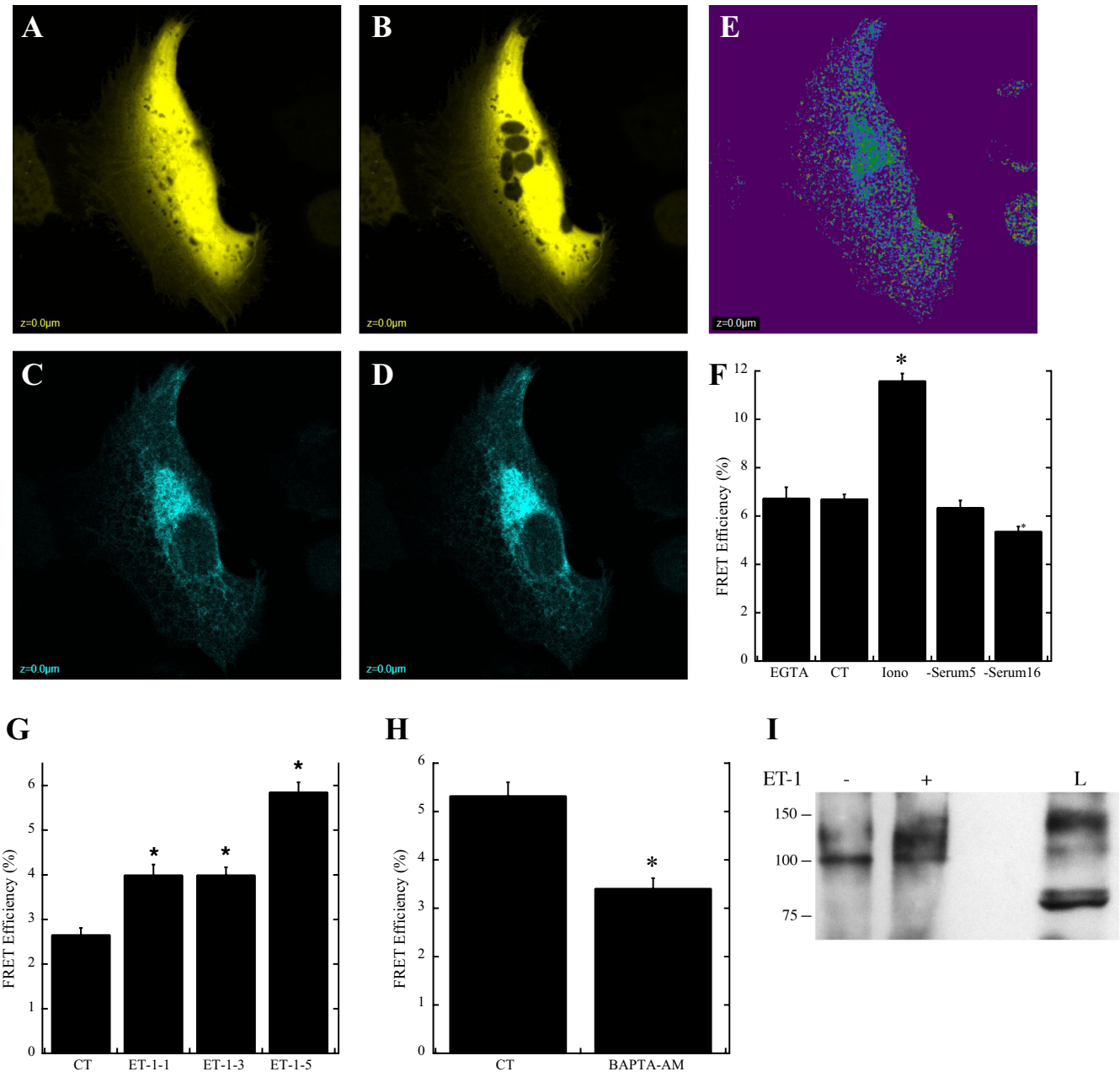

Fig. 4. Characterization of NHE1 interactions with CaM in response to ionomycin and ET-1. Stable cell lines expressing human NHE1-cerulean were transiently transfected with yellow fluorescent protein (YFP)-CaM. Cells were characterized by excitation (for cerulean) at 458 nm and collection of emission at 464–505 nm or excitation (for YFP) at 514 nm and collection of emission at 522–590 nm. *A–E*: images of cells were treated with 0.5 μM ionomycin for 3 min prior to fixation. *A* and *B*: excitation and emission for cerulean in NHE1-cerulean-containing cell. *B*: areas that were photobleached using 10 maximal-intensity illuminations on the laser illuminator at 458 nm. *C* and *D*: emission examined at 522–590 nm in cells excited at 514 nm before (*C*) and after (*D*) photobleaching. *E*: Förster resonance energy transfer (FRET) efficiency generated by the SP5 Leica software showing FRET between NHE1 and CaM. *F*: summary of effect of 0.5 μM ionomycin (Iono) and serum withdrawal on efficiency of FRET between NHE1-cerulean and YFP-CaM. $*P < 0.0001$. Cells were maintained in medium containing normal serum. Serum was withdrawn for 5 h (−Serum5) or 16 h (−Serum16). *G*: effect of 1 nM ET-1 on FRET between YFP-CaM and NHE1-cerulean. Cells were subjected to serum withdrawal for 24 h prior to treatment with ET-1 for 1, 3, or 5 min. *H*: effect of treatment with 50 μM BAPTA-AM for 30 min. Values are means ± SE of ≥20 cells. $*P < 0.0001$. *I*: immunoprecipitation of CaM-NHE1 complex. Anti-CaM antibodies were used to immunoprecipitate the CaM-NHE1 complex. Experimental cells were treated with 1 nM ET-1 as described above. Immunoblotting was carried out with anti-NHE1 antibodies. L, sample of cell lysate prior to immunoprecipitation. Results are typical of 3 experiments.

only with relatively mild acidification with lower concentrations of NH_4Cl. Higher concentrations of NH_4Cl caused greater acidification of cells and maximal activity of NHE1, which was not further enhanced by ionomycin (not shown).

We also examined the effect of CaM antagonists on activity of the Na^+/H^+ exchanger (Fig. 3*B*). When cells were treated with W7 or TFP, recovery from an acute acidosis was significantly inhibited.

Characterization of CaM binding to NHE1 in vivo. To determine whether stimulation of NHE1 activity by ionomycin or ET-1 was correlated with binding of CaM to the NHE1 cytosolic domain, we transiently transfected CaM linked to YFP (Venus) into the stable cell lines containing NHE1-cerulean; a cell line containing dectin-cerulean was used as a control. We then examined changes in the efficiency of FRET between CaM and NHE1. Fluorescence recovery after photobleaching was used. Figure 4, *A–E*, illustrates one example of this type of experiment. Stably transfected cells display NHE1 distributed over the surface of the cells. Ten pulses of a maximal-intensity laser were able to bleach fluorescence of the YFP-CaM protein. This resulted in an increased fluorescence of the CFP, indicative of FRET between the CaM and NHE1 proteins. Ionomycin enhanced the FRET between CaM and the NHE1 protein (Fig. 4*F*). In this series of experiments we also examined the effect of externally applied EGTA and serum withdrawal. External EGTA application did not reduce the interaction between NHE1 and CaM. However, withdrawal of serum significantly reduced the interaction between NHE1 and CaM after 16 h (Fig. 4*F*). Control AP-1 cells expressed dectin-cerulean; however, no FRET between dectin and CaM was detected with ionomycin treatment (not shown).

We also determined whether ET-1 treatment enhanced the interaction between CaM and NHE1. For these experiments, serum was initially withdrawn overnight prior to treatment with ET-1. Withdrawal of serum resulted in a reduced level of interaction between the proteins. Addition of ET-1 restored the interaction between NHE1-cerulean and YFP-CaM (Fig. 4*G*). This occurred rapidly, within 1 min, and increased with 5 min of treatment. Longer (10 min) treatments resulted in no interaction between the proteins compared with the control (not shown). In the control cell line AP-1 expressing dectin-cerulean, no FRET between dectin and CaM was detected with ET treatment (not shown).

As a test of the requirement for intracellular Ca^{2+}, cells were maintained in normal medium with serum, and we examined the effect of addition of 50 μM BAPTA-AM for 30 min on the interaction between CaM and NHE1 (Fig. 4*H*). This resulted in a significant decrease in the interaction between the two proteins.

To confirm that treatment with ET-1 resulted in increased NHE1-CaM complex, we treated cells with ET-1 and immunoprecipitated the complex using anti-CaM antibody. Figure 4*I* demonstrates that more NHE1 was immunoprecipitated in cells treated with ET-1 than controls.

DISCUSSION

The Na^+/H^+ exchanger is a pH regulatory plasma membrane protein that is critical in cell growth and differentiation and important in pathologies of the heart and in tumors (see Refs. 13 and 11 for reviews). An association of NHE1 with CaM has been suggested earlier (2), and Ca^{2+} and CaM have been suggested to be important regulators of NHE1 (2, 20, 44). However, demonstration of their mode of action in vivo and their link to hormonal regulation of NHE1 in vivo is lacking. The present study is the first to examine the in vivo association of CaM with NHE1 by these stimuli with a linkage to regulation by ET-1. ET has been previously demonstrated to activate NHE1 activity in several cell types (6, 15, 19, 21, 38, 43).

Initially, we coupled the fluorescent protein cerulean to the NHE1 cytoplasmic domain. We previously coupled a triple HA tag to the NHE1 COOH terminus without negative effects on function (25), so we suspected that coupling of this tag would not be detrimental. We found that this was the case: NHE1 function was maintained, and the protein was properly expressed and targeted.

To study NHE1 regulation by ET-1, it was also necessary to introduce the ET receptor into AP-1 cells, which are a derivative of CHO cells, as noted by others (9, 32). We confirmed that the receptor was expressed and also that it was coupled by examination of the activity of downstream ERK. Interestingly, we found a biphasic activation of the downstream effects, with a peak of ERK activation at ~5 min followed by a decline in activation. A decline in activation of ERK1/2 over such periods of time has been noted previously (5, 50). We did not find that stimulation with ET-1 activated p38, although anisomycin was able to activate p38, confirming that the protein was functionally responsive in these cells. Others have noted that p38 activation can be smaller and take a much longer than ERK activation (50). Because we were interested in the time course of events that correlated with the activation of the NHE1 protein, this was not pursued further.

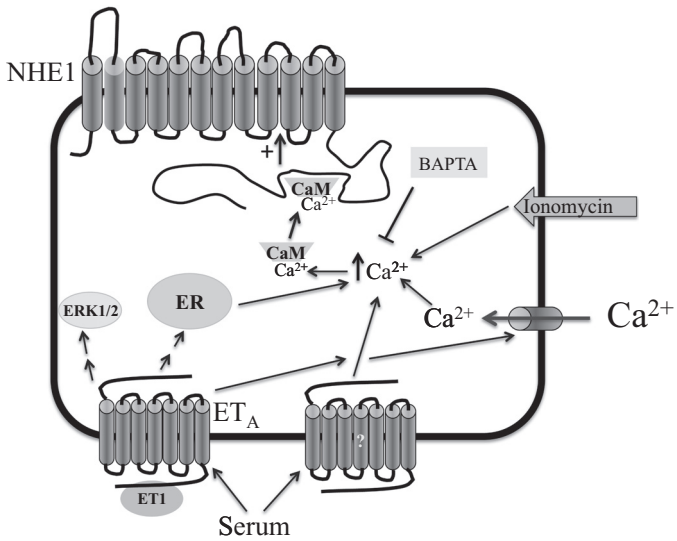

Fig. 5. Putative model of NHE1 regulation by ET and Ca^{2+}. ET-1 binds to the ET_A receptor, which elicits an elevation of intracellular Ca^{2+}, likely through extracellular and intracellular sources (34). Elevation of intracellular Ca^{2+} causes formation of a Ca^{2+}-CaM complex, which binds to the autoinhibitory domain of NHE1, relieving NHE inhibition and stimulating activity. Ionomycin can act directly to increase intracellular Ca^{2+}, while serum may act through ET receptors or other receptors to increase intracellular Ca^{2+}. BAPTA-AM can chelate Ca^{2+} and prevent elevation of intracellular Ca^{2+}. ER, endoplasmic reticulum.

We initially confirmed that addition of ionomycin stimulated NHE1 activity and CaM antagonists inhibited NHE1 activity, demonstrating a role for Ca^{2+} and CaM in regulation of the protein in this system. Next, results demonstrated an in vivo interaction between CaM and NHE1. This in vivo interaction was maintained at a certain level by the presence of serum. Withdrawal of serum resulted in a decrease in the level of NHE1-CaM interaction over a period of hours. Precisely which component of serum was responsible cannot be said at this time; however, it is clear that the decline in activation can be reversed by elevation of intracellular Ca^{2+} through addition of ionomycin. Ca^{2+} is confirmed to be an important regulator of the in vivo interaction between NHE1 and CaM, as addition of the intracellular Ca^{2+} chelator BAPTA-AM reduced the level of NHE1-CaM interaction.

Treatment of cells with ET-1 stimulated NHE1 activity. Treatment with 1 nM ET-1 for 5 min resulted in a significant increase in NHE1 activity. While the amount of stimulation was relatively modest, we used a low dose of ET-1, as opposed to others who used up to 100 times this concentration. We found that coupling of NHE1 to CaM was also increased by 1 nM ET-1 over this same time period. These results demonstrate that ET can act through promotion of an interaction of NHE1 with regulatory CaM. We expect that ET-1 acted through the ET receptor, likely via increasing intracellular Ca^{2+}. A Ca^{2+}-CaM complex is necessary for CaM binding to the NHE1 cytosolic domain (2, 20, 44). ET has been demonstrated to increase intracellular Ca^{2+} levels in several cell types through intracellular signaling mediated via the ET_A receptor (27, 28, 48, 51). It is likely that at least part of its mechanism of activation of NHE1 is through elevation of intracellular Ca^{2+}, which complexes with CaM, which then binds to the NHE1 cytosolic domain. We confirmed that cells with an ET receptor respond to stimulation by ET-1 with an increase in intracellular Ca^{2+}, supporting this hypothesis. A simple schematic of the proposed pathway is shown in Fig. 5.

One earlier report also demonstrated an in vivo association of NHE1 with CaM (42). Bioluminescence resonance energy transfer (BRET) was used to demonstrate that 5-HT_{1A} receptor activation mediates binding of CaM to NHE1. The results of the present study represent a novel investigation into activation of NHE1 by ET and by Ca^{2+} directly in vivo. FRET was used as opposed to BRET. Both techniques have their advantages and disadvantages. While BRET has the advantage of not requiring external excitation of a donor, FRET has the advantage of having a higher light output and is not as affected by the cellular environment or compartmentalization. Because the signal is usually stronger, it allows selection of single cells or regions of the cell to be examined, as in the present study (3). The development of new proteins such as cerulean and Venus, with more distinct separation of their excitation and emission spectra, has further extended its usefulness. This present work has used FRET to demonstrate the novel observations that ET, Ca^{2+}, and serum enhance the association of CaM with NHE1. The study also verifies that the association of regulatory CaM is dynamic. Regulation of NHE1 by CaM has been shown to have important physiological consequences. For example, we recently demonstrated that transgenic mice with an activating mutation in the CaM-binding domain develop cardiac hypertrophy (30). Future studies will examine the in vivo interactions between NHE1 and CaM in the pathological state.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

X.L. and D.P. performed the experiments; X.L. and L.F. analyzed the data; X.L. and L.F. interpreted the results of the experiments; X.L., D.P., and L.F. prepared the figures; X.L., M.M., and L.F. edited and revised the manuscript; X.L., D.P., M.M., and L.F. approved the final version of the manuscript; X.L. and L.F. are responsible for conception and design of the research; L.F. drafted the manuscript.

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