Calmodulin-Binding Autoinhibitory Domain Controls "pH-Sensing" in the Na^+/H^+ Exchanger NHE1 through Sequence-Specific Interaction†

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Received June 27, 1997; Revised Manuscript Received August 15, 1997⊗

ABSTRACT: The calmodulin (CaM)-binding domain reduces the affinity of the Na^+/H^+ exchanger NHE1 for intracellular H^+ by exerting an autoinhibitory function in quiescent cells. We replaced this domain (aa 637−656) with homologous segments from other NHE isoforms (NHE2 and 4) or functionally similar regions from other sources (Na^+/Ca^{2+} exchanger, CaM-dependent protein kinase II, plasma membrane Ca^{2+}-pump, or CaM-binding peptide Trp3). The NHE-1-, NHE2-, and NHE4-segments bound CaM with K_ds of 16, 130, and 27 nM, respectively. These chimeric molecules were expressed in the exchanger-deficient cell PS120. NHE1 with incorporated NHE2-segment was activated in response to Ca^{2+}-mobilizing agents ionomycin and thrombin resulting in an alkaline shift of the intracellular pH (pH_i)-dependence of $^{22}Na^+$ uptake, as was the case with the intact rat NHE2. In contrast, incorporation of the NHE4-segment or other CaM-binding segments induced a constitutive alkaline shift of pH_i-dependence with concomitant abolishment of Ca^{2+}-dependent activation, indicating that these segments could not function as an autoinhibitory domain in NHE1. Detailed analyses revealed that Leu639, Lys651 and Tyr652, conserved in the NHE1- and NHE2-segments, but not in the NHE4-segment, are important for the autoinhibition. Furthermore, ^{125}I-labeled CaM-binding peptide from NHE1 was efficiently crosslinked to the NHE1 protein, suggesting that the inhibitory domain physically interacts with part(s) of the molecule. Together, these findings support the notion that the reduction of H^+ affinity in Na^+/H^+ exchange occurs through a mechanism involving a highly sequence-specific interaction of the inhibitory domain with its putative acceptor in NHE1.

The Na^+/H^+ exchanger isoform 1 (NHE1)[1] is a plasma membrane transporter that regulates the intracellular pH (pH_i) and cell volume in virtually all cells (for review, see refs 1−3). It is activated in response to a variety of stimuli including growth factors, Ca^{2+}-mobilizing agonists, and hyperosmotic stress (*4*−*6*). The Ca^{2+}-dependent activation of NHE1 has been well documented and analyzed (for review, see ref 3). NHE1 has been shown to contain a CaM-binding region within its carboxyl-terminal regulatory domain, which fully overlaps the autoinhibitory domain (*7*−*9*). Deletion or mutation in this CaM-binding domain induced a constitutive activation of NHE1 with resultant increase in the affinity for intracellular H^+ (an alkaline shift of pH_i-dependence of exchange activity) and disappearance of the responsiveness to a rise in intracellular Ca^{2+} concentration. This autoinhibitory domain of NHE1 could be important for the NHE1 regulation in general, because NHE1 responds to all known stimuli only through a change in its pH_i-sensitivity (*4*−*6*, *10*). Such a p*K* change is believed to occur at the "H^+-modifier site" that is distinct from the H^+ transport site (*11*), although these two sites have not experimentally been well characterized. At present, little is also known about the mechanism by which the autoinhibitory domain of NHE1 inhibits H^+ binding to the H^+-modifier site. On the other hand, it is not known whether other isoforms, NHE2, NHE4, and NHE5 (*12*−*15*) are regulated by Ca^{2+} in a CaM-dependent manner. NHE3 (*12*, *16*) does not seem to be regulated by Ca^{2+}, although it binds CaM (*17*).

The mechanism of autoinhibition in NHE1 may be considerably different from that postulated for soluble enzymes such as myosin light chain kinase (*18*) or CaM-dependent kinases I, II, and IV, in which the CaM-binding autoinhibitory domains have been identified (*19*−*21*). These kinases are inactivated in the absence of Ca^{2+}. In these enzymes, the CaM-binding region partly overlaps the autoinhibitory domain which is considered to serve as a pseudosubstrate and thus block the substrate-binding site of the enzyme. In contrast, the autoinhibitory function is incomplete in the case of NHE1, because it is fully active even in quiescent cells as long as the cytosol is acidified. Thus, the autoinhibitory domain decreases the accessibility of activating H^+ to the H^+-modifier site of NHE1. We previously found that a charge reversal mutation within the inhibitory domain (substitution of four positive to four negative amino acids)

† This work was supported by a Grant-in-Aid on Priority Area 321 and a Grant-in-Aid for Scientific Research C (06680640) from the Ministry of Education, Science and Culture of Japan and by Special Coordination Funds Promoting Science and Technology (Encouragement System of COE).

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⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: NHE1, Na^+/H^+ exchanger isoform 1; NHE2, Na^+/H^+ exchanger isoform 2; NHE4, Na^+/H^+ exchanger isoform 4; CaM, calmodulin; pH_i, intracellular pH; pH_o, extracellular pH; NCX1, Na^+/Ca^{2+} exchanger isoform 1; EIPA, 5-(*N*-ethyl-*N*-isopropyl)amiloride; N1-CBD, NHE1 CaM-binding domain peptide; N2-CBD, NHE2 CaM-binding domain peptide; N4-CBD, NHE4 CaM-binding domain peptide; XIP, Na^+/Ca^{2+} exchanger inhibitory peptide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; aa, amino acid(s).

S0006-2960(97)01547-X CCC: $14.00

completely abolished its autoinhibitory function (*8*), suggesting that positive charges of basic amino acids may be important for the autoinhibition. This observation raised the possibility that CaM-binding segments from other proteins which have many basic amino acids may equally be effective in reducing the affinity of the regulatory site for activating H^+.

In the present study, we identified CaM-binding regions in the Na^+/H^+ exchanger isoforms 2 (NHE2) and 4 (NHE4) in a portion of the molecule homologous to that in NHE1. We examined the effect of replacement of the NHE1 CaM-binding domain with the homologous segments from NHE2 and NHE4 or the CaM-binding segments from other proteins. We found that the NHE1 CaM-binding domain was functionally replaced only by the NHE2-segment. Amino acid substitution experiments based on the sequence comparison among NHE1, NHE2, and NHE4 revealed the existence of several residues essential for the autoinhibition in NHE1. These data indicate that the autoinhibition in NHE1 is due to a highly sequence-specific interaction of the inhibitory domain with its putative acceptor.

EXPERIMENTAL PROCEDURES

Materials. 5-(*N*-Ethyl-*N*-isopropyl)amiloride (EIPA) was a gift from New Drug Research Laboratories of Kanebo, Ltd. (Osaka, Japan). ^{22}NaCl, [7-^{14}C]benzoic acid, and ^{125}I-Denny Jaffe reagent were purchased from Dupont NEN. The NHE1-specific polyclonal antibody (RP-cd) was described previously (*7*). In this study, the following four peptides were synthesized based on the published amino acid sequences: N1-CBD, NH_2-RNNLQKTRQRLRSYNRHTLVA-amide (21-mer) (*22*); N2-CBD, NH_2-SRNLYQIRQRTL-SYNRHNLTA-COOH (21-mer) (*14*); N4-CBD, NH_2-TRNMYQVRQRTLSYNKYNLKP-amide (21-mer) (*12*); XIP, NH_2-RRLLFYKYVYKRYRAGKQRG-COOH (20-mer) (*23*). These peptides were >95% pure as determined by high-pressure liquid chromatography. All other chemicals were of the highest purity available.

Cells, Culture Conditions, and Stable Expression. The Na^+/H^+ exchanger-deficient cell line (PS120) (*24*) and the corresponding transfectants were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 25 mM $NaHCO_3$, 7.5% (v/v) fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL). Cells were maintained at 37 °C in presence of 5% CO_2. The exchanger-deficient PS120 cells (5×10^5 cells/100-mm dish) were transfected with each plasmid construct (20 μg) by the calcium phosphate co-precipitation technique. Cell populations that stably express NHE1 variants were selected by the "H^+-killing" procedure as described (*25*).

Isolation of cDNAs of NHE2 and NHE4. Total RNA was prepared from rat stomach using Tri-zol (Life Technologies). Poly(dT)$_{13}$-primed first-strand cDNAs were synthesized from total RNA and then amplified by RT-PCR using the following primers (5′ → 3′): for NHE2, sense, TTGAGT-TAATAGCGCTGTCCCCCAGCG, and antisense, GGGTTT-GAGTAACAAGTCACAACCATG; for NHE4, sense, CCT-CGGTTGGGTAATGCAGCTGGAAAC, and antisense, TCATCCCTACCCTCAGAAAAGGCAGGG. PCR products were further amplified using the following nested primers: for NHE2, sense, TGAGGGTGCCCAGATCCCT-ACACTGGC, and antisense, CATTCACCACAAGTCT-GCTTTGGCTTC; for NHE4, sense, TGCCGCAGAGGCG-TGGCTCACATCAGG, and antisense, TTGTAATAAATC-TACATTTCTGTGCCC. The nested PCR products were cloned into pCRII vector (Invitrogen), and their DNA sequences were determined on a Perkin Elmer ABI model 373S autosequencer. Amino acid sequences deduced from these nucleotide sequences were the same as those of the original clones (*12*, *14*), although some changes in the nucleotide sequences were observed. These cDNAs were cloned into the mammalian expression vector pECE.

Construction of NHE1 Mutants. Plasmids carrying cDNA of human NHE1 deleted of the 5′-untranslated region (pEAP-Δ5′) and its internal deletion mutant (Δ637−656) of CaM-binding domain were described previously (*7*, *25*). For construction of chimeric exchangers whose CaM-binding domain (aa 637−656) was replaced by the functionally similar segments of other proteins, we used plasmid pEAP-Δ5′AN containing two unique restriction sites (*Afl*II and *Nde*I) (cf. Figure 2), whose construction was described in detail previously (*9*). For incorporation of the exogenous segments, pairs of sense (72-mer) and antisense (70-mer) oligonucleotides corresponding to these segments were synthesized (cf. Figure 2), phosphorylated with polynucleotide kinase, annealed, and cloned into *Afl*II and *Nde*I restriction sites in pEAP-Δ5′AN.

For construction of plasmids containing amino acid substitutions in the CaM-binding domain (cf. Figure 6), we used both pEAP-Δ5′AN and pEAP-Δ5′AN with the NHE4-segment inserted into *Afl*II and *Nde*I restriction sites as templates for PCR amplification. The sense primer contained an exogenous *Afl*II site and the CaM-binding domain sequences with substituted nucleotides, whereas the antisense primer contained a sequence corresponding to aa 743−749 of NHE1. Using these primers, DNA fragments were generated by PCR, digested, and then inserted into *Afl*II and *Apa*I (corresponding to aa 698 of NHE1) sites of pEAP-Δ5′AN. DNA sequences of incorporated oligonucleotides or PCR-amplified regions were confirmed by sequencing.

Measurements of $^{22}Na^+$ Uptake and pH_i. EIPA-sensitive $^{22}Na^+$ uptake was measured in stable transfectants grown up to confluence in 24-well dishes after cells had been acidified by a NH_4Cl prepulse technique as described (*17*). Serum-depleted cells were loaded with NH_4Cl for 30 min at 37 °C in NaCl standard solution [compositions: 20 mM Hepes/Tris (pH 7.4), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM glucose] containing 0−30 mM NH_4Cl. Cells were then rapidly washed once with choline chloride standard solution [compositions: 20 mM Hepes/Tris (pH 7.4), 120 mM choline chloride, 2 mM $CaCl_2$, and 1 mM $MgCl_2$] and incubated in the same medium for 40 s. $^{22}Na^+$ uptake was then started by adding the choline chloride standard solution containing ^{22}NaCl (37 kBq/mL) (final concentration, 1 mM) and 1 mM ouabain. Forty seconds later, cells were rapidly washed four times with ice-cold phosphate-buffered saline to terminate $^{22}Na^+$ uptake. For some wells, the choline chloride solution additionally contained 0.1 mM EIPA. When the effects of ionomycin and thrombin were measured, these agents were present in the choline chloride solution during washing and $^{22}Na^+$ uptake. pH_i was estimated by measuring the distribution of [^{14}C]benzoic acid (74 kBq/mL) (*26*) under the same conditions as those used for $^{22}Na^+$ uptake measurement, except

that the uptake medium contained [^{14}C]benzoic acid and nonradioactive NaCl. pH_i at an acidic extracellular pH was measured essentially as described previously (*8*, *9*).

In some experiments, we measured pH_i-dependence of $^{22}Na^+$ uptake by K^+/nigericin pH_i clamp method (*9*, *27*). Serum-depleted cells in 24-well dishes were preincubated for 30 min at 37 °C in Na^+-free choline chloride/KCl medium [composition: 20 mM Hepes/Tris (pH 7.4), 140 mM choline chloride + KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, and 5 μM nigericin]. KCl concentration was varied from 1 to 140 mM, while the total concentration of KCl plus choline chloride was maintained at 140 mM. $^{22}Na^+$ uptake was started by adding the same choline chloride/KCl solution containing ^{22}NaCl (37 kBq/mL) (final concentration, 1 mM), 1 mM ouabain, and 100 μM bumetanide. After 1 min, cells were rapidly washed four times with ice-cold phosphate-buffered saline to terminate $^{22}Na^+$ uptake. pH_i was calculated from the imposed $[K^+]$ gradient by assuming the equilibrium $[K^+]_i/[K^+]_o = [H^+]_i/[H^+]_o$ at equilibrium and an intracellular $[K^+]$ of 120 mM.

Measurements of Dansyl-Calmodulin Fluorescence and Circular Dichroism Spectrum. Dansylation of CaM was carried out using bovine brain CaM (1 mg) and dansyl chloride as described previously (*7*). Dansyl-CaM fluorescence was monitored at 25 °C under stirring on a spectrofluorometer (Hitachi, MPF-4) in a mixture containing 10 mM Hepes/Tris (pH 7.2), 30 mM NaCl, 200 nM dansyl-CaM, and different concentrations of NHE-derived peptides in the presence of 0.1 mM $CaCl_2$. Excitation wavelength was 340 nm and the slit width was 5 nm. Fluorescence was measured in a range of emission wavelengths between 400 and 600 nm. Circular dichroism spectra of these NHE-derived peptides (100 μM) in 10 mM Hepes/Tris (pH 7.4) were recorded on a Jasco J/500A spectropolarimeter.

Photoaffinity Cross-Linking of Calmodulin-Binding Peptide to NHE1 Protein. NHE1 CaM-binding peptide N1-CBD (50 μg) was incubated for 2 h at 25 °C in the dark with ^{125}I-Denny-Jaffe reagent (3.7 MBq, DuPont NEN) in 200 μL of 0.1 M sodium phosphate (pH 8.0) and then 200 μL of 0.2 M glycine/Tris (pH 8.5) was added to terminate the labeling. SDS−PAGE on a 20% gel confirmed exclusive ^{125}I-labeling of the peptide with an expected M_r of ~3000. The NHE1 protein was immunoaffinity-purified from the lysate of stable transfectants of NHE1 grown to confluence in fifteen 24.5 cm × 24.5 cm rectangle dishes and harvested in 20 mM Hepes/Tris (pH 7.4), 100 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 0.8 mM benzamidine. Cells were solubilized with 1% octaethyleneglycol mono-*n*-dodecyl ether ($C_{12}E_8$, Nikko Chemicals, Japan) in 100 mL of the same harvesting buffer and centrifuged for 30 min at 100000*g*. The supernatant was applied to a protein A-Sepharose column (0.5 mL bed) that had been pretreated with NHE1-specific antibody (RP-cd). The column was washed with 100 mL of modified harvesting buffer containing 0.5% $C_{12}E_8$ and 0.5 M NaCl and then with 10 mL of the original harvesting buffer, and then the protein A-Sepharose beads were recovered into the 1.5 mL tube. The beads with the immune complex (50 μL) were mixed with ^{125}I-labeled N1-CBD (final concentration, 10 μM) in 400 μL of a solution containing 20 mM Hepes/Tris (pH 7.4), 100 mM NaCl, and 5 mM EDTA. This mixture was placed on a 48-well culture dish and then exposed to light (average 366 nm) at 2 cm distance by using a hand-held UV lamp. In some experiments (Figure 7), the cell extract with $C_{12}E_8$ was directly photoaffinity labeled. Proteins were then extracted from these by boiling for 5 min in SDS−PAGE sample buffer containing 1% SDS and analyzed on SDS−PAGE on a 7.5% gel. The gel was subjected to silver staining (Wako Chem. Co.) for protein detection, while ^{125}I-labeled proteins were visualized by Bioimage analyzer (Fuji Film Co.).

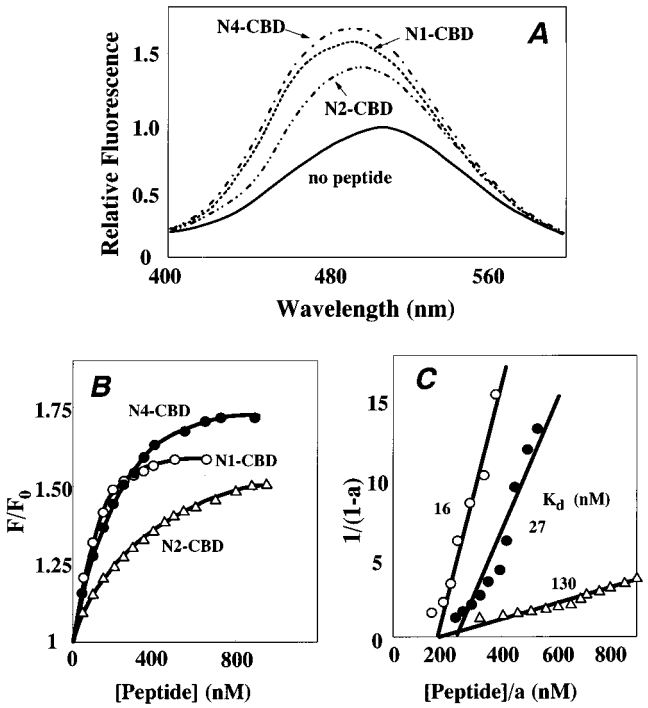

FIGURE 1: CaM binding by peptides derived from NHE2 and NHE4 homologous to CaM-binding segment of NHE1. (A) Effect of exchanger-derived peptides on fluorescence emission spectra of dansyl-CaM. Emission spectra of dansyl-CaM (200 nM) were measured in the presence of $CaCl_2$ with 1 μM of either N1-CBD (- - -), N2-CBD (−••−) or N4-CBD (−•−), or without peptide (—). (B) Fluorescence intensity (F) at 490 nm in the presence of peptides relative to that of dansyl-CaM alone (F_o) is plotted against the concentration of peptides added ([peptide]). (C) The data in B were analyzed as described previously (*7*, *42*). The fractional degree of saturation (a) of dansyl-CaM fluorescence was calculated as $a = (F - F_o)/(F_\infty - F_o)$, where F_∞ is the fluorescence intensity at the saturated level of peptides. Plots of $1/(1 - a)$ against [peptide] divided by a give straight lines. The reciprocal of slope gives dissociation constants (K_d) for dansyl-CaM, whereas the x axis intercepts for these peptides were close to CaM concentration (200 nM) used in this experiment, indicating a 1:1 binding stoichiometry.

Others. Protein concentration was measured by bicinchoninic assay system (Pierce Chemical Co.) using bovine serum albumin as a standard.

RESULTS

Effect of Replacement of NHE1 CaM-Binding Domain by Exogenous Segments. In NHE1, the high-affinity CaM-binding site is located in a segment between aa 635 and aa 659 (N1CBD) (*7*). We found that segments of NHE2 (aa 607−627; N2CBD) and NHE4 (aa 597−617; N4CBD) homologous to this segment of NHE1 also bound CaM (Figure 1). Synthetic peptides for all these segments enhanced the fluorescence intensity of dansyl-CaM with a blue shift of the emission spectrum in the presence of Ca^{2+} (Figure 1A), but not in its absence (data not shown). Fluorescence titration permitted us to estimate the apparent dissociation constant (K_d) for CaM to be 16, 130, and 27

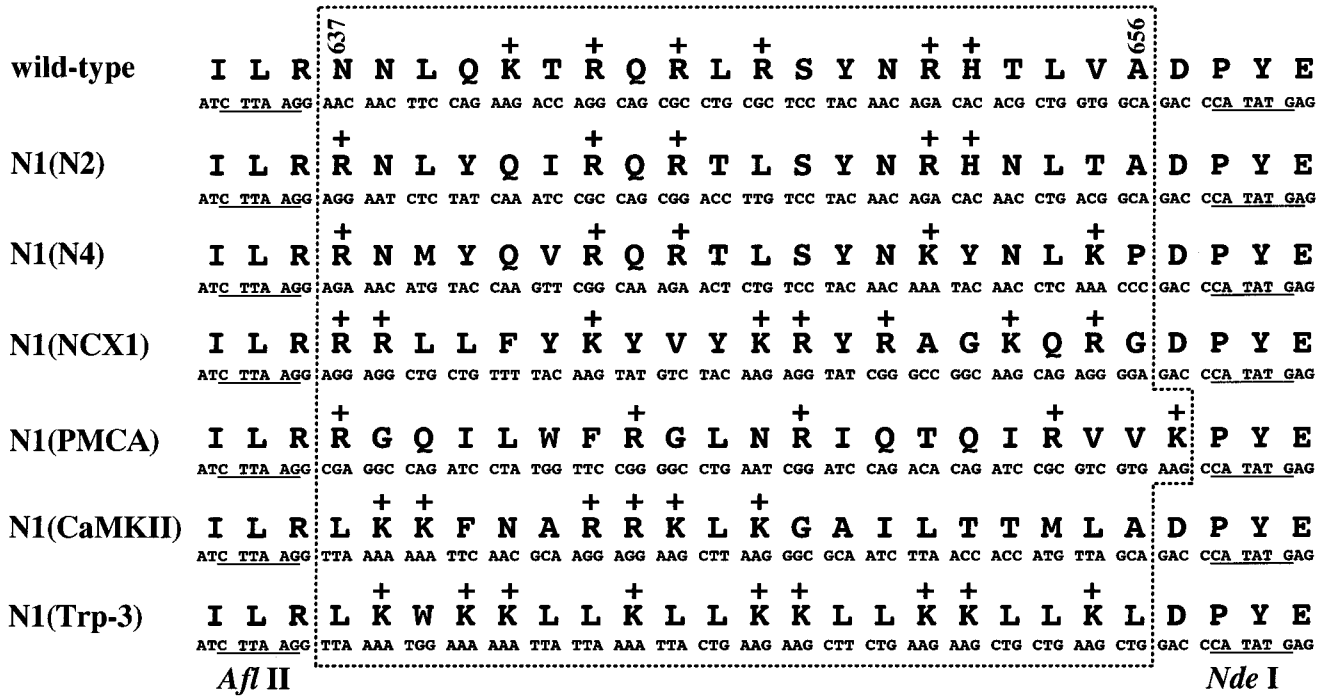

FIGURE 2: Schematic representation of chimeric NHE1 constructs containing exogenous CaM-binding segments. Asn-637 to Ala-656 of NHE1 was replaced by other exogenous CaM-binding segments: N1(N2) from rat NHE2 (*14*), N1(N4) from rat NHE4 (*12*), N1(NCX1) from Na^+/Ca^{2+} exchanger (*23*), N1(PMCA) from plasma membrane Ca^{2+} pump (*30*), N1(CaMKII) from CaM-dependent protein kinase II (*28*), and N1(Trp-3) from CaM-binding model peptide Trp-3 (*31*). DNA sequences used for the chimeric construction are shown below the amino acid sequences.

nM for N1-CBD, N2-CBD, and N4-CBD, respectively (Figure 1B and C). The value for N1-CBD was close to the value (20 nM) obtained previously for the fusion protein containing most of the NHE1 cytoplasmic domain (*7*). All these peptides had structures consisting of β-sheet and random-coil, as determined by circular dichroism spectra (data not shown). We asked whether these NHE CaM-binding domains were functionally interchangeable. To address this question, we generated NHE1 chimeras with its CaM-binding domain replaced with those from NHE2 and NHE4, as well as with various CaM-binding segments from other sources; Na^+/Ca^{2+} exchanger (NCX1) inhibitory peptide XIP, a peptide from CaM-dependent protein kinase II, a peptide (23 residues of C28R2) from plasma membrane Ca^{2+} pump, and CaM-binding model peptide Trp-3 (Figure 2). XIP binds CaM with $K_d = 200$ nM and inhibits Na^+/Ca^{2+} exchange activity with $IC_{50} = 1.5$ μM (*23*). The peptide from CaM-dependent protein kinase II binds CaM with high affinity ($K_d = 0.1$ nM) and inhibits this enzyme strongly (*28*, *29*). C28R2 binds CaM ($K_d = 0.1$ nM) and inhibits the Ca^{2+} pump activity with $IC_{50} = 1$ μM (*30*). Trp-3 binds to CaM with extremely high affinity ($K_d = 0.2$ nM) (*31*).

First, we measured the resting pH_i in various mutant transfectants placed in an acidic medium ($pH_o = 6.0$) to examine their ability to control pH_i. We also measured EIPA-sensitive $^{22}Na^+$ uptake in corresponding mutant transfectants in normal uptake medium after the prepulse with 30 mM NH_4^+ which gives activity close to V_{max} (*9*) (Figure 3A). Previously we showed that plot of one of these parameters against the other was useful to estimate the pH_i-sensitivity of a large number of NHE1 mutants whose expression levels in cells were significantly varied (*9*). In cells expressing chimera with the NHE2 CaM-binding segment, the relationship between pH_i and exchange activity

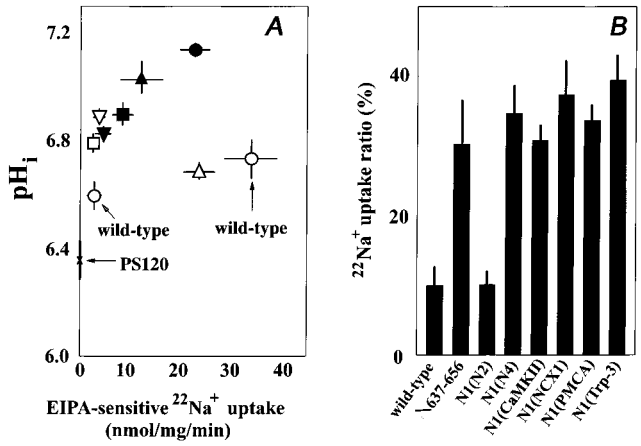

FIGURE 3: pH_i-sensitivity of wild-type and various chimeric NHE1s. (A) pH_i at $pH_o = 6.0$ and EIPA sensitive $^{22}Na^+$ uptake (30 mM NH_4^+ prepulse) were measured (see text) using cells expressing the wild-type NHE1 (○), Δ637−656 (●), N1(N2) (△), N1(N4) (▲), N1(NCX1) (□), N1(PMCA) (▽), N1(CaMKII) (■), or N1(Trp-3) (▼), or no transfected PS120 cells (×) as described under Experimental Procedures. For the wild-type NHE1, two populations of cells with two different V_{max} values of $^{22}Na^+$ uptake were used. Data are means ± SD of at least three independent experiments (B) The ratio between EIPA-sensitive $^{22}Na^+$ uptake activities measured in cells prepulsed with 3 and 30 mM NH_4^+. Data are means ± SD of at least three independent experiments.

was found to be similar to that for the wild-type transfectant, indicating that the replacement does not significantly affect pH_i-sensitivity of NHE1. In contrast, cells expressing chimeras containing the NHE4 CaM-binding segment or other exogenous segments had elevated levels of resting pH_i, which seem to be comparable to that for the mutant (Δ637−656) deleted of the CaM-binding domain of NHE1, when the difference in the expressed exchange activities (V_{max}) of these chimeras were considered. Thus, among these exog-

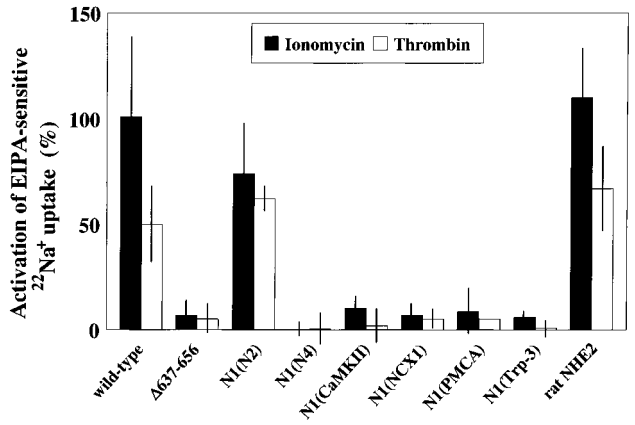

FIGURE 4: Effect of ionomycin and thrombin on the rate of EIPA-sensitive $^{22}Na^+$ uptake in cells expressing NHE1 variants. EIPA-sensitive $^{22}Na^+$ uptake was measured in cells transfected with various NHE1 variants in the absence or presence of ionomycin or thrombin as described under Experimental Procedures. Percentage activation of the $^{22}Na^+$ uptake activity was plotted in the *ordinate* (means ± SD of at least three independent experiments).

enous segments tested, only the NHE2-segment exerted an autoinhibitory effect on pH_i-sensitivity of NHE1.

The same conclusion was also obtained from another approach. We measured initial rates of $^{22}Na^+$ uptake into cells acid-loaded to pH_i of 7.23 ± 0.09 (n = 20) and <6.2, respectively, by prepulses with 3 and 30 mM NH_4^+, and then we calculated the ratio between these activities for each NHE1 mutant (Figure 3B). Because expression levels of mutant exchangers can be normalized by the activity in cells prepulsed with 30 mM NH_4^+ (see above), this ratio is the activity of a particular mutant at pH_i of 7.23 (3 mM NH_4^+ prepulse), which approximately reflects the pH_i-sensitivity of each mutant (cf. Figure 5). The ratio was low for both the wild-type NHE1 and the chimera with the NHE2-segment, whereas it was high for chimeras containing the NHE4-segment or other exogenous segments whose levels were similar to that for the deletion mutant (Δ637−656) (Figure 3B). The higher pH_i-sensitivity of the chimera with the NHE4-segment compared with the chimera with the NHE2-segment was confirmed when the pH_i-dependence of EIPA-sensitive $^{22}Na^+$ uptake of these mutants was directly measured in resting cells (compare Figures 5A and B).

Figure 4 shows the effect of Ca^{2+}-mobilizing agents ionomycin and thrombin on the Na^+/H^+ exchange in cells partially acidified by 5 mM NH_4^+ prepulse. Replacement of NHE1 CaM-binding segment with other exogenous segments, except the NHE2-segment, abolished early activation of exchange activity by these Ca^{2+} mobilizing agents. As shown typically in Figure 5A and B, ionomycin-induced alkaline shift of $^{22}Na^+$ uptake was retained in the chimera with NHE2-segment, whereas it was abolished in the chimera with NHE4-segment. Thus, among these various exogenous CaM-binding segments only the NHE2-segment is functionally interchangeable with the autoinhibitory CaM-binding domain of NHE1. We expressed the rat NHE2 cDNA in PS120 cells and measured the effect of ionomycin on $^{22}Na^+$ uptake activity. Ionomycin activated NHE2 by inducing an alkaline shift of exchange (Figures 4 and 5C), indicating that NHE2, like NHE1, is a Ca^{2+}-activatable isoform. We also tried to express the NHE4 protein in PS120. However, it was not possible to isolate cell clones expressing NHE4 with a high Na^+/H^+ exchange activity, which seems to be

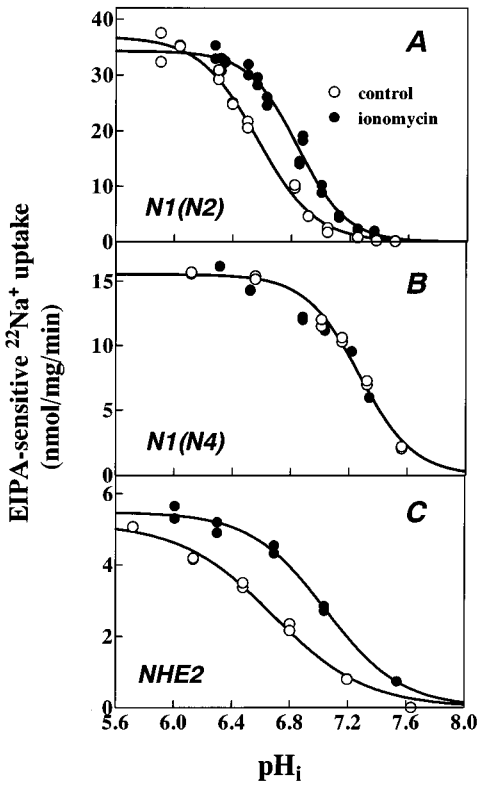

FIGURE 5: Effect of ionomycin on pH_i-dependence of EIPA-sensitive $^{22}Na^+$ uptake in cells expressing NHE1 chimeras or intact NHE2. The pH_i-dependence of the rate of $^{22}Na^+$ uptake was measured using cells transfected with NHE1 chimeras [(A) N1-(N2); (B) N1(N4)] or intact rat NHE2 (C) in the absence or presence of 5 μM ionomycin as described under Experimental Procedures.

consistent with the report that NHE4 did not exhibit exchange activity in isoosmotic solution (*32*).

Effect of Amino Acid Substitution with NHE1 CaM-Binding Domain on Exchange Activity. We predicted that amino acids commonly present in NHE1 and NHE2 but absent in NHE4 would account for the difference in the effects of the NHE2- and NHE4-segments. Amino acid comparison revealed that Leu639, Arg651, and His652 of NHE1 are conserved in the CaM-binding segment of NHE2 but not in that of NHE4 (Figure 2). Figure 6 shows the effect of substitution of these amino acids on pH_i-sensitivity and ionomycin-induced activation of NHE1. The pH_i-sensitivity of mutant exchangers were estimated as described above by measuring the ratio between $^{22}Na^+$ uptake activities in cells prepulsed with 3 and 30 mM NH_4^+ (cf. Figure 3B). We also measured the ratio between EIPA-sensitive $^{22}Na^+$ uptake activities in cells pH_i-clamped at 6.8 and 5.6 by a K^+/nigericin method. A single amino acid substitution of Leu639 of NHE1 by methionine (which is present in NHE4) considerably increased these parameters for the pH_i-sensitivity, suggesting that autoinhibitory function of the NHE1 CaM-binding domain was impaired by this substitution (Figure 6A). The same amino acid substitution also markedly reduced ionomycin-induced activation of the mutant NHE1 (Figure 6B). Any one of other hydrophobic amino acids [isoleucine, valine, tryptophan (Figure 6), phenylalanine, and tyrosine (data not shown)] could not functionally replace leucine completely. Substitution of Arg651/His652 of NHE1 by Lys/Tyr (which is present in NHE4) also significantly reduced the function of the NHE1 CaM-binding domain. Opposite substitutions, Met639→Leu together with

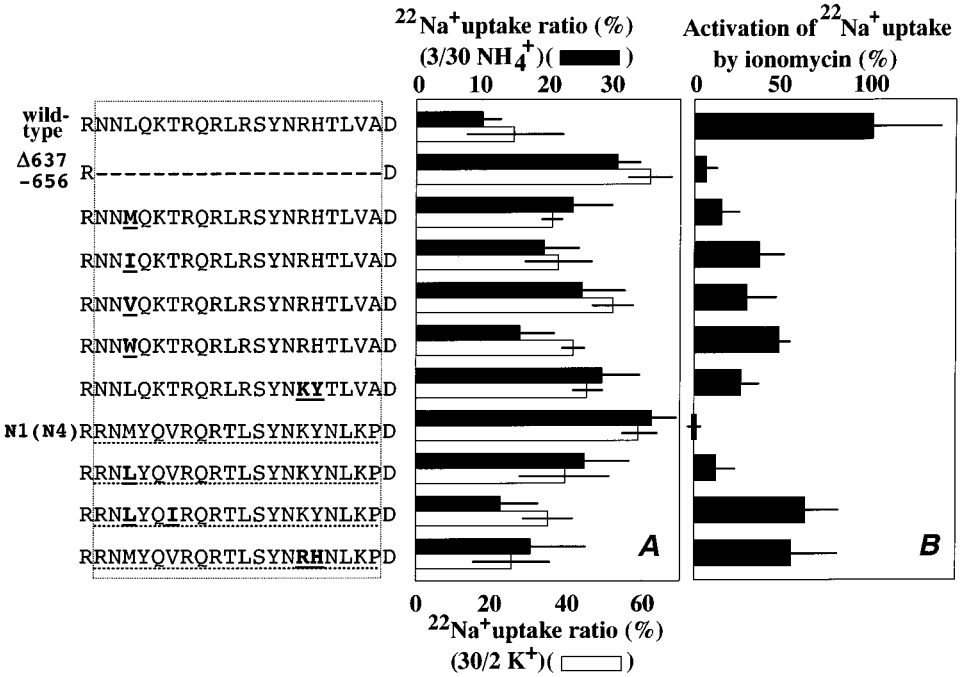

FIGURE 6: Effect of amino acid substitution in the CaM-binding domain on pH_i-sensitivity and ionomycin-induced activation of NHE1. Amino acid sequences on the left are those for the NHE1 CaM-binding domain (first seven sequences) or the chimera with N4-segment (N1(N4)) (last four sequences) with substitutions. Substituted amino acids (bold) are underlined. (A) The ratio between EIPA-sensitive $^{22}Na^+$ uptake activities measured in mutant transfectants prepulsed with 3 and 30 mM NH_4^+ (closed bars) and the ratio between EIPA-sensitive $^{22}Na^+$ uptake activities measured in the same mutant transfectants pH_i-clumped at $pH_i = 6.8$ and 5.6 (open bars) (see text). Data are means ± SD of at least three independent experiments. (B) Ionomycin-induced activation of EIPA-sensitive $^{22}Na^+$ uptake. Data are represented as the percentage activation (means ± SD of at least three independent experiments).

Val642→Ile (which is present in NHE2) or Lys651/Tyr652→Arg/His, in the chimeric exchanger containing the NHE4-segment partially restored both the pH_i-sensitivity and the ionomycin-induced activation, although substitution of Met639→Leu alone in the same chimera was not as effective. All these results suggest that Leu639, Arg651, and His652 play an important role as the key residues for the autoinhibitory function of NHE1 CaM-binding domain.

Photoaffinity Cross-Linking of NHE1 CaM-Binding Peptide. The sequence-specific autoinhibition exerted by the CaM-binding domain raised the possibility that this domain interacts specifically with other portion(s) of the NHE1 molecule. To study such an interaction, we used photoaffinity cross-linker Denny-Jaffe reagent. Immunoaffinity-purified NHE1 was mixed with ^{125}I-labeled N1-CBD and then exposed to UV light (Figure 7). N1-CBD was rapidly crosslinked to the NHE1 protein under light, reaching a plateau in >9 min after the start of light exposure. In order to examine the specificity of crosslinking, the immunoaffinity-purified NHE1, crude cell extract and their mixture were separately exposed to UV light in the presence of ^{125}I-labeled N1-CBD, which were then subjected to SDS−PAGE, silver-stained (Figure 7A), and visualized by autoradiography (Figure 7B). N1-CBD was cross-linked efficiently to the NHE1 protein but poorly to other contaminating proteins or proteins in the crude cell extract, except some low molecular weight proteins including the immunogloblin heavy chain (IgG). The ^{125}I-radioactivity incorporated into IgG or other proteins was less than 10% of that incorporated into the NHE1 protein, when it was normalized by the amount of each protein estimated by densitometry (data not shown). Thus, N1-CBD was cross-linked much more efficiently to NHE1 than to other proteins. To further examine the specificity of photoaffinity cross-linking, purified NHE1 was

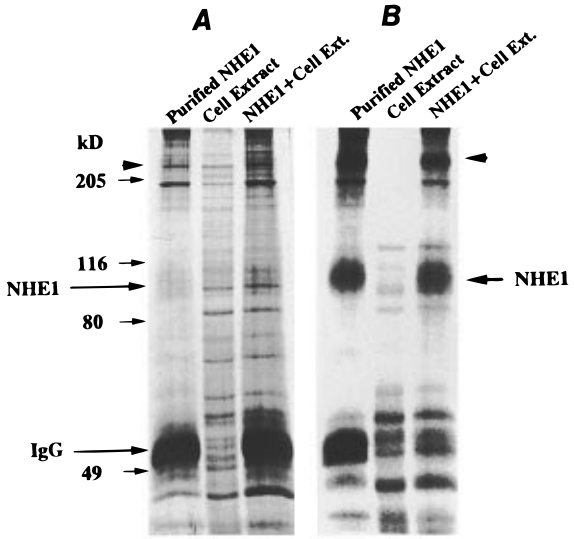

FIGURE 7: Photoaffinity cross-linking of CaM-binding peptide to the NHE1 protein. ^{125}I-labeled N1-CBD was incubated with either immunoaffinity-purified NHE1 protein (see Experimental Procedures), crude cell extract (75 μg/mL), or a mixture of both (total volume, 400 μL each) and was then exposed to light for 10 min. Supernatant was removed and NHE1 protein was eluted with 50 μL of SDS−PAGE sample buffer from protein A-Sepharose beads (lanes 1 and 3). For lanes 1 and 3, 20 μL samples were analyzed by SDS−PAGE (7.5% gel). For the cell extract (lane 2), 0.5 μg of protein was analyzed. Proteins were visualized by silver staining (A), while ^{125}I-labeled proteins were visualized by Bioimage analyzer (B). Arrows and arrowheads represent the NHE1 monomers and oligomers (*43*), respectively.

incubated with ^{125}I-labeled N1-CBD under light in the presence of excess nonradioactive N1-CBD or XIP. Nonradioactive N1-CBD, but not XIP, markedly reduced ^{125}I incorporation into the NHE1 protein (Figure 8), indicating

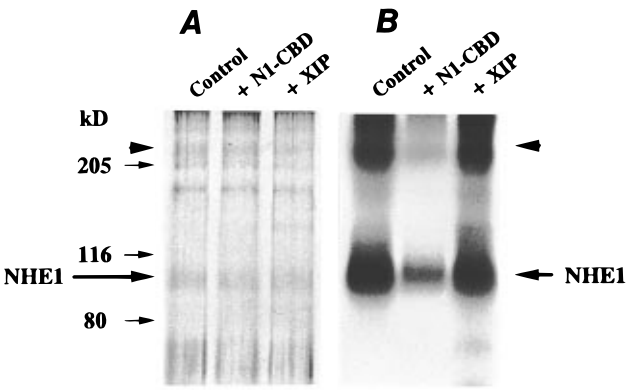

FIGURE 8: Effect of presence of excess nonradioactive peptides on photoaffinity cross-linking. ^{125}I-labeled N1-CBD was mixed with immunoaffinity-purified NHE1 protein (see Experimental Procedures) in the presence of 200 μM of nonradioactive N1-CBD or XIP and then exposed to light for 10 min. Proteins were visualized by silver staining (A), while ^{125}I-labeled proteins were visualized by Bioimage analyzer (B). Arrows and arrowheads represent the NHE1 monomers and oligomers, respectively.

that cross-linking mostly occurs in a specific manner. These data suggest that N1-CBD interacts physically with other portion(s) of the NHE1 molecule.

DISCUSSION

In the present study, to obtain insight into the mechanism for the autoinhibition by the CaM-binding domain of NHE1 we tested the effect of the replacement of this domain with the homologous segments from NHE2 and NHE4 or CaM-binding segments from other sources. We found that the NHE2-segment functions as an autoinhibitory domain in NHE1. Consistent with this finding, rat NHE2, like human NHE1, was found to be activated in response to ionomycin, suggesting that the NHE2-segment functions as a Ca^{2+}-dependent autoinhibitory domain in NHE2. In contrast, the NHE4-segment was not effective in NHE1 despite its high sequence homology with the NHE2-segment. The same negative results were obtained with the CaM-binding segments from Na^+/Ca^{2+} exchanger, plasma membrane Ca^{2+}-ATPase, and CaM-dependent protein kinase II, and CaM-binding peptide Trp-3. Previously, the Na^+/Ca^{2+} exchanger autoinhibitory peptide XIP was shown to be a potent inhibitor of both the plasma membrane and sarcoplasmic reticulum Ca^{2+}-ATPases (*33*). XIP also inhibited the gastric H^+/K^+-ATPase (S. Wakabayashi, unpublished data). In addition, the CaM binding peptides from myosin light chain kinase, CaM-dependent protein kinase II and protein kinase C were reported to be mutually interchangeable within these enzymes, although the strength of inhibition was varied (*34*). In contrast to these findings, N1-CBD did not inhibit Na^+/Ca^{2+} exchanger, plasma membrane and sarcoplasmic reticulum Ca^{2+}-ATPases, or H^+/K^+-ATPase (S. Wakabayashi, unpublished data). Thus, the CaM-binding autoinhibitory domain of NHE1 seems to be highly specific to NHE1, although we did not test the effect of this segment on activities of other NHE isoforms.

A single amino acid substitution of Leu639→Met in the NHE1 CaM-binding domain greatly reduced the autoinhibition and Ca^{2+}-induced activation of NHE1 (Figure 6), indicating that Leu639 is critically important. Other hydrophobic amino acids could not fully replace it functionally. On the other hand, a single amino acid substitution of Met639→Leu in the NHE4-segment incorporated into NHE1 did not render the NHE4-segment autoinhibitory, whereas substitution of Met639→Leu together with Val641-Ile (Ile is originally present in NHE2-segment) partially restored the autoinhibition and ionomycin-induced activation. Thus the effect of Leu639 substitution in this chimera was influenced by surrounding amino acids. Importance of hydrophobic amino acids in the autoinhibition has been recognized in several other proteins such as myosin light chain kinase (*35*) and K^+ and Na^+ channels (*36*, *37*). Arg651/Tyr652, which are fairly distant from Leu639 but conserved in both the NHE1 and NHE2 CaM-binding segments, are also important for the function of the CaM-binding domain in NHE1, because substitution of Arg651/His652→Lys/Tyr (originally present in NHE4) significantly decreased the autoinhibition and ionomycin-induced activation and because substitution of Lys651/Tyr652→Arg/His in the chimera containing the NHE4-segment partially restored the autoinhibition and Ca^{2+}-induced activation. We previously reported that charge reversal mutation of Lys641, Arg643, Arg645, and Arg647 to four negative residues (Glu) abolished the autoinhibitory function of the NHE1 CaM-binding domain (*9*). However, single or several double substitutions of these amino acids with Gln did not significantly affect the function of this domain (see ref 7 and S. Wakabayashi, unpublished result). Thus some positive charge from these basic amino acids is also important probably for charge−charge interaction. All these results suggest that the autoinhibition occurs in a manner specific to the sequence of the autoinhibitory domain of NHE1.

The results from cross-linking experiments with ^{125}I-labeled N1-CBD suggest that the NHE1 protein contains a region(s) recognizing the autoinhibitory domain. The region(s) interacting with the autoinhibitory domain in NHE1 is currently unknown. Recently we have provided evidence that an 80-amino acid cytoplasmic region (aa 515−595, subdomain I) adjacent to the last membrane-spanning segment is required for the maintenance of pH_i-sensitivity of NHE1 within a high physiological range. We found that deletion of subdomain I from NHE1 caused a drastic acidic shift of pH_i-dependence of exchange activity. Furthermore, subdomain I was found to be involved in a similar acidic shift of the pH_i-dependence caused by cell ATP depletion. Such ATP depletion-induced shift of the pH_i-dependence was significantly less in deletion mutants lacking the CaM-binding autoinhibitory domain as compared to the wild-type, suggesting that subdomain I can be a candidate for the target of interaction with the autoinhibitory domain. The pH_i-sensitivity of NHE1 is thought to be regulated through a change in the H^+ affinity of the H^+-modifier site (see the introduction), although the location of the latter in the NHE1 molecule is still unknown. It is possible that subdomain I has a stimulatory interaction with the H^+-modifier site, increasing its H^+ affinity, and that ATP depletion blunts such interaction. Alternatively, subdomain I itself may contain the H^+-modifier site, and a conformational change in this subdomain could modulate the H^+ affinity of the latter. However, the relationship between the H^+-modifier site, subdomain I, and the CaM-binding autoinhibitory domain is currently unclear.

It is important to note that the autoinhibition is incomplete in NHE1. The Na^+/H^+ exchange occurs even in the unstimulated cells, as long as pH_i becomes lower than the

resting level. Recently, the molecular basis of autoinhibition was deduced from the three dimensional structures of several kinases (for review see ref 38) including CaM-dependent kinase I (*39*) and *Caenorhabditis elegans* twitchin kinase (*40*). In these enzymes, the inhibitory domain extensively interacts with the catalytic core and completely inactivates the enzyme. Such a tight interaction of the inhibitory domain with the active site does not appear to occur in NHE1. The interaction of the autoinhibitory domain with its acceptor would alter the H^+ affinity of the H^+-modifier site indirectly through a conformational change of the NHE1 protein. Such an indirect interaction seems to occur also in the plasma membrane Ca^{2+}-pump which exhibits residual activity in the presence of Ca^{2+} but in the absence of CaM (for review, see ref 41).

ACKNOWLEDGMENT

We thank Dr. Bernhard Schmitt and Dr. Bénédict Bertrand for their participation during the early stage of this work.

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BI9715472