The ubiquitous plasma membrane Na\(^+\)/H\(^+\) exchanger (NHE1) is rapidly activated in response to various extracellular signals. To understand how the intracellular Ca\(^{2+}\) is involved in this activation process, we investigated the effect of Ca\(^{2+}\) ionophore ionomycin on activity of the wild-type or mutant NHE1 expressed in the exchange-deficient fibroblasts (PS120). In wild-type transfectants, a short (up to 1 min) incubation with ionomycin induced a significant alkaline shift (~0.2 pH unit) in the intracellular pH (pH\(_i\)) dependence of the rate of 5-(N-ethyl-N-isopropyl)amiloride-sensitive \(\mathrm{Na}^+\) uptake, without changes in the cell volume and phosphorylation state of NHE1. Mutations that prevented calmodulin (CaM) binding to a high affinity binding region (region A, amino acids 636-656) rendered NHE1 constitutively active by inducing a similar alkaline shift in pH\(_i\), dependence of Na\(^+\)/H\(^+\) exchange. These same mutations abolished the ionomycin-induced NHE1 activation. These data suggest that CaM-binding region A functions as an "autoinhibitory domain" and that Ca\(^{2+}\)/CaM activates NHE1 by binding to region A and thus abolishing its inhibitory effect. Furthermore, we found that a short stimulation with thrombin and ionomycin had apparently no additive effects on the alkaline shift in the pH\(_i\) dependence of Na\(^+\)/H\(^+\) exchange and that deletion of region A also abolished such an alkaline shift induced by a short thrombin stimulation. The results strongly suggest that the early thrombin response and the ionomycin response share the same activation mechanism. Based on these data and the results shown in the accompanying paper (Bertrand, B., Wakabayashi, S., Ikeda, T., Pouysségur, J., and Shigekawa, M. (1994) J. Biol. Chem. 269, 13703-13709), we propose that CaM is one of the major "signal transducers" that mediate distinct extracellular signals to the "pH\(_i\) sensor" of NHE1.

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1 The abbreviations used are: pH\(_i\), intracellular pH; NHE1, Na\(^+\)/H\(^+\) exchanger isoform 1; CaM, calmodulin; pH\(_o\), extracellular pH; [Ca\(^{2+}\)\(_i\)], intracellular Ca\(^{2+}\) concentration; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; Mes, 2-(N-morpholino)ethanesulfonic acid; Mops, 3-(N-morpholino)propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; aa, amino acids(s).

Regulation of the intracellular pH (pH\(_i\)) and the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) is crucial to a variety of cell functions such as cell proliferation, secretion, and contraction. Both pH\(_i\) and [Ca\(^{2+}\)\(_i\)] are finely controlled by various extracellular stimuli including hormones, neurotransmitters, and growth factors. Many of these agents bind to their specific receptors in the cell membrane, leading to phosphatidylinositol 4,5-bisphosphate hydrolysis and subsequent inositol triphosphate and diacylglycerol formation (1). Inositol triphosphate releases Ca\(^{2+}\) from internal stores, whereas diacylglycerol, together with Ca\(^{2+}\), is believed to activate protein kinase C. One consequence of such signaling cascades in numerous cell types is stimulation of the plasma membrane Na\(^+\)/H\(^+\) exchanger and subsequent cytoplasmic alkalization (2-4), most prominent in the absence of bicarbonate (5).

The Na\(^+\)/H\(^+\) exchanger (NHE1 human isoform) that has recently been cloned (6) and characterized (7-9), is a ubiquitous amiloride-sensitive electroneutral transporter that can be activated in response not only to hormones and growth factors but also to other stimuli such as hyperosmotic stress (2-4). The activation of NHE1 results from increased affinity of the allosteric modifier site of the exchanger for the intracellular H\(^+\) (10-12). At present, however, little is known about the molecular mechanism of regulation of this pH\(_i\) sensor. Previous studies indicate that protein kinase C is involved in this activation of NHE1 (2, 3). Several lines of evidence have suggested that Ca\(^{2+}\) also activates Na\(^+\)/H\(^+\) exchange. However, the effect of intracellular Ca\(^{2+}\) has been controversial. Ca\(^{2+}\) ionophore has been reported to stimulate Na\(^+\) influx in human foreskin fibroblast (HSWP cells) (13) and increase pH\(_i\) in thymic lymphocyte (14), human skin fibroblast (WS-1 cells) (15), and human platelets (16). On the other hand, Ca\(^{2+}\) ionophore has been reported not to increase pH\(_i\) in other types of cells such as 3T3 fibroblasts (17), human foreskin fibroblasts (12), and smooth muscle cells (18). These inconsistent data may result from the use of different cell types or different experimental conditions.

In the accompanying paper (19), we found that NHE1 is a novel member of the calmodulin (CaM)-binding proteins and that two CaM-binding sites are located in the middle of the carboxyl-terminal cytoplasmic regulatory domain. We have presented evidence that the high affinity CaM-binding region A (aa 636-656) is involved in the activation of NHE1 in response to growth factors and osmotic stress. In view of a micromolar range of intracellular CaM concentration, we thought that direct binding of Ca\(^{2+}\)/CaM to region A may be a key event in the Ca\(^{2+}\)-induced activation of NHE1.

In the present work, we studied the effect of an ionomycin-induced [Ca\(^{2+}\)\(_i\)] increase on the Na\(^+\)/H\(^+\) exchange activity in PS120 fibroblasts expressing the wild-type and mutant NHE1. Here we show that the ionomycin-induced [Ca\(^{2+}\)\(_i\)] increase elicits an alkaline shift in pH\(_i\) dependence of Na\(^+\)/H\(^+\) exchange without changes in the cell volume and phosphorylation state of NHE1. From the analysis with transfectants having mutations in the high affinity CaM-binding region A, we propose that region A exerts an inhibitory effect on protonation of the allosteric modifier site and that direct binding of Ca\(^{2+}\)/CaM to it...
CaM-binding Site and Ca\textsuperscript{2+} Regulation of NHE1

**EXPERIMENTAL PROCEDURES**

**Materials**—The amiloride derivative, 5-(N-ethyl-N-isopropyl)amiloride (EIPA) was a gift from New Drug Research Laboratories of Kanebo, Ltd. (Osaka, Japan). \textsuperscript{32}NaCl and \textsuperscript{[\textsuperscript{14}C]benzoic acid were purchased from Du Pont NEN. All other chemicals were of the highest purity available.

**Cells and Culture Conditions**—The Na\textsuperscript{+/H\textsuperscript{+}} antiporter-deficient cell line PS120 (20) and the corresponding transfectants were maintained in Dulbecco's modified Eagle's medium (H21, Life Technologies, Inc.) containing 25 mM NaHCO\textsubscript{3} and supplemented with 7.5% (v/v) fetal calf serum, gentamycin (50 units/ml), and streptomycin (50 \mu g/ml). Cells were maintained at 37°C in presence of 7.5% serum.

**Construction and Stable Expression of NHE1 Mutant Molecules**—The plasmid including cDNA coding for the NHE1 human isoform with deleted of the 5'-untranslated region was described previously (plasmid designated pEAP-35') (7). The plasmid (pE6998) coding for NHE1 deleted of the carboxy-terminal cytoplasmic tail (aa 699-815) has also been described (7). Construction of a deletion mutant (637-656; aa 637-656 were deleted) and a charge reversal mutant (1K3R4E; Lys-641, Arg-643, Arg-645, and Arg-647 were replaced by 4 glutamic acids) and stable expression of the corresponding mutant exchangers were performed as described in the accompanying paper (19).

**Measurement of pH\textsubscript{r} Dependence of \textsuperscript{22}Na\textsuperscript{+} Uptake**—pH\textsubscript{r} dependence of \textsuperscript{22}Na\textsuperscript{+} uptake was measured as described previously (11) with slight modifications. Stable transfectants grown to confluence in 24-well dishes were incubated for 5 h in a serum-free, bicarbonate-free H21 medium buffered with 20 mM HEPES (pH 7.4) to maintain the Na\textsuperscript{+/H\textsuperscript{+}} exchanger in the resting state. Cells were then rapidly washed once with choline chloride standard solution (composition: 20 mM HEPES/Tris (pH 7.4), 120 mM NaCl, 5 mM KCl, 2 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 5 mM glucose). Cells were then rapidly washed once with choline chloride standard solution (composition: 20 mM HEPES/Tris (pH 7.4), 120 mM choline chloride, 2 mM CaCl\textsubscript{2}, and 1 mM MgCl\textsubscript{2}) with or without 5 mM ionomycin and incubated for 40 s in the same medium. \textsuperscript{22}Na\textsuperscript{+} uptake was started by incubating cells in choline chloride standard solution containing 1 mM \textsuperscript{[\textsuperscript{32}P]NaCl (37 kBq/ml) and 1 mM ouabain with or without 5 mM ionomycin. In some wells, the \textsuperscript{22}Na\textsuperscript{+} uptake medium also contained 0.1 mM EIPA. Forty s after, cells were rapidly washed four times with ice-cold phosphate-buffered saline to terminate \textsuperscript{22}Na\textsuperscript{+} uptake. Cells were then solubilized with 0.1 N NaOH, and radioactivity was measured with a γ-counter. pH\textsubscript{r} was estimated by measuring the distribution of \textsuperscript{[\textsuperscript{14}C]benzoic acid (37 kBq/ml) (21) under the same conditions as those used for \textsuperscript{22}Na\textsuperscript{+} uptake measurement, except that the uptake medium contained \textsuperscript{[\textsuperscript{14}C]benzoic acid and nonradioactive NaCl.

**Measurement of Intracellular Na\textsuperscript{+} Concentration**—For measurement of the intracellular Na\textsuperscript{+} concentration, cells were equilibrated with 110 kBq/ml of \textsuperscript{[\textsuperscript{32}P]NaCl for 4 h at 37°C in a serum-free H21 medium buffered with 20 mM HEPES (pH 7.4). Cells were further incubated for 30 min in NaCl standard solution containing 110 kBq/ml of Na\textsuperscript{2+} and 3 mM NaCl. Cells were then rapidly washed once with choline chloride standard solution with or without 5 mM ionomycin and incubated for another 40 s in the same medium. Cells were then washed four times with ice-cold phosphate-buffered saline to stop \textsuperscript{22}Na\textsuperscript{+} equilibration and solubilized with 0.1 N NaOH. Radioactivity was measured with a γ-counter. The intracellular Na\textsuperscript{+} concentration was calculated by assuming 5 μl of cell water for the protein (21).

**Measurement of Cell Volume**—Cell volume was measured by cell sizing using a Coulter Counter (model ZBI; Coulter Electronics) associated with a Coulter Channelizer (model C1000). The averaged cell volume was calculated from volume distribution curves. Before cell volume measurement, cells were trypsinized and resuspended in NaCl standard solution. Cell volume did not significantly change during 2 h after trypsinization.

**Immunoprecipitation and Phosphopeptide Mapping**—\textsuperscript{32}P labeling of cells, immunoprecipitation of NHE1, and phosphopeptide mapping were carried out as described previously (8, 22, 23).

**Other Procedures**—Preparation of a rabbit polyclonal antibody (RP-cd) was described in the accompanying paper (19). Protein concentration was measured by bicinchoninic acid assay system (Pierce Chemical Co.) using bovine serum albumin as a standard.

**RESULTS**

Ca\textsuperscript{2+} Ionophore Induces an Alkaline Shift in pH\textsubscript{r} Dependence of \textsuperscript{22}Na\textsuperscript{+} Uptake—Fig. 1 shows the time courses of EIPA-sensitive \textsuperscript{22}Na\textsuperscript{+} uptake by PS120 cells expressing the wild-type NHE1 and \textsuperscript{22}Na\textsuperscript{+} uptake proceeded linearly at least for the first minute. When cells were preloaded with 3 mM NH\textsubscript{4}Cl, ionomycin stimulated the rate of EIPA-sensitive \textsuperscript{22}Na\textsuperscript{+} uptake by 5-fold (Fig. 1A). In contrast, when cells were preloaded with 30 mM NH\textsubscript{4}Cl to produce a strong intracellular acidification, ionomycin did not stimulate \textsuperscript{22}Na\textsuperscript{+} uptake (Fig. 1B), indicating that ionomycin did not change the maximal rate (V\textsubscript{max}) of \textsuperscript{22}Na\textsuperscript{+} uptake. The ionomycin-induced activation at low NH\textsubscript{4}Cl concentration was not due to a change in the intracellular Na\textsuperscript{+} concentration, because the latter, as measured as described under “Experimental Procedures,” was 9.6 ± 0.8 and 10.5 ± 0.4 mM (means ± S.D., n = 3) after treatment with and without ionomycin, respectively.

Under the conditions of Fig. 1A, however, ionomycin produced a significant cytosolic acidification (∼0.2 pH unit) (Fig. 1C), which accounts for part of the observed activation of \textsuperscript{22}Na\textsuperscript{+} uptake. We also found that when different batches of wild-type transfectants were loaded with 3 mM NH\textsubscript{4}Cl, the ionomycin-induced activation of \textsuperscript{22}Na\textsuperscript{+} uptake and cytosolic acidification varied from one batch of cells to the other. To avoid these complications, we measured both \textsuperscript{22}Na\textsuperscript{+} uptake and pH\textsubscript{r} under the same experimental conditions using the same batch of cells and always analyzed \textsuperscript{22}Na\textsuperscript{+} uptake rates as a function of pH\textsubscript{r} with a change in the V\textsubscript{max} value. Since ionomycin rapidly (within 30 s) increased
The rate of \(^{22}\text{Na}^+\) uptake was plotted as a function of pH. EIPA-sensitive mutants provided convincing evidence that the rise in \([\text{Ca}^{2+}]_i\) stimulates the \(\text{Na}^+/\text{H}^+\) exchange activity by increasing pH sensitivity of the exchanger.

**Ionomycin Does Not Change Cell Volume**—Since the ionomycin-induced increase in \([\text{Ca}^{2+}]_i\), has been reported to induce cell shrinkage, which may lead to exchanger activation (14, 24), we checked the effect of ionomycin on the volume of cells expressing the wild-type NHE1. As shown in Fig. 3, ionomycin did not change the cell volume during the first 10 min, whereas sucrose (100 mM) induced a rapid, significant loss (10%) of cell volume. Thus, cell shrinkage does not appear to be involved in the ionomycin-induced activation of NHE1 at least under our conditions.

**Ionomycin Does Not Increase Phosphorylation of NHE1**—One possible mechanism for the \(\text{Ca}^{2+}\)-induced activation of NHE1 could be phosphorylation of critical regulatory site(s) in the NHE1 molecule (8, 9). We checked whether ionomycin increased phosphorylation of NHE1. Cells expressing the wild-type NHE1 were depleted of serum, labeled with \(^{32}\text{P}\) and then stimulated for 1 min with 5 \(\mu\text{M}\) ionomycin or for 20 min with 2 units/ml \(\alpha\)-thrombin as described under “Experimental Procedures.” The exchanger was subsequently immunoprecipitated with a specific antibody (RP-cd). As shown in Fig. 4A, 1-min treatment of cells with ionomycin did not significantly increase phosphorylation of the exchanger, whereas thrombin markedly increased it after the 20-min treatment; \(^{32}\text{P}\) radioactivity incorporated into the exchanger, relative to the control value, was 0.99 \(\pm\) 0.17 and 1.60 \(\pm\) 0.44 (means \(\pm\) S.D., \(n = 3\)) for the ionomycin- and thrombin-stimulated cells, respectively. It is important to point out that during the early phase (up to 1 min) of thrombin stimulation, phosphorylation of the exchanger did not increase significantly (data not shown), which is consistent with the previous observation (8). Fig. 4B shows the result of the phosphopeptide mapping analysis. As described previously (9, 22 and 23), four major phosphopeptides (P1–P4) together with additional minor spots (a, b, and c) appeared in the maps. The minor spots may represent the partially digested fragments as described previously (23). \(^{32}\text{P}\) Incorporation into two phosphopeptides (P3 and P4) relative to those into P1 and P2 increased in response to thrombin. However, ionomycin neither increased \(^{32}\text{P}\) incorporation into these major phosphopeptides nor produced any additional major phosphopeptide. These findings strongly suggest that phosphorylation of NHE1 did not occur during the ionomycin-induced rapid activation of NHE1.

**Mutations in CaM-binding Region A Abolish Ionomycin-induced Activation of NHE1**—In the accompanying paper (19), we identified two CaM-binding regions of NHE1 that had high and intermediate affinities for CaM. To examine whether the high affinity CaM-binding region A is involved in the ionomycin-induced activation of NHE1, we analyzed pH dependence of \(^{22}\text{Na}^+\) uptake by mutant exchangers having a defect in CaM binding. We used the deletion mutant of region A (Δ637–656)
rate were compared among four different transfectants, it was the rapid thrombin-induced alkaline shift in pH dependence of mutations of CaM-binding region A reduced the cytoplasmic pH dependence of \( \%a^+ \) uptake similar mechanisms.

Thus, we extensively repeated the same experiments under slightly

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fectants

and a mutant with charge reversal mutation of this region (1K3R4E), which lost the CaM-binding ability (19). As shown in Fig. 5 (A and B), these mutations almost completely abolished the ionomycin-induced alkaline shift in pH dependence of the rate of EIPA-sensitive \( 2^2Na^+ \) uptake. In addition, deletion of the cytoplasmic tail (aa 636–815) (plasmid A635 (23)) also produced the same effect (data not shown). In sharp contrast, a mutant deleted of the carboxy-terminal tail (aa 699–815) (plasmid pEA698 (7)) that retained CaM-binding regions, preserved the ionomycin-induced alkaline shift (Fig. 5C). These findings suggest that direct binding of Ca\(^{2+}\)/CaM to region A stimulated the exchange activity by increasing pH\(_s\) sensitivity.

**Mutations in CaM-binding Region A Abolish Thrombin-induced Activation of NHE1**—In the previous study (11), a brief stimulation of Chinese hamster lung fibroblastic cells (CCL39, a parental cell line of PS120) with \( \alpha \)-thrombin and insulin has been shown to induce an alkaline shift in pH dependence of the rate of amiloride-sensitive Na\(^+\)/H\(^+\) exchange with a built-in modifier activity by increasing pH\(_s\) sensitivity.

Mutations in CaM-binding Region A Abolish Thrombin-induced Activation of NHE1—In the previous study (11), a brief stimulation of Chinese hamster lung fibroblastic cells (CCL39, a parental cell line of PS120) with \( \alpha \)-thrombin and insulin has been shown to induce an alkaline shift in pH dependence of the rate of amiloride-sensitive Na\(^+\)/H\(^+\) exchange with a built-in modifier activity by increasing pH\(_s\) sensitivity.

To confirm that the pH\(_s\) sensitivity of Na\(^+\)/H\(^+\) exchange was indeed different among the wild-type and mutant transfectants, we measured a change in the resting value of pH\(_s\) in cells placed at various pH\(_s\). As shown in Fig. 8, pH\(_s\) of the wild-type transfectant was 0.1–0.4 pH unit higher than that of the exchanger-deficient cell line PS120 over the range of pH\(_s\), from 5.6 to 7.4. Interestingly, pH\(_s\) of the cells expressing two mutant exchangers (A637–656 and 1K3R4E) was 0.2–0.4 pH unit higher than that of the wild-type transfectant. For this experiment, we used wild-type and mutant transfectants showing comparable \( V_{max} \) values of Na\(^+\)/H\(^+\) exchange. Therefore, the data strongly suggest that mutants of CaM-binding region A possess higher pH\(_s\) sensitivity, compared to the wild-type exchanger. These findings demonstrate that mutations of CaM-binding region A constitutively activate the exchanger by increasing its pH\(_s\) sensitivity.

It should be noted that despite constitutive activation of mutants, their maximal activities were lower than that of the wild-type NHE1 (cf. Figs. 2, 5, and 6). This difference appears to be due to the reduced number of NHE1 expressed in the plasma membrane of mutant transfectants (data not shown). High copy-numbered mutant cells with high constitutive activity could be eliminated during the "H\(^+\)" killing selection because of the toxicity of high pH\(_s\) to cell growth.

**DISCUSSION**

The NHE1 molecule can be separated into two distinct domains, an amino-terminal transport domain (T) that catalyzes amiloride-sensitive Na\(^+\)/H\(^+\) exchange with a built-in modifier site (pH sensor) and a carboxyl-terminal cytoplasmic regulatory domain that determines the set point value of the modifier site (7) (cf. Fig. 9). Protonation of the modifier site is generally considered to activate the exchanger. In the present work, we studied the mechanism by which [Ca\(^{2+}\)] regulates activity of
of EIPA-sensitive 2ZNa+ uptake was normalized by the maximal rate from five independent experiments with wild-type transfectants and plotted against pHi.

uptake comparable to those of mutant transfecteds were selected by cell population of wild-type transfectants with a 13714
bonate-free H21-culture medium containing 10
then incubated for 5
Mops and 10
rn
Ca2+/CaM with region A and via interaction of R with aa
interaction between aa 637—635 and the H+ sensor (23). We propose that regulation of NHE1 by extra-cellular signals occurs both via interaction of Ca2+/CaM with region A and via interaction of R with aa 567—635.

A "constitutively" activate the exchanger, as opposed to the inhibition expected from the overall structural distortion. In addition, a large deletion of the cytoplasmic tail (aa 699—815) did not influence the NHE1 functions including the pHi sensitivity of Na+/H+ exchange and the ionomycin- growth factor-induced alkaline shift in the pHi dependence (see "Results" and Ref. 7). Therefore, we think that the observed mutation-induced effects are attributable to the loss of function of a limited segment (region A) of the cytoplasmic domain.

One could also argue that the ionomycin-induced activation of NHE1 may be due to cell shrinkage caused by an increase in [Ca2+], (14, 24). However, cell shrinkage did not occur in response to ionomycin under our experimental conditions, although osmotic response to a hypertonic solution occurred normally (Fig. 3). Therefore, cell shrinkage did not contribute to the observed ionomycin-induced activation of NHE1. It is also possible that an increase in [Ca2+], will activate protein kinase(s) such as Ca2+/CaM-dependent protein kinase or protein kinase C, which may eventually lead to phosphorylation and activation of NHE1. However, we detected neither a significant increase in the phosphorylation of NHE1 within 1 min after addition of ionomycin (Fig. 4A) nor ionomycin-induced formation of new phosphopeptides in the peptide map analysis (Fig. 4B). Thus direct phosphorylation of NHE1 is not involved in the rapid response to ionomycin.

Receptor occupancy by growth factors and hormones induces a rapid increase in [Ca2+]. We examined how a short stimulation with -thrombin affected the NHE1 activity. We found that: (i) thrombin and ionomycin had apparently no additive effects on the alkaline shift in the pHi dependence (see "Results"), (ii) deletion of CaM-binding region A completely abolished the thrombin-induced alkalization (Fig. 6), and (iii) thrombin did not increase phosphorylation of NHE1 after a short stimulation (1 min) (see "Results"). These data suggest that direct interaction of Ca2+/CaM with region A is also the main mechanism for the activation of NHE1 in the early phase of thrombin response.

It is important to note that the mutations abolishing CaM binding to region A (aa 636—656) reduced the cytoplasmic alkalinization induced by a long (10—20 min) stimulation with
thrombin only by 50% (see Fig. 7 in the accompanying paper (Ref. 19); see also Ref. 23). On the other hand, deletion of aa 567–635 (plasmid Δ567–635) resulted in a complete loss of such thrombin-induced alkalization and a drastic acidic shift in the pH dependence of Na+/H+ exchange (7, 23). The latter result suggests that aa 567–635 of the cytoplasmic domain is required for the maintenance of high pH sensitivity, as well as for the full activation of the exchanger by thrombin. Therefore, activation of NHE1 in response to growth factors seems to be mediated via at least two independent mechanisms involving interaction of two different regions of the cytoplasmic domain with the modifier site (pH sensor) within the NH2-terminal transporter domain (T), as illustrated in our working model (Fig. 9). Amino acids 636–656 are involved in Ca2+/CaM-dependent, rapid activation of NHE1, whereas aa 567–635 are involved in the slow and long lasting activation induced by growth factors.

In our previous paper, we proposed that aa 567–635 mediates growth factor signals through the effect of a putative regulatory protein (CaM) phosphorylation (22). Protein phosphorylation is involved in this R-mediated regulation, because a potent phosphatase inhibitor okadaic acid or protein kinase C activator phorbol ester has been shown to induce a long lasting activation of NHE1 (2, 3, 9, 27), even when the cytoplasmic tail (aa 636–815) was deleted (23). The R-mediated mechanism may involve phosphorylation of R itself, because all major phosphorylation sites in the NHE1 molecule have been shown to map to the cytoplasmic tail (aa 636–815), yet deletion of this region (plasmid Δ5638) still preserves 50% of cytoplasmic alkalization in response to growth factors including thrombin (23). Thus growth factors seem to regulate activity of NHE1 by the Ca2+/CaM-dependent and the R-mediated mechanisms, both of which do not require direct phosphorylation of the exchanger molecule. In addition to these, direct phosphorylation of the NHE1 cytoplasmic tail could provide another mechanism for activation of the exchanger. The latter mechanism will require further investigation.

In this study, we investigated the Ca2+-dependent activation of NHE1. It is interesting to compare the amino acid sequence of its NH2-terminus to those of the other recently cloned NHE isoforms (NHE2, NHE3, and NHE4) (6, 28–31). The amino-terminal transport domain with 12 (or 11) transmembrane helices and the cytoplasmic domain containing 12 (or 11) transmembrane helices contains many of the amino acids of the exchanger (trout P-NHE), which does not have a sequence homology to region A of NHE1, has been shown to exhibit a higher pH sensitivity, as compared to the human NHE1 (33). In contrast, an epitHELial isoform (rat NHE3) has been reported to possess a lower pH sensitivity (34). Such variation in the pH sensitivity could be due to the variation in the sequence of the specific cytoplasmic segments that interact with the modifier site within the amino-terminal transport domain.

In conclusion, the present data support the notion that the NHE1 activation induced by a relative short incubation with Ca2+ ionophore or thrombin occurs by reversing the inhibition by the CaM-binding autoinhibitory domain through the direct binding of Ca2+/CaM. These data suggest that CaM is one of major signal transducers that mediate distinct extracellular signals to pH sensor of NHE1. The regulation of NHE1 via Ca2+/CaM binding could be particularly important for muscle cells such as cardiac myocytes. These cells will require rapid extrusion of H+ produced in response to increases in [Ca2+] and metabolic activity associated with contraction. The Na+/H+ exchange stimulated rapidly through the direct binding of Ca2+/CaM would serve as a potent H+ extrusion system during contraction.

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