Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 5431–5435, June 1994
Biochemistry

The cytoplasmic domain of the Na^+/H^+ exchangers (NHEs) dictates the nature of the hormonal response: Behavior of a chimeric human NHE1/trout βNHE antiporter

(catecholamines/protein kinase A/protein kinase C/signal transduction)

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Communicated by Robert W. Berliner, February 28, 1994

ABSTRACT **Studies of the effect of cAMP on the cloned Na^+/H^+ exchangers (NHEs) are difficult to interpret as variable results have been reported for the different isoforms when expressed in various cell types. We took advantage of the fact that the human NHE1 and the trout erythrocyte βNHE, when expressed in the same cell line, PS120, respond differently to cAMP (NHE1 is insensitive, βNHE is activated) to analyze the molecular mechanisms of cAMP activation. We constructed both a chimera between NHE1 and βNHE and a set of βNHE mutants to delineate the critical parts of the molecule involved in the activation process. NHE1 becomes cAMP stimulated when its cytoplasmic domain is replaced by the cytoplasmic domain of βNHE; thus, the cytoplasmic C terminus of βNHE, which contains two cAMP-dependent consensus sequences, is essential to confer cAMP dependence. Serine to glycine substitution of only one of the two protein kinase A (PKA) consensus sites decreased by 60% the ability of cAMP to activate Na^+/H^+ exchange. Simultaneous Ser to Gly substitution of the two PKA consensus sites decreased the cAMP-mediated activation by 72%. The residual activation required a cytoplasmic fragment (aa 559–661) that contains four sequences considered likely as putative PKA consensus sites. The results obtained with the chimeric NHE also demonstrated that if the cytoplasmic C terminus is crucially involved in the hormonal activation, the rate of Na^+/H^+ exchange so induced can be modulated by the nature of the interaction between the N- and C-terminal domains.**

Na^+/H^+ exchangers (NHEs) are plasma membrane transport proteins found in a broad range of biological systems and have multiple functions. Four isoforms have been recently cloned and functionally evaluated after transfection in fibroblasts: NHE1, NHE2, and NHE3 from mammalian species (1–4) and βNHE from trout erythrocytes (5). Each isoform can be activated by a variety of stimuli including hormones, growth factors, and osmotic stress.

There are multiple unanswered questions concerning the regulation by hormones and second messengers (reviewed in ref. 6). Thus, the data on the effect of cAMP on NHEs are difficult to interpret. In renal and intestinal epithelia, apical Na^+/H^+ exchange is inhibited by cAMP analogs and a hormone capable of increasing cAMP content, while the basolateral Na^+/H^+ exchange activity remains unaffected. These results suggest a difference in the NHEs located, respectively, in the apical and the basolateral membranes. In agreement with that, a consensus sequence for cAMP is present in the cytoplasmic C terminus of NHE2, which appears to be localized on the apical surface of epithelial cells (3), while NHE1, the basolateral isoform, does not possess such a consensus sequence (1). In addition, as expected, when NHE1 is transfected in the PS120 fibroblasts, there is no effect of cAMP on the activity of the exchanger. However, when NHE1 is transfected into the OK (opossum kidney) cell line it is inhibited by cAMP, suggesting then some indirect regulation that is cell type specific (7). Unexpectedly also, there is no effect of cAMP on the activity of the apical isoform NHE2 (which contains cAMP consensus sites) when it is transfected into the PS120 fibroblasts. Thus, cAMP effects on mammalian NHEs are widely variable; they could be explained in some cases by the presence of putative protein kinase consensus sequences in the individual NHE isoform but, in other cases, they seem to be dependent on the cell type that expresses the NHE. The cAMP effect observed on the trout erythrocyte exchanger βNHE appears less ambiguous. The endogenous exchanger is strongly activated by cAMP and catecholamines (8–10). Cloning of this isoform showed that βNHE has two cAMP-dependent protein kinase consensus sequences in the C terminus, which are separated by only 4 amino acids (5). When transfected in PS120 fibroblasts, it remains activatable by cAMP and catecholamines. In addition, the cytoplasmic region between aa 559 and 759, which contains the two consensus sites, is required for cAMP regulation, since deletion of this region abolishes the response to catecholamines and cAMP, while the antiporter retains the ability to exchange Na^+ for H^+ and to be activated after protein kinase C (PKC) stimulation (5).

To obtain more insight into the relationship between the primary structure of NHEs and their regulation by cAMP, we constructed a chimera that exchanges the cytoplasmic domains of NHE1 and βNHE and also a set of point mutants in βNHE that abolishes protein kinase A (PKA) consensus phosphorylation sites. Analysis of these molecules expressed in PS120 fibroblasts demonstrates that the cytoplasmic domain of βNHE contains all the information to confer the cAMP response.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis of βNHE. cDNA T encoding the cAMP-activatable Na^+/H^+ antiporter (βNHE) was cloned into the eukaryotic expression vector pECE and called pET (5). Site-directed mutagenesis of cDNA T was carried out using the transformer site-directed mutagenesis kit (Clontech). To perform mutagenesis reactions, cDNA T was subcloned into the phagemid pBluescript SK and then the restriction fragment *Bst*XI/*Bst*XI was deleted to reduce the size of the plasmid and to eliminate an *Alf* III restriction site incompatible with the utilization of the transformer kit (Fig.

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Abbreviations: NHE, Na^+/H^+ exchanger; PKC, protein kinase C; PKA, protein kinase A; pH_i, intracellular pH; DMT, double mutant.

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1). After mutagenesis reactions, the *Hin*dIII/*Hin*dIII restriction fragment of cDNA T, containing the points of mutation, was exchanged with the *Hin*dIII/*Hin*dIII fragment of the wild-type pET.

The chimera Mermaid-NHE was constructed with the transmembrane domain of NHE1 and the cytoplasmic domain of βNHE. The cDNA coding for NHE1, subcloned into the vector pECE, was named pEAPΔ5′ (11). Cytoplasmic regions of βNHE and NHE1 are encoded by the *Msc* I/*Msc* I restriction fragments of pET and pEAP (Fig. 1). To construct Mermaid-NHE, the *Msc* I/*Msc* I fragment of pEAPΔ5′ was substituted with its pET *Msc* I/*Msc* I counterpart. Before carrying out the substitution, the *Msc* I restriction site of pET located at position 1734 was eliminated by a silent point mutation to avoid deletion of the *Msc* I(1575)/*Msc* I(1734) fragment. All mutations and subsequent ligations were verified with a T7 sequencing kit (Pharmacia).

Cell Culture and Expression of Na^+/H^+ Antiporters. Chinese hamster lung fibroblast PS120 cells [a derivative of CCL39 cells, which are Na^+/H^+ antiporter deficient (12)] and the corresponding transfectant cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (H21; GIBCO) containing 25 mM $NaHCO_3$ supplemented with 10% calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37°C in the presence of 5% CO_2/95% air. Cells were transfected (10 μg of plasmid per 2 × 10^5 cells) by the calcium phosphate precipitation method (13). Three days after transfection, cells were subjected to the H^+-killing test to eliminate cells that do not express a functional Na^+/H^+ antiporter (14). This test was repeated five times over a period of 3 weeks, and the resulting stable transfectant cells were used for experiments.

Determination of Intracellular pH (pH_i) Changes. Cells in 24-well plates were incubated 5 h in serum-free H21 medium to maintain the NHE in the resting state. Then they were washed once and equilibrated for 1 h in freshly prepared Hepes-buffered DMEM (pH 7.7) containing 1 μCi of [^{14}C]benzoic acid per ml (1 Ci = 37 GBq) with or without the Na^+/H^+ antiporter stimulating factors. At the indicated times, [^{14}C]benzoic acid incubation medium was aspirated and cells were rapidly washed four times with ice-cold phosphate-buffered saline (pH 7.4). Radioactivity was recovered from cells lysed in 0.1 M NaOH. The difference in pH_i from stimulated and unstimulated cells was calculated from the equilibrium distribution of the weak acid as described (15).

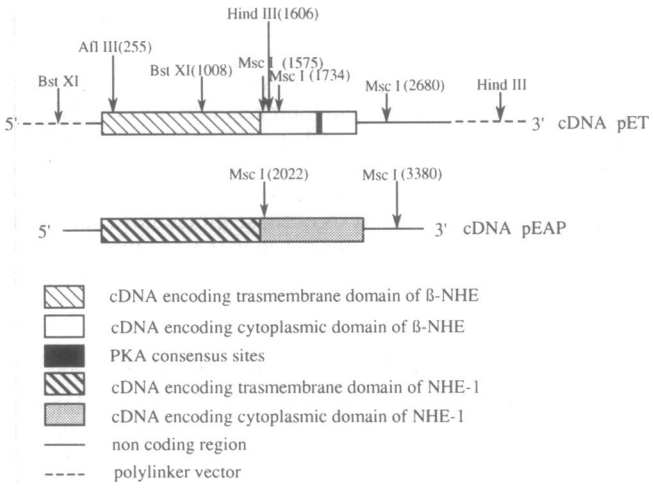

FIG. 1. Schematic representations of cDNA encoding βNHE (pET) and NHE1 (pEAPΔ5′). Noncoding regions are represented by solid bars and coding regions are represented by boxes. Mutagenesis of βNHE PKA consensus sites was carried out on the *Bst*XI(1008)/*Eco*RI fragment of pET subcloned into pBluescript. After mutagenesis, the *Hin*dIII/*Hin*dIII fragment was isolated and exchanged with the *Hin*dIII/*Hin*dIII fragment of the wild-type pET to get a complete mutated cDNA. To construct Mermaid-NHE, the *Msc* I/*Msc* I fragment of NHE1 was exchanged with its βNHE counterpart. The *Msc* I site of pET, located at position 1734, was first eliminated by a silent point mutation.

RESULTS

Grafting the βNHE Cytoplasmic Domain to NHE1 Confers cAMP Dependence to NHE1. Fig. 2 shows the effects of exogenous 8-BrcAMP on the pH_i of exchanger-deficient PS120 fibroblasts stably transfected with different forms of exchanger. The cells were incubated in the absence of bicarbonate at an external pH (pH 7.7) above the set point value. Thus, before addition of cAMP, all the NHEs are quiescent. It has been shown (5) that under these conditions, addition of cAMP to PS120 cells not transfected with an exchanger induced an intracellular acidification due to metabolic acidosis. Fig. 2*a* shows that a similar acidification was observed in PS120 cells transfected with the human exchanger NHE1, indicating, as expected, that NHE1 is not activated by cAMP. Conversely, in cells transfected with the trout erythrocyte exchanger βNHE, cAMP resulted in a marked alkalinization, which can be prevented by amiloride, indicating as shown previously that βNHE is activated by cAMP. To check the role of the cytoplasmic domain of βNHE in this cAMP-dependent activation, a chimera was

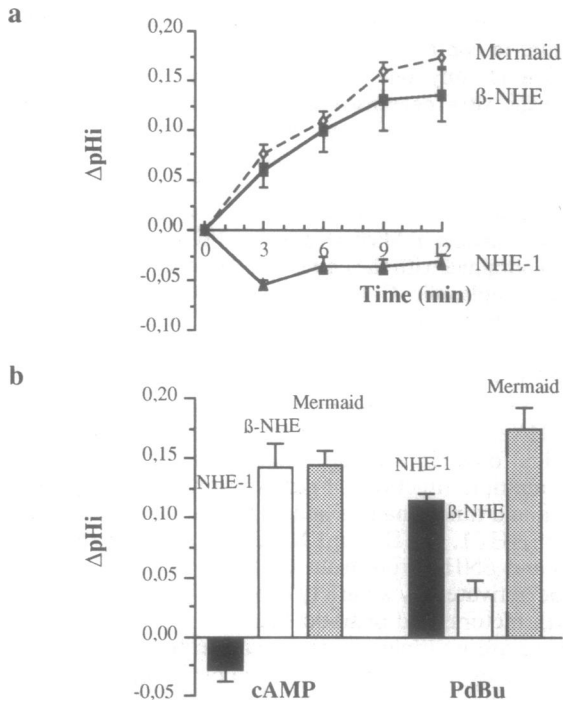

FIG. 2. (*a*) cAMP effects on human NHE1, trout erythrocyte βNHE, and chimeric Mermaid-NHE exchangers. Antiporter-deficient PS120 cells transfected with the different exchangers were incubated 5 h in serum-free H21 medium to maintain the NHE in the resting state. The cells were further incubated for 1 h at 37°C in bicarbonate-free H21 medium buffered with Hepes (pH 7.7). Cells were then placed in the same medium containing [^{14}C]benzoic acid at 1 μCi/ml. 8-BrcAMP (1 mM) and 3-isobutyl-1-methylxanthine at 50 μM were added at time 0; pH_i was estimated at several time intervals. Data represent means ± SE of four determinations. (*b*) Differential effects of cAMP and phorbol ester (PdBu; phorbol 12,13-dibutyrate) on stimulation of NHE1, βNHE, and Mermaid-NHE. Changes in pH_i were measured, as defined above, 12 min after addition of activators (200 nM PdBu). Error bars (means ± SE) are based on four determinations.

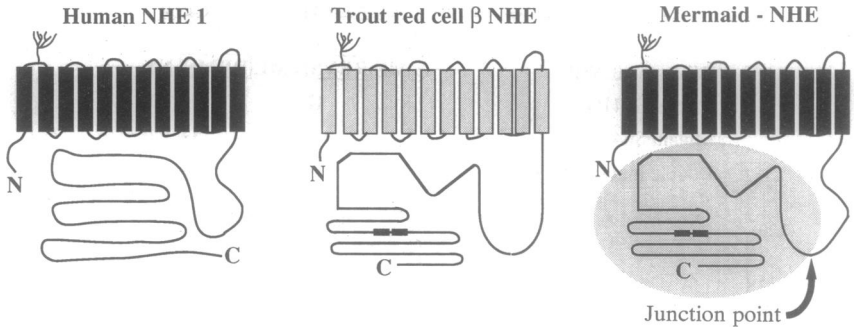

FIG. 3. Topographic representation of the human antiporter NHE1, the trout erythrocyte antiporter βNHE, and the chimeric antiporter constructed from NHE1 and βNHE. In this chimera, NHE1 sequences are represented by dark lines and βNHE sequences are represented by lines in the light stippled circle. As this chimera is constructed with the head of the human antiporter and the tail of the fish antiporter, it has been called Mermaid-NHE.

constructed with the transmembrane domain of NHE1 and the cytoplasmic domain of βNHE (Fig. 3; see also Fig. 1). As in this chimera, the head is from the human and the tail is from the fish exchangers; it has therefore been called Mermaid-NHE. Fig. 2 shows that Mermaid-NHE, when transfected in fibroblasts, was activated by cAMP with a magnitude similar to that of βNHE, indicating that the βNHE cytoplasmic domain totally controls the regulation by β agonists. The activation is inhibited by amiloride (data not shown). It is noteworthy that Mermaid-NHE was also activated by phorbol ester, but the rate of exchange was much greater than that of βNHE and similar to that of NHE1 (Fig. 2*b*).

Two Serine Residues Are Critically Involved in the Activation Process by cAMP. The results obtained with Mermaid-NHE indicate that the cytoplasmic domain of βNHE is essential to mediate activation by cAMP and other cAMP-dependent protein kinase activators such as catecholamine and forskolin. This cytoplasmic region contains two cAMP-dependent protein kinase consensus sequences RRVS and RRMS (5). These PKA consensus sites are spatially closely related, the two serines being located at positions 641 and 648, respectively. To examine the participation of these PKA sites in hormonal activation, we first constructed three antiporter mutants of βNHE characterized by serine to glycine substitution either at position 641 (S641G mutant) or at position 648 (S648G mutant) or at both positions (DMT for double mutant) (Table 1). These three mutant antiporters were then stably expressed in the exchanger-deficient cell line PS120 and selected for their capacity to regulate pH_i upon acid load. Fig. 4 shows that mutation of only Ser-641 or of only Ser-648 strongly reduced the response of the antiporter to exogenous cAMP. It is noteworthy that the transient acidosis observed after addition of cAMP is due to the fact that cell acidosis, which is induced by the addition of cAMP to PS120, then cannot be immediately balanced, as for βNHE, by the sluggish Na^+/H^+ exchange capacity of the mutated antiporters. The extent of cytoplasmic alkalinization of S641G and S648G mutants, measured 10 min after addition of cAMP, represent, respectively, 45% and 36% that of the wild type. The two sites are thus necessary for full activation of the antiporter, with roughly an equivalent participation level. However, the simultaneous mutation of the two serines, corresponding to the loss of the two PKA consensus sites, failed to fully eliminate PKA regulation of the antiporter; βNHE/DMT was still slightly activated by cAMP (Fig. 4), the activation level being then 28% of that observed with βNHE.

Conversely, it is noteworthy that mutation of serines did not modify the level of activation by the PKC pathway; Fig. 5 shows that all the mutants were activated by phorbol esters with the same efficiency as the wild-type βNHE.

Localization of a Complementary Zone Conferring Some cAMP Dependence. Thus, the two PKA consensus sequences are critically involved in the antiport activation by cAMP, but when these PKA sites are suppressed by mutation of serines, cAMP is still able to slightly activate the antiporter. To characterize the cytoplasmic element(s) that contributes to this residual activation, we constructed a set of truncations within the cytoplasmic domain of βNHE slightly upstream or downstream of the two PKA consensus sites of βNHE/DMT and expressed the truncated cDNAs in PS120 fibroblasts. Fig. 6 confirms, as previously demonstrated, that deletion of the last 200 amino acids (Δ559 mutant) abolishes the ability of cAMP to activate the antiporter. Conversely, DMT/Δ661 or DMT/Δ696 mutants, which are obtained by smaller C-terminal deletions (the last 98 and 63 aa, respectively), retained the ability to be slightly activated by cAMP and their response is almost identical. Thus, the residual cAMP-dependent activation observed after suppression of the PKA sites likely involves the cytoplasmic region between aa 559 and 661.

It is noteworthy that Δ559, which has lost the ability to be activated by cAMP, can still be activated by phorbol esters and other PKC activators (5). Thus, the distal region of the cytoplasmic domain corresponding to the last 200 aa is not critical for PKC activation.

DISCUSSION

In the present study, we have analyzed molecular mechanisms of cAMP regulation of the NHE. Starting from the fact

Table 1. Amino acid sequences of βNHE and derivative mutants between aa 638 and 648

pET cDNA	AGG	AGG	GTC	AGT				AGG	AGG	ATG	AGC
βNHE	Arg	Arg	Val	Ser-641	Val	Met	Glu	Arg	Arg	Met	Ser-648
Mutated cDNA	AGG	AGG	GTC	GGT				AGG	AGG	ATG	AGC
βNHE/S641G	Arg	Arg	Val	*Gly-641*	Val	Met	Glu	Arg	Arg	Met	Ser-648
Mutated cDNA	AGG	AGG	GTC	AGT				AGG	AGG	ATG	GGC
βNHE/S648G	Arg	Arg	Val	Ser-641	Val	Met	Glu	Arg	Arg	Met	*Gly-648*
Mutated cDNA	AGG	AGG	GTC	GGT				AGG	AGG	ATG	GGC
βNHE/DMT	Arg	Arg	Val	*Gly-641*	Val	Met	Glu	Arg	Arg	Met	*Gly-648*

In the first row, the sequence of wild-type pET is represented. Single nucleotide changes T → G in codon 641 and A → G in codon 648 have Ser-641 and -648 substituted by glycines. DMT was obtained in two steps, performing first a mutation to obtain S641G and then a mutation to construct S641G + S648G = DMT.

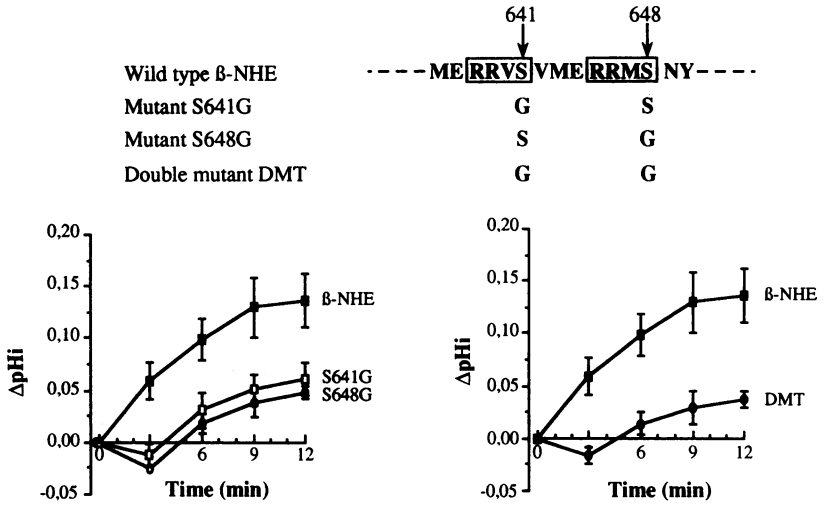

FIG. 4. cAMP effects on βNHE and on three βNHE mutants. These mutants were obtained by point mutation by substituting serine with glycine: S641G, S648G, and DMT. Single nucleotide changes in codon 641 (AGT → GGT) and in codon 648 (AGC → GGC) allowed substitution of serine by glycine. DMT has been obtained in two steps, performing first a mutation to obtain S641G and then a second mutation to construct S641G + S648G = DMT. Changes in pH_i were measured as defined in the legend to Fig. 2.

that the human NHE1 and the trout erythrocyte βNHE, when expressed in the same cell line, PS120, respond differently to cAMP (5), we have constructed both a chimera between NHE1 and βNHE and a set of mutants to delineate the critical parts of NHEs involved in cAMP regulation.

One important result is that NHE1, which normally is not cAMP regulated when expressed in PS120 fibroblasts, becomes responsive to cAMP when its cytoplasmic domain is replaced by the cytoplasmic domain of βNHE. Thus, the cytoplasmic C terminus of βNHE is essential to confer the cAMP-dependent activation of the antiporter. It has been shown (Fig. 6) that after truncation of the cytoplasmic segment corresponding to the deletion of the last 200 aa (Δ559) βNHE is no longer activated by cAMP and catecholamines, although it retains its ability to mediate amiloride-sensitive Na^+/H^+ exchange and to be activated by phorbol esters and PKC activators (5). Thus, all the determinants for activation by cAMP are localized in this fragment (aa 559–759), which in particular contains two typical cAMP-dependent protein kinase consensus phosphorylation sites. The results reported in this paper showed that these PKA consensus sites are indeed involved in the cAMP-mediated activation process: the point mutation of only one of the two serines decreases by 60% the ability of cAMP to activate the antiporter (Fig. 4). Of note is the fact that the mutation of serines does not change the Na^+/H^+ exchange capacity induced by phorbol ester (Fig. 5), indicating that the decrease in activity reported above is not related to a decrease in the amount of exchanger protein expressed in the plasma membrane. The removal of the two PKA consensus sites, obtained by the simultaneous mutation of the two serines, however, did not fully abolish cAMP regulation. A residual activation (≈28% of the control) persists. By examining selective deletion mutations, we showed that this residual activation required a fragment of the cytoplasmic tail (aa 559–661), which encompasses the two typical PKA consensus sequence phosphorylation sites RRXS. It has recently been proposed that not only RRXS but also RXS and RXXS sequences could possibly represent cAMP-dependent protein kinase consensus sequences (16). As this section of the βNHE molecule contains four such putative consensus sites (Ser-583, -609, -611, and -659), the residual activation could be related to the presence of these sites.

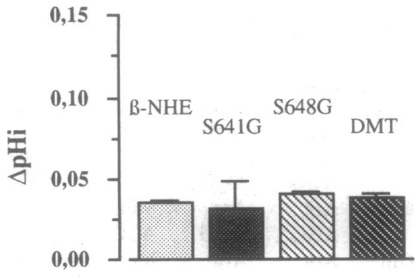

FIG. 5. Effect of phorbol ester on βNHE and three mutants obtained by point mutation of Ser-641 and/or -648. Changes in pH_i were measured as defined in Fig. 2, 12 min after addition of phorbol 12,13-dibutyrate (200 nM). Error bars (means ± SE) are based on triplicate determinations.

Thus, from these results it clearly appears that the difference in the ability of cAMP to activate βNHE and NHE1 is exclusively due to a difference in the structure of the cytoplasmic domains of the two NHEs, in particular the presence or absence of typical cAMP-dependent consensus sequences. Likely, phosphorylation of the cytoplasmic region of the exchanger by the cAMP-dependent protein kinase is crucial to activate the exchanger protein. How phosphorylation regulates the rates of Na^+/H^+ exchange is not yet understood at the molecular level. It is thought (1, 6, 11, 17) that such regulation of NHE is mediated through reversible phosphorylation-dependent interaction of the C-terminal cytoplasmic domain with the transporting domain. Then the results obtained with Mermaid-NHE would mean that both NHE1 and βNHE transmembrane domains have the same ability to interact with the cytoplasmic domain of βNHE. It must be noted that in NHE1 and βNHE, the membrane-spanning regions are highly homologous: first, they have 75% amino acid identity (5); second, they share the unique property among NHE isoforms to have the N-linked glycosylation consensus sequence in the first extracellular loop, a feature of NHE1 isoforms (1, 5, 6). Characterization of a region(s) within the transmembrane domain likely involved in transduction of the cAMP signal would need the construction of a chimera between the cytoplasmic domain of βNHE and the transmembrane domain of NHE3, the isoform that is most different from the others. If the cAMP response of βNHE can be transferred to NHE3, then it is likely that the putative intracellular loop between membrane segments 4 and 5 and between membrane segments 5a and 5b, which are the most highly conserved regions within the NHE family, are involved in this coupling with the cytoplasmic domain.

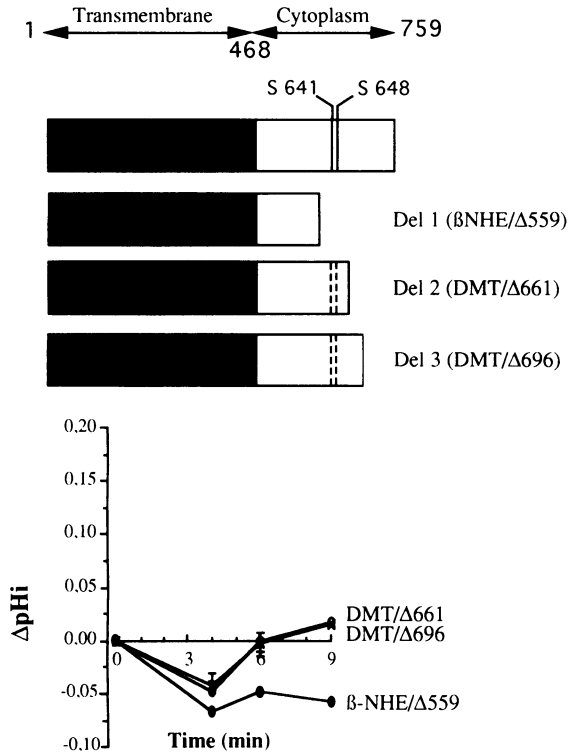

FIG. 6. Effects of cAMP on various deleted forms of βNHE. PS120 cells were stably transfected by trout erythrocyte exchanger cDNA coding for the complete protein (βNHE, 759 aa), coding for the first 559 aa (Δ559) or coding for the mutant DMT truncated at aa 661 (DMT/Δ661) or at aa 696 (DMT/Δ696). DMT is the wild-type βNHE in which Ser-641 and -648 have been replaced by glycine, thus suppressing the two cAMP-dependent consensus sequences. Changes in pH_i were measured as defined in Fig. 2.

The cytoplasmic tail is crucially involved in activation of NHEs by cAMP as shown above, but it is also required for activation by PKC activators, as demonstrated in NHE1 (11). Comparison of the effects of phorbol ester and cAMP on Mermaid-NHE allows us to obtain some additional information. Fig. 2 shows that transfected NHE1, while it is insensitive to cAMP, was strongly activated by phorbol ester; βNHE, on the other hand, was stimulated by both agonists but it transported at a much slower rate after phorbol ester activation than after cAMP activation [the same pattern is observed when erythrocytes are stimulated by PKA and PKC agonists (18)]. The comparison of phorbol ester and cAMP effects on Mermaid-NHE shows that the chimera had an unexpected behavior; it was activated by cAMP as βNHE, but it retained a strong reactivity to phorbol ester as NHE1. In other words, the βNHE cytoplasmic tail, when activated by cAMP, induced the same rate of exchange through the NHE1 and the βNHE transmembrane domains. Conversely, when activated by phorbol ester, the βNHE cytoplasmic tail induced a rate of exchange that was much smaller when mediated by the βNHE than by the NHE1 transmembrane domains. This cross-reactivity indicates that the kinetic properties of the antiport are determined not only from its capacity to receive the hormonal signal but also from cross-talk between cytoplasmic and membrane domains, which modulates the signal. In summary, our results obtained after transfection into the same fibroblast cell line of NHE1, βNHE, Mermaid-NHE, and a set of βNHE mutants clearly indicate first that the ability of NHEs to be activated by cAMP depends on the presence of PKA consensus sequences in the protein, suggesting that activation is caused by phosphorylation of the exchanger itself. This suggestion will be evaluated when a specific βNHE antibody becomes available. Second, the rate of exchange so induced would likely be modulated by the nature of interaction between the N- and C-terminal domains. The role of the PKA consensus sequences observed in NHEs physiologically inhibited by cAMP—i.e., NHE2 and NHE3—remains to be considered.

We thank D. Roux for technical advice and D. Grall for technical assistance on cell culture. This work was supported by grants from the Commissariat à l'Energie Atomique, the Centre National de la Recherche Scientifique (CNRS URA 638, CNRS UMR 134), and the Fondation pour la Recherche Médicale.

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