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Cloning and expression of a cAMP-activated Na^+/H^+ exchanger: Evidence that the cytoplasmic domain mediates hormonal regulation

(Na^+/H^+ exchange/catecholamines/protein kinase A/protein kinase C/signal transduction)

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ABSTRACT **The ubiquitous plasma membrane Na^+/H^+ exchanger (termed NHE1) is activated by diverse hormonal signals, with the notable exception of hormones acting through cAMP as second messenger. Therefore, the Na^+/H^+ exchanger found in the nucleated trout red cell is of particular interest since it is activated by catecholamines, forskolin, and cAMP analogues. We report here that a cloned cDNA encoding the red cell exchanger restores functional Na^+/H^+ activity when transfected into Na^+/H^+ antiporter-deficient fibroblasts (i.e., it regulates intracellular pH in a Na-dependent and amiloride-sensitive manner). This red cell exchanger represents an additional form of Na^+/H^+ exchanger (termed βNHE), which is characterized by a specific cytoplasmic domain involved in activation by the cAMP-dependent signaling pathway. After transfection in the same cellular context, βNHE, but not NHE1, is activated by cAMP or by hormones that increase cAMP levels. Comparison of the amino acid sequences of exchangers shows that βNHE, but not NHE1, contains two clustered consensus motifs for phosphorylation by a cAMP-dependent protein kinase (protein kinase A; PKA). A deletion mutant devoid of the C-terminal region of the cytoplasmic loop containing the two PKA sites restores Na^+/H^+ activity but is no longer activated by cAMP analogues or catecholamines. In red blood cells, the Na^+/H^+ exchanger is also activated by another pathway involving protein kinase C (PKC). Expression of βNHE in fibroblasts shows that these two independent signaling pathways impinge on two distinct domains of the exchanger. The cytoplasmic segment containing PKA consensus sites, which is crucial for cAMP activation, is unnecessary for stimulation by PKC activators.**

The amiloride-sensitive Na^+/H^+ exchanger, first analyzed in kidney (1), is a plasma membrane transport protein that participates in intracellular pH homeostasis (2). It is also activated by a number of external signals (osmotic pressure, sperm, phorbol esters, growth factors, hormones, and neurotransmitters) and, through such activation, plays a major role in cell volume regulation, mitogenesis, secretion, etc. (3–5). How do distinct types of external signals lead to exchanger activation, and are there multiple forms of the Na^+/H^+ exchanger? The only form of eukaryotic exchanger characterized at the molecular level was studied by cloning a cDNA encoding a human antiporter (6, 7) and then isolating similar cDNAs from several different mammalian species including rabbit (8), pig, and hamster (L. Counillon and J.P., unpublished results). This ubiquitous Na^+/H^+ exchanger, now referred to as NHE1, is expressed in the basolateral membranes of polarized epithelial cells (8) and can be activated at least by two independent signaling pathways (9). NHE1, however, is probably not the only Na^+/H^+ exchanger, since another form is postulated to be localized in the apical membrane of epithelial cells (8, 10–12), as evidenced by kinetic experiments showing differences in amiloride sensitivity and regulation. Indeed three additional isoforms, NHE2–NHE4, have been cloned and found to be differentially expressed in kidney and intestinal cells (13, 14). None of these eukaryotic antiporters share homology with the Na^+/H^+ exchanger protein in *Escherichia coli* (15).

It was of interest to consider the Na^+/H^+ exchanger found in nucleated trout red cells (termed βNHE) since, in contradistinction to NHE1 and to the apical antiporter, it is activated by cAMP and other cAMP-dependent protein kinase (protein kinase A; PKA) activators such as catecholamines or forskolin (16–19). Thus, it is likely to represent an additional form of antiporter. The trout red cell exchanger is also activated by a distinct signaling pathway involving protein kinase C (PKC) (19).

We report here the isolation and characterization of a cDNA clone encoding this cAMP-activated Na^+/H^+ antiporter§. In opposition to NHE1, it contains two typical consensus sites for phosphorylation by PKA. After transfection into an antiporter-deficient fibroblast cell line, the expressed protein responds to both PKA and PKC activators, as observed in the trout red cell. Truncation of the C-terminal region of the cytoplasmic segment, which contains the two PKA consensus sites, abolishes the capacity of the antiporter to be activated by cAMP and other PKA activators, while it retains the ability to exchange Na^+ for H^+ and to be activated following PKC stimulation.

EXPERIMENTAL PROCEDURES

cDNA Library Screening. Poly(A)$^+$ RNA from hematopoietic tissue (cephalic kidney) of the trout *Salmo gairdneri* was prepared according to standard procedures (20, 21). A size-selected cDNA library primed with oligo(dT) was constructed in the λgt10 vector by using a commercially available kit (RiboClone; Promega). Double-stranded cDNAs >1.9 kilobases (kb) were selected by agarose gel electrophoresis, electroeluted, and ligated in the *Eco*RI cloning site of the arms of the λgt10 vector. Recombinants (4×10^5) were screened with a 1.9-kb ^{32}P-labeled *Bam*HI fragment of the human Na^+/H^+ antiporter cDNA c28 (6). Hybridization conditions were 50% formamide/4× standard saline citrate (SSC)/5× Denhardt's solution/1% SDS/sonicated salmon

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Abbreviations: PKA, protein kinase A (cAMP-dependent protein kinase); PKC, protein kinase C; pH_i, intracellular pH; MPA, N^5-methyl-N^5-propylamiloride.

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§The nucleotide sequence of the trout red blood cell exchanger cDNA has been deposited in the GenBank data base (accession no. M94581).

sperm DNA (100 μg/ml) at 42°C, four 15-min washes in 2× SSC/0.1% SDS at 42°C, and two 5-min washes in 0.5× SSC/0.1% SDS at 55°C. Positive clones were plaque purified after three rounds of screening.

cDNA Cloning and Sequencing. Four clones (with inserts of 2.1–3.2 kb) designated A, C, F, and M were subcloned into pBluescript KS^+ or SK^+ (Stratagene) for restriction mapping and sequencing (see Fig. 1). Sequencing was performed by the dideoxy chain-termination method, using a T7 sequencing kit (Pharmacia), from restriction fragments of cDNA by using universal primers and synthetic oligonucleotide primers complementary to the cDNA. Several restriction fragments were rescued with R408 helper phage (Stratagene), and single-stranded DNA was sequenced.

Cell Culture and Transfection. The Chinese hamster lung fibroblast line CCL39, the Na^+/H^+ antiporter-deficient derivative PS120 (22), and the corresponding transfectants were maintained in Dulbecco's modified Eagle's medium (H21; GIBCO) containing 25 mM $NaHCO_3$ supplemented with 7.5% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). Cells were maintained at 37°C in the presence of 5% CO_2. cDNA T, which is composed of the coding region for the trout red cell Na^+/H^+ antiporter as well as 60 base pairs (bp) of 5′ noncoding region and 800 bp of untranslated sequence, was inserted into the eukaryotic expression vector pECE (23). This expression plasmid was called pET. The construction of the trout red cell Na^+/H^+ antiporter deletion mutant (pETΔ559) was carried out by digestion of cDNA T with *Pvu* II [on the coding region (see Fig. 1)] and *Sma* I (on the polylinker of Bluescript) followed by ligation with T4 ligase in the *Sma* I cloning site of pECE. The PS120 cell line (2×10^5 cells per 100-mm dish) was transfected with each plasmid construct (20 μg) by using the calcium phosphate coprecipitation technique (21). Three days after transfection, the cells were subjected to an "acid-load selection test" to eliminate cells that do not express a functional Na^+/H^+ antiporter (24). This treatment was repeated five times over a period of 3 weeks, and the resulting stable transfectant cells were used for experiments.

Measurement of the Na^+/H^+ Antiporter Activity. *$^{22}Na^+$ uptake.* Amiloride-sensitive $^{22}Na^+$ uptake was measured by the NH_4^+-loading method. Cells, plated in 24-well dishes, were incubated for 1 hr in 50 mM NH_4Cl/70 mM choline chloride/5 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/15 mM Hepes, pH 7.5, then quickly washed twice with 120 mM choline chloride/5 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/15 mM Mops, pH 7.0, and incubated in $^{22}Na^+$ uptake medium (120 mM choline chloride/5 mM KCl/1 mM $MgCl_2$/1.8 mM $CaCl_2$/15 mM Hepes, pH 7.5/1 mM ouabain/carrier-free $^{22}NaCl$ at 1 μCi/ml (1 Ci = 37 GBq). After washing three times with cold saline, cells were solubilized in 0.1 M NaOH, and radioactivity was assayed by γ counting.

Intracellular pH (pH_i) measurements. pH_i dependence of the antiporter was performed as follows: a change in pH_i was produced by loading the cells with different concentrations of NH_4^+ (media with 0–50 mM NH_4Cl). Determination of pH_i was estimated from the distribution of [7-^{14}C]benzoic acid as described (25).

RESULTS

The Cytoplasmic Domain of the Trout Red Blood Cell Exchanger Contains Two PKA Consensus Sites. The sequence of cDNA T, which encodes the trout red cell Na^+/H^+ exchanger (Fig. 1), predicts a protein of 759 amino acids (M_r = 85,118). A hydropathy plot of the sequence reveals that the protein consists of two distinct domains: (*i*) an amphipathic N-terminal domain (467 amino acids), which contains 10 putative transmembrane-spanning segments using the method of Engelman *et al.* (26) or 12 according to Kyte and Doolittle (27), and (*ii*) a highly hydrophilic C-terminal domain, likely corresponding to a large cytoplasmic region of the molecule (6). The C-terminal domain contains two typical consensus sites for phosphorylation by PKA [Arg-Arg-Xaa-Ser (28)] that are very close together (Ser-641 and Ser-648). Fig. 2 compares the amino acid sequence of the protein with that of the human Na^+/H^+ exchanger (NHE1). It appears that the trout red cell protein, which is slightly shorter than NHE1 (759 vs. 815 amino acids), exhibits a hydrophobicity plot that is similar to NHE1. The transmembrane segments and the cytoplasmic domain exhibit 74% and 48% amino acid identity with their respective counterparts in NHE1. The two putative glycosylation sites in NHE1 also exist in the red cell Na^+/H^+ exchanger. Interestingly, the PKA consensus sequences observed in trout red cell protein do not exist in the cytoplasmic domain of the human exchanger. Notably, the red cell antiporter is activated by cAMP, whereas NHE1 is not.

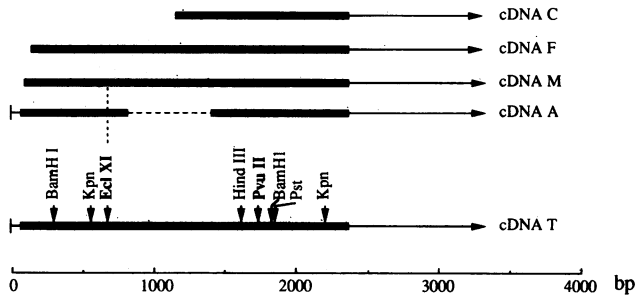

FIG. 1. Partial restriction map and cloning strategy for the trout red cell Na^+/H^+ exchanger. Four clones, A, F, M, and C, were isolated and sequenced. The coding region is shown as a solid black bar. The largest clone (M) contains 3200 nucleotides including the 3′ untranslated sequence and a poly(A)$^+$ tail but lacks an ATG translation initiation codon. The shortest clone (A) contains 60 nucleotides of 5′ noncoding sequence, the ATG translation initiation codon, and the 3′ untranslated sequence. The sequence of clone A is identical to that of all the other clones, but it contains an internal deletion of 669 bp in the coding region. To reconstruct a cDNA coding for the entire Na^+/H^+ antiporter, clones A and M were ligated together at the common *Ecl*XI site. The resulting cDNA was designated cDNA T. The coding region of this clone was completely sequenced three times on both strands. A scale (in bp) is shown below clone T.

Expression of the cDNA in Mutant Cells Restores Functional Na^+/H^+ Antiport Activity. To show that cDNA T encodes a protein that functions as a Na^+/H^+ exchanger, it was subcloned into the eukaryotic expression vector pECE, which was then transfected into the exchanger-deficient fibroblast cell line PS120. As indicated by the high number of cells that survive an acid load, cDNA T encodes a functional Na^+/H^+ antiporter. Indeed, cells transfected with sperm DNA never gave rise to colonies. Stable transfectants were then selected by five successive acid loadings as described (24). Fig. 3*A* shows the rate of $^{22}Na^+$ uptake in transfected cells after cytoplasmic acidification to functionally activate the antiporter. Na^+ influx is strongly inhibited by amiloride and by the amiloride analog N^5-methyl-N^5-propylamiloride (MPA). Fig. 3*B* illustrates the dose–response curves for both amiloride and MPA (IC_{50} = 5 μM and 0.4 μM, respectively). Fig. 3*C* illustrates the pH_i dependence of the amiloride-sensitive $^{22}Na^+$ uptake in PS120 cells transfected either with the cDNA encoding the trout red cell antiporter or with the cDNA encoding the human antiporter. For both antiporters, similar allosteric activation by internal H^+ was observed. The red cell exchanger, however, shows a shift of the pH_i dependence to the alkaline side. These results demonstrate that red cell cDNA T does indeed encode a Na^+/H^+ antiporter that is sensitive to amiloride and activated by intracellular H^+.

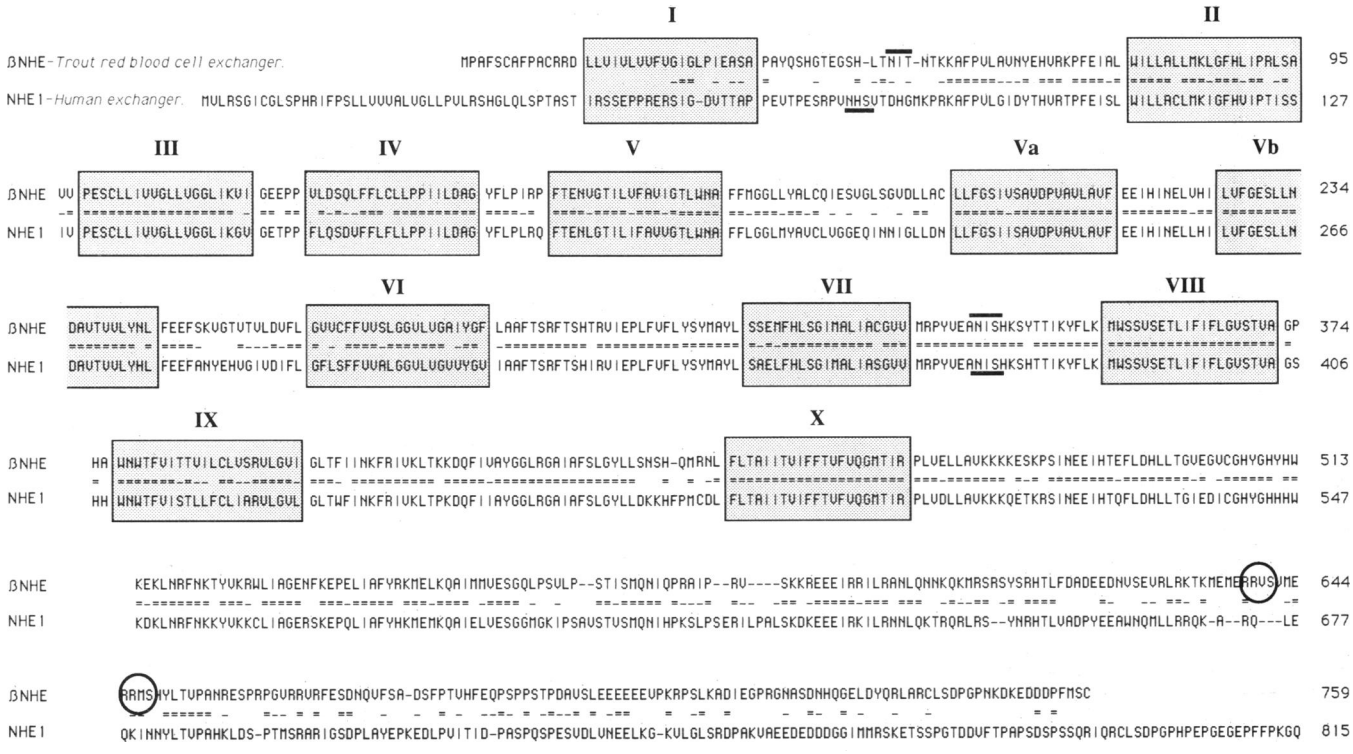

FIG. 2. Alignment of the amino acid sequences of the human growth factor-activatable Na^+/H^+ exchanger (NHE1) (6, 7) and the trout red cell Na^+/H^+ exchanger (βNHE). The 10 putative membrane-spanning domains are boxed as well as the two additional domains named Va and Vb predicted by using the Kyte and Doolitle program (27). Amino acid numbers are shown. Identical amino acids are marked by equal signs, and conservative substitutions are indicated by dashes. Note the conservation of the N-linked glycosylation sites (underlined and overlined). The two consensus PKA sites are circled.

The Cytoplasmic Domain Containing the PKA Consensus Sites Is Essential to Mediate Hormonal Activation. Fig. 4 shows the effects of exogenously added 8Br-cAMP on the pH_i of cells incubated in the absence of bicarbonate at an external pH (7.7) above the set point value. Under these conditions, Na^+/H^+ exchangers are quiescent. Addition of cAMP to exchanger-deficient PS120 cells induced an intracellular acidification, due to metabolic acidosis. A similar acidification was observed in PS120 cells transfected with the human exchanger (pEAP-Δ5′), indicating that this Na^+/H^+ exchanger is not activated by cAMP (at pH 7.7, the magnitude of the cAMP-induced acidification is insufficient to stimulate Na^+/H^+ exchange; see Fig. 3*C*). Conversely, in cells transfected with the trout red cell exchanger (pET), cAMP treatment resulted in a marked intracellular alkalinization. This reversion in pH_i is due to the stimulation of transmembrane Na^+/H^+ exchange because the alkalinization is prevented by amiloride or MPA (data not shown). This difference in behavior between the human exchanger and the trout red cell antiporter suggests that the ability of cAMP to activate the red cell Na^+/H^+ exchanger is related to the presence of typical PKA phosphorylation sites on this antiporter (i.e., Ser-641 and Ser-648).

We next examined whether cAMP can activate the antiporter after removal of the terminal region of the cytoplasmic domain, which contains the consensus sites for PKA. Toward this end, a cDNA deletion mutant (TΔ559) devoid of the last 200 amino acids (559–759) was generated, cloned into the pECE expression vector, and stably expressed in the antiporter-deficient cell line PS120. Deletion of the C-terminal cytoplasmic domain was shown to preserve both the ability of the exchanger to catalyze amiloride-sensitive Na^+/H^+ exchange and the characteristics of the H^+-modifier site; the pH_i dependence was shifted only slightly (0.05 pH unit) to the alkaline side (data not shown). Interestingly, Fig. 4 shows that PS120 cells expressing the deleted antiporter do not exhibit any alkalinization in response to cAMP. This result, confirmed by the lack of response to forskolin and isoproterenol (Fig. 5), suggests that the truncated Na^+/H^+ antiporter (TΔ559) is no longer activated by cAMP. As the trout red cell exchanger is activated by phorbol esters (19), we also examined whether PKC activators (α-thrombin and phorbol esters) stimulate both the wild-type antiporter and the deletion mutant (Fig. 5). The results demonstrate that, confirming the hypothesis, the region of the cytoplasmic loop between amino acids 559 and 759 is crucial for cAMP-dependent activation of the exchanger, but it is not required for PKC-dependent activation since intracellular alkalinization occurs following thrombin and phorbol ester addition to all transfectants.

DISCUSSION

In the present study we have cloned, sequenced, and functionally expressed a Na^+/H^+ exchanger present in a nucleated erythrocyte (trout red cell). It has been demonstrated *in vitro* (16, 17) and also *in vivo* (30, 31) that this antiporter is activated by catecholamines through increases in intracellular cAMP, which leads to stimulation of PKA. cAMP does not activate Na^+/H^+ exchange in other cells except in rat hepatocytes (40) and in another nucleated erythrocyte (frog red cell) (32). On the contrary, cAMP inhibits the activity of exchangers in the apical membranes of renal (33, 34), intestinal (35), and gall bladder epithelium (36) and has no effect on the exchangers in the basolateral membrane (37). These differences in regulatory mechanisms indicate the probable existence of different isoforms of proteins. However, it is also possible that there is only one form of the exchanger and that the differences in regulation are dictated by the cell type and different signaling networks.

The first goal of this work was to determine, by molecular cloning and functional expression, whether or not the trout

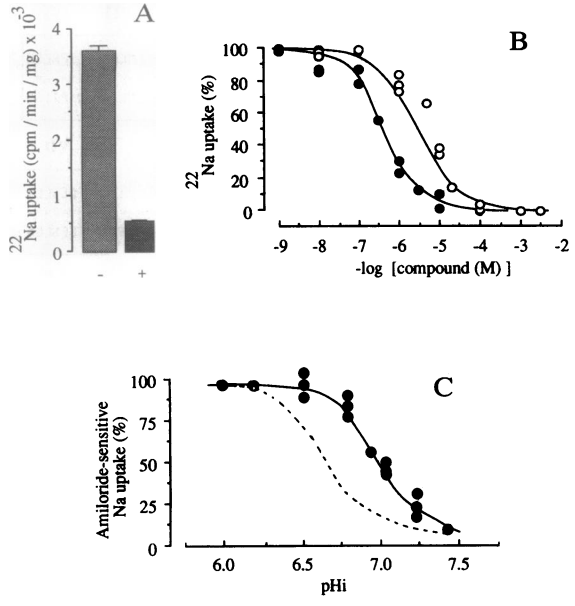

FIG. 3. $^{22}Na^+$ uptake studies in fibroblasts expressing the trout cDNA T. $^{22}Na^+$ uptake was conducted in PS120 cells transfected with pET (cDNA T subcloned into the eukaryotic vector pECE). (*A*) $^{22}Na^+$ uptake (1 min) was measured in acid-loaded cells as described in *Experimental Procedures* in the absence (−) or presence (+) of 1 mM amiloride. (*B*) Dose–response curves for inhibition of $^{22}Na^+$ uptake by amiloride (○) and by an amiloride analog, MPA (●). (*C*) pH_i dependence of the amiloride-sensitive $^{22}Na^+$ uptake of the trout red cell exchanger (●) stably expressed in PS120 cells. The dashed line indicates the pH_i dependence of the human Na^+/H^+ exchanger stably expressed in the same cells (data from ref. 29). The initial rate of amiloride-sensitive $^{22}Na^+$ uptake is represented as the percentage of the maximal rate in each experiment.

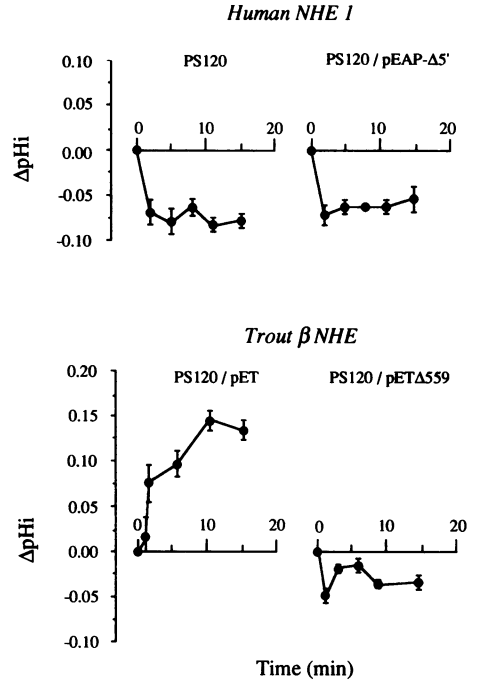

FIG. 4. cAMP effects on human and trout exchangers. Antiporter-deficient cells (PS120) and the different transfectants (PS120/pEAP-Δ5', PS120/pET, and PS120/pETΔ559) were incubated overnight in the serum-free H21 medium to maintain the Na^+/H^+ exchanger in the resting state. The cells were further incubated for 1 hr at 37°C in the bicarbonate-free H21 medium buffered with 7 mM Hepes (pH 7.7). Cells were then placed in the same medium containing [^{14}C]benzoic acid at 1 μCi/ml. 8Br-cAMP (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 the means ± SE of triplicate determinations.

red cell exchanger represents a form of antiporter distinct from NHE1, which is ubiquitously expressed and localized in the basolateral membrane of epithelial cells (6, 8). The trout red cell exchanger has a similar hydrophobicity plot to NHE1 and is also predicted to have 10–12 membrane-spanning domains and a long cytoplasmic C terminus. Recently, three distinct isoforms of the Na^+/H^+ exchanger have been cloned from kidney, intestinal, and stomach epithelia cells, NHE2–NHE4 (13, 14). When compared individually, each of the transmembrane segments of the trout red cell NHE isoform is much more homologous to the corresponding regions in NHE1 than to the corresponding segments of NHE2, NHE3, and NHE4. The transmembrane domains of the trout NHE and the human NHE1 exhibit a strong homology: 74% identity at the amino acid level. However, in the cytoplasmic domain of the trout NHE isoform, the homology with NHE1 falls to 48%, and a marked divergence is apparent: in contrast to the human NHE1 isoform, the trout Na^+/H^+ exchanger possesses two consensus sites for phosphorylation by PKA at Ser-641 and Ser-648. The trout Na^+/H^+ exchanger expressed in PS120 cells is stimulated by cAMP and by all the activators of cAMP-dependent protein kinase tested (i.e., catecholamines and forskolin). In marked contrast, NHE1 does not respond to these signals when expressed in the same cells. The results are consistent with the difference in amino acid sequences outlined above (i.e., two consensus sequences for PKA are present in the trout red cell antiporter and none are present in NHE1). The pivotal role of these consensus sites in activation by cAMP is further emphasized by the result obtained with the mutant exchanger devoid of these sites. Removal of the PKA consensus sites abolishes the capacity of the antiporter to be activated by cAMP, forskolin, and catecholamines. It reflects the existence in certain cells of a form of the exchanger with typical PKA consensus sites, which confer activation by β-adrenergic agonists. This exchange protein could be called βNHE to

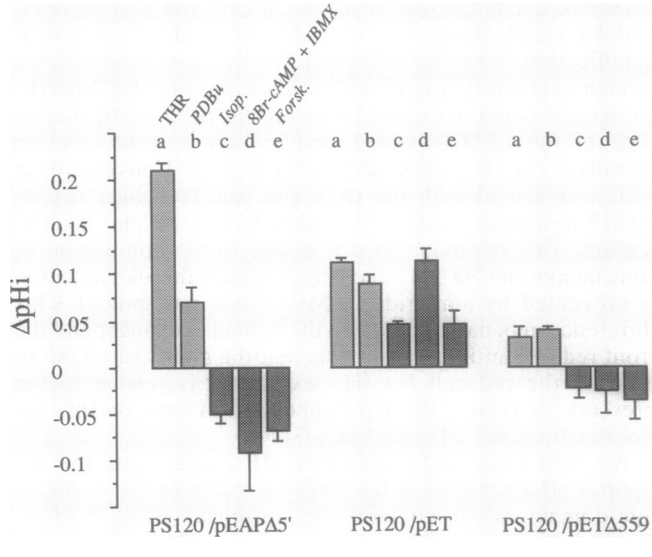

FIG. 5. Differential effects of PKA and PKC activators on the stimulation of human (pEAP-Δ5'), trout red cell (pET), and truncated trout red cell (pETΔ559) exchangers. The changes in pH_i were measured, as defined in Fig. 4, 10 min after addition of the kinase activator. PKC activators were 10 nM α-thrombin (THR; bar a) and 200 nM phorbol 12,13-dibutyrate (PDBu; bar b). PKA activators were 0.5 μM isoproterenol (Isop.; bar c), 1 mM 8Br-cAMP plus 50 μM 3-isobutyl-1-methylxanthine (8Br-cAMP + IBMX; bar d), and 5 nM forskolin (Forsk.; bar e). Error bars (mean ± SE) are based on triplicate determinations.

distinguish it from the well-characterized NHE1 and the presumed apical forms called NHE2–NHE4.

The second goal addressed in this work concerns the multiple pathways leading to exchanger activation. In fibroblasts, epidermal growth factor and α-thrombin activate NHE1 by independent signaling mechanisms [i.e., through receptor tyrosine kinase (epidermal growth factor) or a PKC-dependent pathway (α-thrombin)]. However, both agonists stimulate phosphorylation of NHE1 at serine residues (7), and the phosphorylation pattern was shown to be identical in epidermal growth factor- and thrombin-stimulated cells, suggesting that the two pathways converge on the same kinase (9). As observed in trout red cell, βNHE expressed in fibroblasts is also activated by two independent signaling mechanisms [i.e., through a cAMP-dependent pathway (catecholamines and forskolin) or through a PKC-dependent pathway (thrombin and phorbol esters)]. Do these pathways interact with different sites on βNHE to induce activation or do they converge early on a common activating protein as an "Na^+/H^+ exchanger kinase"? A clear-cut answer has been obtained by use of the deletion mutant. As shown here, the cytoplasmic domain containing PKA consensus sites is essential for cAMP-induced activation. Conversely, βNHE is activated by a PKC-dependent pathway. Thus, the two signaling pathways impinge on different domains of βNHE to activate exchange activity. Our results also indicate that partial deletion of the cytoplasmic C-terminal domain does not markedly alter the "pK" of βNHE with respect to intracellular H^+ dependence. The data agree with findings obtained for NHE1 where only total deletion of the cytoplasmic domain affects the affinity of the internal H^+ modifier site (29).

The antiporter cDNA clone described here will be a valuable tool for answering other important questions related to the peculiar regulatory properties of the cAMP-dependent Na^+/H^+ exchanger. Indeed, it has been shown previously that βNHE is desensitized despite the continued presence of cAMP (38) and that desensitization leads to refractoriness (19). Moreover, the activity of βNHE is controlled by molecular oxygen through a change in the quaternary structure of hemoglobin (39).

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