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Rapid Report

Kinetic characterization of a stably expressed novel Na^+/H^+ exchanger (NHE-2)

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We have recently reported the molecular cloning, sequencing and tissue distribution of a novel Na^+/H^+ exchanger (NHE-2). The cDNA for NHE-2 was cloned by screening a rat intestinal cDNA library. This clone was unique due to the fact that it lacks the first two transmembrane domains which are present in the other Na^+/H^+ exchanger isoforms (NHE-1, NHE-3, NHE-4). This structural change in the cDNA offered a unique opportunity to study in detail the properties of this stably expressed cDNA in chinese lung fibroblasts that lack the Na^+/H^+ exchanger (PS120) cells. Amiloride-sensitive Na^+ uptake was linear up to 2 min in PS120 cells transfected with the cDNA. Kinetics of the amiloride-sensitive Na^+ uptake showed a V_{max} of 24.7 ± 5 nmol/μl ICW per min and a K_m of 33.1 ± 2.0 mM. The inhibitory constant (K_I) for amiloride and its analogue 5-*N*-ethyl-*N*-isopropylamiloride (EIPA) was 0.15 μM and 0.66 μM, respectively. Epidermal growth factor, a known stimulator of NHE-1, also upregulated the expressed NHE-2. These results characterize the kinetic properties of this unique exchanger and suggests that the first two transmembrane domains of the Na^+/H^+ exchanger isoforms are not essential for the expression of amiloride-sensitive Na^+ uptake.

The Na^+/H^+ exchangers belong to a family of transporters which catalyzes the electroneutral exchange of Na^+ with H^+ [1–3]. Na^+/H^+ exchanger-1 (NHE-1) is ubiquitous in its distribution as it is involved in intracellular pH and volume regulation [4]. Human NHE-1 has been cloned by Pouyssegur's group in Nice-France [4]. Subsequently, NHE-1, 3 and 4 were cloned from rat tissues by Orlowski et al. [3] and rabbit NHE-1 and NHE-3 by Tse et al. [2,5]. NHE-3 is believed to be involved in Na^+ absorption as its distribution is mainly in the intestinal and renal epithelial cells [5]. The hydropathy plot of rat NHE-1, 3 and 4 reveals thirteen transmembrane domains (designated A–M) which are common to all the NHE isoforms [3]. We have recently cloned a unique Na^+/H^+ exchange isoform designated NHE-2 which is widely expressed in tissues of the gastrointestinal tract. The cDNA is 4 kb in length with an open reading frame of 2091 bases. NHE-2 shows 47.8%, 41.2% and 56.2% amino acid identity to NHE-1, 3 and 4, respectively. Hydropathy plot of the predicted protein shows eleven transmembrane domains. When compared with the other isoforms it lacks domain A, B and half of domain C which are common to the NHE-1, 3 and 4. Therefore, it was of interest to determine this structural alteration in the cDNA with its function as expressed stably in the PS120 cells which lack Na^+/H^+ exchange activity.

Materials and methods. The Na^+/H^+ exchanger deficient Chinese hamster lung fibroblast cell line PS120 was a gift from J. Pouyssegur, Center de Biochimie, Nice (Nice, France). EIPA was a gift from Ian Blair, Vanderbilt University (Nashville, TN). EGF was obtained from Collaborative Research (Bedford, MA). Amiloride and ouabain octahydrate were obtained from Sigma (St. Louis, MO). Sodium-22 was obtained from NEN (Boston, MA). 3-*O*-Methyl-D-[1-^{3}H]glucose and D-[1-^{14}C]mannitol were obtained from Amersham (Arlington Heights, IL). All other chemicals were of the highest purity available and were obtained from Sigma.

Cell culture and transfection. The Na^+/H^+ exchanger-deficient PS120 fibroblast cell line and the corresponding transfectants were maintained in Dulbecco's Modified Eagle's Medium (GIBCO) containing 10% fetal calf serum (FCS), penicillin (100 units/ml) (PC), and streptomycin sulfate (100 μg/ml) (SM). The

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cells were grown in 100-mm dishes (Corning) at 37°C in the presence of 5% CO_2 and passaged by trypsinization (1:10) at 48-h intervals. PS120 fibroblast cells were transfected as follows: cells were trypsinized, seeded at $5 \cdot 10^5$ cells per 10 cm plate and incubated overnight in 10 ml of growth medium. 20 μg of an expression plasmid (PCMV4) containing cloned NHE-2 cDNA was mixed with 0.5 ml of 0.25 M $CaCl_2$, 0.5 ml of 2X BBS and left at 22°C for 20 min. The calcium phosphate-DNA suspension (1 ml) was added dropwise to the plate of cells and the mixture was swirled gently and incubated for 24 h at 37°C under 5% CO_2. The medium was removed and cells were rinsed twice with growth media, refed and incubated for 24 h at 37°C under 5% CO_2. The cells were split and incubated for an additional 24 h before acid selection for transport studies [6]. Experiments were conducted with cells taken between 4 and 7 passages.

Acid selection of PS120 cells. After each thawing of cryopreserved stocks, the cells were passaged three times and acid selected as described below to reconfirm the stable transfection.

Confluent NHE-2-transfected PS120 cells grown in 100-mm dishes were used for acid selection. The culture medium was replaced with 50 mM NH_4Cl and 72 mM choline in Hepes-Tris buffer containing 4.9 mM KCl, 1 mM $CaCl_2$, 2.5 mM $MgSO_4$, 20 mM Hepes-Tris adjusted to pH 7.5. After 1 h incubation at 37°C, the monolayers were rinsed twice with 122 mM choline chloride in Hepes buffer (pH 7.0), then incubated for another hour in 122 mM NaCl in Hepes buffer (pH 7.5). The cells were then rinsed with the culture medium (DMEM + 10% FCS) and returned to standard culture conditions. Non-transfected PS120 monolayers were treated in a similar fashion and did not recover from the lethal acid load.

Transport measurements. All buffers were made in Hepes-Tris buffer (pH 7.4) described above. 0.5% albumin was added to the washing, acid loading and transport buffers to prevent cellular detachment during the prolonged incubations.

Cells grown to confluence in 35-mm dishes were used for the transport assays. The monolayers were rinsed twice with 122 mM choline chloride (Washing buffer) then acid loaded for 25 min at 37°C in 50 mM NH_4Cl and 70 mM choline chloride (Acid loading buffer). After a 5-min equilibration period at room temperature, the acid loading buffer was aspirated. After two rapid rinses with washing buffer, Na^+ uptake was initiated with the addition of 0.7 ml of transport buffer containing 1 mM NaCl (^{22}Na: 1500 cpm/μl), and 122 mM choline chloride in the presence or absence of amiloride. The uptake was stopped at various time points with ice-cold stop buffer containing 122 mM choline chloride, 0.2 mM amiloride and 1 mM ouabain.

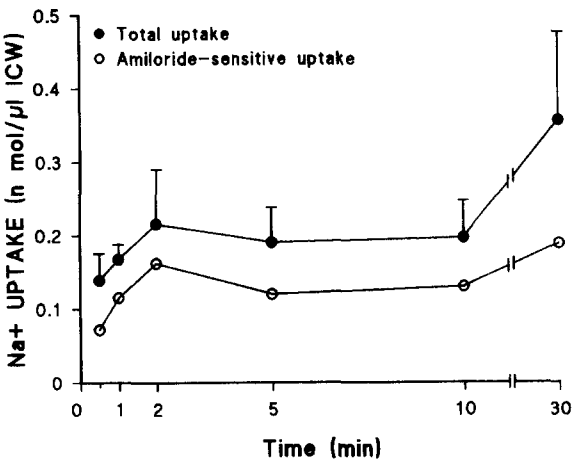

Fig. 1. Effect of amiloride on 1 mM Na^+ uptake stimulated by proton gradient. Cells were incubated with or without amiloride (25 μM). Each point is the mean value of triplicate experiments. Na^+ uptake was plotted against time scale.

Kinetics of amiloride-sensitive Na^+/H^+ exchange were carried out in transport buffer containing 0–92.5 mM NaCl with the appropriate concentrations of choline chloride to maintain isotonicity.

The effect of epidermal growth factor (EGF) was determined in monolayers that were serum deprived for 4 h prior to the addition of EGF (200 ng/ml). Control cells were treated in a similar fashion except that the vehicle only (sterile water) was added. Preincubation with EGF was carried out for 1 h prior to acid loading.

At the end of the experiment, the monolayers were solubilized in 0.1 M NaOH overnight and subjected to liquid scintillation counting (LS 4000, Beckman Instruments, Palo Alto, CA).

Determination of intracellular space. 3-*O*-Methylglucose equilibrium uptake was used to determine intracellular water space according to a well established method [7].

Analysis of data. All values were expressed as nmol of sodium uptake per μl of intracellular water space, and expressed graphically as the mean $\pm$ 1 S.D. Each data point represents the mean values of three or four different experiments run in triplicate.

Results. Time-course of Na^+ uptake. Fig. 1 depicts total and amiloride-sensitive Na^+ uptake at 1 mM Na^+ concentration under outwardly directed pH gradient generated by NH_4Cl loading. Uptake was determined at 30 s, 1 min, 2 min, 5 min, 10 min and 30 min. Na^+ uptake was linear up to 2 min. Amiloride at 25 and 100 μM inhibited Na^+ uptake, in a similar fashion (data not shown).

Kinetics of amiloride-sensitive Na^+ uptake. V_{max} and K_m of amiloride-sensitive Na^+ uptake were analyzed using a nonlinear computerized model of Michaelis-Menten kinetics (Enzfitter, by R.J. Leatherbarrow,

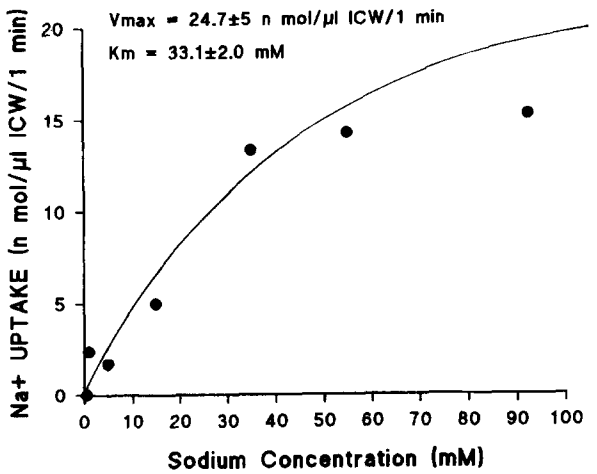

Fig. 2. Kinetics of amiloride-sensitive Na^+/H^+ exchange. The kinetics data were derived from the amiloride-sensitive Na^+ uptake (total uptake − uptake in the presence of amiloride) which was determined at 1 min. Each point is the mean of triplicate values. The amiloride-sensitive component was plotted against Na^+ concentration. The Michaelis constant (K_m), and maximal velocity (V_{max}) values were analyzed using a computerized model of Michaelis-Menten kinetics. Similar results were obtained in three independent experiments.

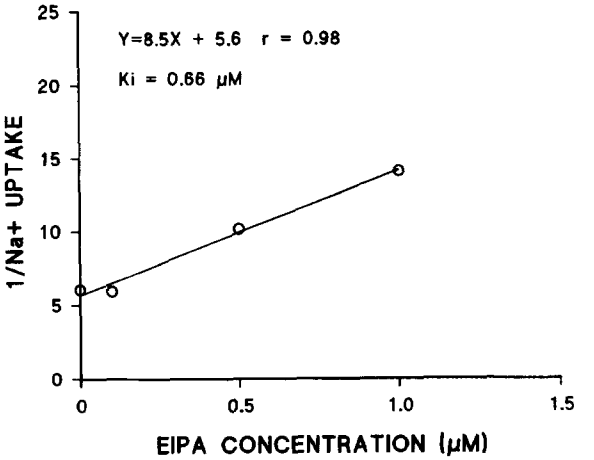

Fig. 4. A Dixon plot of 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) inhibition on 1 mM Na^+ uptake stimulated by proton gradient. The transport reaction was stopped at 1 min. Each point represents the mean of four experiments. The calculated K_I for EIPA was approx. 0.66 µM. Similar results were obtained with 50 mM of Na^+ (data not shown).

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As seen in Fig. 2, V_{max} of amiloride-sensitive Na^+/H^+ was 24.7 ± 5 nmol/µl ICW per min. K_m value was 33.1 ± 2.0 mM.

The effect of varying concentrations of amiloride on Na^+/H^+ exchange activity. Fig. 3 depicts the effect of varying concentrations of amiloride (0.01–100 µM) on the uptake of 1 mM Na^+ under outwardly directed H^+ gradient. The inset depicts a Dixon plot of the inverse of Na^+ uptake versus 0–0.25 µM amiloride concentration. The plot is linear suggesting a single amiloride binding site with K_I of 0.15 µM.

The effect of varying concentrations of EIPA on Na^+/H^+ exchange activity. The effect of varying concentrations of EIPA (0.1 nM–100 µM) on the uptake of 1 mM Na^+ under outwardly directed H^+ gradient was studied. Fig. 4 depicts a Dixon plot of the inverse of Na^+ uptake versus 0–1 µM EIPA concentration. The plot is linear suggesting a single EIPA binding site with K_I of 0.66 µM. Na^+ uptake at 1 and 100 µM of EIPA were similar at 0.08 nM/µl ICW (data not shown).

Effect on EGF of amiloride-sensitive Na^+/H^+ exchange activity. To determine the effect of EGF on amiloride-sensitive Na^+ uptake, experiments were designed to examine Na^+ uptake in the presence and absence of 100 µM amiloride in the setting of an outwardly directed H^+ gradient. As seen in Fig. 5, the

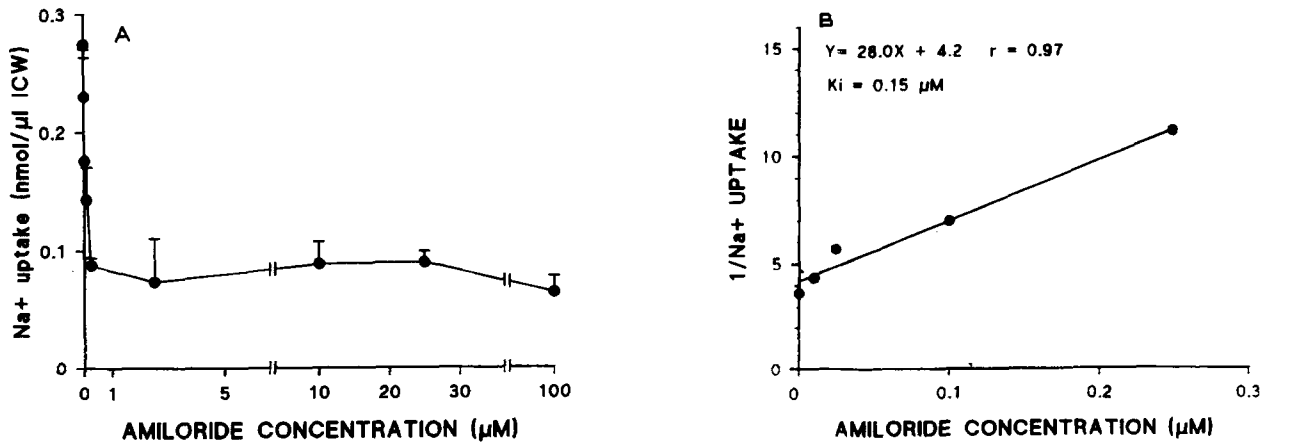

Fig. 3. (A) Effect of various concentrations of amiloride on Na^+ uptake stimulated by proton gradient. (B) A Dixon plot of 1/Na versus amiloride concentrations. Transport reaction was stopped at 1 min. Each data point represents the mean values of three different samples. Similar results were obtained in five experiments. The calculated K_I for amiloride was 0.15 µM.

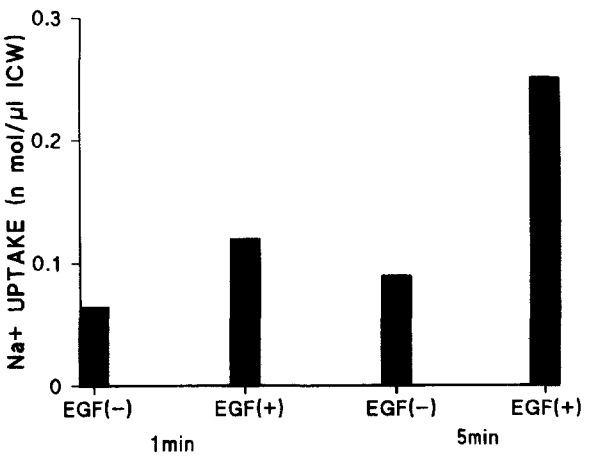

Fig. 5. Effect of EGF (200 ng/ml) on 1 mM Na^+ uptake. Amiloride-sensitive Na^+ uptake was determined in cells incubated with or without EGF. Transport reactions were stopped at 1 and 5 min with stop buffer.

amiloride-sensitive component was significantly greater with EGF administration compared with controls at both 1 min and 5 min time points ($p < 0.05$).

Discussion. The present studies are the first to characterize the kinetic properties, inhibition profile and activation by epidermal growth factor of a unique Na^+/H^+ exchanger (NHE-2). NHE-2 was cloned from a rat intestinal cDNA library using the *Bam*H1-*Bam*H1 fragment of a human fibroblast Na^+/H^+ exchanger (NHE-1) as a screening probe. NHE-2 is uniquely expressed in the gastrointestinal tract and it recognizes two transcripts of 4.4 and 4.6 kb in these tissues. This observation suggested that NHE-2 is alternatively spliced. NHE-2 has a total of 3959 bases, the 5′ end has a 546 base untranslated region and 3′ end has a 1322 base untranslated region with an open reading frame of 2091 bases. The 3′ end and the open reading frame share sequence homology with the other Na^+/H^+ exchanger isoforms, however, the 5′ end of NHE-2 does not share sequence homology with other Na^+/H^+ exchangers, again suggesting that the predominant transcript of this clone is alternatively spliced. The hydropathy plot of NHE-2 using the Kyte-Doolittle algorithm using a window of 11 amino acids suggested eleven transmembrane domains within the N-terminus, whereas NHE-1, 3 and 4 contain 13 transmembrane domains. Despite missing the first two transmembrane domains NHE-2 transfected in PS120 cells resulted in expression of amiloride-sensitive Na^+ uptake, suggesting that the amiloride sensitivity in the N-terminus is present beyond the first two domains. The protein encoded by NHE-2 cDNA is sensitive to amiloride, however, it is relatively resistant to amiloride analog EIPA. The kinetics of amiloride-sensitive Na^+ uptake showed a V_{max} of 24.7 ± 5 nmol/μl ICW per min and a K_m of 33.1 ± 2.0 mM. The C-terminus of NHE-2 contains regulatory domains including potential phosphorylation site. NHE-1 is activated by oncogenic transformations and by several external signals including epidermal growth factor, α-thrombin, okadaic acid and phorbol esters. Activation results from increased affinity of the antiporter for intracellular H^+ at the allosteric modifier site [8]. The functional domains involved in the activation of Na^+/H^+ exchanger (NHE-1) by epidermal growth factor is thought to reside in the C-terminal cytoplasmic regulatory domain that determines the set point for the internal modifier site and mediates growth factor signals with the H^+ sensor via phosphorylation. We therefore undertook studies to determine whether epidermal growth factor also mediates activation of NHE-2 in a similar manner to the NHE-1. At physiological concentrations, epidermal growth factor also upregulated amiloride-sensitive Na^+ uptake in PS120 cells, suggesting activation of NHE-2 by mitogens. The present studies suggest important structure-function relationship of this novel Na^+/H^+ exchanger.

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