A mechanism for the activation of the Na/H exchanger NHE-1 by cytoplasmic acidification and mitogens

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Eukaryotic cells constantly have to fight against internal acidification. In mammals, this task is mainly performed by the ubiquitously expressed electroneutral Na⁺/H⁺ exchanger NHE-1, which activates in a cooperative manner when cells become acidic. Despite its biological importance, the mechanism of this activation is still poorly understood, the most commonly accepted hypothesis being the existence of a proton-sensor site on the internal face of the transporter. This work uncovers mutations that lead to a nonallosteric form of the exchanger and demonstrates that NHE-1 activation is best described by a Monod–Wyman–Changeux concerted mechanism for a dimeric transporter. During intracellular acidification, a low-affinity form of NHE-1 is converted into a form possessing a higher affinity for intracellular protons, with no requirement for an additional proton-sensor site on the protein. This new mechanism also explains the activation of the exchanger by growth signals, which shift the equilibrium towards the high-affinity form.

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INTRODUCTION

The NHE-1 isoform of the Na⁺/H⁺ exchanger is expressed at the plasma membrane of all mammalian cells and tissues, and exerts the crucial function of protecting cells from internal acidifications (for a review, see Counillon & Pouyssegur, 2000). This transporter belongs to the gene family of Na⁺/H⁺ exchangers, whose conserved sequences are present in archaeobacteria, eubacteria, lower eukaryotes, plants and animals. At physiological intracellular pH, NHE-1 displays a very low basal activity. When the cytoplasm becomes acidic, it activates sharply and reaches its maximal velocity in about one pH unit (Aronson *et al*, 1982; Paris & Pouyssegur, 1984). This allosteric activation profile prevents the disruption of the transmembrane sodium gradient and cytoplasmic alkalization, while ensuring a very efficient response to even

moderate intracellular acidifications. NHE-1 sensitivity to internal pH variations is increased in a wide variety of situations, such as osmotic shock (Bianchini *et al*, 1995) or stimulation by hormones and mitogens (Paris & Pouyssegur, 1984). This is thought to represent a permissive step for hormonal response and cell proliferation (Ganz *et al*, 1989).

NHE-1 possesses an amino (N)-terminal transmembrane domain responsible for transport, and a large cytosolic carboxy (C)-terminal end that interacts with signalling proteins to modulate the exchanger's response to acidifications (Fig 1A) (Wakabayashi *et al*, 1992). Although the mechanism of allosteric activation of NHE-1 is not yet understood, the hypothesis of the existence of an alternate proton-binding site possessing a sensor function is commonly accepted. To gain insight into this mechanism of activation, we constructed a collection of mutated exchangers bearing substitutions of conserved charged residues located in intracellular loops (ILs) (Fig 1B). Charges may finely tune the access of hydrated protons to their binding and transport sites, or form salt bridges involved in allosteric transitions (Schirmer & Evans, 1990).

This strategy allowed us to identify a low-affinity point mutant of NHE-1 exhibiting a loss of allosteric behaviour. On the basis of these data and the characterization of a C-terminal deletion mutant, we show that NHE-1 activation by intracellular protons does not require the presence of an additional proton-sensor site, but instead follows a Monod–Wyman–Changeux mechanism.

RESULTS

Construction and characterization of WT and mutant NHE-1s

Mutants were constructed with the human NHE-1 cDNA (Sardet *et al*, 1989) inserted into a modified polycistronic pECE vector (SV40 promoter) possessing a neomycin resistance gene. The 11 different mutants characterized in this work are shown in Fig 1B. They were stably expressed in NHE-deficient cells (Pouyssegur *et al*, 1984) using neomycin selection instead of H⁺ killing techniques to avoid possible biases due to the functional consequences of our mutations on the exchangers. Apart from mutant E253K, which exhibited a very low level of expression, all mutants were expressed to similar levels (Figs 2A,B), and were functional, with affinities for external sodium similar to those of wild type (Fig 2C). The electrophoretic mobility of the precursor and mature glycosylated forms (Counillon *et al*, 1994) as well as dimer formation (Fafournoux *et al*, 1994) were similar between

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Fig 1 | (A) Topological model of NHE-1 (Wakabayashi *et al.*, 2000). Crucial residues for the allosteric response are circled on the model. They are present in two re-entering internal loops (Il₂ and Il₄), and on Il₅, which is close to the C-terminal regulatory region of the protein. (B) Sequences alignment of the internal loops of the mammalian NHEs. The first Il is not depicted because of its small size and low conservation. Charged residues are in bold letters. The numbers correspond to the human NHE-1 sequence.

mutants and wild-type NHE-1 (Fig 2A). To measure the effect of these mutations with high accuracy, we used rapid kinetics of ²²Na⁺ uptake, in which intracellular pH was clamped at the desired values using the ionophore nigericin (see Methods). These experiments were performed on growth-factor-stimulated (20% serum) or -starved cells. The wild-type NHE-1 displayed characteristic cooperative activation (Fig 3A) with a Hill coefficient of 1.35 ± 0.07 in the absence of growth factors, and 1.69 ± 0.02 in the presence of 20% serum (Table 1). The E184H (Il₂), E248Q (Il₃), R327K and R330Q (Il₄), K438E and K443E (Il₅) mutations did not result in detectable changes (data not shown) were not studied further. By contrast, the R180K (Il₂), R327E and E330M (Il₄) substitutions resulted in a decreased sensitivity of the exchanger to intracellular protons (Table 1), but were still sensitive to factors. The fact that mutations in loops 2 and 4 modify the cooperative behaviour of NHE-1 shows that these regions are important for the allosteric regulation of the exchanger. Recently, Wakabayashi *et al.* (2003) showed that the positive charge carried by R440 in Il₅ is required for a normal allosteric response. In accordance with this finding, we discovered that the D448K substitution in the same loop, which replaces a negative by a positive charge, has no significant effect under resting conditions, but slightly increases the sensitivity for protons in the presence of growth factors (Table 1). To summarize, four positions in a total of nine exhibited changes when mutated. These positions are located in intracellular loops 2, 4 and 5 (Fig 1).

The R327E mutant

The R327E substitution provoked a dramatic loss of cooperativity (Fig 3B,C). In the absence of serum, this mutant exhibits michaelian behaviour (Hill coefficient of 1.02 ± 0.05 , to compare with 1.35 ± 0.07 for wild type). Nonlinear fitting with a hyperbolic function provided us with an affinity constant of $3.6 \pm 0.7 \times 10^{-6}$ M ($r^2 = 0.986$). This corresponds to a half activity at pH 5.44, well below the physiological range of wild-type NHE-1 and in accordance with the apparent affinity constant obtained using the Hill plot of the same data ($10^{-5.42}$ M) (Table 1). As shown in Fig 3, this mutant is activated, although moderately, by growth factors (Hill coefficient of 1.15 ± 0.06 , Table 1). Based on these data, we concluded that this mutation stabilizes the transporter in the low-affinity conformational state of the allosterical mechanism. We therefore decided to test the models used at present for cooperativity, using this affinity parameter and our data as experimental constraints.

The proton-sensor site model

This model implies that the transport site is modulated by an additional site possessing a sensor function. As detailed in the supplementary information online, this mechanism is defined by four affinity constants: two for the transport site (K_t and K_t'), depending on whether the transport site is protonated or not and, symmetrically, two for the sensor site (K_s and K_s'), depending on whether the sensor site is protonated or not. The saturation

Fig 2 | Levels of expression, maximal velocities and affinities for external sodium for wild-type and mutant NHE-1s. (A) Western blots were prepared from membranes of cells transfected with wild-type and mutated transporter with either normal (exemplified by the E330Q mutant) or altered phenotypes (R180K, E253K, R327E, E330M and D448K). Note the presence of the intracellular immature form at 82 kDa, the fully glycosylated mature form at 110 kDa, and of their dimeric counterparts around 200 kDa. The PS120 lane corresponds to membranes prepared from untransfected cells. (B) Maximal velocities of wild-type and mutated exchangers, measured by $^{22}\text{Na}^+$ uptake for an external Na^+ concentration of 120 mM. The R327E mutant apparently has a smaller $V_{\max}$ value because of its low affinity for extracellular protons. (C) Comparison of K_m values for extracellular sodium. Note that despite small individual variations, wild type and mutants exhibit very similar K_m values.

function of this model is defined by the following equation (see supplementary information online for details):

$$\frac{V}{V_{\max}} = \bar{Y} = \frac{K_s'[\text{H}^+] + [\text{H}^+]^2}{K_s'K_t + [\text{H}^+](K_s' + K_t') + [\text{H}^+]^2} \text{ with } K_t'K_s = K_tK_s'.$$

Qualitatively, a high affinity for the transport site when the sensor is protonated ($K_t' < K_t$) implies a low affinity for the sensor in the unprotonated form of the exchanger ($K_s > K_s'$). This leads to the prediction that a small fraction of the exchanger would be active in its physiological range of action. Moreover, fitting $V/V_{\max}$ values to this equation provided us with negative or absurd

Fig 3 | Kinetic characterization of WT NHE-1 and the R327E mutant. (A) Growth factors induced activation of WT NHE-1 for intracellular H^+ concentrations between 10^{-7} and 10^{-6} M: cells were acidified using the technique described in the Methods and the activity of the exchanger was measured by rapid kinetics of $^{22}\text{Na}^+$ uptake. Filled diamonds, NHE-1 plus 20% serum; filled squares, NHE-1 in cells deprived of serum for 12 h. (B, C) Comparison between WT NHE-1 and the R327E mutant in the absence (B) or in the presence (C) of growth factors. Filled diamonds, NHE-1; open circles, R327E. Note the michaelian behaviour of the R327E mutant in the absence of growth factors (B), and the different scales used for graphs (B) and (C). Lines are obtained from the fits, and error bars correspond to standard errors.

Table 1 | Hill number values and apparent affinities derived from the Hill plot of our experimental data for wild-type and mutant exchangers

Mutant	– Growth factors			+ Growth factors		
	Hill coefficient	Apparent K_d (pH units)	r^2	Hill coefficient	Apparent K_d (pH units)	r^2
R180K	1.32 ± 0.04	5.67 ± 0.16	0.998	1.67 ± 0.06	6.22 ± 0.26	0.996
R327E	1.02 ± 0.05	5.42 ± 0.25	0.991	1.19 ± 0.06	5.54 ± 0.3	0.987
E330M	1.20 ± 0.05	5.85 ± 0.22	0.995	1.40 ± 0.12	6.12 ± 0.69	0.963
D448K	1.24 ± 0.01	5.88 ± 0.25	0.999	1.79 ± 0.1	6.21 ± 0.04	0.991
WT	1.35 ± 0.07	5.89 ± 0.32	0.984	1.69 ± 0.02	6.4 ± 0.07	0.999

Decimal logarithms of $V/V_{\max}/(1 - V/V_{\max})$ were plotted as the decimal logarithm of intracellular proton concentrations. Hill coefficients and apparent K_d values were obtained from the parameters of the equations given by linear regression of the Hill plots, using experimental points taken around the X-axis.

numbers for some of the equilibrium constants, indicating that this model cannot depict NHE-1 activation (see supplementary information online for details).

A sequential mechanism (Koshland *et al*, 1966) for a dimer, in which the protonation of one subunit modifies the affinity of the second one, is similar to a proton-sensor model in which the two proton-binding sites would be able to transport (see supplementary information online). This model also gave unsatisfying fits.

The Monod–Wyman–Changeux model

Unlike the previous models, our data gave good fits when applied to a Monod–Wyman–Changeux concerted model (Monod *et al*, 1965) for a dimer. In this model, the transporter oscillates between two distinct conformations possessing, respectively, a high and a low affinity for protons. Its saturation function is described by the following equation:

$$V/V_{\max} = \bar{Y} = \frac{\alpha(1 + \alpha) + L_0 c \alpha(1 + c \alpha)}{L_0(1 + c \alpha)^2 + (1 + \alpha)^2}$$

where K_h is the affinity constant for the high-affinity form, K_l is the affinity constant for the low-affinity form, $\alpha = [H^+]/K_h$ and $c = K_h/K_l$, and $L_0 = [\text{low-affinity form}]/[\text{high-affinity form}]$ (unprotonated forms).

We calculated a microscopic affinity constant of $1.7 \pm 0.14 \times 10^{-8} \text{ M}$ ($r^2 = 0.991$) for the high-affinity form, the low-affinity constant obtained from the R327E mutant being $3.6 \pm 0.7 \times 10^{-6} \text{ M}$. The ratio of the low- to the high-affinity form is the allosteric constant L_0 and equals 5952 ± 513 . Therefore, this model correctly predicts that at physiological pH, the most abundant form of the exchanger is the low-affinity form, resulting in a very low basal exchanger activity. When the proton concentration rises, the small population of high-affinity exchanger is preferentially protonated. Because the equilibrium is defined by the ratio between the unprotonated forms of the exchanger (thermodynamic constant L_0), the protonation of the high-affinity form will spontaneously trigger the conversion for the low-affinity inactive exchanger in a fully active high-affinity form.

Mechanism for growth factor activation

To challenge this model, we tested its ability to provide a unifying explanation for the growth factor activation of NHE-1, by fitting

the activation curve of wild-type NHE-1 stimulated by 20% serum. Growth factor activation fitted with a tenfold decrease of the L_0 value (from 5952 ± 513 in unstimulated cells, to 535 ± 69 , $r^2 = 0.999$ in stimulated cells), but not with changes in the microscopic constants for the low- and high-affinity forms for protons.

Therefore, growth signals result in a new equilibrium, which is shifted towards the high-affinity form, making the system more sensitive to acidification.

The C-terminal truncation mutant

The deletion of the C-terminal region of NHE-1 from residue 566 completely abolishes growth factor activation (Wakabayashi *et al*, 1992). Because mitogens shift the allosteric equilibrium towards the high-affinity form of NHE-1, it is tempting to hypothesize that the cytosolic region of NHE-1 serves as a regulator, which enables signalling proteins to modify this conformational balance. We therefore expressed a mutant of NHE-1 with the C-terminal regions necessary for growth factor activation deleted, based on the rationale that this might also lock the transporter in the high- or low-affinity conformation. This mutant, which has a much lower level of expression than wild type (Fig 4A), possesses michaelian behaviour (Fig 4B, Hill coefficient of 1.01 ± 0.01 ($r^2 = 0.999$)). Nonlinear fitting provided an affinity constant of $2.4 (\pm 0.2) \times 10^{-6} \text{ M}$ ($r^2 = 0.998$), in good accordance with that of the R327E mutant ($3.6 \times 10^{-6} \text{ M}$). This suggests that this C-terminal truncation stabilizes the transporter in the low-affinity conformation.

DISCUSSION

The present work provides a coherent quantitative explanation for the activation of NHE-1 by intracellular acidifications. This mechanism simply requires the oscillation of dimeric NHE-1 between two conformations differing only in the microscopic affinities of their proton transport site. In this model, the two conformations have the same affinity for extracellular sodium. This explains the michaelian behaviour of the coupling cation at steady state. The proposed mechanism does not fit with the existence of higher-order oligomers, and totally excludes monomers (see additional online data). The existence of a dimer has been previously reported both from biochemical (Fafournoux *et al*, 1994) and kinetic arguments (Otsu *et al*,

Fig 4 | (A) Expression of the wild-type NHE-1 and the deletion mutant ($\Delta\text{C-ter}$) of the regulatory domain. In all, 10 μg of purified membrane proteins are loaded in the WT lane, and 80 μg in the $\Delta\text{C-ter}$ lane. The arrow shows the mature glycosylated form of the deletion mutant. (B) Dose–response curve of the deletion mutant for intracellular protons: due to the low expression of this mutant, increased $^{22}\text{Na}^+$ activities and uptake times were used (see Methods). Note the hyperbolic curve, with a half maximum at 2.4×10^{-6} M. (C) Mechanism of activation of NHE-1 by cytoplasmic acidifications and mitogens. This model predicts a symmetrical dimer, which oscillates between a low- and a high-affinity form for intracellular protons. Sodium binding is michaelian because the affinity of this ion is identical for the two conformations. The equilibrium is largely in favour of the low-affinity form, resulting in low basal activity and cooperative activation. Growth signals modify the C-terminal end. This increases the sensitivity of the system by shifting the equilibrium towards the high-affinity form.

1989). This study, which addressed a different question, reaches the same conclusion using different experimental tools and independently provides a strong piece of evidence for a dimeric functional unit.

Previous experiments using various chimeric (Borgese *et al.*, 1994; Wakabayashi *et al.*, 1995) and deletion mutants (Wakabayashi *et al.*, 1992) had led to the conclusion that the modulation of this allosteric response by intracellular signalling pathways is both modular and complex (Bianchini *et al.*, 1995). Recently published crystal structures of ion channels have shown that extremely precise adjustments of the polypeptide chain are required for correct ion coordination (Doyle *et al.*, 1998; Dutzler *et al.*, 2002). Based on this observation, it was difficult to understand how very different modifications of the regulatory end of NHE-1 (for a review, see Counillon & Pouyssegur, 2000) could systematically and precisely lead to similar increases in the

affinity of a hypothetical sensor site. Our work reveals a much simpler explanation, which unifies a large body of observations (Fig 4C). Growth signals modify the balance between two pre-existing thermodynamical states of NHE-1. This changes the sensitivity of the system for protons, with no physical modification of the binding sites themselves. In this model, the C-terminal region of NHE-1 may be viewed as an allosteric regulatory subunit, which opens Na^+/H^+ exchange activation to higher integration levels via its interaction with the effectors of intracellular signalling.

METHODS

Cell culture and transfection. Exchanger-deficient fibroblasts (Pouyssegur *et al.*, 1984) were grown as described by Touret *et al.* (2001). Transfections were performed using the calcium phosphate precipitation method. Cellular populations stably expressing the mutants were selected using $500 \mu\text{g ml}^{-1}$ G418.

Site-directed mutagenesis. Double-stranded mutagenesis (Quick-change site-directed mutagenesis, Stratagene) was performed as described by Poët *et al.* (2001) using oligonucleotides bearing the appropriate codon changes (MWG Biotech), on a modified polycistronic pECE vector (SV40 promoter) containing human NHE-1 cDNA (inserted between the *HindIII* and *EcoRI* sites) followed by a neomycin resistance gene. Mutations were verified by automated sequencing (MWG Biotech).

Construction of the C-terminal deletion mutant of NHE-1. The above-mentioned construct was digested using the *AgeI* (codon 551) and *EcoRI* restriction enzymes. The remaining plasmid containing the NHE-1 transmembrane region, and essential PIP2- and CHP-binding sites (Aharonowitz *et al.*, 2000; Pang *et al.*, 2001) was recircularized on a double-stranded adapter containing a stop codon. For western blotting, an HA epitope tag was inserted just before the stop codon.

Western blotting. Unless stated, 30 μg of membrane proteins prepared from PS120 cells transfected with WT or mutant NHE-1 s were run on 7.5% acrylamide gels (Biorad mini gel system). Immunoblots were revealed as described by Counillon *et al.* (1994), using commercial monoclonal antibodies against the NHE-1 C-terminal end (Chemicon) and the HA tag (Sigma clone HA-7).

Measurement of initial rates of Na^+/H^+ exchange. Cells seeded on 24-well plates were acidified, incubated for 5 min in sodium-free solutions containing 2.5 μM nigericin (Sigma), 140 mM KCl, and calibrated in a range of pHs varying from 5.2 to 7.2, in the presence of 20 mM of HEPES, MOPS or MES buffers (Bidet *et al.*, 1987). Following 10 min incubation in the same solutions in which the nigericin had been replaced by 50 mg ml^{-1} of BSA, the cells were rinsed in sodium-free medium (120 mM choline chloride, pH 7.4) (Touret *et al.*, 2001). Linear sodium uptake was then carried out for 15–30 s in the same medium containing $0.25 \mu\text{Ci ml}^{-1}$ $^{22}\text{Na}^+$. Uptake was stopped by four rapid rinses in ice-cold PBS. Cells were solubilized in 0.1 N NaOH, and radioactivity was measured by scintillation counting. NHE-1 initial rates were calculated as the amiloride-sensitive $^{22}\text{Na}^+$ accumulations, which were in the order of magnitude of a few thousand cpm at the plateau. Growth factor activation was measured by the addition of 20% fetal calf serum 10 min before the acidification. Na^+/H^+ exchange activity in the absence of growth factors was measured on serum-starved cells for 12 h. The

affinities for extracellular sodium and maximal velocities were determined using different isotopic dilutions in the ²²Na⁺ uptake medium (0.5 μCi ml⁻¹) (Touret *et al*, 2001). No significant sodium accumulation could be detected in the untransfected PS120 cells when tested in the same conditions. We also verified that the uptake remained linear for the time course and sodium concentration used for the characterization of this mutant.

Due to its low activity, the ΔC-ter deletion mutant was characterized using 2 μCi ml⁻¹ of ²²Na⁺ and 5-min uptake times. Under these conditions, the amiloride-sensitive uptake was comparable to that of wild type and other mutants.

Data analysis and treatment. Data were compiled using Microsoft Excel software, and fitted using Sigmaplot 2001 (Jandel) with built-in or user-defined equations. The experimental points (with standard errors) correspond to the compilation of at least three independent experiments, with each experimental point determined at least in duplicate. Constants obtained from fitting our data are provided with the error of the fits and *r*² goodness-of-fit factors.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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