

Species differences in bacterial NhaA Na⁺/H⁺ exchangers

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ABSTRACT

Bacteria have adapted their NhaA Na⁺/H⁺ exchangers responsible for salt homeostasis to their different habitats. We present an electrophysiological and kinetic analysis of NhaA from *Helicobacter pylori* and compare it to the previously investigated exchangers from *Escherichia coli* and *Salmonella typhimurium*. Properties of all three transporters are described by a simple model using a single binding site for H⁺ and Na⁺. We show that *Helicobacter pylori* NhaA only has a small acidic shift of its pH-dependent activity profile compared to the other transporters and discuss why a more drastic change in its pH activity profile is not physiologically required.

Keywords: NhaA, *Helicobacter pylori*, *Escherichia coli*, *Salmonella typhimurium*, cation/proton antiport, solid supported membrane

Abbreviations: ACMA, 9-Amino-6-Chloro-2-Methoxyacridine; EcNhaA, NhaA Na⁺/H⁺ antiporter from *E. coli*; HpNhaA, NhaA Na⁺/H⁺ antiporter from *H. pylori*; IPTG, isopropyl-beta-D-thiogalactopyranoside; SSM, solid-supported membrane; StNhaA, NhaA Na⁺/H⁺ antiporter from *S. typhimurium*.

HIGHLIGHTS

- The *H. pylori* NhaA Na⁺/H⁺ exchanger was previously reported to be pH-insensitive
- We investigate *H. pylori* NhaA by SSM-based electrophysiology
- *H. pylori* NhaA shows a similar profile to *E. coli* NhaA with a small acidic pK shift
- NhaA exchangers from enterobacteria can be explained by the same mechanism

1. INTRODUCTION

Transmembrane exchange of sodium ions for protons exists in virtually all organisms and is essential for cell survival as it controls the intracellular concentrations of these two ions and the cellular volume [1,2]. According to the transporter classification system [3], most Na^+/H^+ exchangers belong to the superfamily of monovalent cation/proton antiporters (CPA) [1].

The current prototype for the CPA family antiporters is the Na^+/H^+ antiporter NhaA from *Escherichia coli* (EcNhaA) [4]. EcNhaA belongs to the electrogenic CPA2 subgroup of the CPA superfamily and catalyzes the exchange of 2 H^+ for 1 Na^+ [4]. The crystal structure of EcNhaA was solved almost a decade ago [5] and has served as the basis for a tremendous number of studies that have tried to provide a better understanding into the structural basis of the EcNhaA transport mechanism.

A general property of Na^+/H^+ exchangers seems to be a highly pH regulated activity profile. This was initially explained in EcNhaA by allosteric regulation of transporter activity coordinated by a so-called “pH sensor” that activates EcNhaA only above a certain pH value [2]. More recently, an alternate model of pH regulation in EcNhaA was introduced [6], following solid-supported membrane (SSM)-based electrophysiological investigation of EcNhaA. SSM-based electrophysiology [7,8] is a powerful tool that allows the investigation of bacterial membrane transporters and channels that are normally inaccessible to classical electrophysiological measurements. It was proven that [6] the observed substrate dependencies of EcNhaA can be described by a simple kinetic model based on a single substrate binding site which is alternatively occupied by a sodium ion or two protons (Figure 1A). Remarkably, the same model was recently used to explain the pH dependence of the NhaP1 transporter from *Methanocaldococcus jannaschii*, the prototype of the electroneutral CPA1 family, suggesting that it can be generally applied to all CPA Na^+/H^+ antiporters [9].

The physiological role of EcNhaA is to enable growth of *E. coli* in high salinity media or in the presence of lithium [4]. Close homologues of EcNhaA have been found in other Enterobacteriaceae, including *Vibrio cholerae* [10], *Salmonella enteritidis* [11], *Salmonella typhimurium* [12] and *Helicobacter pylori* [13]. We have recently characterized NhaA from *Salmonella enterica* serovar Typhimurium LT2 (StNhaA) using solid-supported membrane (SSM)-based electrophysiology and found a transport activity profile very similar to that of EcNhaA in respect to the transition from low to high pH [12], in line with the high sequence identity between the two (92%), though the transporter has a higher activity in the alkaline.

NhaA from *Helicobacter pylori* (HpNhaA) shares a lower sequence similarity to EcNhaA due to the presence of 10 extra aminoacid residues at the N-terminus and 40 extra residues in the cytoplasmic loop between transmembrane helices (TMs) VIII and IX in HpNhaA [13,14]. Interestingly, the pH dependence of HpNhaA is reported to be drastically different to that of EcNhaA, with almost no down-regulation occurring at neutral or acidic pH as shown by 9-Amino-6-Chloro-2-Methoxyacridine (ACMA) dequenching experiments [13,14]. As such a pH-independence in the transport activity of HpNhaA would contradict our current model of pH regulation in Na^+/H^+ antiporters [6,9], we investigated the activity of HpNhaA using SSM-based electrophysiology. This approach could assess whether HpNhaA indeed has a significantly different pH regulation compared to its *E. coli* and *S. typhimurium* counterparts or whether this contradiction is a consequence of the experimental method used so far. Finally, we compare the activity profiles of the EcNhaA, StNhaA and HpNhaA exchangers and discuss them under the terms of the competition model of Na^+/H^+ antiport.

2. MATERIAL AND METHODS

Plasmids and bacterial strains. The HpNhaA gene, codon-optimized for *E. coli* expression and containing a C-terminal 6-His tag, was synthesized by Genscript (Genscript, Piscataway,

NJ, USA) in the pET-22b(+) expression vector (Merck Millipore, Billerica, MA, USA). For expression in the Na⁺/H⁺ exchanger deficient strain KNabc [15], the gene was cloned into the pTrcHis2 TOPO expression vector (Life technologies, Darmstadt, Germany) using the mutagenic primers HpNhaA_for: ATCCATGGCCATGAATCTGAAGAAGACGG and HpNhaA_rev: ATGAATTCGCGACCTTCAATGCGACC. Extraneous aminoacids at the N-terminus introduced by cloning were removed using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Waldbronn, Germany) and the mutagenic primers HpNhaA_QC_for: GATTAAATAAGGAGGAATAAACCTAGGCCATGAATCTGAAG and HpNhaA_QC_rev: CTCAGATTCATGGCCTAGGTTTATTCCTCCTTATTTAATC.

Protein expression and purification. Protein intended for purification was produced in *E. coli* BL21(DE3) and purified using Ni²⁺-affinity chromatography as previously described [16]. Reconstitution into proteoliposomes was performed as described [6] at a lipid-to-protein ratio (LPR) of 10 (w:w).

For ACMA dequenching measurements, KNabc cells were grown in LBK medium (10 g/L tryptone, 5 g/L yeast extract, 67 mM KCl) at 37°C until A₆₀₀ = 0.8. At this point they were either harvested without induction or induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG), left to overexpress HpNhaA for 2h and then harvested.

SSM-based electrophysiology. Electrophysiological measurements were performed as described [6]. Non-activating solutions contained 25 mM MES, 25 mM MOPS, 25 mM Tris, 5 mM MgCl₂ and 300 mM KCl. Activating solutions contained 25 mM MES, 25 mM MOPS, 25 mM Tris, 5 mM MgCl₂, *x* mM NaCl and (300-*x*) mM KCl. All solutions were titrated to the desired pH using HCl or Tris. Peak currents were corrected for solution-exchange effects by subtracting the peak currents recorded at pH 5, where no transporter activity was apparent.

ACMA dequenching in everted vesicles. Everted vesicles were prepared as described [17] and resuspended in buffer containing Tris 10 mM (titrated to pH 7.5 with HCl), 250 mM sucrose

and 140 mM choline chloride. Total protein concentration was measured using the Bradford assay [18]. Fluorescence was measured using a Hitachi F4500 Fluorimeter (Hitachi High-Technologies Corporation, Tokyo, Japan) at $\lambda_{exc}=410$ nm and $\lambda_{em}=480$ nm. Dequenching assay was performed in buffer containing 10 mM MES (titrated to the indicated pH using Tris), 145 mM KCl, 5 mM MgCl₂, 2 μ M ACMA. 200 μ g (total protein) of everted vesicles were added to 1 mL external buffer. Acidification of the vesicles was induced using 5 mM Tris-lactate (at the corresponding pH). After reaching steady-state fluorescence, dequenching was induced by adding either 0.5 or 5 mM NaCl. Finally, the pH gradient was dissipated by addition of 20 mM NH₄Cl. Dequenching was calculated by: $\% \text{ dequenching} = \frac{F_{deq} - F_{min}}{F_{fin} - F_{min}} \cdot 100$, where F_{deq} is the steady-state level of fluorescence achieved after dequenching, F_{min} is the steady-state level of fluorescence before Na⁺ addition, and F_{fin} is the steady-state level of fluorescence after dissipation of the pH gradient.

SDS-PAGE and Western blot. SDS-PAGE was performed on everted vesicles essentially as described [19]. Proteins were transferred to a PVDF membrane and incubated with Penta-His Antibody (QIAGEN, Hilden, Germany). The secondary antibody used was Polyclonal Sheep anti-mouse IgG antibody (GE Healthcare Life Sciences, Freiburg, Germany). Detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Bonn, Germany).

Kinetic analysis and numerical methods. The steady-state solution for the kinetic model (Fig. 1A) was calculated as described previously [6]. This calculation yields turnover or activity of the transporter at given Na⁺ and H⁺ concentrations and was used in a global fit to the electrophysiological data set.

3. RESULTS

Proteoliposomes containing reconstituted HpNhaA were analyzed using SSM-based electrophysiology. Proteoliposomes were allowed to adsorb to the SSM, then transport was initiated by fast Na^+ concentration jumps. This gave rise to negative transient currents of up to 5 nA in amplitude (Fig. 1B) with similar characteristics to those previously reported for EcNhaA [6,20] and StNhaA [12]. The negative polarity of these currents is in accordance to the overall transport of one negative charge per transport cycle to the interior of the liposomes.

pH dependence of HpNhaA transient currents. Recorded peak currents were used as a measure of transporter activity and were plotted for two different Na^+ concentration jumps across the pH range 5.5 to 9.5. Note that, unlike the ACMA dequenching assays, the pH in this case is symmetrical across the proteoliposome membrane.

We could readily observe that transport activity of HpNhaA follows a bell-shaped curve, showing down-regulation at both acidic and alkaline pH values (Fig. 1C). Activity of HpNhaA was maximal at pH 8 under saturating (100 mM) Na^+ concentrations.

Na^+ dependence of HpNhaA transient currents. We further measured the activity of HpNhaA in response to varying Na^+ concentration jumps at three different pH values (6.5, 8 and 9.5). In all three cases (Figure 1D) the peak currents follow a hyperbolic Michaelis-Menten type dependence that allowed the determination of apparent K_m values (Table 1) for Na^+ . The drastic increase of the K_m value from pH 8 to pH 6.5 shows that competition between Na^+ and H^+ binding is clearly observed in HpNhaA.

The kinetic model accurately describes HpNhaA. Our kinetic model for Na^+/H^+ antiport (Figure 1A) was used to determine kinetic parameters of HpNhaA antiport. A symmetrical model was employed with identical K_D^{Na} and pK , describing Na^+ and H^+ binding, respectively, for the inside and outside open forms and equal forward and backward rates of

the conformational transitions [6]. An excellent fit was obtained for all experimental results (Figure 1C-D), yielding the same K_D^{Na} value as for EcNhaA and a pK value which is shifted by less than one pH unit into the acidic. For comparison we also fitted data obtained previously for StNhaA to the kinetic model [12] and included the obtained kinetic parameters together with those from EcNhaA in Table 1.

ACMA dequenching measurements. Given that our electrophysiological investigation found a clear pH dependence in the activity profile of HpNhaA, it was important to assess why previously performed ACMA dequenching experiments show a pH independence of the activity profile above pH 6.5. To this end, we employed the ACMA dequenching assay under different conditions of protein expression and Na^+ levels.

Two different *E. coli* everted vesicle preparations that expressed HpNhaA were used. One preparation (Figure 2A, rightmost lane) came from *E. coli* cells induced with 1 mM IPTG and left to express the protein for 2h. The second preparation (Figure 2A, middle lane) originated from *E. coli* cells that were not induced with IPTG and could express only reduced levels of HpNhaA corresponding to the “leakiness” of the used plasmid.

Under different conditions of transporter expression and Na^+ concentrations it can be seen (Figure 2B) that the pH dependence shown in the ACMA dequenching assay was changed. The everted vesicles containing highly overexpressed HpNhaA showed a pH-insensitive behavior after addition of 5 mM Na^+ at pH 6.5 – 8.5, as previously reported [13]. By comparison, the preparation where HpNhaA was expressed to a lower level showed a decrease in activity at acidic pH that was highly pronounced when the experiment was performed using 0.5 mM Na^+ . Everted vesicles isolated from KNabc cells that were not transformed with HpNhaA showed no dequenching across the used pH range (data not shown).

To characterize the different pH profiles we fitted a theoretical titration curve to the experimental data and quantified the pH shift at the different conditions by apparent pK values. It is obvious from the figure that depending on the conditions used the pH profile of HpNhaA determined by the ACMA dequenching assay can be shifted by up to one pH unit.

4. DISCUSSION

Figure 3A shows a comparison of the kinetic properties of EcNhaA, StNhaA and HpNhaA. They show distinct differences in the value of the pH optimum and the width of the profiles. In respect to the highly different habitats the three organisms populate the shift in pH profile of only ~ 1 pH unit is very moderate.

Transport mechanism and pH regulation in HpNhaA. An interesting property of HpNhaA is the drastic pH dependence of its K_m for Na^+ , which it has in common with other bacterial Na^+/H^+ exchangers (see Table 1 and [6,9]). This has been explained by competition of Na^+ and H^+ for a common binding site and a kinetic model derived from these results explains indeed the pH profiles of EcNhaA [6] and StNhaA [12] as well as HpNhaA as shown in Figure 1. Competition of Na^+ and H^+ for a common binding site is described by the formula [21]:

$$K_m = K_D^{Na} (1 + 10^{n(pK-pH)})$$

which gives K_m as a function of pH and the Na^+ dissociation constant K_D^{Na} of the binding site. The Hill coefficient n takes into account that NhaA transports 2 H^+ for 1 Na^+ .

Figure 3B shows a plot of the experimental K_m values from Table 1. The function given above was fitted to the data points determined for HpNhaA using a fixed value of n = 1 and 2. The result of the fit with n = 1 is shown in the figure (black line); n = 2 did not yield a satisfying fit. The values obtained from the fit of $K_D^{Na} = 3.8$ mM and $pK = 7.9$ are in excellent agreement with those given in Table 1 from the global fit of the kinetic model (see Figure 1).

This analysis confirms the competition mechanism for acidic down-regulation of NhaA as put forward previously for EcNhaA [6]. The fact that a much better fit was obtained for $n = 1$ shows that there is little cooperativity in H^+ transport or that only one H^+ is kinetically relevant. This also supports the kinetic model of Figure 1A, where also only one H^+ was considered.

Experimental results of StNhaA and EcNhaA are shown in Figure 3B and compared to the predictions of the function given above (dashed lines). Here the K_D^{Na} and pK given in Table 1 were used together with $n = 1$. The good agreement observed confirms the validity of the competition concept. Indeed, the kinetic model based on this principle (a single binding site for Na^+ and H^+) fully explains the substrate dependency of HpNhaA over the entire experimental range (Figure 1).

Adaptation of HpNhaA to its acidic environment. *H. pylori* colonizes the gastric mucosa, where the pH can be as low as 2. Therefore, it came as no surprise that in contrast to the strong pH dependence of EcNhaA, the Na^+/H^+ exchange activity of *H. pylori* was reported to be pH independent between pH 6.5 and 8.5 [13]. It was suggested that this was an adaptation of *H. pylori* to its acidic environment and that HpNhaA may excrete protons using a putatively positive inside potential in acidophilic bacteria [13]. In contrast, the activity profile of HpNhaA determined from our electrophysiological analysis shows a bell shaped pH dependence with a maximal activity at pH ~ 8 . How can these conflicting experimental results and interpretations be explained?

In the first place, *H. pylori*, although living in an acidic environment, has evolved strategies to maintain a cytoplasmic pH close to neutral and a membrane potential of -100 mV. It does so by periplasmic buffering using the products of the urease reaction to keep the periplasmic pH at ~ 6.1 [22]. Therefore, no *a priori* requirement for a drastic pH adaptation of HpNhaA exists. Secondly, external pH is not relevant for NhaA activity, because its activity is down-

regulated via competition at the cytoplasmic Na^+ uptake site. Indeed we show that HpNhaA has an activity of $> 60\%$ of its maximal activity at the reported near neutral cytoplasmic pH of *H. pylori*. Therefore, a moderate acidic shift by 0.8 pH units of HpNhaA compared to the *E. coli* transporter (Figure 3A) makes perfect physiological sense.

A further discrepancy concerns the bell shaped pH profile of HpNhaA shown in Figure 1B, which is different from the previously reported flat pH dependence from pH 8.5 to pH 6.5 [13] determined in ACMA dequenching measurements on everted vesicles. A control dequenching measurement using our preparation and the same conditions is shown in Figure 2B (Induced Na^+ 5 mM) and confirms this finding. The lack of alkaline down-regulation has a simple explanation: in the dequenching assay the interior of the vesicles is always kept at a more acidic pH than the exterior by the activity of the *E. coli* respiratory chain. In contrast, the electrophysiological assay was performed at symmetrical pH where high pH at the interior of the proteoliposomes (H^+ uptake side) leads to a reduction of the transport activity (Figure 1C).

To clarify the difference of the pH profile at the acidic side we performed dequenching assays at reduced expression and reduced Na^+ concentration (Figure 2B). The first and most important information from these measurements is that conditions can be found where HpNhaA is clearly pH dependent in the dequenching assay. Not surprisingly a lower Na^+ concentration leads to a stronger acidic down-regulation and an alkaline shift of the apparent pK (pK_{app}) of 0.4 – 0.5 (Figure 2B) because H^+ competes more effectively for the cation binding site slowing down Na^+ uptake into the vesicles. But more importantly, also a reduction in expression density leads at high and low Na^+ to a stronger acidic down-regulation and an alkaline shift of pK_{app} of 0.6 – 0.7 indicating an insufficient dynamic range of the assay. Although the observed pK_{app} shift is admittedly too small to fully explain the

discrepancy between electrophysiological and dequenching measurements, it definitely contributes to the differences observed with the two techniques.

The dequenching assay has been extensively used for the characterization of EcNhaA and its mutant variants in everted vesicles. Acidic down-regulation was observed for the wild type, while a pH independent behavior was found for the G338S mutant [23] that was later shown to be an acidic-shifted EcNhaA variant using SSM-based electrophysiology [6]. We report a similar result, namely that the acidic-shifted HpNhaA exchanger displays a flat pH profile down to pH 6.5. We conclude that the low pK of EcNhaA G338S and HpNhaA shifts acidic down-regulation to a pH range inaccessible to dequenching measurements.

Species dependence of NhaA Na⁺/H⁺ exchangers. Although living in different environments the enteric bacteria compared in this study have NhaA Na⁺/H⁺ exchangers with remarkably similar kinetic properties (Table 1). Their transport mechanism is the same and the mechanism of acidic down-regulation works in all cases via substrate competition. The most prominent difference among them is a pK shift of 1.2 pH units from *S. typhimurium* to *H. pylori*. Na⁺ affinity, on the other hand, is identical in the three organisms. An interesting feature of HpNhaA is the high value of $k_2/k_1 = 20$ (Table 1) corresponding to a high H⁺ translocation rate. This leads to a wider pH profile and a higher activity at low pH.

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FIGURE LEGENDS

Figure 1. SSM-based electrophysiological investigation of HpNhaA. A, Kinetic model for Na^+/H^+ exchangers [6]. The substrates Na^+ or H^+ bind to the outward open (C_o) or inward open (C_i) form. Substrate binding is assumed to be in rapid equilibrium described by the parameters pK and K_D^{Na} . The reorientation of the carrier takes place with the rate constants k_1 and k_2 . B, Transient currents induced by a concentration jump of 100 mM Na^+ on HpNhaA proteoliposomes at different pH values. C-D, pH (C) and Na^+ (D) dependence of HpNhaA peak currents. Peak currents were corrected for solution-exchange effects and normalized to the peak current of a 100 mM Na^+ concentration jump at pH 8.0 recorded on the same sensor. Solid lines show a simultaneous fit of the data in C and D to the kinetic model in A. Data in C-D are average of recordings using three individual sensors \pm SD.

Figure 2. ACMA dequenching assay for HpNhaA. A, Western blot of preparations employed in the ACMA dequenching assay using an anti-His antibody. Equal amounts (100 μg) of total protein were added to all lanes. Left lane shows membranes from *E. coli* KNabc cells that were not transformed with HpNhaA expression plasmid; middle lane shows membranes from cells that were harvested without induction and the right lane membranes from HpNhaA overexpressing cells induced with 1 mM IPTG. B, pH dependence of ACMA dequenching on everted vesicle preparations from (A) in response to either 0.5 or 5 mM Na^+ . Solid lines are fits of a theoretical titration curve to the experimental data ($\% \text{Dequenching} = A \cdot \frac{10^{-pK}}{10^{-pH} + 10^{-pK}}$, where A is a scaling factor and $pK = pK_{\text{app}}$ given in the figure). Data in B are the average of three individual experiments \pm SD.

Figure 3. Comparison of NhaA exchangers from different species. (A) pH profile obtained from electrophysiological analysis at 100 mM Na^+ concentration and symmetrical pH. Transport mode corresponds to the reverse transport direction compared to the physiological

activity. Solid lines are presented as guides. (B) Plot of the K_m values given in Table 1. The solid black line is a fit to the data using the expression derived for competition of Na^+ and H^+ for a common binding site with a fixed Hill coefficient $n = 1$ and the determined parameters $K_D^{Na} = 3.8 \text{ mM}$ and $pK = 7.9$. The dashed lines correspond to EcNhaA (red) and StNhaA (blue) and are a plot of the same function with $n = 1$, and the K_D^{Na} and pK from Table 1. Data for StNhaA and EcNhaA were taken from [6,12].

Table 1. Kinetic parameters determined for the different NhaA subtypes.

| | K_m (mM) | | | | pK | K_D^{Na} (mM) | k_2/k_1 |
|----------|---------------|----------------|-----------------|------------------|-----|--------------------|-----------|
| HpNhaA | 4 ±1 (pH 9.5) | 7 ±4 (pH 8.0) | - | 101 ±5 (pH 6.5) | 8.0 | 3 | 20 |
| EcNhaA* | 7 ±1 (pH 9.0) | 11 ±1 (pH 8.5) | 102 ±7 (pH 7.5) | 178 ±10 (pH 7.0) | 8.8 | 3 | 7 |
| StNhaA** | - | 16 ±7 (pH 8.5) | - | 144 ±40 (pH 7.0) | 9.2 | 3 | 7 |

* All Parameters taken from [6,20]

** K_m values taken from [12]

Figure1
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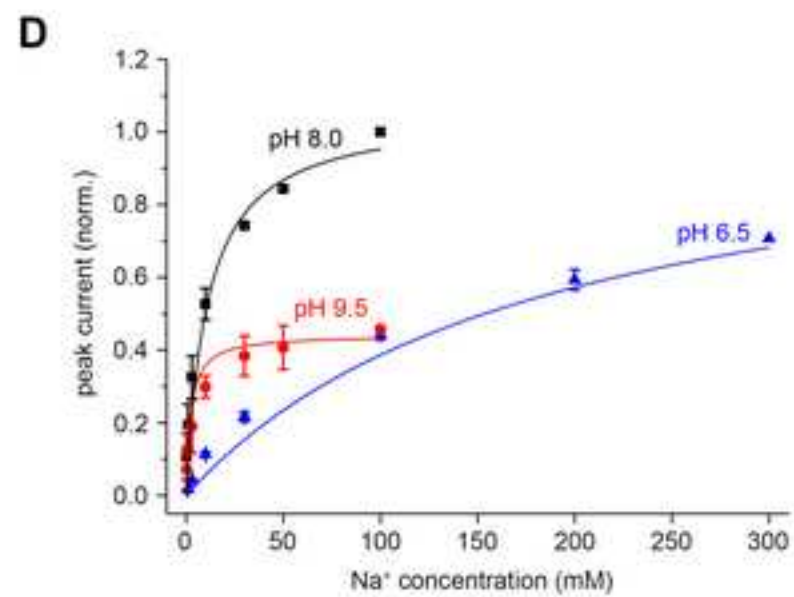
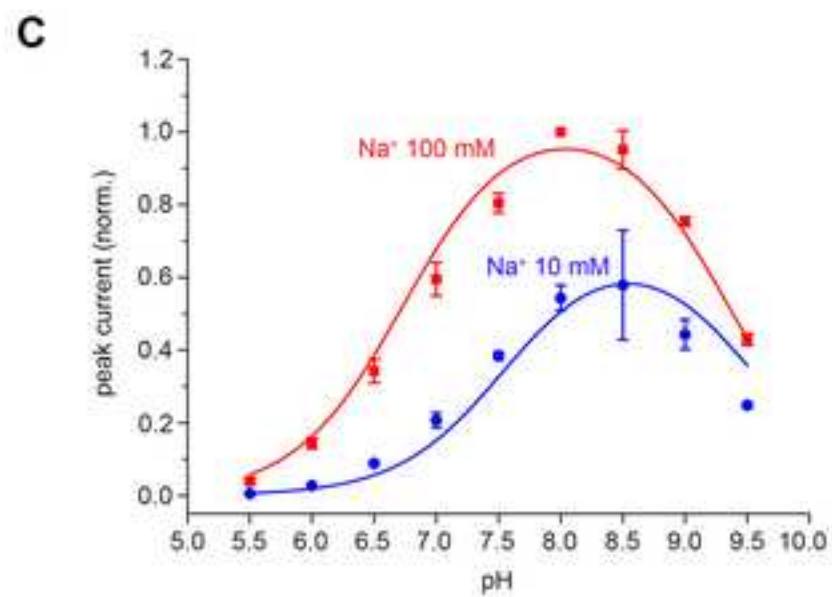
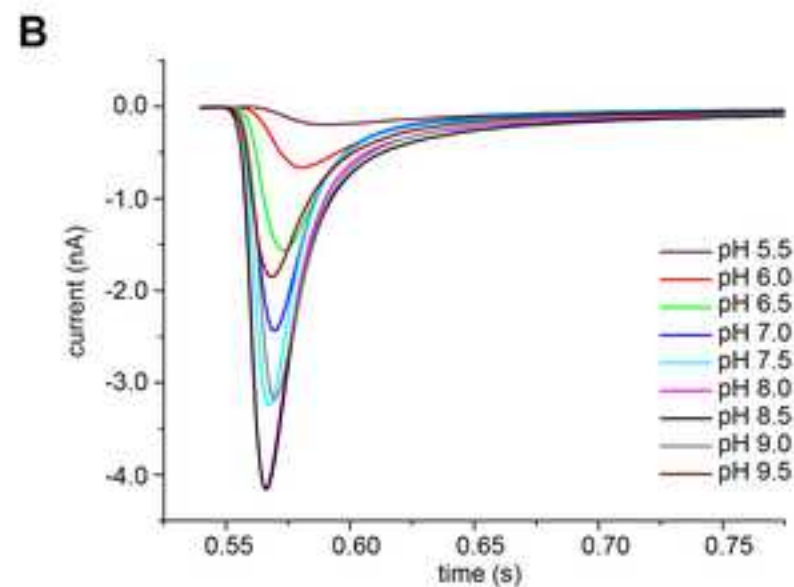
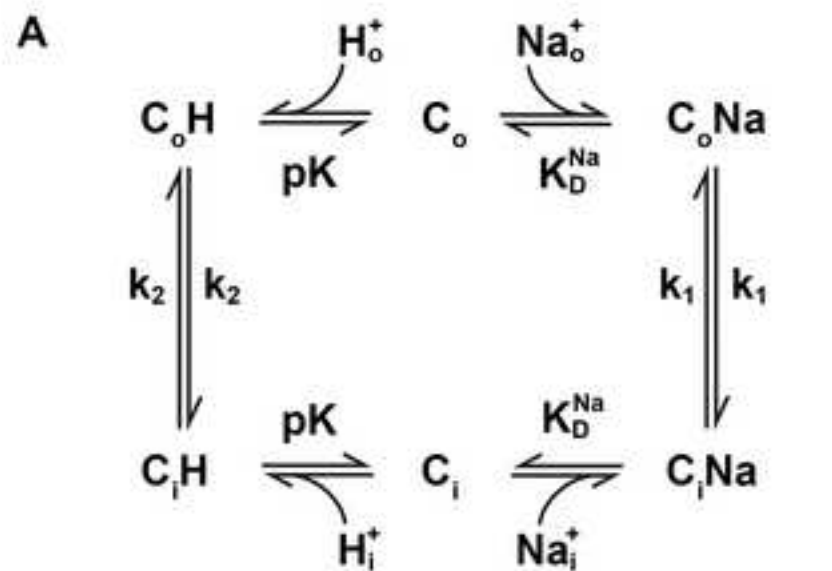
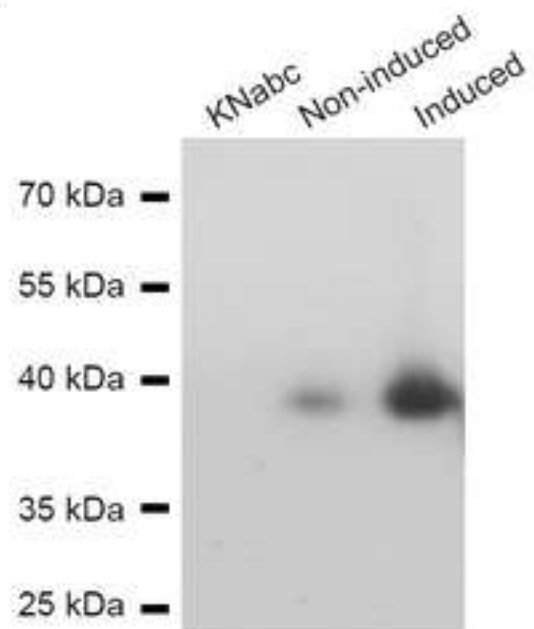


Figure2

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A



B

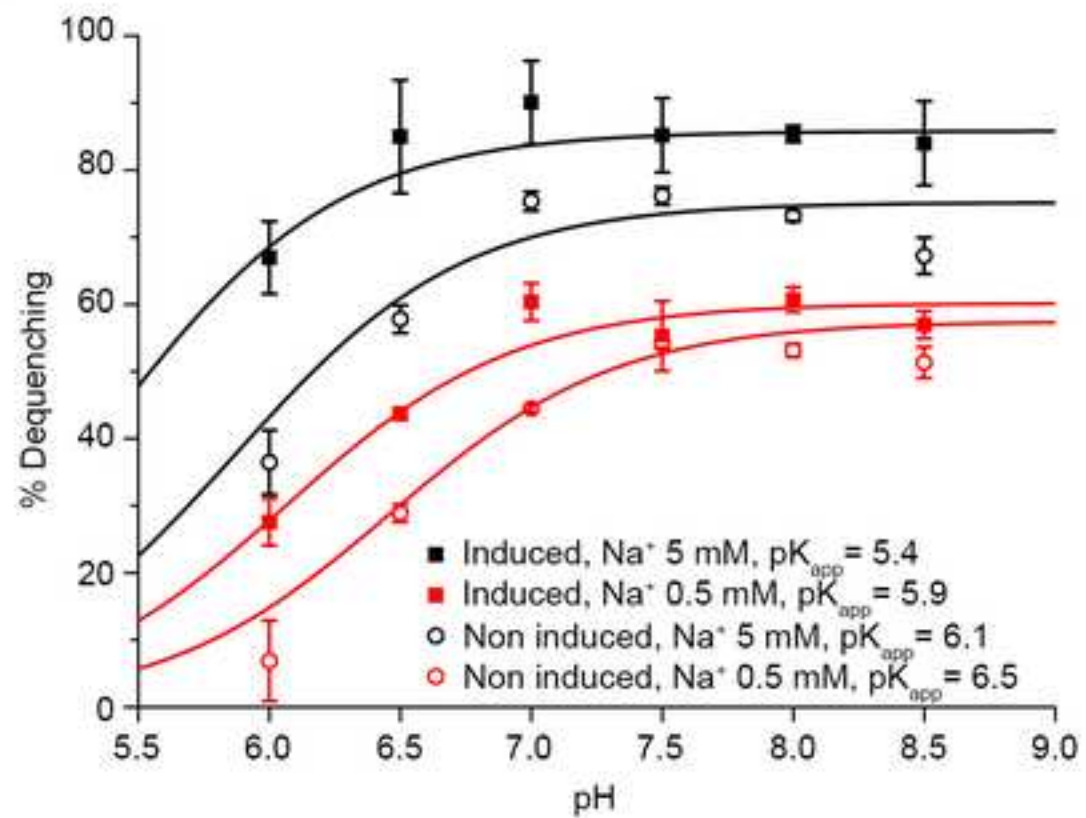


Figure3
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