REGULATION OF Na\textsuperscript{+}/H\textsuperscript{+} ANTIPORTER IN TROUT RED BLOOD CELLS

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Summary

The trout red blood cell Na\textsuperscript{+}/H\textsuperscript{+} antiporter (\betaNHE) displays two interesting properties: it is the only NHE known to be activated by cyclic AMP, and the activation process is followed by a desensitisation of the transport system itself.

Cloning and expression of \betaNHE have provided significant information about Na\textsuperscript{+}/H\textsuperscript{+} activation, in particular that activation by cyclic AMP is directly dependent upon the presence of two protein kinase A consensus sites in the cytoplasmic tail of the antiporter. Expression of \betaNHE in fibroblasts demonstrates that the protein kinase A (PKA) and protein kinase C (PKC) activation pathways are independent and do not converge on a common kinase. Moreover, the hydrophilic C-terminal fragment is essential to the mediation of the various hormonal responses. NHE1 (the human ubiquitous isoform) is not activated by cyclic AMP, but a ‘NHE1 transmembrane domain/\betaNHE cytoplasmic domain’ chimera is fully activated by cyclic AMP.

In red cells, activation of \betaNHE is the result of phosphorylation by PKA of at least two independent sites. Desensitisation, inhibited by the phosphatase inhibitor okadaic acid, may consist of the dephosphorylation of one of these two sites. Furthermore, Calyculin A (CLA), another specific protein phosphatase inhibitor, induces in unstimulated cells a Na\textsuperscript{+}/H\textsuperscript{+} exchange activity whose exchange properties are very different from those of the adrenergically stimulated antiporter. It is suggested that CLA may be able to revive ‘sequestered’ antiporters.

We propose that the molecular events underlying \betaNHE desensitisation could be similar to those involved in rhodopsin desensitisation. Antibodies were generated against trout red cell arrestin in order to analyse the binding of arrestin to the activated exchanger. Recombinant trout arrestin was produced in a protease-deficient strain of Escherichia coli and its functionality tested in a reconstituted rhodopsin assay.

Key words: Na\textsuperscript{+}/H\textsuperscript{+} exchanger, protein kinase A, protein kinase C, protein phosphatase, desensitisation, arrestin, erythrocyte.

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Introduction

The Na\textsuperscript{+}/H\textsuperscript{+} Exchanger (NHE) is a plasma membrane transport protein found in a wide range of biological systems. The stoichiometry of the exchange is 1 H\textsuperscript{+} to 1 Na\textsuperscript{+}, and thus the exchanger is electrically neutral. The Na\textsuperscript{+}/H\textsuperscript{+} exchange process is driven by the combined chemical gradients of Na\textsuperscript{+} and H\textsuperscript{+}; under normal physiological conditions, there is a large driving force favouring net entry of Na\textsuperscript{+} in exchange for internal H\textsuperscript{+}. The antiporter can also mediate Li\textsuperscript{+}/H\textsuperscript{+} exchange as well as self exchange for Na\textsuperscript{+} and Li\textsuperscript{+} (Na\textsuperscript{+}/Li\textsuperscript{+}). Protons play a key role in the regulation of the antiporter. Extracellular protons can inhibit the exchange by competing with external Na\textsuperscript{+}. Conversely, intracellular protons activate the exchange with kinetic parameters in accordance with an allosteric model suggesting the presence of two distinct internal proton-binding sites interacting cooperatively. This behaviour can be explained by the probable existence of a second cytoplasmic H\textsuperscript{+} binding site termed a ‘modifier’. The binding of a proton would increase the affinity of the transporter site for protons and therefore increase the rate of exchange. This phenomenon is that, despite favourable conditions, the Na\textsuperscript{+}/H\textsuperscript{+} exchanger is normally quiescent but rapidly becomes activated as soon as the acidity of the cytoplasm increases. The exchanger thus reacts to cell acidification by adjusting the internal pH (pHi) to a ‘set point’ exactly as does a ‘pH stat’. The main function of the antiporter is thus to regulate intracellular pH and to protect the cell against intracellular acidification. In addition, NHE can be activated at a physiological pH by various stimuli (growth factors, hormones, phorbol esters and hyperosmotic shock) which act by increasing the proton affinity of the transport site. Thus, after such stimulation, the dependence of the antiport activity on pHi is shifted into a more alkaline range. Since the ‘set point’ is shifted from its previous pH, Na\textsuperscript{+}/H\textsuperscript{+} exchange helps to maintain pHi at its new value (for reviews, see Clark and Limbird, 1991; Yun et al. 1995).

NHE is involved in various functions including pH homeostasis, volume regulation, cell proliferation and transcellular Na\textsuperscript{+} absorption. The existence of several isoforms of the exchanger had been predicted (1) by the broad range of physiological functions in which it is involved, (2) by the different types of protein kinase regulation in different cell
types and (3) by the variable sensitivity of the exchanger to the diuretic amiloride. NHE is inhibited by amiloride and its derivatives, but the degree of inhibition varies from cell type to cell type and among different plasma membrane domains (apical versus basolateral) (for a review, see Clark and Limbird, 1991). The first Na+/H+ exchanger was cloned by Sardet et al. (1989) and called NHE1. The cloning of NHE1 greatly facilitated the isolation of different isoforms, and four new isoforms have now been cloned: the trout red cell antiporter βNHE (Borgese et al. 1992) and three further mammalian subtypes: NHE2, NHE3 and NHE4 (Orlowski et al. 1992; Collins et al. 1993; Tse et al. 1993). NHE2 and NHE3 are expressed in kidney, colon, small intestine and stomach (Orlowski et al. 1992; Wang et al. 1993; Tse et al. 1993). They represent the apically expressed NHE isoforms described in epithelia, NHE1 being expressed on the basolateral membrane. βNHE, present in the membrane of trout red cells, represents an interesting isoform of the NHE family which possesses the basic properties described for other cell types but also shows certain specific characteristics: (1) it does not regulate intracellular pH; (2) it is activated by adrenergic agonists; and (3) its activation is rapidly followed by its desensitisation (Motais et al. 1990). This antiporter is activated in vivo when the fish finds itself in hypoxic water (Fiévet et al. 1987, 1988; Thomas et al. 1988), thus promoting intracellular alkalization which increases, via a Bohr effect, the affinity of haemoglobin for oxygen (Claireaux et al. 1988; Cossins and Richardson, 1985; Nikinmaa, 1982).

In red cells, the antiporter does not regulate intracellular pH. Anions and protons are passively distributed across the red cell membrane according to a Donnan equilibrium: 

\[
\frac{[Cl^-]_i}{[Cl^-]_o} = \frac{[HCO_3^-]_o}{[HCO_3^-]_i} = \frac{[H^+]_o}{[H^+]_i}. 
\]

When the acid–base status is disturbed, this equilibrium is modified by the redistribution of anions (HCO₃⁻ and Cl⁻) by the anionic exchanger (band 3), which explains the linear relationship between erythrocyte pH and extracellular pH. βNHE remains inactivated even when the intracellular pH drops to 6.3, a value outside the normal physiological pH range; below this pH, it becomes active, indicating that the set point in red blood cells (RBCs) is approximately pH6.5 (Motais et al. 1990). At a physiological pH, it has previously been demonstrated, in vitro and later in vivo, that this antiporter is activated by catecholamines, which by increasing intracellular cyclic AMP concentration, stimulate PKA (for a review, see Fiévet and Motais, 1991). Addition of catecholamines to resting RBCs strongly activates the antiporter: the Na⁺ influx increases enormously (100-fold), reaching its maximal value within 2–3 min and then, despite the continuing presence of the activator, the Na⁺/H⁺ exchange declines rapidly as a result of a desensitisation of the transport system (Garcia-Romeu et al. 1988). The phorbol ester PMA (phorbol myristate acetate), a protein kinase C activator, also stimulates βNHE but the activity is only 25% of that induced by catecholamines (Motais et al. 1990). It was intriguing to speculate about this dual activation pathway. Do protein kinase A and protein kinase C act through an ancillary protein or does each kinase promote a separate phosphorylation of a distinct part of the protein? Results obtained from transfected Na⁺/H⁺ (see below) will provide the answer. In this report, we will give a resume of the major results contributing to the understanding of the regulation of βNHE, with special emphasis on the phenomenon of desensitisation.

**Molecular identification of βNHE**

βNHE was cloned from trout cephalic kidney, the fish haematopoietic tissue, and later from circulating erythrocytes (Borgese et al. 1992). βNHE is a protein of 759 amino acids (predicted size 85 kDa), with a hydrophobic profile very similar to those of all NHEs (Fig. 1). The antiporter consists of two distinct domains: an amphipathic N-terminal domain (467 amino acids) which contains 12 membrane-spanning segments and a very highly hydrophilic C-terminal domain corresponding to a large cytoplasmic region (292 amino acids). Fig. 2 shows the homologies between NHE isoforms. The transmembrane domains are the most conserved regions. It should be noted that the transmembrane Va and Vb helices are the most constant regions, exhibiting 95% homology, suggesting that this region is essential to exchange activity (Fafournoux et al. 1994). βNHE, although not a mammalian antiporter, is the nearest isoform to NHE1, suggesting that βNHE may be a basolateral isoform. Moreover βNHE, like NHE1, is an N-glycosylated protein (Fig. 3), a characteristic not shown by the ‘apical’ isoform NHE3 (Cournillon et al. 1994). The exact glycosylation site (Asn49 or Asn337) has not yet been defined; however, results obtained with NHE1 indicate that glycosylation is at the first site (Asn49).

**Regulation of transfected βNHE**

Functional expression of βNHE was carried out in fibroblast cell line PS120, which lacks an endogenous Na⁺/H⁺ exchange. In PS120 cells, there was no effect of cyclic AMP on the activity of any exchanger other than βNHE (Levine et al. 1993). Stably expressed βNHE is stimulated by cyclic AMP or catecholamines (Borgese et al. 1992). The C-terminal domain of βNHE contains two typical consensus sites for protein kinase A (Arg-Arg-X-Ser) that are very close together (Ser641 and Ser648). There are no such consensus sites on NHE1, which is not activated by cyclic AMP. PKA consensus sites are necessary for the complete stimulation of the antiporter. A truncated βNHE exchanger with the last 200 amino acids deleted (βNHEΔ559) is no longer activated by cyclic AMP. Deletion of the distal C-terminal domain did not reduce the ability of NHE to catalyse Na⁺/H⁺ exchange and preserved the characteristics of the H⁺ modifier site (Wakabayashi et al. 1992). Point mutations of Ser641 and Ser648 were made (serine changed to glycine) to examine the relative involvement of these serines in the activation mechanism. Mutation of either Ser641 or Ser648 causes a 60% decrease in the ability of cyclic AMP to activate the exchanger. The two serines are involved in the activation pathway to the same degree. The simultaneous removal of the two consensus sites did not totally abolish the
activity, but reduced it to 28% of the control value. Using selective deletion mutations, we have shown that the element triggering this residual activity was located in fragment 559–661 of the cytoplasmic tail. As was to be expected, PKC activation of βNHE was unaffected by removal of PKA consensus sites. A significant observation, however, was that the truncated antiporter βNHEΔ559 was still activated by phorbol esters and thrombin. Obviously, PKC determinant elements are not co-located in region 559–759 of βNHE. It is clear that the two protein kinases stimulate exchange activity by acting on different parts of βNHE.

We have seen that region 559–759 contains all the determinants for cyclic AMP activation. Does this region determine the hormonal behaviour of the antiporter? The

Fig. 1. Primary amino acid sequence and deduced topological model of βNHE. The arrow shows the putative N-glycosylation consensus site (Asn49). The two protein kinase A (PKA) consensus sites are underlined, and the position of the membrane is shaded. Δ559 shows the point where the deletion was performed to obtain the deleted antiporter βNHEΔ559.

<table>
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<tr>
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Fig. 2. Amino acid sequence homology between Na+/H+ exchanger isoforms. Amino acid alignments were performed on transmembrane and cytoplasmic regions independently. The homology levels for each region are shown in the two distinct portions of the figure. The shaded part corresponds to the transmembrane-restricted regions. NHE sequences are from Orlowski et al. (1992) and Collins et al. (1993).
cytoplasmic part of βNHE (containing the two consensus sites) was grafted onto the transmembrane domain of NHE1 and the resulting chimera was called ‘mermaid’. In PS120 cells, NHE1 was grafted onto the transmembrane domain of NHE1 and the electrophoretic mobility of NHE1 was determined. Stable expression of βNHE in OK cells could provide new clues to the understanding of cyclic AMP regulation of NHEs.

**Desensitisation of red blood cell Na⁺/H⁺ antiporters**

In trout red cells, as discussed above, the Na⁺/H⁺ exchanger is inactive at physiological pH and remains inactive when the cells become acidic. Thus, unlike other NHE isoforms, βNHE in the membrane is in a non-functional state. However, βNHE can be strongly and rapidly activated (Fig. 4) by addition of β-adrenergic agonists or cyclic AMP analogues to the suspending medium. These stimuli, via a phosphorylation mediated by a cyclic-AMP-dependent protein kinase (PKA), shift the antiport from a non-functional to a functional conformation by changing the characteristics of the internal H⁺ modifier site and the $V_{\text{max}}$ of the exchange (Guizouarn et al. 1993). This cyclic-AMP-dependent phosphorylation responsible for βNHE activation is controlled by an okadaic acid (OA)-insensitive phosphatase (Guizouarn et al. 1993). Note that, conversely, the activation of NHE1 is controlled by an OA-sensitive phosphorylation (Sardet et al. 1991). As illustrated in Fig. 4, when the non-functional antiporter has been forced into the activated state by PKA-dependent phosphorylation, Na⁺ influx reaches a maximal value $2\text{ min after adrenergic stimulation and then falls abruptly and exponentially towards the unstimulated value (}t_{1/2}=30\text{ min).}$ This decrease in Na⁺/H⁺ activity does not reflect a desensitisation of elements involved in the transmission of the hormonal signal (e.g. β-adrenergic receptor and/or G-protein); indeed, it also occurs when an exogenous cyclic AMP analogue...
is used to stimulate Na⁺/H⁺ antiport directly, thus bypassing receptor-mediated phenomena at the cell surface (Garcia-Romeu et al. 1988). This decline in activity also does not reflect a simple activation/deactivation transition of the antiporter, but a transition from an active to a refractory state. βNHE can no longer be immediately reactivated by a fresh challenge with catecholamine or cyclic AMP. Several hours without stimulation are necessary for the exchanger to recover its ability to respond to cyclic AMP or catecholamines (Guizouarn et al. 1993). The decline in activity thus reflects a desensitisation of the transport system itself (Garcia-Romeu et al. 1988). This βNHE desensitisation is blocked and reversed by OA, indicating control by an OA-sensitive phosphatase of the NHE desensitisation is blocked and reversed by OA, the transport system itself (Garcia-Romeu 1993). The decline in activity thus reflects a desensitisation of the antiporter in a ‘transporting’ state. Desensitisation would be due to the dephosphorylation of the OA-sensitive site by a protein phosphatase 1 (PP1) (Guizouarn et al. 1993, 1995). Experiments designed to evaluate the phosphorylation state of the antiporter, immunoprecipitated from red cells, at different times after stimulation are in progress.

The refractory state could correspond to a recycling of the desensitised transporters in the membrane, and some data obtained using Calyculin A (ClA) support this possibility (Guizouarn et al. 1995). ClA is a phosphatase inhibitor that is 10–100 times more potent than OA as a PP1 inhibitor. It is also 10 times more potent than OA in blocking desensitisation. Furthermore, ClA, unlike OA, induces a large Na⁺/H⁺ exchange activity in unstimulated cells. The characteristics of CIA-induced and PKA-induced Na⁺/H⁺ exchange are very different. Moreover, simultaneous addition of maximal concentrations of ClA and catecholamine produces an additive stimulation of the Na⁺/H⁺ exchange consistent with the interpretation that these agents act on two distinct pools of exchangers (Guizouarn et al. 1995). Since cloning of βNHE showed that only one isoform is present in circulating red cells, it seems likely that ClA is able to unmask antiporters normally inaccessible to activation by PKA or PKC. A simple explanation is that ClA activation corresponds to the recruitment of the refractory sequestered antiporters via an effect on the cytoskeleton. Indeed, ClA is known to induce large modifications of microtubules and microfilaments (Gurland and Gundersen, 1993; Chartier et al. 1996). The use of βNHE antibodies in microscopic studies will be essential to evaluate the validity of this interpretation.

**Is arrestin involved in Na⁺/H⁺ desensitisation?**

Our knowledge of the mechanisms implicated in the desensitisation process mainly derives from the study of two G-protein-coupled receptor systems: the light receptor or rhodopsin and the β-adrenergic receptor. In both systems, a cytosolic protein termed arrestin plays a key role in the desensitisation process. Arrestin is a 48kDa protein which binds to activated phosphorylated rhodopsin or β-receptors (β-arrestin) (Wilden et al. 1986; Shinohara et al. 1987; Lohse et al. 1990, 1992). This binding precludes receptor–G-protein interaction and thus interrupts the activation process. To investigate a possible role of arrestin in the desensitisation of the trout red cell antiporter, we have used immunochemical techniques to demonstrate the presence of an arrestin-like protein in nucleated red cells (Mirshahi et al. 1989). Prior to this demonstration, arrestin was assumed to be localised exclusively in photosensitive cells. This red cell arrestin binds to photoactivated rhodopsin just as the retinal arrestin does, supporting its possible involvement in some undefined desensitisation process (Scheuring et al. 1990). To investigate the physiological role of arrestin in red cells, several approaches have been used. First, molecular cloning demonstrates that three different isoforms of arrestin coexist in circulating trout red cells: TRCarr1, TRCarr2 and TRCarr3 (Jahns et al. 1992). TRCarr standing for Trout Red Cell arrestin. The three TRCarrs exhibit very high homology with each other (97%) and are highly homologous to the other vertebrate arrestins cloned so far: retinal arrestin (Wilden et al. 1986), retinal cone (C) arrestin (76%; Shinohara et al. 1987), β-arrestin 1 (82%; Lohse et al. 1990; Parruti et al. 1993) and β-arrestin 2 (52%; Stern-Marr et al. 1993; Attiamadal et al. 1992; Rapoport et al. 1992). Two polyclonal antibodies were then generated by a protein fusion technique, one raised against the N-terminal part of the protein and the other against the C-terminal part. These antibodies are able to immunoprecipitate TRCarr not only from the cytoplasm but also from the particulate (membrane) fraction of RBCs (Fig. 5). The variation of arrestin bound to the membrane was examined as a function of time after catecholamine stimulation. No significant modification of arrestin distribution during the desensitisation process was detected (Jahns et al. 1996). However, the immunoprecipitation protocol used is not sufficiently sensitive to exclude the
possibility that only a few arrestins from the cytoplasmic pool are implicated in the regulation of the Na+/H+ antiporter. For a modified experimental approach (*in vitro* interaction), a large amount of TRCarr was necessary, but previous isolation of native arrestin from RBCs had been found to yield denatured and aggregated protein. We succeeded in producing TRCarr using a recombinant expression strategy with bacterial hosts: the pET expression system was used to produce recombinant TRCarr (rTRCarr) in the *E. coli* strain B L-21 (Jahns et al. 1996). rTRCarr was engineered with a histidine tag (6His) at the C-terminal part of the protein. This tag was shown to allow protein purification on a Ni²⁺ affinity-chromatography column (Hochuli et al. 1987). rTRCarr protein can be labelled by either the N- or the C-terminal-specific anti-TRCarr antibodies, proving the integrity of the purified expressed protein (Jahns et al. 1996). The functional integrity of the recombinant protein was tested in a reconstituted rhodopsin assay. Fig. 6 shows that rTRCarr binds to phosphorylated and light-activated rhodopsin from bovine retinal rod outer segment, but that there was also an additional significant binding of rTRCarr to the non-photoactivated rhodopsin. The binding of rTRCarr to the light-activated rhodopsin, however, was much more pronounced than that to the non-photoactivated rhodopsin. Moreover, addition to the samples of 1 mg ml⁻¹ heparin, which has previously been shown to inhibit arrestin–rhodopsin interactions (Gurevich et al. 1994), completely blocked the binding of rTRCarr to rhodopsin, even after photoactivation. This indicates that rTRCarr is a suitable tool for further investigation of the functional role of TRCarr in the trout erythrocyte and may also be helpful in the identification of its appropriate target in these cells.

**References**


epidermal growth factor and okadaic acid activate the Na\(^+\)/H\(^+\) exchanger, NHE-1, by phosphorylating a set of common sites. J. biol. Chem. 266, 19166–19171.


