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Regulation of Na^+/H^+ Exchanger by Urogastrone, a Potent Activator of Cell Proliferation

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Summary

We tested the effects of epidermal growth factor (EGF) on Na^+/H^+ exchanger (NHE) activity using urogastrone for treatment of Wistar rats and rat kidney tissue slices. NHE activity was monitored in isolated kidney brush border membrane vesicles by following fluorescence quenching of acridine orange. A significant increase of NHE activity was detected as early as 5 min after addition of urogastrone to rat kidney slices *in vitro*. In Wistar rats treated with urogastrone we also found increased NHE activity (by about 12 %). Both changes of NHE activity were the result of a significant rise of V_{max} value and an apparent decrease in K_m value in *in vitro* experiments. The rise of NHE activity caused by urogastrone was sensitive to the inhibitors of transcription and translation. The presence of phosphatase inhibitor, NaF, elevated NHE activity of non-stimulated as well as of urogastrone-stimulated exchanger, suggesting that phosphorylation plays an important role in Na^+/H^+ exchange. Osmolarity of the medium seems to regulate NHE activity in such a manner that both hyper- and hypoosmolar conditions inhibited NHE activity. The absence of Ca^{2+}ions produced a 60 % decrease of NHE activity. The chemical modification of histidine residues with diethyl pyrocarbonate or SH groups with N-ethylmaleimide inhibited NHE activity.

Key words

Na^+/H^+ exchanger • Kidney brush border membrane vesicles • Epidermal growth factor • Urogastrone

Introduction

Na^+/H^+ exchangers (NHEs) are integral membrane proteins that mediate the electroneutral exchange of intracellular H^+ for extracellular Na^+ across plasma membranes. Molecular cloning studies have revealed six distinct mammalian NHEs isoforms (NHE1-NHE6), which are products of distinct genes dispersed throughout the mammalian genome (Wakabayashi *et al.* 1997).

NHE-3 is markedly expressed in the kidney, localized to the apical membranes of renal proximal tubules (Biemesderfer *et al.* 1993) and intestinal epithelia, specifically targeted to the apical membrane (Hoogerwerf *et al.* 1996). Knockout mice for the NHE-3 isoform exhibit a decrease in blood pressure, appear to be mildly acidotic, and show absorptive defects in the kidney and intestine, suggesting that NHE-3 is the major absorptive Na^+/H^+ exchanger in the kidney and intestine. Furthermore, the lack of the exchanger impairs the acid-base balance and Na^+-fluid volume homeostasis (Schultheis *et al.* 1998).

NHEs are regulated by a variety of agents that target primarily tyrosine kinase and by agonist of Ser/Thr

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kinases including protein kinase A (PKA) and protein kinase C (PKC). An increase in cytosolic Ca^{2+} concentration and changes in cell volume affect NHE activity. The regulatory mechanisms involve phosphorylation of NHE by PKA and/or PKC, as well as by calmodulin (CaM) kinase and mitogen activated protein (MAP) kinases (Fliegel and Frohlich 1993). This regulation involves interaction with associated proteins such as calmodulin, calcineurin B homologue protein, the NHE-3 regulatory factor (Weinman *et al.* 1993), as well as action of heterotrimeric and small GTP-binding proteins (Lin *et al.* 1996). Some data demonstrated effects of products of phosphoinositide metabolism (Khurana *et al.* 1996) and vesicular traffic (D′Souza *et al.* 1998).

It is also known that epidermal growth factor (EGF), the growth factor required for kidney tubulogenesis (Weller *et al.* 1991), affects sodium transport in kidneys *via* basolateral membrane receptors (Vehaskari *et al.* 1989).

In the rat, it appears that kidney tissue contains mRNA of four NHE isoforms. The amount of respective protein varies in the following order: NHE-3, NHE-1, NHE-2 and NHE-4. NHE-3 would be the prime candidate for the transporter controlling Na^+ reabsorption in the proximal tubule since it mediates Na^+ movement across the apical membrane (Kelly *et al.* 1997).

The aim of the present study was to investigate the kinetic parameters of NHE-3 from rat kidney and the influence of EGF on its kinetic parameters by *in vitro* and *in vivo* experiments. Urogastrone, a potent activator of cell proliferation, was used as a source of EGF. NHE activity was monitored in brush-border membrane vesicles (BBMV) isolated from the rat kidney cortex. We addressed the problem of regulation of NHE-3 by following the effects of Ca^{2+} ions, protein phosphorylation, changes in osmolarity and inhibitors of transcription and translation under *in vivo* and *in vitro* conditions. Involvement of cysteine and histidine residues in the activity of the transporter was tested by using chemical modifiers of SH and histidine residues, respectively.

Methods

Material

Acridine orange was obtained from Eastman Kodak (Rochester, NY, USA). Urogastrone, valinomycin, ATP, diethyl pyrocarbonate (DEPC) and Hepes were from Sigma (St. Louis, USA). Tetramethylammonium hydroxide (TMA), N-ethylmaleimide (NEM) and EGTA were obtained from Fluka (Buchs, Swiss). All other chemicals were of analytical grade.

Preparation of brush-border membrane vesicles

BBMV were isolated from the kidney cortex of Wistar male rats (150-200 g) according to the Mg^{2+}/EGTA precipitation method of Biber *et al.* (1981). Briefly, rat kidney cortex slices were homogenized with Ultra-turax homogenizer in an isoosmotic medium containing 5 mM EGTA. BBMV were purified by two successive precipitations with 12 mM $MgCl_2$ and differential centrifugation. Applying this method, a high yield of BBMV was obtained. The vesicles thus obtained were largely oriented with the right side out. The sidedness and sealedness of the BBMV were tested as described by Turrini *et al.* (1989). The orientation of the vesicles was predominantly right-side-out (94±4 %, n=5). Isolated and purified vesicle preparations were resuspended in 5 mM Hepes-Tris buffer (pH 7.0), 150 mM mannitol, and 150 mM K-gluconate to the protein concentration of 10 mg/ml. Vesicles were used for enzyme activity determination immediately following isolation, or were kept in liquid nitrogen until further use. The purity of BBMV was checked by measuring the activity of a specific marker enzyme, alkaline phosphatase, according to King and Armstrong (1934). The alkaline phosphatase was enriched 8.04±0.08 fold as compared to the homogenate. Contamination with basolateral membranes was negligible as measured by the Na^+/K^+ ATPase activity, which remained the same as in kidney cortex homogenates (Berner and Kinne 1976).

The protein concentration was measured by the method of Bradford (1976) using bovine serum albumin as standard.

Measurement of brush-border NHE activity

NHE activity in BBMV was determined from the efflux of protons which causes fluorescence quenching of acridine orange (Mackovic *et al.* 1986). The measurement was started by dilution of 10 µl (10 µg of proteins) of vesicles into 2 ml buffer (5 mM Tris-Hepes, pH 7.0, 150 mM mannitol, 150 mM TMA-gluconate, 2.5 µM valinomycin, 6 µM acridine orange) and incubated at 25 °C. Due to the transmembrane proton gradient acridin orange accumulated in the vesicles, resulting in a net decrease of acridine orange fluorescence (during 3 min). Proton efflux was induced by the addition of Na-gluconate (10-50 mM) and measured as an increase in fluorescence in spectrofluorometer (excitation 493 nm,

emission 525 nm). Fluorescence quenching was expressed relatively to the fluorescence observed in the absence of Na-gluconate (TMA-gluconate was added in the same concentration in order to compensate for constant osmolarity).

A typical record of acridine orange fluorescence and its modulation by Na^+ is shown in Figure 1.

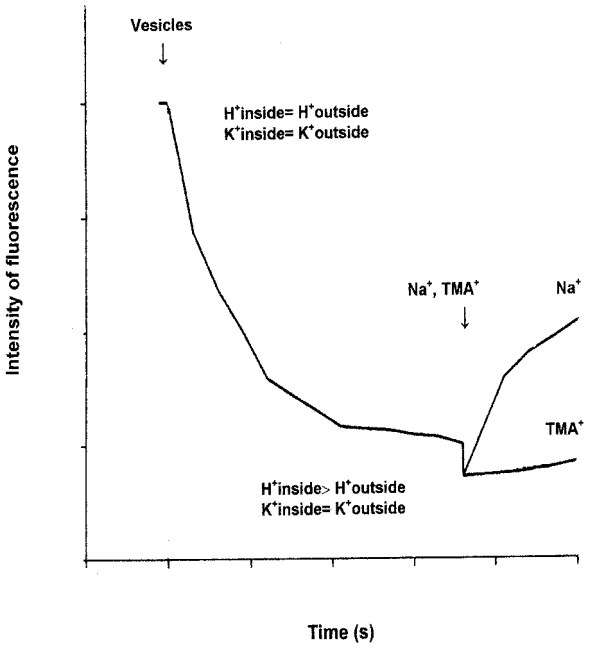

Fig 1. *Typical record of acridine orange fluorescence and its modulation by Na^+*

Experiments in vitro

Kidney cortex slices were prepared from one-month-old Wistar rats. Animals were sacrificed by cervical dislocation and their kidneys were immediately removed. Thin slices (0.1-0.4 mm) of kidney cortex were cut using Steadie-Riggs microtome. Incubations were carried out in Krebs-Ringer buffer ($NaHCO_3$ 10 mM, pH 7.4, NaCl 127 mM, KCl 5.1 mM, $CaCl_2$ 2.3 mM, $MgSO_4$ 1.3 mM, glucose 10 mM, ATP 2 mM) for the estimation of control NHE activity.

In the experiments where NHE activity was followed after stimulation with urogastrone, slices were incubated in the buffer containing urogastrone in the final concentration of 62 μg/ml at 37 °C for 5, 15, 30, and 60 min, respectively, or urogastrone was repeatedly added at time intervals 0, 5, 15, 30 and 60 min. After incubation, BBMV were isolated as described above.

For the inhibition of intracellular phosphatases 0.5 and 1.0 mM NaF was added prior to urogastrone administration.

For the inhibition of protein synthesis, kidney cortex slices were first incubated in Krebs-Ringer buffer in the presence of 50 μg/l of actinomycin D or 100 μg/l of cycloheximide for 30 min at 37 °C and then urogastrone was added. In order to test the involvement of Ca^{2+} in the regulation of NHE activity slices were incubated in standard Krebs-Ringer buffer (control) or in Krebs-Ringer buffer without Ca^{2+} and supplemented with 5 mM EGTA, and then urogastrone was administered.

In order to test the effects of osmolar conditions on NHE activity BBMV were incubated in Krebs-Ringer buffer (isoosmolar), or in Krebs-Ringer buffer containing an appropriate concentration of mannitol in order to attain hyperosmolarity (500 mOsm). Hypoosmolar condition (100 mOsm) was achieved by dilution of the Krebs-Ringer buffer. For the chemical modification of cysteine and histidine residues of rat kidney NHE, BBMV were incubated at 37 °C for 10 min either with NEM or DEPC. The final concentration of the respective inhibitor is indicated where appropriate.

Experiments in vivo

Urogastrone (500 μg/kg) was administered to Wistar rats by intraperitoneal injection (i.p.) at 0, 24 and 48 h after beginning of the experiment. Two hours after the last injection, rats were sacrificed and BBMV were prepared. Actinomycin D in 0.9 % NaCl (0.1 mg/kg), or cycloheximide in 0.9 % NaCl (0.2 mg/kg) were administered i.p. 30 min before urogastrone was administered and then repeated each 12 h in the course of 48 hours. Control animals received the same volume of saline at the indicated time intervals.

Statistics

Statistical significance of the difference was tested by the Wilcoxon-Mann-Whitney test at the significance level $p<0.05$.

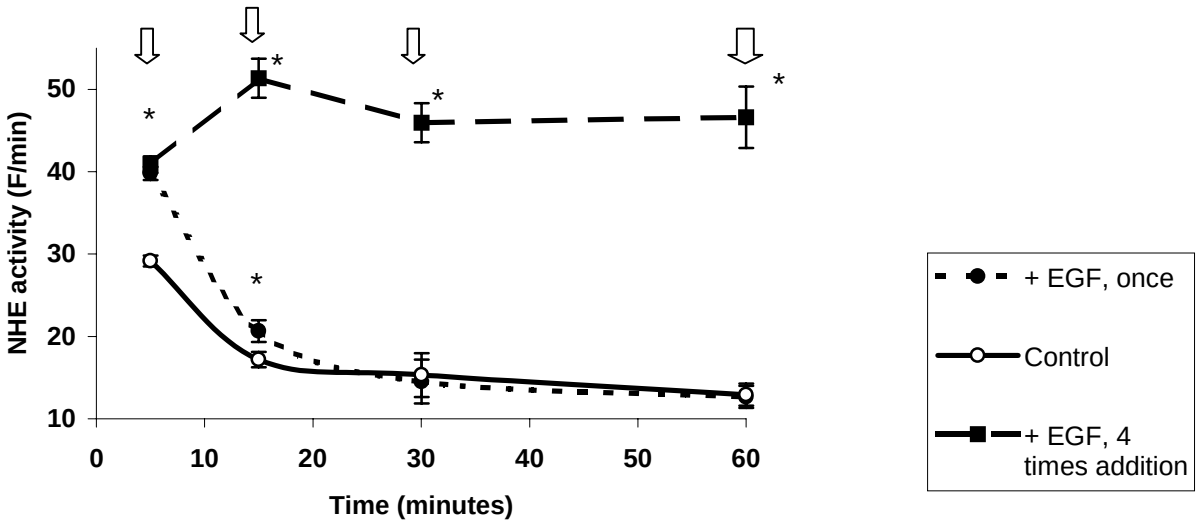

Fig. 2. *The Na^+/H^+ exchange activity of stimulated and non-stimulated NHE during 60 min. The NHE activity was monitored during 60 min of incubation without urogastrone (—o—), with urogastrone added once to the reaction mixture at time 0 (--●--), or with urogastrone repeatedly added to the reaction mixture at time 0, 5, 15, 30, and 60 min of incubation (indicated by arrows) (— —■— —). The NHE activity is presented as mean ± S.E.M. from 5 membrane preparations. * significant difference compared to the control values.*

Results

Stimulation of NHE by urogastrone

Kidney tissue slices were incubated in the buffer with 62 μg/ml urogastrone for 5, 15, 30 and 60 min, and NHE activity was measured subsequently. As shown in Figure 2, urogastrone enhanced NHE activity by 35 % five minutes after addition to the incubation buffer when compared with the NHE activity in the control sample. Sixty minutes after stimulation with urogastrone, the activity of NHE was similar to the activity in control samples. If urogastrone was added to the kidney tissue

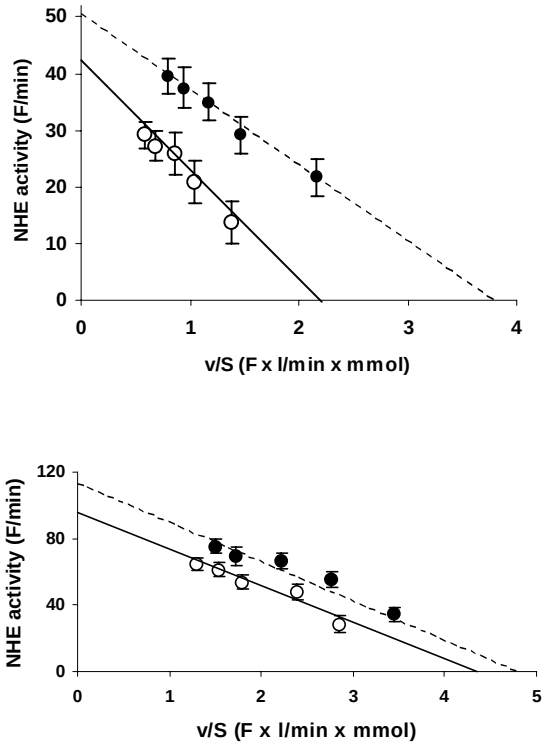

Fig. 3. *Kinetic analysis of the stimulated and non-stimulated NHE in in vivo and in vitro experiments.* ***Upper panel****: BBMV were isolated from rat kidney tissue slices incubated with urogastrone (62 μg/ml) in vitro; —o— represents non-stimulated NHE activity with calculated kinetic parameters: V_{max} = 42.25±1.37 ΔF/min, K_m = 19.60±1.51 mM; --●-- represents stimulated NHE activity with kinetic parameters: V_{max} = 50.82±1.48 ΔF/min; K_m = 13.50±0.96 mM.* ***Lower panel****: BBMV were isolated from the kidneys of Wistar rats treated with urogastrone (500 μg/kg) in vivo; —o— represents non-stimulated NHE activity with calculated kinetic parameters: V_{max} = 93.70±3.50 ΔF/min, K_m = 20.53±1.50 mM; --●-- represents stimulated NHE activity with kinetic parameters: V_{max} = 104.50±4.20 ΔF/min; K_m = 22.00±1.63 mM. NHE activity was measured at different Na^+ concentrations (10-50 mM), and is presented as means± S.E.M. from 5 experiments.*

slices repeatedly, at time 0, 5, 15, 30 and 60 min, NHE activity remained at the level of about 135 % of the control values (Fig. 2). This fact suggests that degradation of EGF may have led to the loss of the enhancing effect occurring during 60 min of the incubation period.

The effect of urogastrone on NHE activity was also studied in *in vivo* experiments (described in the Methods). NHE activity significantly increased from 93.70±3.50 ΔF/min in control animals to 104.50±4.20 ΔF/min in animals treated with urogastrone.

In order to ascertain whether the observed activation of NHE results from increase in the affinity of NHE for substrates or from the higher number of active NHEs, we determined the kinetic parameters of NHE from the proximal tubule BBMV. Kinetic parameters, K_m and V_{max}, were determined for stimulated and non-stimulated NHE *in vitro* and *in vivo* with Na^+ concentrations between 10 and 50 mM. The results showed that NHE exchanged Na^+ ions with H^+ ions according to the simple Michaelis-Menten kinetics, and we therefore calculated the kinetic parameters from Eadie-Hofstee diagram. Urogastrone caused a significant decrease of K_m value (from 19.60±1.51 mM to 13.50±0.96 mM), and significant increase of V_{max} (from 42.25±1.37 ΔF/min to 50.82±1.48 ΔF/min) as shown by the experiment *in vitro*. However, in experiments *in vivo* we found that treatment with urogastrone significantly increased V_{max}, but K_m value remained apparently unchanged.

Table 1. The effects of inhibitors of transcription and translation on urogastrone stimulated NHE activity.

	***In Vitro* NHE activity ΔF/min**	***In Vivo* NHE activity ΔF/min**
Urogastrone	39.75±0.75	104.50±4.20
Actinomycin D	28.85±1.00*	56.67±0.84*
Cycloheximide	35.10±0.80*	47.19 ±0.91*

*Urogastrone, actinomycin D, and cycloheximide were administered to Wistar rats or added to rat kidney tissue slices as described in the Methods. NHE activity is presented as means ± S.E.M. from 5 membrane preparations. * significant difference compared to urogastrone-stimulated NHE activity in the absence of inhibitors.*

Stimulation of NHE by urogastrone increased V_{max} of the exchanger suggesting that protein synthesis may be included in the regulation mechanism. In order to investigate whether transcription or translation takes place in the stimulation of NHE by urogastrone we added inhibitors, actinomycin D and cycloheximide, prior to the stimulation with urogastrone *in vitro* and *in vivo*. Both inhibitors significantly decreased NHE activity *in vitro*, as well as *in vivo* (Table 1).

Previous experiments showed that long-term regulation of NHE by urogastrone is regulated by transcription and translation. For the study of short-term regulation of NHE activity we examined the effects of phosphorylation and Ca^{2+} ions on NHE activity. The role of phosphorylation in the activation of NHE by urogastrone was indirectly monitored by prolonging the phosphorylated state using inhibitor of phosphatases, NaF.

As shown in Figure 4, the presence of phosphatases inhibitor affects non-stimulated as well as urogastrone-stimulated NHE. NaF in a concentration of 0.5 mM increased non-significantly basic, non-stimulated NHE activity by 7-10 %, whereas 1 mM NaF caused a significant increase in non-stimulated NHE activity by 58 to 69 % compared to the control activity. The significant increase by 10-18 % of stimulated NHE activity was found in the presence of 0.5 mM NaF, whereas 35-45 % increase was observed in the presence of 1 mM NaF. The inhibition of phosphatases by NaF was more evident on NHE activity in the longer time of incubation (60 min) than in the shorter period (5 min).

Growth factors have been shown to induce a very rapid and transient rise in cytoplasmic calcium. Although a calmodulin-binding regulator box was not identified in the NHE-3 sequence, it has been shown that NHE-3 is also regulated by calmodulin (Levine *et al.* 1995). In order to follow the effect of Ca^{2+} ions on NHE, the activity of the exchanger was monitored in the presence (2.3 mM) and in the absence of Ca^{2+} (0 mM Ca^{2+} and presence of 5 mM EGTA). The absence of Ca^{2+} ions decreased NHE activity what amounted only to 40 % of the activity determined in the presence of Ca^{2+} ions. Similar results were found in the case of NHE stimulated by urogastrone (Table 2).

Hyper- and hypoosmolality affect NHE activity

It has been well documented that NHE activity is regulated by osmolarity. Hypoosmotic shock

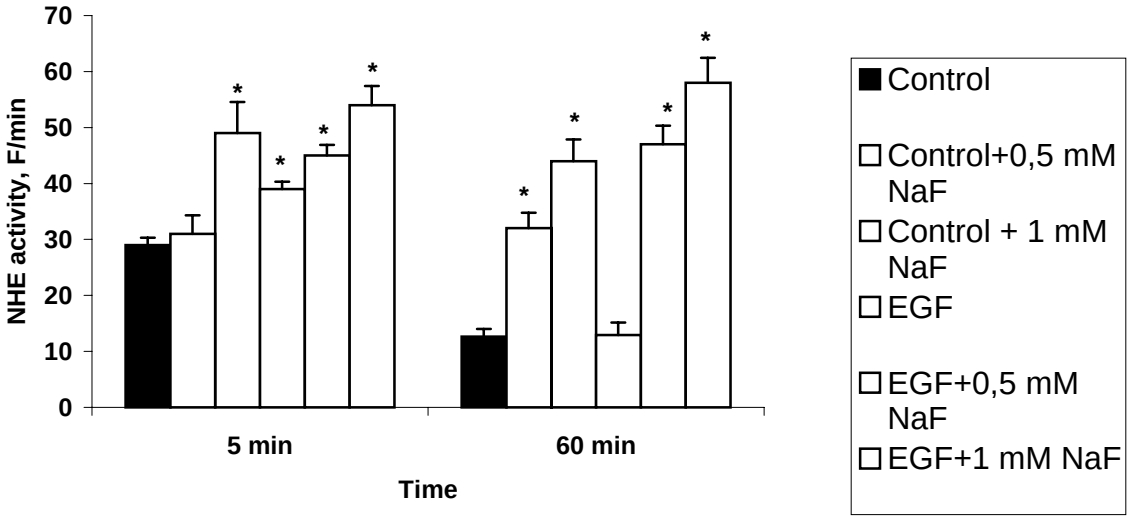

Fig. 4. *The effects of prolonged phosphorylation on NHE activity. NaF, inhibitor of phosphatases, was used in two different concentrations (0.5 and 1.0 mM, respectively) during 5 or 60 min of incubation; NHE activity was shown as mean ± S.E.M. from 5 experiments. * significant difference to the corresponding control values.*

Table 2. Influence of Ca^{2+}, DEPC, NEM and changes of osmolar conditions on NHE activity

	Non-stimulated NHE activity ΔF/min	Stimulated NHE activity ΔF/min
Control	29.36±0.73	39.75±0.75*
Ca^{2+} 0 mM	11.90±0.90*	16.60±0.20*
DEPC, 50 μM	16.40±1.20*	18.95±0.90*
NEM, 1 mM	23.65±0.45*	33.95±0.95*
Hypoosmolarity (100 mOsm)	13.60±1.30*	24.80±0.95*
Hyperosmolarity, (500 mOsm)	21.15±0.55*	29.55±1.15*

*Rat kidney tissue slices were incubated with indicated concentrations of DEPC, NEM, in the absence of Ca^{2+} ions, or in hypo- and hyperosmolar conditions as described in Methods. Data are means ± S.E.M. from 5 membrane preparations. * significant difference compared to the respective control values*

(<300 mOsm) caused a decrease in potasium and sodium content in renal tubule cells (Grantham *et al.* 1974). Recently it was reported that NHE-3 mRNA was downregulated by hypoosmolar infusion in rat colon mucosa (Doble *et al.* 2000). We found that both hypo- and hyperosmolality of incubation medium (Table 2) decreased NHE activity in BBMV.

Chemical modification of NHE

The reaction of DEPC with proteins is highly specific for histidine residues. The inhibition of NHE activity, either non-stimulated or stimulated, suggests that histidine appeared to be important for NHE activity, both basic and after addition of urogastrone (Table 2).

The role of SH groups in the activity of NHE was studied by addition of NEM into the reaction mixture as described in Methods. NEM decreased basic NHE activity by about 19 %. However, if NHE was stimulated by urogastrone, NEM inhibited NHE activity by about 15 % (Table 2).

Discussion

The analysis of the mRNA content in kidney tissue showed that four different mRNA messages for NHE are present in the kidney, namely NHE-1, NHE-2, NHE-3 and NHE-4. NHE-3 is located in the apical membrane, while NHE-1 was found in the basolateral membrane (Orlowski and Grinstein 1997). We isolated BBMV from rat kidney cortex and studied the NHE form present there. According to the literature (Counillon and Pouyssegur 2000), NHE present in BBMV belongs to the NHE-3 isoform. Our results have shown that NHE present in BBMV isolated from the rat kidney follows Michaelis-Menten kinetics. Using Na^+ as substrate in a concentration between 10 and 50 mM, we determined K_m and V_{max} (K_m=19.60 mM, V_{max}=42.25 ΔF/min).

It has been shown that epidermal growth factor regulates NHE activity. In intestinal epithelia, receptors for EGF are present on the basolateral membrane and regulate brush-border NHE on apical membrane (Donowitz *et al.* 1994).

We addressed the question whether EGF may serve as a regulator of NHE activity by using urogastrone as a source of EGF. Urogastrone was administered in the dose of 500 μg/kg body weight to experimental animals *in vivo*, or in dose of 62 μg/ml for treatment of kidney slices *in vitro*. We found that urogastrone is a stimulator of NHE activity *in vitro*. This stimulation is due to a decrease in K_m value and an increase in V_{max}. Our experiments *in vivo* showed that urogastrone stimulated NHE activity by increasing V_{max}. The decrease in the K_m value (observed *in vitro*) from 19.60 to 13.50 mM suggests that there is an increase in the affinity of the exchanger for sodium ions. However, the regulation of NHE-3 appears to be reflected in the changes in V_{max} (observed *in vitro* and *in vivo*) implying that the agonist increases the activity of the exchanger, either by changing the turnover or the number of active exchangers present in the plasma membrane either by translocation of active forms or activation of dormant forms.

On the other hand, short-term regulation of activity could be simply explained by direct phosphorylation of the antiporters. The primary amino acid sequence reveals the existence of consensus sites for phosphorylation by PKA and PKC, as well as multiple sites suitable for phosphorylation by CaM kinase and Ser/Thr kinases (Fliegel and Frohlich 1993). Tyrosine phosphorylation of the exchanger has not been reported (Wakabayashi *et al.* 1997). Although the precise mechanism of regulation of NHE activity by phosphorylation is not completely elucidated, it is well known that phosphorylation is very important in the regulation of antiporters activity. In our experiments, we used an inhibitor of phosphatases (NaF) in order to prolong the existence of the phosphorylated form of the exchanger. The NHE activity was measured in BBMV isolated from kidney slices that were either stimulated or not stimulated by urogastrone. This experiment revealed that the activity of non-stimulated antiporters was higher in the presence of phosphatases inhibitor. The same effect was found if NHE was stimulated by urogastrone and its activity was measured in the presence of NaF. Taken together, these results suggest that phosphorylation is an important mechanism of regulation for constitutive NHE activity as well as for a stimulated exchanger.

The presence of second messenger Ca^{2+} ions is very important for NHE activity. NHE activity is regulated by phosphorylation with PKC that is activated by diacylglycerol (DAG). For activation of PKC by DAG Ca^{2+} ions are required (Orlowski and Grinstein 1997). We found that basic constitutive, as well as stimulated NHE activities were reduced by about 60 % in the absence of Ca^{2+} ions.

Osmolarity affects NHE in such a manner that either a lower or a higher osmolar environment inhibits its activity, and this is similar to the published data obtained with NHE-3 in the colon mucosa (Doble *et al.* 2000).

The presence of histidine and SH groups modifiers (DEPC and NEM) causes a decrease of NHE activity, basic and stimulated by urogastrone, suggesting that these groups play an important role in the import of sodium ions and export of H^+ ions in kidney brush-border membranes.

We also attempted to answer the question whether stimulation of NHE by urogastrone is transcriptionally or translationally regulated. Therefore, we monitored the exchanger activity in the presence of an inhibitor of transcription (actinomycin D) or cycloheximide, which is an inhibitor of translation. By an *in vitro* procedure we found that neither actinomycin D nor cycloheximide affect constitutive NHE activity. However, the activity of the exchanger stimulated by urogastrone was affected by the presence either of actinomycin D or cycloheximide in such a manner that actinomycin D caused a 27 % and cycloheximide a 12 % decrease of antiporter activity. Using *in vivo* experiments, we found that urogastrone-stimulated antiporter activity decreased to 54 % of the controls if actinomycin D were administered to the experimental rats, and to 45 % of the control if cycloheximide were administered to the animals.

Our study shows that urogastrone activates NHE from BBMV. This activation is related to the changes in kinetic parameters of NHE, and is dependent on transcription and translation. We also found that NHE activity is regulated by Ca^{2+} concentration, osmolarity, phosphorylation and histidine and SH modifiers.

Acknowledgements

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