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Regulation of the Na^+/H^+ exchanger under conditions of abolished proton gradient: isosmotic and hyperosmotic stimulation

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Activation of the Na^+/H^+ exchanger following isosmotic and hyperosmotic stimuli was investigated in an osteoblast cell line (RCJ 1.20). The pH dependence of the transporter activity was studied under conditions of abolished proton gradient ($pH_i = pH_o$) across the membrane. The isotonic response is Na^+_o dependent, increases towards higher pH-values, displaying a sigmoidal dependence on $pH_{i=o}$ (Hill coefficient ≈ 1.8) and is controlled by pH_o. The greater than first order dependence on pH suggests that H^+_o inhibits the exchange beyond the rate expected from competition with the Na^+_o alone. This may be due to the existence of an external H^+ regulatory site with a negative cooperative effect on the intra- or extracellular transport site. The hyperosmotic activation is Na^+_o independent, parallels the sigmoidal pH dependence of the isosmotic stimulus (Hill coefficient ≈ 2.0) and is mediated through an increase of the V_{max} without a change in the intracellular proton sensitivity.

Na^+/H^+ exhanger; Hyperosmotic stimulus; Intracellular/extracellular pH; Bone cell

1. INTRODUCTION

The ubiquitous Na^+/H^+ exchanger is involved in intracellular pH regulation and cell volume control [1,2]. It possesses an extracellular and intracellular transport site for protons and sodium in addition to a proton regulatory site on the intracellular side [3]. The regulatory internal site controls the rate of exchange at different cytosolic pH (pH_i) in cooperation with the internal transport site and inhibits transport at high values of pH_i [4]. Different stimuli, including growth factors, intracellular calcium and phorbol esters can modulate the exchange rate of the antiporter [5,6]. Similarly, a hyperosmotic stimulus in various cell types results in cell volume shrinkage and a regulatory volume increase accompanied by an alkalinization of the cytosol due to the activation of the exchanger [7]. The hyperosmotic stress was shown to selectively decrease Na^+ and increase H^+ sensitivities at the intracellular site, causing an alkaline shift in the pH dependence of the exchanger [8–10].

The significance of the proton electrochemical gradient on the function of the Na^+/H^+ exchanger has not been yet fully assessed. In order to distinguish between modulation of the Na^+/H^+ exchange by pH or the transmembrane proton gradient we have studied the isosmotic and hyperosmotic stimulation of the antiporter under conditions of an abolished proton gradient. Our data present for the first time the pH dependence of the exchange process under the initial constrain of $pH_i = pH_o$ in a well established bone cell line [11]. The results suggest a regulatory role of an external H^+ inhibitory site on the transport process. Hyperosmotically stimulated cells display an increase of the exchange rate due to elevation of the V_{max}.

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2. MATERIALS AND METHODS

2.1. *Cells*

A cell line of rat calvaria, RCJ 1.20 [11], was cultured in modified Eagle's medium (MEM) containing 7.5% fetal calf serum (FCS), 4.5 mg/ml glutamine, 50 U/ml penicillin and 200 μg/ml of streptomycin. Cells at subpassages 3–10 were seeded at an initial density of 1.2×10^5 cells/cm^2 and maintained at 37°C under an atmosphere of 5% CO_2 in air. Three to four days after plating, cells were collected from confluent cultures by a short trypsinization (0.05% trypsin and EDTA 0.02%). The proteolytic digestion was stopped by 2% FCS and the cells were briefly sedimented at $7000 \times g$ and resuspended in MEM containing 0.2% FCS. Cells were restored from trypsinization by incubation at 37°C for 1 h prior to the fluorimetric experiments.

2.2. *Fluorimetric procedures*

Cells in suspension were loaded for 30 min at 37°C with 2–3 μM BCECF, a fluorescent pH probe. Subsequently, cells were washed twice ($7000 \times g$) and suspended at $5–6 \times 10^7$ cells/ml in a HEPES-buffered solution. Each fluorimetric measurement employed $1–2 \times 10^6$ cells/ml under continuous stirring at 37°C. Fluorescence ratios of BCECF/AM (excitation, 440 and 500 nm; emission, 530 nm, slits 4 nm) were monitored with an SLM 8000 spectrofluorimeter. Fluorescence background under the conditions of BCECF/AM experiments was < 5%. BCECF-loaded cells were used for 30 min utmost and the leakage during this interval was less than 10%. In-

tracellular pH calibration was performed by the nigericin method [12] or by lysing the cells with 50 μM digitonin and varying the pH of the solution. The calculated pH based on the digitonin procedure was corrected for the shift in the excitation maximum by a factor of 0.08 ± 0.01 pH units ($n = 10$). Accordingly, in all experiments the corrected pH is presented. The pH_i of RCJ bone cells suspended in a HEPES-buffered medium at an extracellular pH (pH_o) of 7.3 was 7.11 ± 0.01 ($n = 28$, $T = 37°C$).

2.3. *Intracellular pH clamp*

BCECF-loaded cells were washed twice with a 150 mM K^+ (or 30 Na^+/120 K^+) solution. The pH of the enriched K^+ medium was adjusted to the desired level (6.2–7.4). An intracellular pH equilibration to external pH was achieved by incubating the cells for 6 min at 37°C with 2 μM nigericin. Ionophore scavenging was accomplished by incubating the cells with 5 mg/ml BSA for 4 min at 37°C followed by one step of washing and resuspending the cells in a similar pH solution containing 5 mg/ml of BSA. External solutions containing 150 mM KCl or 30 mM NaCl/120 mM KCl were used, as indicated. During the experimental procedures, pH_i clamped cells were suspended in various solutions at iso- or hyperosmotic osmolality at the desired external pH. The initial pH_i remained constant at the desired level for 60 min, at least.

2.4. *Solutions and Materials*

Na^+ solution contained (in mM): 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES and 0.2% FCS at pH 7.3. The hyperosmotic effect was produced by 200 mOsm sucrose, an optimal hyperosmotic stress, above which a saturation of the exchange rate is observed. Replacement of sucrose by sorbitol or mannitol does not affect the response to the hypertonic stimulus. The osmolarity of media was adjusted to 300 ± 5 mOsm (as measured by a Wescor 5100 vapor pressure osmometer). 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF/AM) was purchased from Molecular Probes, Eugene, OR. Nigericin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), BSA and amiloride were obtained from Sigma. The results are presented either as representative tracings of fluorimetric experiments of at least three recordings or as the mean ± S.D. The error bar is absent when the standard deviation is smaller than a symbol. Curves were fitted by a non-linear regression method.

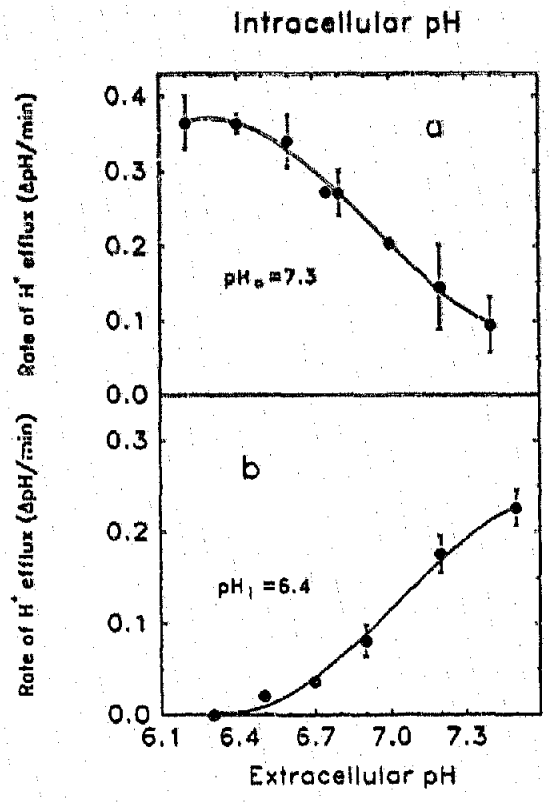


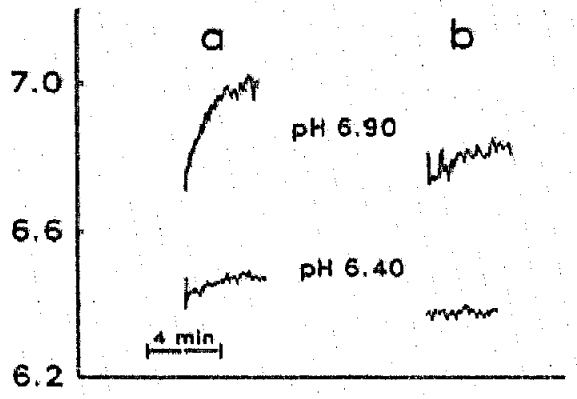
Fig. 1. Effect of external and internal pH on the sodium-proton exchange. (a) BCECF-loaded cells were clamped at different pH_i in 145 mM K^+ medium. The activation of the sodium-proton exchanger was fluorimetrically recorded following cell resuspension in a 145 mM Na^+_o medium at $pH_o = 7.3$. (b) The initial alkalinization rate of cells clamped at $pH_i = 6.4$ was measured following suspension in a 145 mM Na^+_o solution at various pH_o.

3. RESULTS

The pH response to a hyperosmotic stress was sodium-dependent and was inhibited by amiloride (0.33 mM). The exchange rate was not affected by DIDS (50 μM), furosemide (0.5 mM), ouabain (0.1 mM) or by replacement of the external chloride with gluconate. Substituting Na^+ by Li^+ in the external medium maintained the pH_i increase at a lower rate, whereas a similar substitution by K^+ and Rb^+ abolished the cytosolic alkalinization. Therefore, the sodium-proton exchanger is the main transporter of RCJ bone cells underlying the pH_i changes in the response to a hyperosmotic stress in bicarbonate free medium. In order to characterize the regulation of the antiporter under isosmotic and hyperosmotic stimulations we varied the external and internal proton concentrations.

RCJ cells at pH_i range of 6.2–7.4 (initially clamped by the nigericin technique) were suspended in an isomotic Na^+ medium at $pH_o = 7.3$. The dependence of the initial rate of alkalinization on the pH_i (Fig. 1a) is sigmoidal with a pK of 6.7, similar to previous reports [10]. In a complementary study, cells initially clamped at $pH_i = 6.4$ were suspended in external solutions containing different proton concentrations within the physiological pH range of 6.3–7.5 (Fig. 1b). A linear relationship between the initial alkalinization rate and pH_o was observed in the physiological pH range of 6.7–7.5, as expected for the Na^+/H^+ exchanger [13]. These responses were inhibited by 0.33 mM amiloride. The results are consistent with the presently accepted model assuming the existence of a transport and regulatory ion binding site on the cytoplasmic side of the exchanger, and of an external transport site [3].

RCJ cells were clamped at the desired pH_i by nigericin and suspended in isotonic solutions at $pH_i = pH_o$. The isosmotic solutions contained 145 mM or 30 mM Na^+ (Fig. 2a,b), in the pH range of 6.2–7.4. The equalization of internal and external pH abolishes the electrochemical gradient of protons across the cell membrane, leaving sodium as the remaining gradient. The alkalinization response studied under these conditions was abolished by amiloride (0.33 mM). The isotonic response is dependent on the extracellular Na^+ concentration (Na^+_o), increasing towards higher pH values and displays a sigmoidal behavior (Fig. 3a,b).



pH time course of the Na^+/H^+ exchange induced by ctivation at different values of $pH_i = pH_o$. RCJ bone cells ed at the desired pH. (a) The cells at different pH_i were n a $pH_o = pH_i$ 145 mM Na^+_o solution and the alkalinizate recorded. (b) Cells prepared by a similar procedure were spended at $pH_i = pH_o$ in Na_o = 30 mM solution.

dependence of the sodium proton exchange at ial settings of $pH_i = pH_o$ resembles the ice of the exchanger activity on pH_o (at a con-H_i). If this resemblance reflects a similar ng mechanism, it means that under conditions pH_o the external binding site outweighs the ef-ie internal regulatory site.

ffect of pH and Na_o^+ on the hyperosmotic ac-of the Na^+/H^+ exchanger was examined in pH cells in the pH range of 6.2–7.4. Following , cells were suspended in hyperosmotic media 200 mOsm) under $pH_i = pH_o$ conditions at 5 or 30 mM Na^+ (Fig. 3c,d). The hyper- and c activations yield a similar sigmoidal de-e. The difference between the initial alkalinizas of isosmotic or hyperosmotic stimuli in the of high and low Na_o yields identical maximal n rates of 0.08–0.09 ΔpH/min (Fig. 3e,f). osmotic modulation of the exchanger's rate is lent of the extracellular sodium in the range of nM.

arison of isosmotic versus hyperosmotic V_{max} resence of either 145 or 30 mM Na^+ (Fig. 3) a statistical significant difference ($P<0.05$). f maximal activation rates of the transporter osmotic and hyperosmotic conditions at 145 30 mM NaCl were not significantly different. coefficients of the isotonic and hyperosmotic t 145 Na_o^+ were 1.84 ($r=0.95$, $n=6$) and 1.99 , $n=6$) respectively (Fig. 3c,d). The Hill coeffi-the low sodium curves were not calculated in a limited number of experimental points.

ler to examine whether the pH clamping in the sence of K_o^+ had an effect on the following activation in the presence of Na_o^+ we performed ional set of experiments and carried out the pH g in a 30 mM NaCl/120 mM KCl instead of a

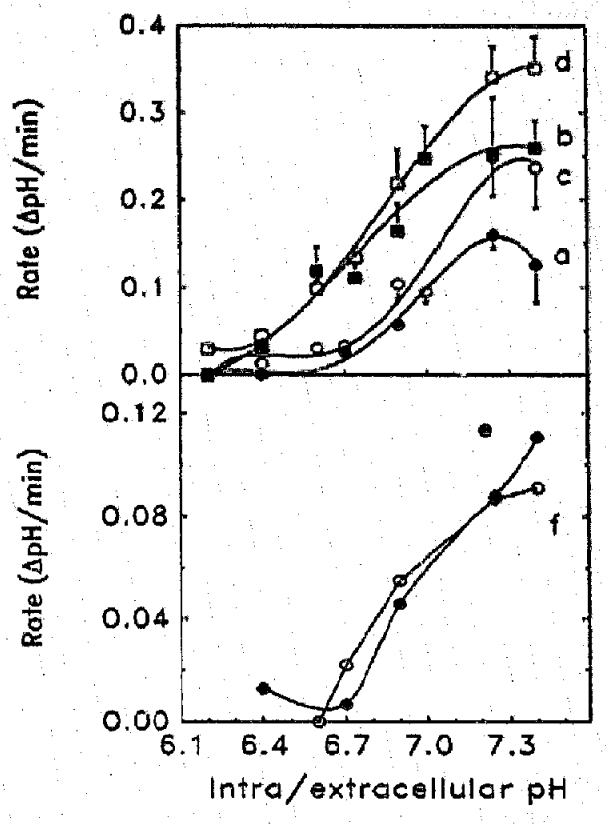


Fig. 3. The pH dependence of the initial rate of alkalinization induced by isosmotic and hyperosmotic activation of $pH_i = pH_o$. BCECF-loaded cells were clamped at the desired internal pH by nigericin in a Na^+-depleted K^+ medium. The cells were resuspended in a solution of a pH identical to the intracellular one ($pH_i = pH_o$) containing 30 mM Na^+/120 mM K^+ (b) or 145 mM Na^+ (d). The upper figure describes the pH dependence of the isosmotic activation of the Na_o^+/H_i^+ exchanger in 30 mM (a) and 145 mM Na^+ (c). The hyperosmotic activation was performed by adding the cells to a hyperosmotic solution containing 200 mOsm sucrose and 30 mM Na^+/120 mM K^+ (b) or by adding the cells to a solution of 200 mOsm sucrose and 145 mM Na^+ (d). The difference between the hyper- and isosmotic activations of the exchanger in the presence of 30 mM Na^+ (e) and 145 mM Na^+ (f) are given in the lower figure.

150 KCl solution. Then, the cells were resuspended in an identical 30 mM NaCl/120 mM KCl medium. Under these conditions no alkalinization was observed in the pH region of 6.3–7.4, reflecting a quiescent exchanger. The consequent addition of a hyperosmotic load leads to the activation of the exchanger with increasing rates of alkalinization towards higher pH values (Fig. 4). The rates show a sigmoidal dependence with a Hill coefficient of 2.24 ($r=0.89$, $n=6$). Maximal rates of activation were 0.055–0.065 ΔpH/min, comparable to the initial alkalinization rates of unclamped cells following the addition of 200 mOsm sucrose to a 145 Na_o^+ medium at pH 7.3. The results are similar to the findings obtained by the previous experimental procedure (Fig. 3e,f). It should be emphasized that the curves given in Fig. 3e,f are calculated by the subtraction of two experimental plots which limits the accuracy of the curve,

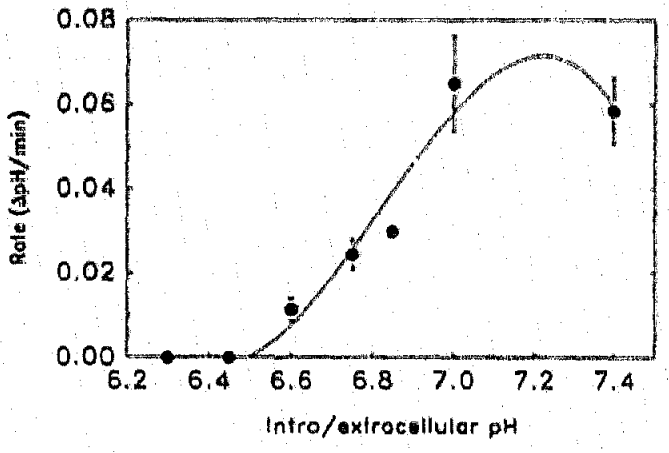


Fig. 4. The pH dependence of the initial rate of alkalinization by hyperosmotic activation at $pH_i = pH_o$. Similar experimental procedures to those described in Fig. 3 were used with the exception that the initial clamping ($pH_i = pH_o$) was carried out in isosmotic medium containing 30 mM Na^+/120 mM K^+ and cells were resuspended in an identical solution containing 200 mOsm sucrose.

as compared to a plot obtained from a direct experimental determination (Fig. 4). It is evident from Fig. 4 that maximal saturable rates of osmotic activation occurs at the physiological pH region of 7.0–7.4.

4. DISCUSSION

The sigmoidal increase of the exchange rate upon lowering pH_i (at constant pH_o), the first order dependence of the sodium activation curve and the exchanger electroneutrality support the existence of an intracellular regulatory proton binding site [3]. The linear increase of the exchange process upon elevation of the extracellular H^+ concentration (H_o^+) in the range of 6.2–8.0 (at a constant pH_i) was interpreted in terms of a simple Michaelis–Menten competition kinetics between H_o^+ and Na_o^+ on the external transport site [4]. This last dependence on pH_o indicates that the competition between H_o^+ and Na_o^+ on the external transport site is dominating the exchange kinetics, though the intracellular proton regulatory site has its maximal effect at the low pH region (6.2–6.4). Thus, at the maximal activity of the regulatory site at $pH_i = 6.3$ the rate of exchange vanishes due to the overriding inhibitory effect of the high H_o^+ at the external site. The resuspension of $pH_i = pH_o$ clamped cells in a Na^+ containing solution leads to the activation of the sodium-proton exchanger. This suggests that a proton gradient is not essential for the activation of the exchanger, in line with an identical pH_o dependence of the antiport at either pH_i 6.5 or 7.0 in C_6 glial cells [10]. Furthermore, the activation is pH dependent under the constant thermodynamic driving force of the Na^+ gradient. Comparison of the exchange rate under high (145 mM) and low (30 mM) Na_o^+ shows that the exchange follows the magnitude of the sodium gradient. The pH dependence of the exchanger following the initial equilibration of external and internal proton concentrations discloses an analogous trend to that obtained when maintaining pH_i at 6.4 and varying pH_o in the range of 6.3–7.4, though it displays a sigmoidal dependence (Figs. 3a,b).

The greater than first order dependence on pH ($pH_i = pH_o$) suggests that H_o^+ inhibits the exchange beyond that expected from the competition with Na_o^+ alone. This may be due to the inhibitory effect of a proton regulatory binding site with a negative cooperative effect on the transport sites. The inhibition may be achieved either by decreasing the apparent affinity for Na_o^+ and increasing the affinity to H_o^+, or alternatively it may be due to an increase in the apparent affinity for intracellular Na^+ and a decrease of the affinity for the cytosolic H^+. This is in line with a previous proposal [14] which suggests the binding of H^+ to an inhibitory extracellular site (of low affinity for Na^+). The suggestion does not eliminate the possibility of the existence of an additional effect of H_o^+ on a rate limiting step in the turnover cycle of the sodium-proton exchanger.

The hyperosmotic activation of the exchanger under a $pH_i = pH_o$ setting is observed at the physiological range of 6.8–7.4, and displays saturation at pH higher than 7.0. It should be stressed that though an isosmotic activity of the exchanger is observed at the pH range of 6.5–7.4, no hyperosmotic activation takes place in the lower region of 6.3–6.8. In view of the similar profiles of iso- and hyperosmotic activations, we may conclude that the hyperosmotic response proceeds through a mechanism similar to the isosmotic one. However, unlike the isosmotic activation it is independent of Na_o^+ in the range of 30–145 mM Na_o^+ [8]. The maximal rates of hyperosmotic activation do not exceed ≈30% of the maximal rates of sodium-proton exchange obtained during isosmotic activation under identical conditions (Fig. 3b,d). This may implicate a distinct pathway of exchanger activation due to a hyperosmotic stress. The pH values of the half maximal rates for isosmotic and hyperosmotic activations are not statistically different. The antiporter responded to the hyperosmotic stress by an increase of V_{max}, reflecting a change in the active number of transporters or an increase in the turnover rate of the exchanger. This result was obtained under an abolished proton gradient and differs from previous findings. Formerly, a hyperosmotic stress administered at different pH_i (at a constant pH_o) caused an increase exchange rate by modifying the intracellular proton sensitivity without a V_{max} alteration [8–10]. These findings may be attributed to an intrinsic property of RCJ bone cells or to an abolished proton gradient effect on the antiporter kinetics.

The mechanism regulating the sodium-proton exchanger during osmotic shrinkage is still unknown. The present work suggests the existence of an external H^+ inhibitory site which regulates the transport site of the

antiporter. It demonstrates the existence of an increase of V_{max} in the exchange rate of hyperosmotically activated bone cells under an abolished proton gradient.

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