Direct characterization of the Na^+/H^+ exchanger in human platelets

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The kinetic properties of the Na^+/H^+ exchanger in human platelets were investigated by direct measurements of pH_i as detected with the fluorescent dye, BCECF. In acid-loaded cells, the antiporter displayed a hyperbolic dependence regarding external Na^+ with an apparent K_m of 38 ± 4 mM (pH_o 7.2 at 25°C) whereas its pH_i-dependent activation between 7.3 to 6.4 did not obey a Michaelian model. External acidification from 7.7 to 6.5 decreased significantly the initial rate of Na^+-dependent H^+ efflux. The amiloride derivative, ethylisopropylamiloride blocked this exchanger and exerted a non-competitive inhibition with respect to Na^+_o ($K_i = 17$ nM). The cation selectivity of the external site of the antiporter was $Na^+ > Li^+ > K^+$ and choline. These results indicate that the BCECF technique allows to evaluate the main features of the Na^+/H^+ exchanger in human platelets, which possesses kinetic properties similar to those reported in other cell types.

Na^+/H^+ exchanger; pH_i; Human platelet

1. INTRODUCTION

The activity of the Na^+/H^+ exchanger is involved in various functions of human platelets, including the regulation of cytosolic pH and the control of platelet volume [1,2]. Determination of the kinetic properties of the exchanger is essential to define its physiological functions. Even though the antiporter has been proposed to be activated by pharmacological agents, including thrombin [3], ADP [4], the calcium ionophore A 23187 [5], arachidonic acid and phorbol esters [6], the direct characterization of the exchanger has never been performed. Up to now, this exchanger has only been characterized using an indirect approach, based on volume changes that are associated with Na^+ uptake in the presence of a weak acid [7]. Thus, the purpose of the present study was to examine the kinetic properties and the ionic specificity of the antiporter in human platelets by direct measurements of pH_i with the aid of the pH-sensitive dye 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF). This dye offers several advantages such as a high sensitivity in the physiological pH range, high fluorescence intensity which limits the cell load and the capacity to enter the cell as its membrane permeant ester. Thus, BCECF is an appropriate dye to evaluate cytosolic pH. It has been successfully used to characterize the Na^+/H^+ exchanger in other cell lines [8-10].

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2. MATERIALS AND METHODS

2.1. *Materials*

BCECF-AM (Molecular Probes, Eugene, OR) and ethylisopropylamiloride (EIPA, a gift from Pharmitalia, Italy) were both dissolved in dimethylsulfoxide ultrapur (Fluka, Switzerland) at concentrations of 1 and 10 mM, respectively. Nigericin (Sigma) was dissolved in ethanol at a concentration of 1.38 mM.

2.2. *pH_i determination*

Platelet-rich plasma (PRP) was obtained by centrifugation of freshly drawn human venous blood containing 1/10 volume of anticoagulant (2.73% citric acid, 4.48% trisodium citrate and 2% glucose). The loading of platelets with BCECF was carried out as previously described [11]. Briefly, PRP was incubated for 20 min at 37°C with 2 μM BCECF-AM, then diluted two-fold with buffer A (145 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 5 mM glucose and 10 mM Hepes, pH 7.2 at 25°C) or buffer B (buffer A where Na^+ has been substituted by *N*-methyl-D-glucamine (NMG^+)) and centrifuged at $270 \times g$ for 10 min at 20°C. The pellet was then resuspended at a final concentration of $8\text{-}9 \times 10^7$ cells/ml in either buffer A or B as desired, supplemented with 1 mM $Ca(NO_3)_2$. All measurements were performed within 3 h after blood sampling. During that time, platelets were kept at room temperature.

Aliquots of the platelet suspension (500 μl) were transferred to a quartz Suprazil micro-cuvette with optical pathways in excitation and emission of 1 and 5 mm, respectively, and thermostated at 25°C. Fluorescence intensities were recorded on a Spex Fluorolog CM111 (Edison, New Jersey, USA) equipped with a 450 W Xenon lamp with wavelength settings of 503 and 532 nm, using 0.9 and 1.8 nm slits for excitation and emission, respectively. Under our experimental conditions, dye leakage did not exceed 10% per h at 25°C. pH_i was determined according to Rink at al. by lysing the cells with 0.1% Triton X-100 at the end of each experiment [9]. Calibration of the pH_i versus fluorescence intensities was performed as previously described [8]. The platelets were suspended in a high K^+-medium (115 mM KCl, 20 mM NaCl, other constituents identical to buffer A, adjusted with KOH to pH ranging from 6.3 to 7.6 at 25°C) and treated with nigericin (2.76 μM), allowing the external pH (pH_o) to set pH_i.

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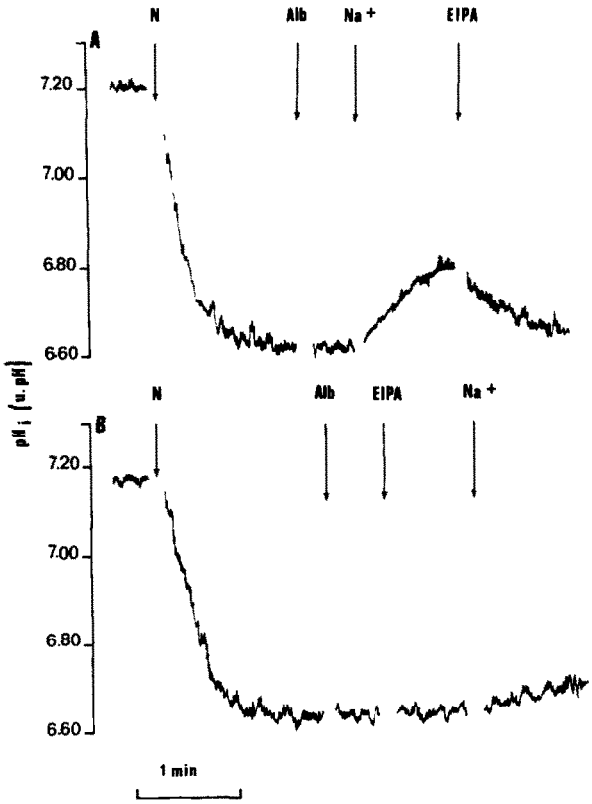

Fig. 1. Activation of the Na^+/H^+ exchanger by an intracellular acidification. Cytosolic acidification was achieved by the addition of 1.38 μM nigericin (N). The apparent nigericin concentration was reduced by the addition of 2 mg/ml serum albumin (Alb). 20 mM NaCl were added either before the addition of 10 μM EIPA (A) or after the incubation for 10 minutes with 10 μM EIPA (B). This experiment is representative of 4 independent experiments.

2.3. *Determination of the buffer capacity of platelets and of the initial rate of Na^+-dependent H^+ efflux.*

The determination of the cellular buffer capacity (β) was carried out by NH_4^+ titration. 10 mM NH_4Cl was added to the cell suspension during the fluorescence recordings and the buffer capacity was determined from the ratio of added base equivalents and the resulting pHi changes, according to the method of Roos and Boron [12].

To induce H^+ efflux, platelets were first acidified by suspension in buffer B to which 1.38 μM nigericin was added. When the decided pH_i was reached, 2 mg/ml bovine serum albumin was added to reduce the apparent nigericin concentration. With this protocol, pH_i remained constant until Na^+ was added. The subsequent recovery of pH_i was measured and shown to be strongly inhibited by the inhibitor of the Na^+/H^+ exchanger, EIPA (10 μM) (Fig. 1). The initial rate of pH_i recovery was calculated as the change in fluorescence intensity as a function of time (i.e. during 15 s corresponding to a linear change). The initial rate of proton efflux in $mmol \cdot l^{-1} \cdot min^{-1}$ was calculated by multiplying the initial rate of pH_i recovery with the buffer capacity. The results are expressed as mean ± SE and n represents the number of independent experiments.

3. RESULTS AND DISCUSSION

Under control conditions, the mean pH_i value averaged 7.36 ± 0.04 ($n = 7$). The buffer capacity of the cells was 28.1 ± 2.25 $mmol \cdot l^{-1} \cdot pH\ units \cdot^{-1}$ ($n = 12$) and remained constant between the pH_i of 6.47 ± 0.08 and 7.36 ± 0.04 (30.20 ± 4.80 and 26.61 ± 2.61 $mmol \cdot l^{-1} \cdot pH\ units^{-1}$, $n = 5$ and 7, respectively).

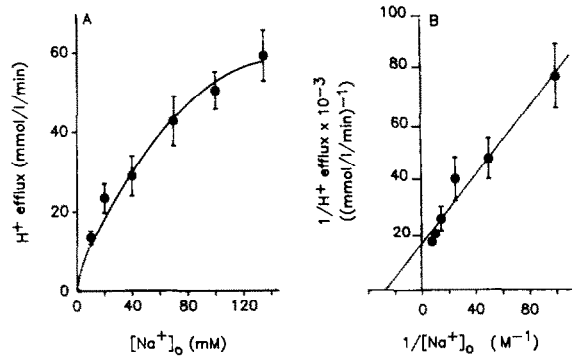

Fig. 2. Control of H^+ efflux by the extracellular Na^+ concentration. Platelets suspended in buffer B at a concentration of 8–9 × 10^8 cells/ml were acidified and then diluted 10-fold by buffers containing Na^+ concentrations ranging from 0 to 135 mM osmotically balanced with *N*-Methyl-D-Glutamine. (A) Dose-response curve of Na^+ action on H^+ efflux. (B) Lineweaver-Burk linearization of data. The line was fitted by the least squares method with a correlation coëfficient of 0.963. Results are means of 5 experiments.

To investigate the effect of external Na^+, the platelet cytosol was acidified to 6.4–6.5 and Na^+/H^+ exchange initiated by platelet suspension in media containing various concentrations of Na^+. The initial rate of H^+ efflux increased with the external Na^+ concentration ($[Na^+]_o$). This Na^+-dependent activation of the exchanger apparently followed simple Michaelis-Menten kinetics (Fig. 2A). This was confirmed by linearizing the data according to the Lineweaver-Burk method (Fig. 2B). Values fit a straight line which gave a maximal rate of recovery with respect to $Na^+{}_o$ of 65 ± 10 $mmol \cdot l^{-1} \cdot min^{-1}$ and indicated that half-maximal stimulation was attained with 38 ± 4 mM $[Na^+]_o$ ($n = 5$). The michaelian kinetics suggest that one Na^+ ion and one H^+ are transported per cycle. The apparent affinity constant for Na^+ showed large variations from one cell type to another (3 to 90 mM) [7, 13–15]. Using a similar approach, Livne et al. have determined in human platelets an apparent K_m of 75 mM at pH_i and pH_o of 6.7 and 7.3, respectively [7].

The cation selectivity of the external site for Na^+ was investigated by measuring the ability of various monovalent cations to induce a H^+ extrusion. In the presence of 20 mM Na^+ at pH_o 7.2 and pH_o 6.4–6.5, the initial rate of H^+ efflux was 21.74 ± 1.78 $mmol \cdot l^{-1} \cdot min^{-1}$ ($n = 5$). H^+ efflux induced by equimolar concentrations of Li^+ and K^+ represented 71 ± 8% and 35 ± 6% of the control value, respectively (15.73 ± 2.41 and 7.87 ± 1.43 $mmol \cdot l^{-1} \cdot min^{-1}$, $n = 5$) whereas choline and NMG^+ were totally ineffective. Among these cations, only Li^+ promoted an EIPA-sensitive pH_i recovery. This suggests that the K^+-induced alkalinization was mediated by residual nigericin, as proposed by Grinstein et al. [13]. A non-specific effect of the hyperosmolarity induced by the addition of these salts was ruled out by the observation

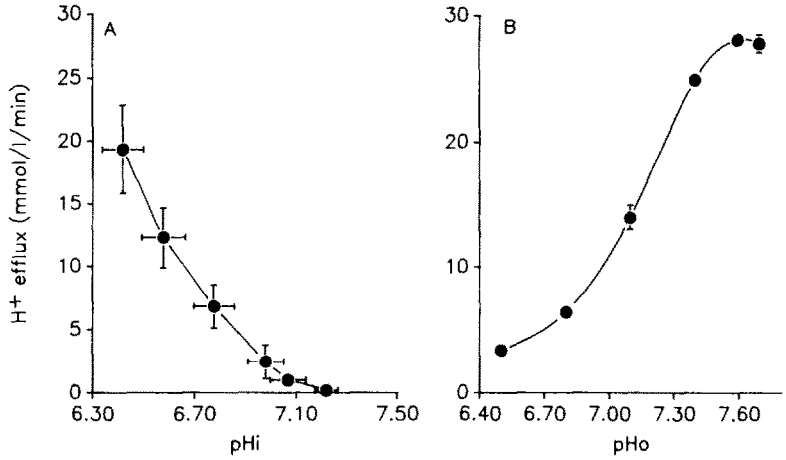

Fig. 3. Effect of intra- and extracellular pH on the Na^+-dependent H^+ efflux. (A) Platelets suspended in buffer B at pH 7.2 were acidified at pH_i ranging from 6.4 to 7.3. H^+ efflux was initiated by addition of 20 mM NaCl. Means of H^+ efflux were plotted against the mean pH_i at 0.2 unit intervals. (B) Platelets were resuspended in buffer B adjusted to pH_o ranging from 6.5 to 7.6 with KOH (1 N) and immediately acidified. Results represent means of 5 experiments.

that 40 mM sucrose and 20 mM tetramethylammonium chloride did not significantly affect the fluorescence signal.

The dependence of the Na^+/H^+ exchanger on pH_i was investigated at pH_o 7.2. The rate of the Na^+-dependent H^+ extrusion elicited by 20 mM Na^+ increased as the cytoplasmic pH decreased (Fig. 3A). Cytosolic acidification from 7.06 to 6.4 enhanced H^+ efflux from 0.5 to 19 $mmol \cdot l^{-1} \cdot min^{-1}$. This activation did not follow Michaelian kinetics, but is compatible with an allosteric regulation by intracellular H^+. This has been attributed to a kinetic control exerted by a modifier site besides the H^+ transport site, first demonstrated in renal membrane vesicles [16]. This property has been later reported to be present in other cell types [10,13]. This dependence of the exchanger activity on pH_i thus underlines its major role in pH_i homeostasis in platelets.

To study the influence of external pH on the exchanger activity, platelets were suspended in media adjusted to various pH_o. In contrast to the pH_i effect, an external alkalinization promoted an activation of the exchanger with a maximum H^+ efflux reached at 7.6 (Fig. 3B). In our experimental conditions, the decrease of pH_o from 7.7 to 6.5 was associated with an intracellular acidification from 6.69 to 6.05. Changes in pH_o from 7.7 to 7.1 were associated with a pH_i decrease from 6.69 ± 0.02 to 6.30 ± 0.01 ($n=4$). If the H^+ efflux were controlled only by pH_i, the expected rates induced by such an intracellular acidification would have been 7.34 ± 2.18 and 21.78 ± 1.78 $mmol \cdot l^{-1} \cdot min^{-1}$ ($n=5$) (Fig. 3A). However, the experimental values differed from the above data (27.82 ± 0.71 and 13.79 ± 1.26 $mmol \cdot l^{-1} \cdot min^{-1}$, $n=4$). If this difference is attributed to a pH_o effect, the external acidification promoted an inhibition of H^+ efflux which markedly reduced the pH_i-dependent H^+ extrusion. These results suggest a mutual interaction between internal and external H^+ sites. Such a hypothesis has been suggested in rat brain synaptosomes, where it has been proposed that the binding of H^+ on the outer face of the exchanger modified the interaction properties of the internal H^+ binding site which controls the antiport activity [17].

The pharmacological properties of the Na^+/H^+ exchanger in platelets was investigated in the presence of a derivative of amiloride, EIPA. The Dixon plot of the data exhibited a linear relationship between the inhibitor concentration and the inverse of H^+ efflux induced by the addition of 20 or 50 mM Na^+ (Fig. 4). The intercepting values on the x-y axis indicated that EIPA did not compete with Na^+, since the apparent inhibition constant (K_i) was not affected by an increase in external Na^+ concentration from 20 to 50 mM (18.47 ±

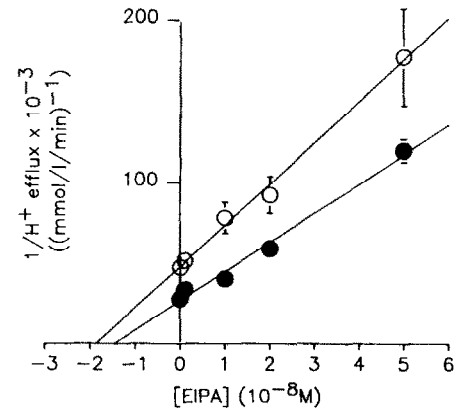

Fig. 4. Inhibition of the Na^+-dependent H^+ efflux by EIPA. Platelets suspended in buffer B were incubated for 10 min with EIPA (0 to 5×10^{-8} M) and then acidified. H^+ efflux was initiated by the addition of 20 (○) or 50 mM NaCl (●). The representation of Dixon was used and lines were fitted by the least squares method with correlation coëfficients of 0.961 and 0.975. Results are means of 4 experiments.

1.10 and 15.70 ± 3.90 nM ($n=4$), respectively), whereas the maximal rate of H^+ extrusion was enhanced from 20.44 ± 1.57 to 38.8 ± 4.07 $mmol \cdot l^{-1} \cdot min^{-1}$ ($n=4$). Our results suggest that EIPA was a more potent inhibitor of platelet Na^+/H^+ exchange than amiloride ($K_i = 8$ μM) [7] and that its K_i is in agreement with those reported in other cell types [18,19]. In addition, EIPA interacted with a site on the transporter which was not affected by Na^+_o. This last observation is consistent with previous results reported in lymphocytes with another 5-(*N,N* disubstituted) amiloride analog: the (5-*N*-methyl-*N*-isobutyl) amiloride (MIA) [20].

In summary, we presented herein the direct characterization of the kinetic and pharmacological properties of the Na^+/H^+ exchanger in human platelets. Using the BCECF technique, we demonstrated that the properties of the antiporter and its cation selectivity were comparable to those reported in other cell types and in platelets when investigated by an indirect approach. We demonstrate that EIPA is a potent inhibitor of the platelet exchanger and acts non-competitively with external Na^+. The present approach should allow a direct evaluation of the characteristics of the exchanger under platelet stimulation and its participation to the physiological and the pathological changes of cytosolic pH.

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