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Biochimica et Biophysica Acta 1278 (1996) 119–124

BBA Biochimica et Biophysica Acta

Identification of a sodium-bicarbonate symport in human platelets

Oscar A. Gende *, Horacio E. Cingolani

Centro de Investigaciones Cardiovasculares, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, 60 Y 120, 1900 La Plata, Argentina

Received 10 January 1995; revised 16 May 1995; accepted 7 August 1995

Abstract

Intracellular pH (pH_i) was measured in human platelets using fluorescent probes. Basal pH_i was higher in HCO_3^--buffered solutions (7.33 ± 0.01) than in nominally HCO_3^- free, Hepes-buffered solutions (7.16 ± 0.01, $P < 0.05$). Addition of EIPA caused a fall in Hepes, but did not inhibit the increase of pH_i when platelets maintained in Hepes were transferred to a CO_2/HCO_3^- buffer. After an intracellular acidosis induced by an NH_4Cl prepulse, the initial velocity of recovery ($d(pH)/dt_i$, in pH units/min) was 3.32 ± 0.69 in Hepes-buffered solution and 2.85 ± 0.88 in HCO_3^- media. Taking into account the differences in buffer capacity, the efflux of acid equivalents after 1.2 min was twice as much in the presence of bicarbonate. The addition of 30 μmol/l EIPA effectively blocked acid efflux ($d(pH)/dt_i = 0.08 \pm 0.04$) in a nominally HCO_3^--free solution, whereas the recovery was reduced but not abolished ($d(pH)/dt_i = 0.37 \pm 0.10$, $P < 0.05$) in the presence of bicarbonate. The stilbene derivative SITS further inhibited the EIPA-resistant pH_i recovery. Removal of external Na^+ inhibited the HCO_3^--dependent recovery whereas depletion of internal Cl^-, did not suppress it. Depolarization of the membrane had no effect on this recovery. The results suggest the contribution of an electroneutral Na^+/HCO_3^- cotransport in the recovery of pH_i following an acid load. Both the Na^+/H^+ antiport and the HCO_3^--dependent mechanism contribute approx. 50% each to the total acid equivalent efflux during the recovery from a pH_i 6.46 ± 0.14 to the basal pH_i in human platelets.

Keywords: pH, intracellular; pH_i regulation; Platelet; Sodium ion/bicarbonate cotransport

1. Introduction

The value of pH_i in eukaryotic cells is higher than would be expected if hydrogen ions were passively distributed across the cell membrane according to their electrochemical gradient. This implies that even in basal conditions there are systems removing acid equivalents from the cytosol. The most widely explored mechanism for acid extrusion is Na^+/H^+ exchange [1]. Recovery after acid loads is assumed to rely on this mechanism.

The identification of bicarbonate-dependent acid extrusion processes in many cellular preparations has prompted a reexamination of the role of HCO_3^- in pH_i homeostasis in human platelets. Two systems that extrude acid equivalents and are dependent on the presence of HCO_3^- have been found in different tissues: (1) an exchange of extracellular Na^+ and HCO_3^- for intracellular H^+ and Cl^- and (2) a cotransport of extracellular Na^+ and HCO_3^- that is independent of Cl^-. The first system, usually called Na^+-dependent Cl^-/HCO_3^- exchange, is electrically silent; the second appears to carry negative charges into the cell with each cycle, suggesting that two or three HCO_3^- ions accompany each Na^+ ion [2,3]. However, a recent report proposes the existence of an electroneutral Na^+/HCO_3^- cotransport mechanism in myocardial tissue [4].

In human platelets, the mechanism currently proposed for acid extrusion is plasmalemmal Na^+/H^+ exchange [5]. The contribution of HCO_3^--dependent mechanisms to intracellular pH regulation in human platelets has recently received some attention [6,7]. This paper shows evidence of the existence of HCO_3^--dependent mechanisms that could account for approx. 50% of the acid transported during the recovery from an acid load. The evidence suggests that an electroneutral Na^+/HCO_3^- cotransport may be the bicarbonate-dependent mechanism playing a role in pH_i regulation of human platelets.

2. Materials and methods

Venous blood was obtained from healthy human volunteers, collected with one sixth volume of ACD (2.5% of

Abbreviations: BCECF-AM, 2′,7-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CDF, 5(6)-carboxy-4,5-dimethylfluorescein diacetate; DMSO, dimethyl sulfoxide; EIPA, ethyl isopropyl amiloride; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid; NMDG, *N*-methyl-D-glucamine; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid.

* Corresponding author. Fax: +54 21 255861.

SSDI 0005-2736(95)00203-0

sodium citrate, 1.5% of citric acid, 2.0% of glucose and 10 μmol/l of prostaglandin E1).

Platelet rich plasma was obtained by centrifugation at 150 × *g* for 20 min and centrifuged at 600 × *g* for 20 min to form a platelet pellet. The pellet was washed twice with a calcium-free buffer (in mmol/l: NaCl 140, KCl 5, EGTA 0.5, aspirin 0.1, glucose 10, Hepes 10, BSA 1%, pH 7.4). After resuspension in the same solution, but omitting EGTA, the platelets were incubated for 15 min in 6 μmol/l BCECF-AM at 37°C.

Some platelets underwent measurement of pH_i after the extracellular dye was washed in a Hepes-buffered solution (in mmol/l: NaCl 140, KCl 5, $CaCl_2$ 1, $MgSO_4$ 1, glucose 10, Hepes 10; pH 7.4). Other platelets were acidified by removal of 25 mmol/l NH_4Cl after 15 min exposure at 37°C. The acidified platelets were kept in a Na-free Hepes-buffered solution (NaCl replaced by NMDG, pH 6.4) until further use.

The kinetics of the pH_i changes was followed by adding 50 μl aliquots of the platelet suspensions to 2 ml of Hepes or bicarbonate-buffered solution. In this media 20 mmol/l $NaHCO_3$ was used to replace Hepes, and NaCl was reduced by 20 mmol/l. Both the stock solutions and the cuvettes were continuously bubbled with a gas mixture of 5% CO_2 and 95% O_2. In some experiments the pH_i was determined using CDF as intracellular fluorescent indicator, and a different protocol was used for the acid load, but similar results were obtained.

Fluorescence of BCECF was monitored in a spectrofluorometer SFM25 (Kontron Instruments, Milano) using wavelengths of 440/503 nm and 535 nm for excitation and emission respectively. Calibration of the fluorescence signals was carried out in a Hepes-buffered solution with 130 mmol/l KCl to replace 130 mmol/l NaCl, and 10 μmol/l nigericin, at defined pH values. Autofluorescence was measured on platelets from the same batch that was not loaded with the dye, and the fluorescence ratio was then calculated as follows: ratio = (fluorescence at 503 nm − autofluorescence at 503 nm)/(fluorescence at 440 nm − autofluorescence at 440 nm). For determinations in the steady-state, the ratio of successive measurements at both excitation wavelengths using an integration time of 2 min was calculated. Kinetic experiments were performed at 503 nm with an integration time of 0.5 s.

The initial rate of pH_i recovery ($d(pH)/dt_i$) was obtained from a least-squares regression of the initial linear segment of the curves from the kinetic experiments.

In order to estimate the intrinsic buffer capacity (β_i), the pH_i change was measured immediately upon exposure of the cells to 10 mmol/l NH_4Cl. β_i was defined as $\Delta[NH_4]_i/\Delta pH_i$. The concentration of ammonium was calculated using a pK_a value of 9.21 [8]. $[HCO_3^-]_i$ was estimated using a re-arrangement of the Henderson-Hasselbach equation. Thus, if $[HCO_3^-]_i$ is known, the total buffer capacity can be calculated at each experimentally measured pH_i.

ANOVA followed by Student-Newman-Keuls test was used for multiple comparison among the groups. Student's *t*-test was used for comparison of two groups of experiments. A P value of 0.05 was used as limit of significance.

3. Results

The pH_i of platelets equilibrated at 37°C in the Hepes-buffered solution was 7.16 ± 0.01. This value was statisti-

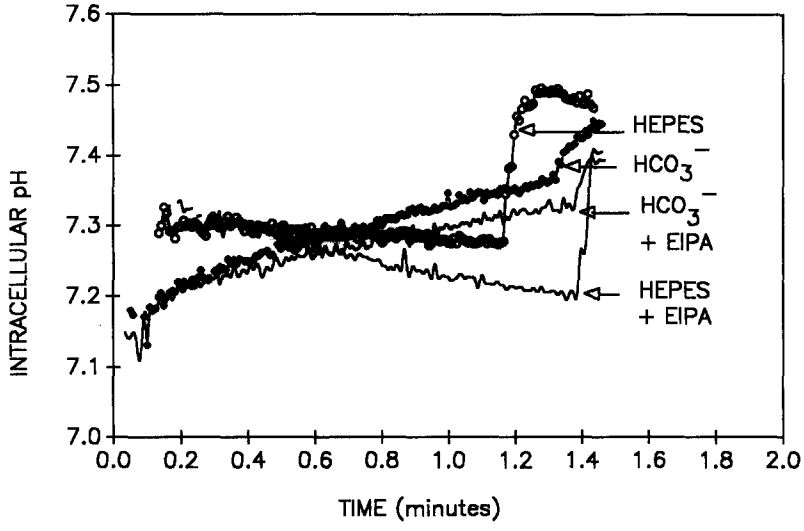

Fig. 1. Time-course of basal pH_i changes in platelets loaded with BCECF after resuspension in Hepes or bicarbonate-buffered solutions at 37°C, pH 7.4. The figure is a composite of data obtained in the absence of inhibitors (symbols), or in the presence of 30 μmol/l EIPA (lines). Note that in the bicarbonate buffered solution the rapid entry of CO_2 was responsible for the initial acidification; the recovery of intracellular pH observed in the presence of EIPA suggests the existence of an acid extrusion mechanism different from Na^+/H^+ exchange and dependent on the presence of HCO_3^-. The arrows indicate the addition of 10 mmol/l NH_4Cl to show that buffer capacity is greater in bicarbonate than in Hepes-buffered solutions.

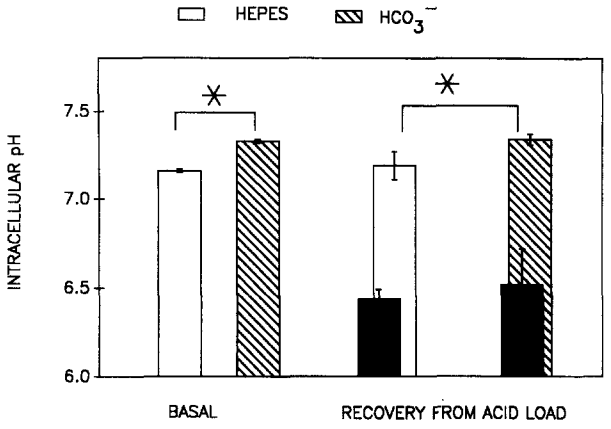

Fig. 2. The bars of the left group show the difference in the steady-state pH_i of platelets maintained at pH_o 7.4. The bars on the right show the pH_i of preacidified platelets at the beginning (black) and at the end of recovery in solutions containing Hepes (open) or bicarbonate (hatched bars). An asterisk indicates statistically significant differences ($P < 0.05$) between the pH_i of platelets in different buffer systems. The initial pH_i of acidified platelets was obtained from the extrapolation at $t = 0$ of the regression line that represents the initial recovery rate.

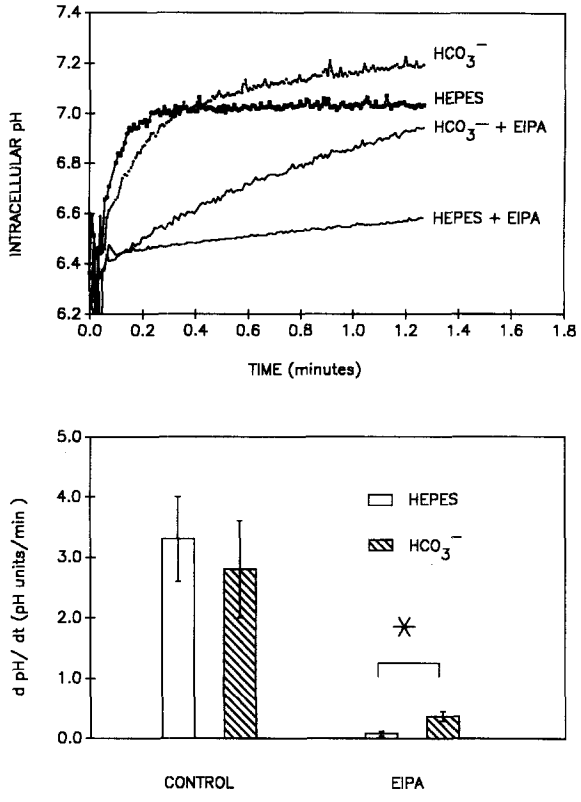

Fig. 3. (Top panel) Time-course of the changes of pH_i in platelets preincubated in 25 mmol/l NH_4Cl, 15 min, washed and resuspended in Hepes or bicarbonate-buffered solutions at 37°C, pH 7.4. The figure is a composite of records obtained in the absence of inhibitors (symbols), or in the presence of 30 μmol/l EIPA (lines) in a representative experiment. In the presence of bicarbonate the recovery of intracellular pH was attenuated but not abolished by 30 μmol/l EIPA, on the other hand, 30 μmol/l EIPA was able to completely block the increase of pH_i in nominally HCO_3^--free solutions. (Bottom panel) Differences in $d(pH)/dt_i$ of platelets in the absence (left group) and in the presence (right) of 30 μmol/l EIPA in solutions containing Hepes (open bars) or bicarbonate (hatched bars). An asterisk indicates a statistically significant difference ($P < 0.05$) between $d(pH)/dt_i$ of EIPA-insensitive recovery in different buffer systems suggesting the existence of an acid extrusion mechanism different from Na^+/H^+ exchange and dependent on the presence of HCO_3^-. The $d(pH)/dt$ of acidified platelets was obtained from the slope of the regression line that fitted the initial points during the recovery.

cally different from the value obtained after the platelets were equilibrated in bicarbonate-buffered media (7.33 ± 0.01, $n = 4$, $P < 0.05$). In this solution CO_2 diffusion causes an initial acidification followed by a recovery that is not inhibited by EIPA (Fig. 1).

Platelets recovered rapidly toward basal values from acidification of pH_i to 6.46 ± 0.14 associated with the removal of 25 mmol/l NH_4Cl after 15 min exposure (Fig. 2). The initial rate of recovery ($d(pH)/dt_i$, in pH units/min) was calculated to be 3.32 ± 0.69 in Hepes-buffered solutions and 2.85 ± 0.88 in the presence of bicarbonate. The acid efflux was effectively blocked in the presence of 30 μmol/l EIPA to 0.08 ± 0.04 in the nominal absence of HCO_3^-, whereas $d(pH)/dt_i$ was reduced to 0.37 ± 0.10 in bicarbonate media (Fig. 3).

Since in the bicarbonate-buffered solution the total buffer capacity (β_t) is higher than in Hepes, we would expect a lower $d(pH)/dt_i$ for a given J_{H+} in the bicarbonate containing solution. This actually occurred. However, to make valid comparisons we took into account the fact that the time course of pH_i change was not the same in both situations. The change of pH_i and the value of the buffer capacity were calculated every 0.5 s using fitted functions (Fig. 4, inset), to calculate the cumulative efflux of acid equivalents. This approach minimizes variation of cell buffer capacity at different pH_i [9] because a fairly narrow excursion of pH_i was included in each calculation. After 1.2 min, when Na^+/H^+ exchanger reached its set point, this mechanism had extruded 31 ± 2 mEq/l of acid, whereas in the same time period the total efflux in the presence of bicarbonate was 65 ± 5 mEq/l ($P < 0.05$, $n = 4$, Fig. 4).

3.1. SITS-sensitivity and Na^+ dependence of HCO_3^- activated pH_i recovery

Fig. 5 shows the effects of EIPA and EIPA plus SITS on the recovery of pH_i from an acid load. In bicarbonate-buffered solutions the EIPA-resistant recovery is further reduced by SITS. The $d(pH)/dt_i$ were 0.14 ± 0.02 and 0.08 ± 0.01 pH units/min in the presence of 60 μmol/l EIPA or EIPA plus 40 μmol/l SITS, respectively. Therefore, in the presence of HCO_3^-, part of the recovery of pH_i is due to an EIPA-insensitive, SITS-sensitive mechanism.

In a bicarbonate-buffered Na^+-free solution, the preacidified platelets do not show any recovery in pH_i. The change of pH_i in a sodium-free solution was not

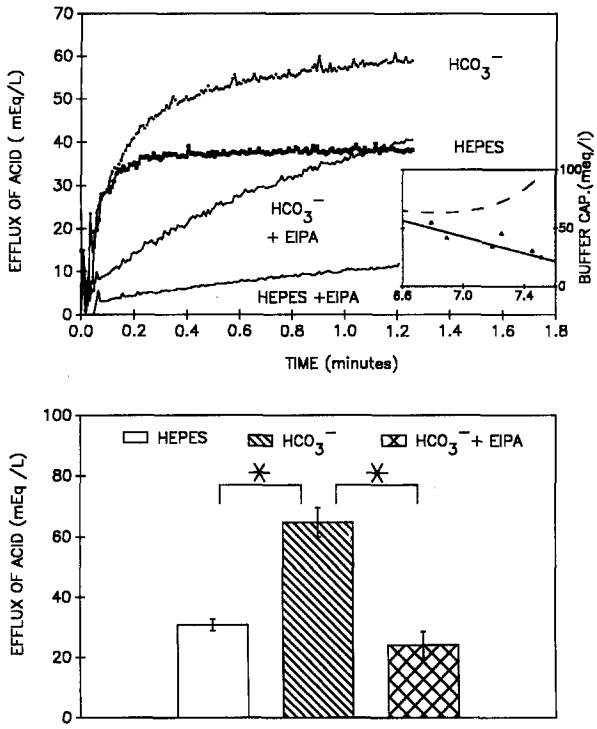

Fig. 4. (Top panel) The efflux of acid equivalents was calculated for the experiment shown in Fig. 3, by the cumulative sum of the product $\Delta pH \times \beta$ calculated every 0.5 s, using the value of β adjusted by the function that the inset shows. (Inset) The intrinsic buffer capacity calculated at different pH_i values fits to the function $\beta_i = 288 - 35.8 \times pH_i$; the total buffer capacity (broken line) was obtained by the addition of $\beta(CO_2) = 2.3 \times [HCO_3^-]_i$. (Bottom panel) Differences in the efflux of acid equivalents from platelets in solutions containing Hepes (open bar) or bicarbonate in the absence (hatched) and in the presence of 30 μmol/l EIPA (double crossed bars). An asterisk indicates a statistically significant difference ($P < 0.05$) between total and EIPA-insensitive efflux suggesting that half of the acid extrusion is due to Na^+/H^+ exchange and half is due to a mechanism dependent on the presence of HCO_3^-.

statistically different from the change observed in the solution containing 140 mmol/l Na^+ in the presence of both SITS and EIPA.

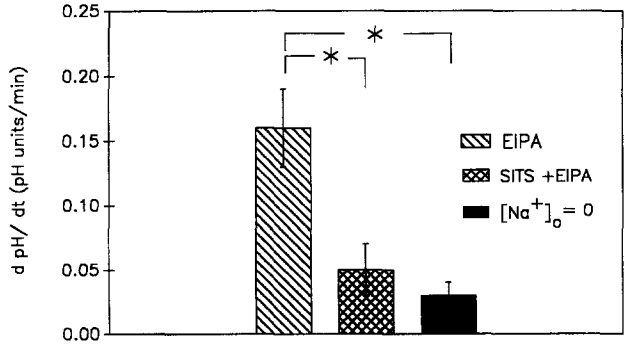

Fig. 5. Initial velocity of recovery in bicarbonate-buffered solutions. The asterisks indicate a significant inhibition of the EIPA-resistant recovery mechanism in the presence of SITS or in a Na^+-free solution ($P < 0.05$, ANOVA). We conclude that in addition to Na^+/H^+ exchange the platelets use a SITS-sensitive, Na^+-dependent mechanism to extrude acid equivalents. Hatched bar: 60 μmol/l EIPA ($n = 12$), double crossed bar: 60 μmol/l EIPA + 40 μmol/l SITS ($n = 12$), black bar: Na^+ free solution ($n = 6$).

3.2. Na^+ and HCO_3^- dependent pH_i recovery is independent of intracellular Cl^-

One candidate for a carrier-mediated influx of Na^+ and HCO_3^- would be the Na^+ dependent Cl^-/HCO_3^- exchange. In a variety of other tissues, this mechanism has been shown to possess an absolute requirement for internal Cl^- [10–13]. Fig. 6 shows that the recovery of pH_i that follows the acid load is not altered by preincubation of the platelets for 30 min in a chloride-free solution (chloride replaced by gluconate). Insensitivity to depletion of intracellular chloride argues against the possibility of a

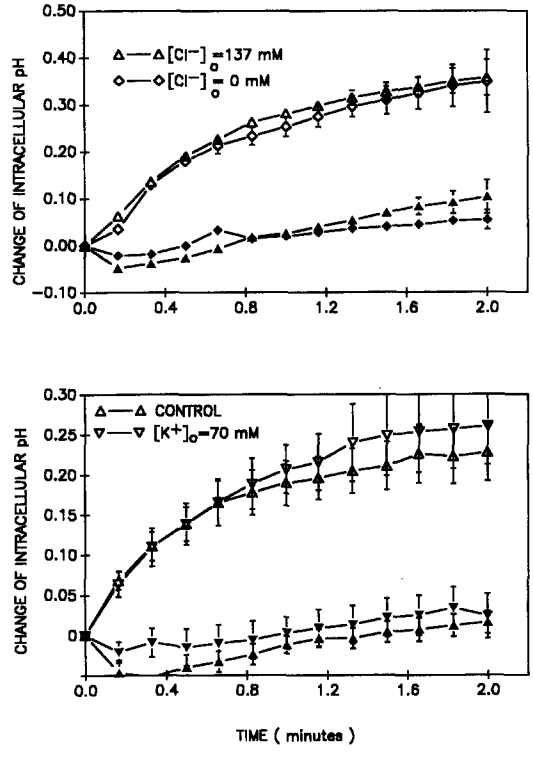

Fig. 6. (Top panel) Recovery after depletion of intracellular chloride. Platelets were preincubated for 30 min in a Cl^--free solution to reduce the $[Cl^-]_i$. Recovery was achieved in the absence (diamonds) or in the presence (triangles) of external Cl^-. In the latter case the inward gradient of Cl^- was greater, but the recovery was not attenuated. The effects of $[Cl^-]_i$ depletion on the total (open symbols) and on the EIPA-resistant recovery (closed symbols) are shown. The initial decrease in pH_i in the presence of EIPA is probably due to CO_2 permeation. Means ± S.E. of six experiments. (Bottom panel) Effect of increased external K^+ (NaCl partially replaced by KCl) on the changes of pH_i of preacidified platelets. This experiment, designed to test the voltage sensitivity of pH_i recovery, shows that depolarization did not accelerate the net transfer of HCO_3^- as would have been expected in a process accompanied by net transfer of negative charges. No differences were observed between recovery in normal (triangles) or high $[K^+]_o$ (inverted triangles) solutions. Closed symbols show the recovery mediated by EIPA-resistant mechanisms. Open symbols indicate the uninhibited recovery. Means ± S.E. of six experiments.

sodium-dependent Cl^-/HCO_3^- exchange playing a role in the recovery.

3.3. Effect of raised $[K^+]_o$

To test the possibility of an electrogenic Na^+/HCO_3^- cotransport playing a role in the recovery, the concentration of KCl in the solutions was elevated from 5 to 70 mmol/l, a procedure that depolarizes the platelets [14]. Fig. 6 shows a comparison between the recovery in normal (i.e., 5 mmol/l) and high (i.e., 70 mmol/l) extracellular potassium concentration. The recovery of pH_i either in the presence or absence of EIPA is not modified by the external K^+ concentration. The absence of an effect argues against an electrogenic transport playing a role in the recovery.

4. Discussion

In this study pH_i was found to be 0.17 pH units higher in the presence of CO_2/HCO_3^- buffers than in nominally HCO_3^--free solution. This observation agrees with the difference of 0.13 pH units found before in human platelets [6].

In the absence of HCO_3^-, pH_i recovery from acid loads rely on a mechanism that shows the characteristics of the Na^+/H^+ antiport already described in platelets [5,15–17] and in a wide variety of mammalian cells [1]. In the experiments performed in Hepes-buffered solution it can be seen that the concentration of EIPA used was high enough to block this exchanger completely. However, an EIPA-resistant, SITS-sensitive pathway seems to contribute to the recovery from acidosis in the presence of bicarbonate.

Several bicarbonate-dependent mechanisms have been described as operating in different tissues: (1) the exchange of intracellular HCO_3^- for extracellular Cl^- a mechanism acting mainly during intracellular alkalosis; (2) the exchange of extracellular Na^+ and HCO_3^- for intracellular H^+ and Cl^-, called sodium-dependent Cl^-/HCO_3^- exchange [10–13,18–21] and (3) a cotransport of extracellular Na^+ and HCO_3^- into the cell that is independent of Cl^- [4,22–28]. The Na^+-independent and the Na^+-dependent Cl^-/HCO_3^- exchanges are electrically silent; on the other hand, the cotransport appears to carry negative charges into the cell with each cycle, suggesting that two or three HCO_3^- ions accompany each Na^+ ion [2,3,22,28]. A recent report, however, proposes the existence of an electroneutral Na^+/HCO_3^- cotransport mechanism in myocardium [4].

Whereas the Na^+-dependent Cl^-/HCO_3^- exchanger seems to be a mechanism active in smooth muscle, glial cells and macrophages, the Na^+/HCO_3^- cotransport has been described as playing a role in transcellular transport in epithelia [8,23.24,27,28], hepatocytes [22,29], glia [30,26], volume regulation of osteosarcoma cells [31], and pH_i regulation of human ciliary muscle cells [25]. Electrically silent HCO_3^- transport mechanisms have been recently described in myocardium [4] and in renal tubular tissue [32].

The main finding of our paper is that in addition to the widely known Na^+/H^+ exchange, a HCO_3^- dependent system contributes to the recovery of pH_i after an acid load. This latter mechanism has not hitherto been detected in platelets possibly because many previous studies of pH_i regulation were conducted using nominally HCO_3^- free solutions. Nevertheless, two recent studies in media of more physiological composition showed that the presence of HCO_3^- does not modify the agonist-evoked cytosolic alkalinization [6,7] but increases steady-state pH_i.

In acidified platelets, the $[HCO_3^-]_i$ could be estimated at 2.46 mmol/l, according to the Henderson-Hasselbach equation. In this situation, the equilibrium potential for HCO_3^- ions can be -55 mV. Since the resting membrane potential of platelets was estimated at -64 mV, pH_i recovery cannot be attributed to an influx of HCO_3^- by passive diffusion.

After a preincubation in Cl^- free medium (Cl^- replaced by gluconate), platelets still showed an EIPA-resistant recovery in the presence of bicarbonate. If we assume that platelets were depleted of Cl_i^- after being kept for 30 min in Cl_o^- free solution, as has been previously reported [33], the recovery of pH_i in platelets does not seem to be dependent on $[Cl^-]_i$. The persistence of recovery in the presence of EIPA, and the insensitivity to the chloride gradient argues against the hypothesis that a Na^+ dependent Cl^-/HCO_3^- exchange is working during the recovery.

Depolarization with high $[K^+]_o$ increased pH_i in hepatocytes [22] and glial cells [26]. In contrast, the lack of changes in the rate of recovery of pH_i following depolarization of platelets by high $[K^+]_o$ suggests that the cotransport may be electroneutral. Therefore, the bicarbonate-dependent pH_i recovery seems to operate through an electrically silent Na^+/HCO_3^- cotransport. The simplest model to explain these results is an influx of Na^- and HCO_3^- in a 1:1 relationship.

The amount of acid extruded from the cell increased during the recovery of pH_i until a stable value was reached. The stimulation of Na^+/H^+ exchange achieved by exposure of the preacidified platelets to normal $[Na^+]_o$ and pH_o in Hepes buffer caused the extrusion of 31 mEq/l. When HCO_3^- was present, the acid extruded after 1.2 min of recovery was almost twice as much as that in the Hepes solution. Thus, when the two mechanisms are working in parallel, 50% of the decrease in intracellular acid equivalents is due to exit through the Na^+/H^+ antiport and 50% by a bicarbonate dependent, chloride independent, SITS sensitive mechanism, the candidate being the Na^+/HCO_3^- symport. After switching the cells from Hepes to HCO_3^--containing buffers β_t increased continuously because of

the gain in $[HCO_3^-]_i$. Therefore, in spite of a substantial HCO_3^- influx, the HCO_3^- activated mechanisms made a modest contribution to the initial rate of pH_i recovery, which was sometimes difficult to detect. The cumulative efflux calculation seems to be devoid of this problem and reflects more closely the participation of the alkalinizing mechanisms during the recovery. The significance of multiple pH regulatory transport systems is obscure. It may be that the primary function of the HCO_3^--dependent mechanism was the regulation of pH_i near neutrality, whereas Na^+/H^+ exchange became active at greater acidification.

From our data it was concluded that a bicarbonate-dependent mechanism is contributing to the recovery of platelet pH_i after acid loads. This mechanism accounts for approximately one half of the acid extrusion during the recovery from acidosis and seems to be a non-electrogenic Na^+/HCO_3^- cotransport.

Acknowledgements

The authors are grateful to Miss Mónica Rando for her technical assistance. This research was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, of which both authors are Established Investigators.

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