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Cl^-/HCO_3^- exchange activity in fMLP-stimulated human neutrophils

Miriam S. Giambelluca *,[1], Oscar A. Gende [2]

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

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ABSTRACT

It is well known that chemotactic agents active Na^+/H^+ exchanger, increasing intracellular pH of neutrophils, but their effect on bicarbonate transporters have not been established yet. To study the effect of fMLP on the activity of Cl^-/HCO_3^- exchange, the rate of pH recovery after acute Cl^- readmission in cell subjected to an alkaline load by CO_2 washout in a Cl-free medium was measured. The activity of the exchanger was reduced to 72% of control when cells were pre-incubated for 5 min with 0.1 μM fMLP and reached 48% of control in steady state after acute exposure. After extracellular bicarbonate or TMA addition the rate recovery of intracellular pH was reduce at 72% and at 84%, respectively. The inhibitory effect on the intracellular pH recovery was not affected by blockers of Na^+/H^+ exchange. We conclude from these studies that an increase of pH_i produced for this chemotactic agent is facilitated by the simultaneous activation of Na^+/H^+ exchange and inhibition of Cl^-/HCO_3^- exchange in neutrophils.

Introduction

To allow cells to control their intracellular pH (pH_i), cells express bicarbonate transport proteins that rapidly and selectively move bicarbonate across the plasma membrane. The importance of transport mechanisms utilizing HCO_3^- has already been studied in neutrophils [1,2]. The present experiments were performed to provide a better understanding of Cl^-/HCO_3^- anion exchanger (AE) regulation in these cells.

The maintenance of pH_i within a narrow physiological range is a fundamental property of almost every cell, since many key cellular processes are highly sensitive to pH_i. Stimulation of human polymorphonuclear leukocytes with soluble or particulate chemotactic agents produces profound morphological and metabolic alterations. The coordinated activation of pH_i regulatory mechanisms along with the NADPH oxidase seems to be essential for sustained microbicidal activity [3–5]. Neutrophils release superoxide upon stimulation generating a spike of intracellular acidification associated with neutrophil activation. Afterward, regulatory mechanisms produce not only a recovery but also an overshot of intracellular pH_i. Proton channels were demonstrably active during the early acidification phase [6] and previous research demonstrated a shift in the set point of Na^+/H^+ exchanger during the respiratory burst resulting in sustained net alkalinization [7,8].

The stimulus–response coupling of fMLP, as many other agonists, has been unequivocally linked to cytoplasmic alkalization, though the role of the bicarbonate transporters in stimulated neutrophils is unclear. Of note, studies conducted in bicarbonate-buffered medium have shown effects on neutrophil function that are at odds with other literature [9,10].

The anion exchanger must normally operate in the direction of net HCO_3^- efflux, because the inward Cl^- gradient drives HCO_3^- outside the cell. As acid loader the efflux of HCO_3^- will offset the Na^+/H^+ exchange limiting the extent of the cytosolic alkalinization.

This paper focuses on the regulation of bicarbonate flux by fMLP – a classical chemotactic agent – and its relation with the alkalization produced by the stimulation of Na^+/H^+ exchanger. Our results indicate that, in contrast with its stimulatory effect on NHE, fMLP inhibits AE suggesting that HCO_3^-/Cl^- and Na^+/H^+ exchanger are inversely regulated during neutrophil activation to allow a net pH_i increase.

Materials and methods

The fluorescent indicator 2′7′-bis(carboxyethyl)-5(6)carboxyfluorescein tetra-acetoxymethyl ester (BCECF-AM) was purchased from Invitrogen (Eugene, OR), N-(2-hydroxyethylpiperazine)-N′-2-ethanesulfonic acid (HEPES), trimethylamine hydrochloride (TMA), 4-acetamido-4-isothiocyanostilbene-2,2-disulfonate (SITS) and methazolamide (MTZ) were from Sigma (St. Louis, MO). All other chemicals were reagent and analytic grades.

Experimental media

HEPES-buffered solution contained (in mM): 140 NaCl, 5 potassium gluconate, 1 $MgSO_4$, 10 glucose and 20 HEPES adjusted to pH 7.4 with NaOH. Bicarbonate-buffered saline solution was similar except for the NaCl concentration that was reduced to 115 mM

* Corresponding author. Fax: +54 0221 483 4833.
E-mail address: miriamgiambelluca@conicet.gov.ar (M.S. Giambelluca).
[1] M.S. Giambelluca is a postdoctoral fellow.
[2] O.A. Gende is an established investigator of Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET).

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and for 25 mM $NaHCO_3$ which was added instead of HEPES. For chloride-free solutions NaCl was replaced by sodium gluconate. Maintenance of pH in bicarbonate-containing solutions was performed by equilibration with an appropriate 5% CO_2–95% O_2 mixture, bubbled in the solutions kept at 37 °C.

Neutrophils isolation

Human neutrophils obtained from fresh peripheral blood drawn by venipuncture were isolated by Histopaque double-gradient centrifugation as described by the manufacturer (Sigma–Aldrich, Procedure 1119). Briefly, neutrophils recovered from the interface between two solutions of Histopaque 1119 and 1077 were washed once in 10 ml of HEPES-buffered solution and suspended at a density of 2×10^7 cells/ml. Viability was greater than 95% as assessed with trypan blue dye exclusion and purity, determined by differential cell counting was greater than 98%.

Determination of pH_i changes

Neutrophils were incubated with 10 μg/ml BCECF-AM for 30 min at 37 °C. Then, dye-loaded cells were separated by centrifugation (700*g*, 10 min), suspended in HEPES-buffered solution, re-incubated for 10 min to complete the hydrolysis, washed, and suspended at a density of 2×10^7/ml and stored on ice. Aliquots of 50 μl of this suspension were diluted in 2 ml of HEPES-buffered solution for measurement of the pH_i changes. The suspension of cells loaded with BCECF was excited at 503 and 440 nm, and the emitted fluorescence was collected at 535 nm. pH_i was calculated in each preparation using a high potassium-nigericin solution (135 mM KCl replaced the same concentration of NaCl in the HEPES solution, with10 μM nigericin, titrated with KOH to 7.8). Small volumes of 0.1 M HCl were added to decrease pH stepwise to 6.5. The relationship between the ratios of fluorescence 503 nm/440 nm and the pH value obtained in each step was linear.

Different protocols were used to impose an intracellular alkalization: CO_2 removal and suspension in a chloride-free, HEPES buffered or the addition or 25 mM trimethylamine hydrochloride (TMA). To avoid activation during centrifugation cycles of cellular suspensions, concentrated solutions were added into the cuvettes during the running of the experiments.

The apparent buffer capacity was estimated using the following equation:

$$\beta_i = \left(\left([\mathrm{TMA}]_o \cdot 10^{(a-b)}\right)/\left(1 + 10^{(a-c)}\right)\right)/\Delta \mathrm{pH}_i$$

where $[TMA]_o$ was 25 mM (the total TMA added), ΔpH_i was the measured change in pH_i produced by this addition, *a* was the extracellular pH, *b* was the final pH_i after TMA addition, and *c* was 9.8 (the p*K* of TMA).

Blockage of ionic transport

Neutrophils were incubated in cuvettes with 2 μM EIPA or 1 μM MTZ. Concentrated cells suspensions were pre-incubated with 1 mM SITS and resuspended in the cuvette at a concentration ten fold lower to avoid color interference.

Statistical analysis

The rate of pH_i change was measured as the slope of a regression line adjusted to the values obtained after the addition of TMA or NaCl. Negative slope was expressed in acidification rate units (ARU = pH units/s 10^4). Data were presented as means ± SEM. Student "*t*" tests were performed using a commercial statistics package. The values of $P < 0.05$ were considered as significant.

Results

Effect of bicarbonate-buffered media on steady-state pH

Addition of 0.1 μM fMLP to neutrophils in HEPES-buffered solution induced a biphasic pH_i change; an initial acidification and subsequent alkalinization which exceeded the pH_i level of the resting state (Fig. 1A).

Neutrophils release superoxide upon stimulation generating a spike of intracellular acidification associated with neutrophil activation. This fast acidification is not an artifact by change of volume and was smaller in cells with higher intracellular bicarbonate (Fig. 1B).

The alkalinization phase was completely abolished when the neutrophils were pretreated with EIPA, a potent inhibitor of Na^+/H^+ exchanger, whereas acidification phase persisted (Fig. 1A).

The pH_i of neutrophils equilibrated in a HEPES-buffered media was higher than the value reached after equilibration in a bicarbonate-buffered media (7.11 ± 0.05 vs. 7.26 ± 0.03, $p < 0.05$, $n = 4$). This result suggests that in steady state there is an active bicarbonate-dependent acidifying process. The stimulation with fMLP produces an increase in pH_i reaching in steady state a value 0.13 ± 0.01 pH units higher in HEPES-buffered media and 0.19 ± 0.03 pH units higher in the presence of bicarbonate ($p < 0.05$, $n = 4$, Fig. 1B and C). The highest difference in the presence of bicarbonate would indicate a reduction of the bicarbonate acidification mechanism due to a modulatory effect of fMLP.

Participation of AE on the pH_i recovery from alkaline overloads

To study the Cl^-/HCO_3^- exchange, aliquots of a suspension of neutrophils previously equilibrated in CO_2/HCO_3^--buffered saline solution were resuspended in a chloride-free (gluconate replaced) HEPES-buffered solution (final concentrations in the cuvette: bicarbonate 0.62 mM, chloride 3.5 mM). The pH_i increased by reduction of CO_2 and stabilized at an alkaline value (7.54 ± 0.05) in the following 10 min. The intracellular bicarbonate was retained or even increased by operation of the anion exchanger in the bicarbonate influx mode. After addition of 50 mM NaCl, pH_i fell rapidly by operation of the exchanger in the bicarbonate efflux mode. The change in pH_i after chloride salts addition could not be imputed to the increase of osmolarity or Na^+ in the solutions, since it was not produced by the addition of 50 mM sodium gluconate, and it could be reproduced with 50 mM LiCl (Table 1). Furthermore, the same changes in osmolarity were produced in controls and treated cells.

Table 1 shows that AE is inhibited only partially by of SITS or MTZ, two well-established inhibitors of bicarbonate transport by the anion exchanger.

fMLP effect on pH_i recovery from alkaline overload

The inhibition of pH_i recovery by fMLP was shown in Fig 2A. As in Fig 1A the pH_i of neutrophils maintained in a solution with low bicarbonate (0.62 mM) responded to fMLP stimulation with a fast acidification. In this part fMLP did not affect the alkalinization process.

When the inward directed chloride gradient was imposed 5 min after fMLP stimulation produced a pH decline of 11 ± 2 ARU in control neutrophils and 8 ± 1 ARU in the presence of fMLP ($p < 0.05$, $n = 4$). Treatment with EIPA did not significantly change the rate of pH_i decline in cells with fMLP.

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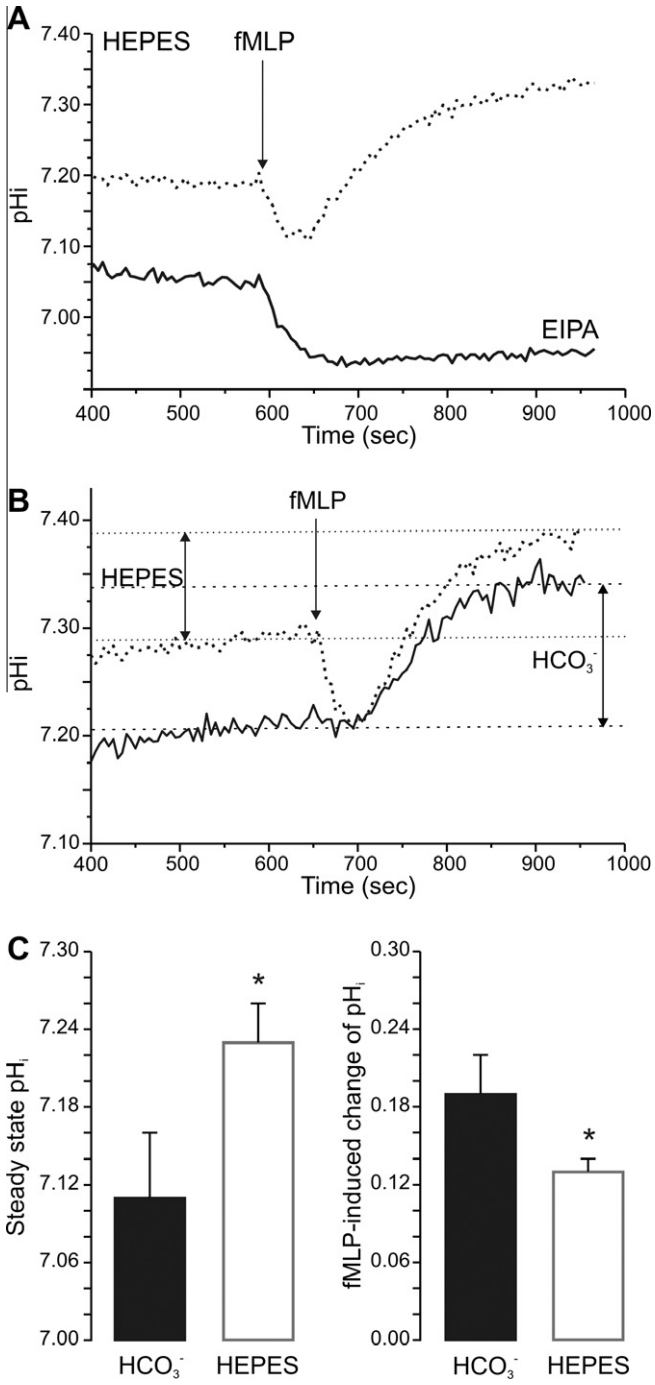

Fig. 1. Effect of fMLP on steady-state pH_i in presence and absence of bicarbonate. (A) Representative experiment showing the pH_i changes induced by 0.1 μM fMLP in neutrophils maintained in a HEPES-buffered saline solution in presence (dotted line) and absence (solid line) of EIPA. (B) Neutrophils maintained in a bicarbonate–CO_2 buffered saline solution (solid line) or in a HEPES-buffered saline solution (dotted line) were stimulated with 0.1 μM fMLP. Double arrows indicate the change in the steady state value of pH_i. The traces are representative of four similar experiments. (C) Mean and SEM of the steady state pH_i and the fMLP-induced change of pH_i. The asterisks indicate statistically significant differences with bicarbonate group ($p < 0.05$).

The agonist produces a transient acidification that distorted records, and it was difficult to evaluate the contribution of the anion exchanger when fMLP was added 10 s before NaCl. In this case we measured the late rate of recovery (i.e., the slope of a linear fit to the values of pH_i acquired between 100 and 400 s after NaCl addition) (Fig. 2C). The rate of pH_i decline achieved by imposing an inward directed chloride gradient was 3.3 ± 0.5 ARU in control experiments and 1.6 ± 0.3 ARU in the presence of fMLP ($p < 0.05$, $n = 4$). A similar degree of inhibition was obtained in the presence of EIPA. In either case, 25 mM TMA was added at the end the experiment and the decline after the jump in pH_i was measured. In the control cells the rate of recovery was 7.8 ± 0.6 ARU and in fMLP it was 6.6 ± 0.3 ARU ($p < 0.05$, $n = 8$). In the presence of EIPA the rates of pH_i recovery were 7.7 ± 0.8 ARU and 6.4 ± 0.6 ARU, respectively ($p < 0.05$, $n = 6$). The treatment with fMLP did not change the buffer capacity of the cells (56.2 ± 6.5 mM/pH unit in control and 53.2 ± 7.6 mM/pH unit in fMLP treated neutrophils, $n = 8$).

Table 1
Acidification rate ($-\Delta$pH/seg. 10^{-4}).

	n	Untreated	Treated	% Inhibition
LiCl	4	7.0 ± 2.5	7.3 ± 2.6	0
Na gluconate	4	7.0 ± 2.5	0.17 ± 0.5	97.5
MTZ (10^{-6} M)	5	12.3 ± 0.7	4.3 ± 0.6	65
SITS (10^{-5} M)	4	11.2 ± 0.4	5.9 ± 1.2	47.3

Data represents means ± SEM. The asterisks indicate statistically significant differences against untreated group.

In a second approach, extracellular bicarbonate was increased in cell maintained in a low chloride HEPES-buffered media by adding 5 mM $NaHCO_3$.

pH_i increased by the increase of extracellular pH and by the operation of the anion exchanger in the bicarbonate influx mode and stabilized at an alkaline value (7.82 ± 0.03) in the following 10 min. After addition of 100 mM NaCl, pH_i fell rapidly, operating the exchanger in the bicarbonate efflux mode. The rate of pH_i change achieved by imposing an inward directed chloride gradient was 22 ± 3 ARU in control neutrophils and 16 ± 3 ARU ($p < 0.05$, $n = 3$) when neutrophils were previously stimulated with fMLP (Fig. 3).

Discussion

Previous studies have suggested that the exchange of Cl^- by HCO_3^- would allow the recovery of pH_i of alkalinized neutrophils [1]. However, neutrophils have an intracellular chloride concentration higher than other cells, resulting in a small driving force for bicarbonate efflux in the case of an alkaline load as the produced by fMLP through stimulation of Na^+/H^+ exchange. Divers agonists increase the efflux of chloride reducing the contents in the cell and increasing the driving force for the efflux of bicarbonate through anion exchanger enhancing acidification tendency. Nevertheless, the teleological role of the anion exchanger in neutrophils is unclear. As acid loader the efflux of HCO_3^- will offset the Na^+/H^+ exchange activation and it would be translated into a metabolic expense by activation of Na/K ATPase. To avoid this energy slippage, the exchangers are restrained when pH_i reaches a set point previous to the value expected by the thermodynamic equilibrium.

The stimulation of NHE by fMLP should be similar in neutrophils equilibrated in solutions buffered with HEPES or HCO_3^- but our experiments showed a greater increase of intracellular pH in the presence of bicarbonate. This result could be due to (1) a reduction in the acidifying activity of Cl^-/HCO_3^- exchanger or (2) the increase of the alkalinizing activity of a transporter of bicarbonate like Na^+/HCO_3^- cotransport. A reduction in the anion exchanger activity would be the most convenient option since an influx of HCO_3^- and Na^+ must be countered by activation of Na, KATPase.

Different intracellular buffer capacities account for the differences in the rate of pH_i changes but presumably this may not affect the steady state pH_i values. Furthermore, the total buffer capacity

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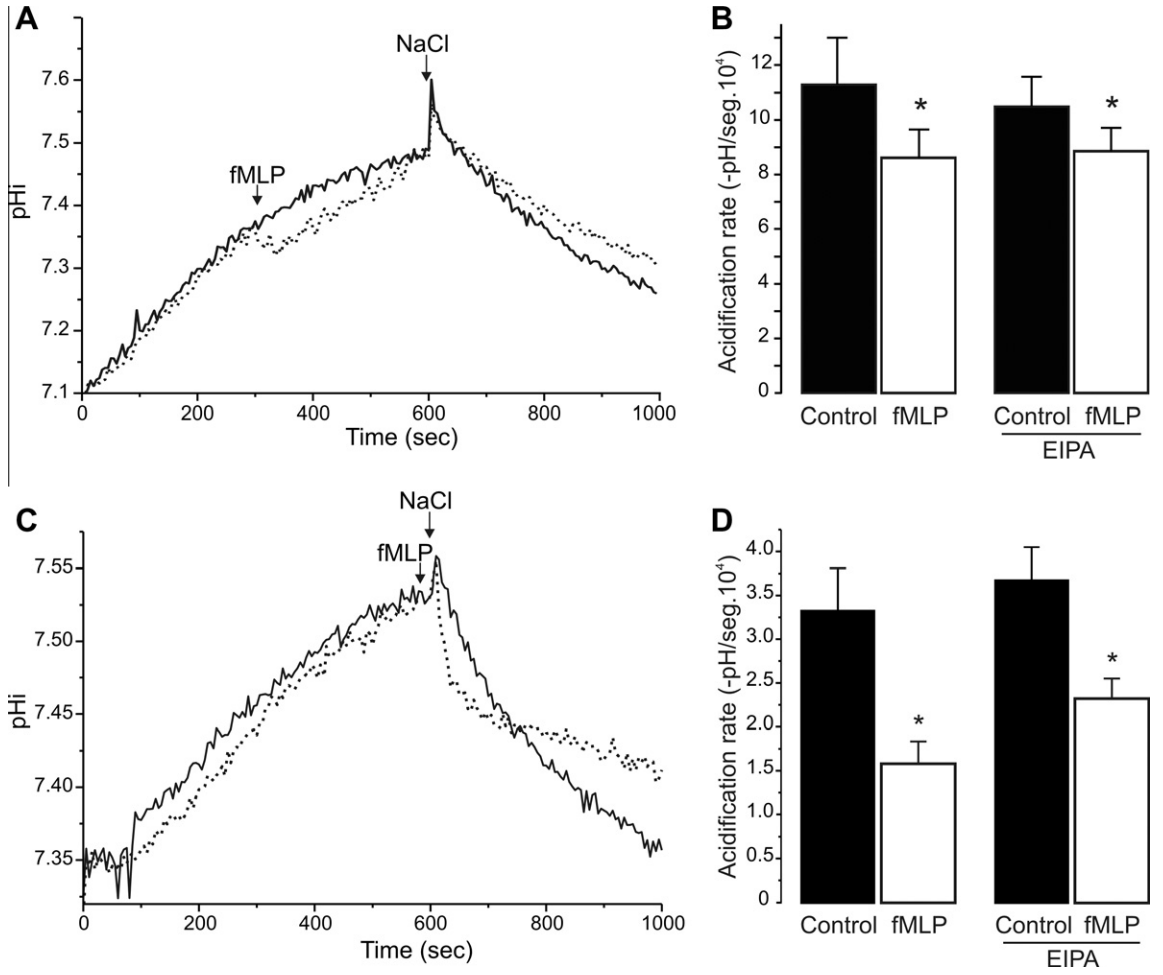

Fig. 2. Effect of fMLP on the recovery from intracellular alkalization. (A) Aliquots of concentrated neutrophils, maintained in the bicarbonate–CO_2 buffered saline solution before assay, was added at a chloride-free HEPES solution resulting in a low Cl^- (3.5 mM), low HCO_3^- (0.62 mM) final medium. The first arrow indicates the addition of fMLP 0.1 μM 5 min before the change in chloride concentration. The second arrow indicates the addition of 50 mM NaCl. (B) Mean and SEM of the initial rate of pH recovery in the absence or presence of EIPA 2 μM. Solid line and filled bars: control; dotted line and empty bars: fMLP. The asterisks indicate statistically significant differences ($n = 4$, $p < 0.05$). C) Representative experiments using a similar protocol that in (A) but 0.1 μM fMLP was added 10 s before the change in chloride concentration as indicated by the arrow. (D) Mean and SEM of the late rate of pH_i recovery in the absence or presence of EIPA 2 μM. The asterisks indicate statistically significant differences ($n = 4$, $p < 0.05$).

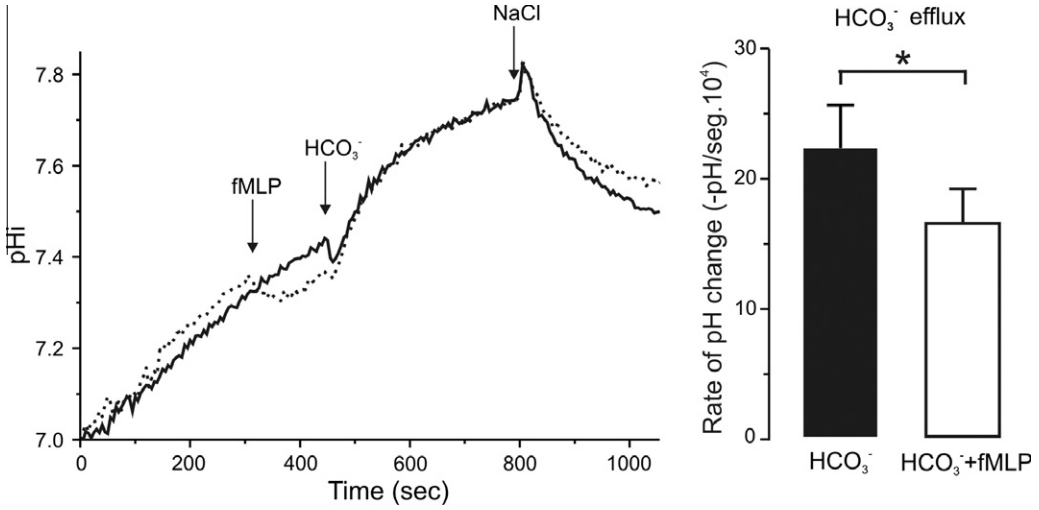

Fig. 3. Effect of fMLP on the changes in pHi after bicarbonate addition. Neutrophils maintained in a chloride-free HEPES buffered saline solution were stimulated at first arrow by 0.1 μM fMLP 300 s before the change in chloride concentration as indicated by upper boxes. At the second arrow 5 mM $NaHCO_3$ was incorporated to the extracellular media. The traces are representative of five similar experiments. Solid line: HCO_3^- + fMLP. Panel B shows the mean and SEM of pH recovery in the absence or presence of 0.1 μM fMLP. The asterisks indicate statistically significant differences ($p < 0.05$).

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is greater in the bicarbonate-containing media and the difference increases at high pH_i values.

The measurement of bicarbonate fluxes requires a previous alkalization because the anion exchanger activity decreases when it reaches a set point previous to the value predicted by the thermodynamic equilibrium. In our first approach this alkalinization was due to a sum of two different mechanisms: CO_2 removal and reversal of chloride gradient. Thus, the change of pH_i after NaCl addition was the only way to study the HCO_3^-. The attenuation of the recovery rate after activation with fMLP could be due to a reduction in bicarbonate efflux, provided that the buffer capacity of the cell was constant. To eliminate the possibility that the fMLP could induce a differential bicarbonate accumulation during alkalization different protocols were used and in every experiment, an attenuation of the pH_i recovery from alkalinization in fMLP-stimulated neutrophils was observed.

The experiments were reproduced in the presence of EIPA or cariporide, two blockers of Na^+/H^+ exchanger, demonstrating that the effect of the agonist on the recovery was independent of other pH_i regulatory mechanisms.

The reduction in the efflux of bicarbonate by fMLP could not be attributed to a reduction of the driving force for the influx of Cl^- because fMLP induces Cl^- loss by Cl^- channels [11], then the efflux of bicarbonate was decreased in spite of the fact that the cellular chloride content was lower.

The inhibitory effect of fMLP seems to be modest, compared with the 65% obtained with MTZ, or 48% with SITS but it could work as a fine adjust when the activity of Na^+/H^+ is low, close to its set point.

Many membrane receptors are coupled through intracellular pathways to Na^+/H^+ exchanger, regulating its activity by changes of its set point. Information on a similar coupling to bicarbonate transporters is scarce. Previous studies demonstrated that second messenger pathways are involved in the regulation of Cl^-/HCO_3^- exchanger activity. In neutrophils stimulated with phorbol myristate acetate, it was suggested a role for anion exchanger in limiting the extent of the cytosolic alkalinization due to other proton-extrusive mechanisms. Unopposed, the latter may result in supraphysiological elevation of pH_i [12]. However, in isolated rat hepatocytes the phorbol dibutyrate treatment directly reduced the activity of AE [13]. Multiple modes of anion exchange mediated by Slc26a6 are negatively regulated by PKC-delta activation [14]. Dopamine receptor stimulation inhibits the activity of the Na^+-independent Cl^-/HCO_3^- exchanger in renal proximal tubular epithelial cells [15]. Stimulation of protein kinase A (PKA) by forskolin and 8-bromo-cAMP was found to reduce Cl^-/HCO_3^- exchange in osteoblasts [16] suggesting that the inhibition of Cl^-/HCO_3^- exchanger activity probably occurs as a result of phosphorylation by PKA. Fully grown mouse oocytes possess robust HCO_3^-/Cl^- exchanger activity that regulates pH_i and confers protection against alkalosis [17]. HCO_3^-/Cl^- exchange was shown to gradually inactivate during cell cycle. The study of the mechanism by which HCO_3^-/Cl^- exchanger activity is inactivated and reactivated during the meiotic cell cycle indicated a negative correlation between MAPK and HCO_3^-/Cl^- exchanger activities during meiosis.

Stimulation of fMLP receptor activates different pathways triggering alkalization by Na^+/H^+ exchange [8]. It is tempting to speculate that HCO_3^-/Cl^- exchange and Na^+/H^+ exchange are inversely regulated during neutrophil activation by other chemotactic agents to allow a pH_i increase. However, this hypothesis deserves further investigation.

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