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Control of cytoplasmic pH by Na^+/H^+ exchange in rat peritoneal macrophages activated with phorbol ester

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The mechanisms underlying cytoplasmic pH (pH_i) regulation in elicited rat peritoneal macrophages were investigated by electronic sizing and fluorescence determinations. Acid-loaded cells rapidly regained normal pH_i by means of an amiloride-sensitive Na^+/H^+ exchange. When stimulated by 12-*O*-tetradecanoyl phorbol 13-acetate, macrophages displayed a biphasic pH_i change: a marginal acidification followed by an alkalinization. The latter results from activation of Na^+/H^+ exchange, since it is Na^+-dependent and prevented by amiloride. When the antiport is inhibited, the full magnitude of the initial acidification can be appreciated. This acidification is independent of the nature of the ionic composition of the medium and probably reflects accumulation of protons generated during the metabolic burst. Under physiological conditions, these protons are rapidly extruded by the Na^+/H^+ antiport.

cytoplasmic pH; Na^+/H^+ exchange; Phorbol ester; Macrophage; (Rat)

1. INTRODUCTION

Macrophages are phagocytic cells which are important not only in host defense against microbial pathogens, but also in a variety of immune responses including antigen processing and presentation, regulation of lymphocyte responsiveness and control of tumor cell proliferation [1]. Exposure of macrophages to a variety of natural and synthetic agents stimulates cell migration, activation of the respiratory burst and lysosomal degranulation [1–3]. The underlying cellular mechanisms and the control of these processes are not well defined.

The importance of the cytoplasmic pH (pH_i) in regulating cell function in a variety of cell types has recently received considerable attention. Changes in pH_i are thought to be essential for fibroblast proliferation [4] and for platelet activation by epinephrine [5]. In human neutrophils, pH_i appears to play an important regulatory role in the chemotactic response [6] and in the generation of the metabolic oxidative burst [7,8]. In these and other cells, control of pH_i is accomplished, at least in part, by an electroneutral exchange of Na^+ for H^+ across the plasma membrane (review [9]).

Little is known about the control of pH_i in resting and activated macrophages. The purpose of the present studies was to establish the existence of an Na^+/H^+ exchanger in the plasma membrane of elicited rat peritoneal macrophages and to examine the response of pH_i to stimulation by TPA, a soluble activator of protein kinase C.

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Abbreviations: TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; BCECF, 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein; NMG^+, *N*-methyl-D-glucammonium$^+$

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2. MATERIALS AND METHODS

2.1. *Reagents and solutions*

The acetoxy methyl ester of BCECF was purchased from Molecular Probes (Junction City, OR). Thioglycolate medium was purchased from Difco (Detroit, MI). Percoll was from Pharmacia (Montreal). Monensin, TPA and Hepes were obtained from Sigma (St. Louis, MO). Solution RPMI 1640 (HCO_3^--free) was from Gibco (Grand Island, NY). Amiloride and 5-*N*-methyl-*N*-propylaminoamiloride were the kind gift of Dr E.J. Cragoe jr, Merck, Sharp and Dohme Laboratories (West Point, PA). NaCl medium contained (in mM) 140 NaCl, 1 KCl, 2 $CaCl_2$, 10 glucose and 10 Hepes-Na (pH 7.3). In K^+ medium and NMG^+ medium, NaCl and Hepes-Na were replaced by the salts of K^+ and NMG^+, respectively. Na-propionate medium contained (in mM) 140 Na-propionate, 1 KCl, 1 $CaCl_2$, 10 glucose and 10 Hepes, pH 6.7. The final osmolarity of these solutions was 290 ± 5 mosM.

2.2. *Cell isolation and characterization*

To elicit macrophages, male Wistar rats (200–225 g, Charles River) were injected intraperitoneally with 5 ml thioglycolate medium [10]. After 7 days, macrophages were collected by peritoneal lavage with 40 ml of medium RPMI 1640 containing 10 mM Hepes-Na (Hepes-RPMI, pH 7.3) and 10 U/ml of sodium heparin (Organon Canada, Toronto). The cells were washed once, then layered over isotonic Percoll (density 1.064 g/ml) and centrifuged at 4°C for 60 min at 800 × g_{av}. The interface band containing the macrophages was collected, washed twice and resuspended in Hepes-RPMI at 7.4–16.7 × 10^6 cells/ml. Centrifugation over Percoll increased the percentage of cells stained with nonspecific esterase, a macrophage marker, from 63.1 ± 8.4 (mean ± SD, n = 12) to 76.2 ± 6.2. By electron microscopy, approx. 90% of the cells in the final suspension were identified as macrophages. The viability of these cells, determined by trypan blue exclusion, was >94%. Macrophages were counted and sized electronically using the Coulter ZM-Channelyzer combination as described [11].

2.3. *Measurement and manipulation of pH_i*

Cytoplasmic pH measurements were performed fluorimetrically as described previously for lymphocytes [12], with minor modifications. Briefly, cells (10^7/ml) were loaded with BCECF by incubation for 10 min at 37°C with 2 μg/ml of the precursor acetoxy methyl ester. The cells were then washed and resuspended in Hepes-RPMI. Even distribution of fluorescence throughout the cytoplasm was ascertained by comparative phase and fluorescence microscopy. Prior to each determination, 2 × 10^6 cells were sedimented and resuspended in 1.6 ml of the indicated medium. Fluorescence was measured at 37°C with magnetic stirring using a Hitachi F-4000 fluorescence spectrometer. Calibration of fluorescence vs pH_i was performed using the K^+/nigericin method of Thomas et al. [13].

Cells were acid-loaded by three different methods: (i) resuspension in NH_4^+-free media following a prepulse of 40 mM NH_4Cl for 15 min at 37°C [14]; (ii) addition of 0.5 μM nigericin, a K^+/H^+ exchanger, to cells suspended in NMG^+ medium [12]; and (iii) suspension of the cells in medium containing the weak acid propionate (e.g. Na-propionate medium) [14].

3. RESULTS AND DISCUSSION

3.1. *Detection of Na^+/H^+ exchange by cell sizing*

The existence of an Na^+/H^+ antiport in rat thymocytes [11] and human neutrophils [15] has been demonstrated by measuring volume changes of cells suspended in propionate-containing media. The protonated form of the weak acid enters the cytoplasmic compartment, where it subsequently dissociates acidifying the cytoplasm. The sustained entry of propionic acid, together with the ensuing activation of Na^+/H^+ exchange, result in intracellular accumulation of Na^+ and propionate$^-$. This is accompanied by uptake of osmotically obliged water leading to cell swelling, detectable by electronic sizing. This approach was used to demonstrate Na^+/H^+ exchange in peritoneal macrophages. The initial median volume of macrophages suspended in propionate media (290 ± 5 mosM) was 431 ± 22 μm^3 (mean ± SE; n = 4). When Na^+ was the main cation in the medium, the cells displayed progressive swelling for at least 8 min (fig.1A,B). Swelling in Na-propionate medium was virtually eliminated by the diuretic

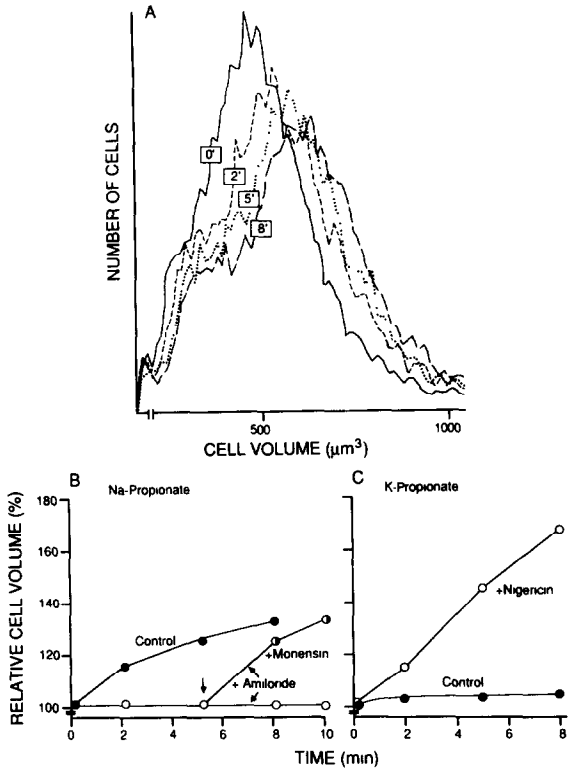

Fig.1. Volume changes of macrophages in propionate solution. (A) Size distribution of cells at increasing times (in min) after resuspension in Na-propionate medium (pH_o = 6.7). Identical aliquots of the cell suspension were sized at the times indicated on the curves using the Coulter ZM-Channelyzer combination; (B) time course of the change in median cell volume after resuspension of macrophages in Na-propionate in the presence (open symbols) or absence (solid symbols) of amiloride (150 μM). Monensin was added where indicated by the vertical arrow to the cells with amiloride; (C) time course of the change in median cell volume after resuspension of macrophages in K-propionate. Where indicated, nigericin (0.5 μM; open symbols) was added at t = 0. Each figure is representative of at least 4 similar experiments.

amiloride (150 μM), an inhibitor of the Na^+/H^+ exchanger (fig.1B). This inhibition could be bypassed by addition of the exogenous Na^+/H^+ exchanging ionophore monensin (fig.1B), indicating that the intracellular acidification and/or an inward Na^+ gradient persisted in the presence of amiloride. The cation specificity of the propionate-induced swelling further indicates the involvement of Na^+/H^+ exchange in this process. The volume of cells suspended in K-propionate medium remained constant for prolonged periods (fig.1C). That acidification occurred and the cells were capable of swelling under these conditions could be demonstrated by the addition of nigericin, which can exchange K^+ for H^+. This ionophore induced marked swelling of the macrophages (fig.1C). Taken together, these studies suggest that an Na^+/H^+ antiport can be activated by acidification of the cytoplasm in rat macrophages.

3.2. *Detection of Na^+/H^+ by pH_i measurements*

The pH_i of rat peritoneal macrophages suspended in Na^+ solution averaged 7.25 ± 0.01 (n = 4). To demonstrate the presence of an Na^+/H^+ antiport, acid loading was achieved by addition of nigericin (0.5 μM) to cells suspended in NMG^+ solution. Under these conditions the ionophore catalyzes the exchange of intracellular K^+ for external H^+, resulting in a cytoplasmic acidification of 0.35 pH units. In the absence of extracellular Na^+ (Na_o^+), only a slow increase in pH_i was recorded thereafter, suggesting the existence of Na^+-independent pH_i-regulatory mechanisms or the presence of significant dye leakage from the acidic cells into the more alkaline medium. In contrast, the addition of 40 mM Na^+ caused a rapid cytoplasmic alkalinization, which was inhibited by amiloride (fig.2a). As expected, the inhibition caused by amiloride was reversed by addition of monensin, which is not affected by the diuretic.

Comparable results were obtained when acidification was achieved by the NH_4^+-prepulse method (fig.2b). A rapid alkalinization was obtained when Na_o^+ was present, which could be inhibited by amiloride (not shown) or by the more specific analog 5-*N*-ethyl-*N*-propylaminoamiloride (EPA in fig.2b). The rapid alkalinization was absent when K^+ or NMG^+ was used to substitute Na^+ (fig.2c). When the cells were suspended in K^+ medium, subsequent addition of nigericin resulted in a rapid alkalinization, due to equilibration of pH_i and pH_o. In contrast, a small further acidification due to K_i^+/H_o^+ exchange was recorded in NMG^+ medium (fig.2c). These results confirm the existence of a cation-selective Na^+/H^+ antiport in the plasma membrane of macrophages.

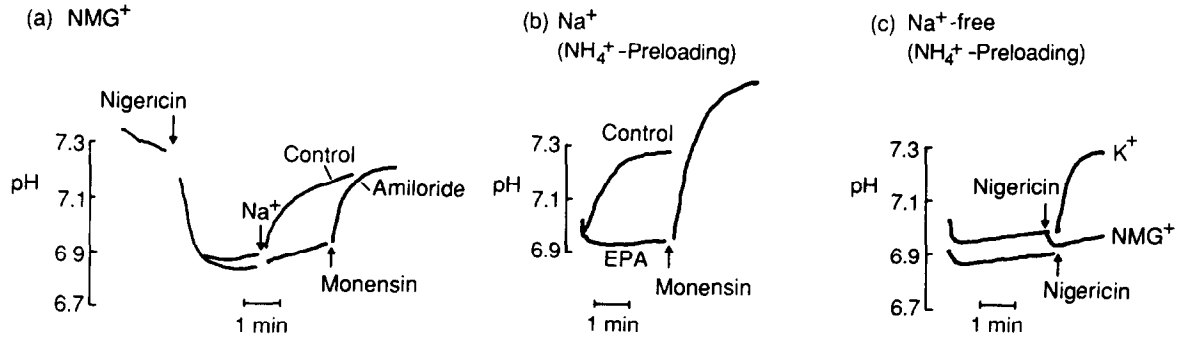

Fig.2. Na^+-induced cytoplasmic alkalinization of acid-loaded macrophages. (a) BCECF-loaded macrophages in NMG^+ medium with or without amiloride (100 μM) were treated with nigericin (0.5 μM) to effect acid loading. Where indicated, NaCl (40 mM) was added. Monensin (5 μM) was added to the amiloride-containing suspension where noted; (b) BCECF-loaded cells were preincubated with 40 mM NH_4Cl for 15 min at 37°C. The trace starts upon resuspension of the cells in NH_4^+-free Na^+ medium with or without 10 μM 5-*N*-ethyl-*N*-propylaminoamiloride (EPA). Where indicated, 5 μM monensin was added to the EPA-containing suspension; (c) BCECF-loaded cells were acid-loaded by the NH_4^+-prepulse technique as in (b) and resuspended in K^+ or NMG^+ medium. Nigericin (0.5 μM) was added where indicated. Traces are representative of 2–4 similar experiments.

3.3. *Effect of activation by TPA on macrophage pH_i*

Addition of TPA to macrophages suspended in Na^+ solution induced a biphasic change in pH_i: a very small but consistent acidification, followed by a moderate alkalinization of 0.055 ± 0.016 ($n = 4$) pH units. The alkalinization is likely due to Na^+/H^+ exchange inasmuch as it can be inhibited by amiloride (not shown) and 5-*N*-ethyl-*N*-propylaminoamiloride (fig.3a). As before, subsequent addition of monensin results in alkalinization. Further evidence that Na^+/H^+ exchange mediated the TPA-induced alkalinization was obtained from ion substitution experiments. Replacement of Na_o^+ by NMG^+ (fig.2b) or K^+ (fig.3c) eliminated the alkalinization, which could be restored by re-addition of Na^+. Impairment of Na^+/H^+ exchange not only eliminated the increase in pH_i, but also accentuated the initial acidification. This decrease in pH_i is not due to reversal of the antiport, since it was observed in the presence of amiloride and its analogs (e.g. fig.3a). If pH_i

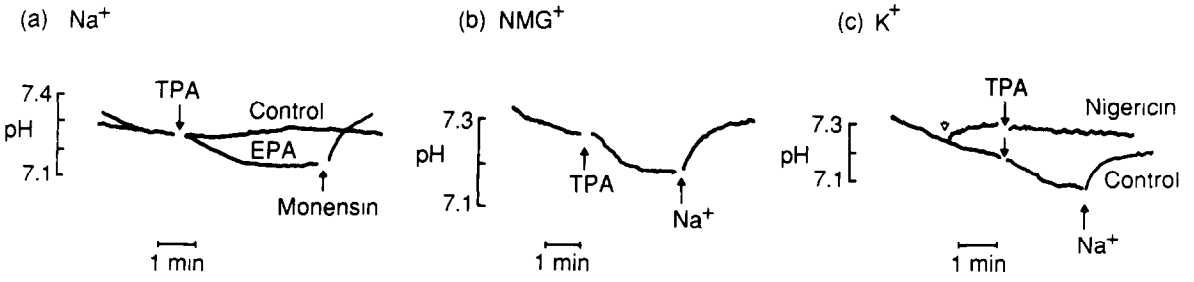

Fig.3. Effect of TPA on pH_i. (a) BCECF-loaded cells were suspended in Na^+ medium with or without 10 μM 5-*N*-ethyl-*N*-propylaminoamiloride (EPA). TPA (2×10^{-8} M) was added where indicated. Monensin (0.5 μM) was finally added to the EPA-containing suspension; (b) cells suspended in NMG^+ medium were stimulated with 2×10^{-8} M TPA. Following stabilization of pH_i, NaCl was added to the suspension. (c) Cells loaded with BCECF were suspended in K^+ medium. The trace is a composite of two experiments. Nigericin (0.5 μM) was added to one of the suspensions (top trace) where indicated by the open arrow. TPA (2×10^{-8} M) was then added to both suspensions. Finally, 40 mM NaCl was added to the control suspension as indicated. Traces are representative of 4 similar experiments.

was clamped by addition of nigericin to cells suspended in K^+ solution, the fluorescence changes induced by TPA were eliminated (fig.3C, top trace), indicating that they are a reliable measure of pH_i.

In summary, the existence of an Na^+/H^+ antiport in the plasma membrane of elicited rat macrophages was demonstrated by both pH_i and cell volume determinations. As reported for other cells [12,16], the macrophage antiport is Na^+-selective, activated by intracellular acidification and inhibited by amiloride and, more potently, by amiloride derivatives alkylated in the 5-nitrogen position of the pyrazine ring. As in neutrophils [17], the antiport in macrophages can be activated by TPA, and the activation is not only the result of the preceding cytoplasmic acidification, since pH_i 'overshoots' to levels more alkaline than the baseline pH_i. These effects of TPA are presumably due to stimulation of protein kinase C, since they were also observed with other β-phorbol diesters, but not with unesterified β-phorbol or with α-phorbol diesters (not shown).

The mechanism underlying the cytoplasmic acidification in elicited macrophages activated by TPA is at present not clear. However, the pH_i response in these cells strongly resembles that reported for human and porcine neutrophils [18], where the acidification is thought to be of metabolic origin. In the absence of Na^+/H^+ exchange, acid generated mainly by the NADPH oxidase and/or the hexose monophosphate shunt is thought to accumulate in the cytoplasm of activated cells [17]. Inasmuch as an analogous respiratory burst is known to occur in macrophages, it is conceivable that the acidification reflects metabolic acid accumulation associated with the generation of superoxide or with related pathways.

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