Cytoplasmic pH Regulation in Macrophages by an ATP-dependent and $N,N'$-Dicyclohexylcarbodiimide-sensitive Mechanism

POSSIBLE INVOLVEMENT OF A PLASMA MEMBRANE PROTON PUMP*

Carol J. Swallow†, Sergio Grinstein§, and Ori D. Rotstein

From the Departments of Surgery, Toronto General Hospital, Toronto, Ontario, M5G 2C4, Canada, The Institute of Medical Science, University of Toronto, Toronto, Ontario, MSS 1A8, Canada, and the Division of Cell Biology, Research Institute, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada

Cytoplasmic pH (pH$_i$) regulation was studied in thioglycolate-elicited murine macrophages using fluorescent probes. Acid-loaded macrophages regained normal pH$_i$, by extrusion of H$^+$ equivalents across the plasma membrane. A fraction of this recovery was due to Na$^+/H^+$ exchange, as evidenced by its partial Na$^+$ dependence and amiloride sensitivity. The residual, Na$^+$-independent pH$_i$ recovery (approximately 50% of the total) persisted in the nominal absence of HCO$_3^-$ and was insensitive to disulfonic stilbenes, ruling out mediation by anion exchange. In contrast, intracellular alkalization and H$^+$ extrusion from the cells were inhibited by N-ethylmaleimide, by N,N'-dicyclohexylcarbodiimide or by prior depletion of intracellular ATP. These observations are consistent with the existence of a H$^+$-pumping ATPase in the plasma membrane of macrophages. The mechanism of activation of the ATP-dependent H$^+$ extrusion process was also investigated. In other systems, Ca$^{2+}$ mobilization has been suggested to signal an exocytic insertion of H$^+$ pumps into the plasma membrane. Acid loading of macrophages was accompanied by an elevation of the cytosolic Ca$^{2+}$ concentration ([Ca$^{2+}$]), measured using indo-1. These results are consistent with a role for Ca$^{2+}$ mobilization in the activation of H$^+$ extrusion.

Because of the extreme sensitivity of most enzymes to the concentration of H$^+$ (OH$^-$), animal cells need to regulate their cytoplasmic pH (pH$_c$) within a narrow range. Active regulation of pH$_c$ is required for two reasons: first, to prevent the cytoplasmic accumulation of metabolically generated acid and second, to counteract the tendency of H$^+$ (equivalents) to enter the cell in response to the internally negative membrane potential. In most animal cells studied to date, pH$_c$ regulation is accomplished by "secondary" active, ion-coupled extrusion of H$^+$ equivalents (see Ref. 1 for review). Three main systems that participate in the regulation of pH$_c$ have been identified. The Na$^+/H^+$ antiport, which is electroneutral and exquisitely sensitive to amiloride and related pyrazine derivatives, promotes the net exchange of external Na$^+$ for internal H$^+$ and is driven by the inward Na$^+$ gradient (2). The two other pH$_c$ regulatory systems involve translocation of H$^+$ equivalents across the plasma membrane in the form of the weak base HCO$_3^-$, in exchange for Cl$^-$. Cl$^-$/HCO$_3^-$ exchange can be either cation-insensitive or dependent on the presence of Na$^+$ (3, 4). Due to the prevailing ion gradients, the latter is expected, on thermodynamic grounds, to function as an acid-extrusion mechanism. In contrast, the Na$^+$-independent Cl$^-$/HCO$_3^-$ exchanger is believed to protect the cell against alkalosis. Despite their different function, both systems are similarly susceptible to inhibition by stilbene disulfonates (3, 4).

There is indirect evidence that in addition to the three systems listed, other pH$_c$ regulatory mechanisms exist in some mammalian cells. Mutant cell lines deficient in Na$^+/H^+$ antiport activity can remain viable for extended periods of time in media nominally devoid of HCO$_3^-$ (5). Similarly, some cell types can survive and even proliferate in the absence of HCO$_3^-$ under conditions where Na$^+/H^+$ antiport activity is precluded by means of inhibitors (6). Such findings suggest that under these conditions, an unidentified process maintains pH$_c$ within the physiological range. In the course of studying pH$_c$ regulation in murine macrophages, we detected a novel mechanism that enables these cells to recover from imposed acid loads. A sizeable component of the recovery was found to be insensitive to amiloride and its analogs and persisted in the absence of extracellular Na$^+$. Moreover, recovery proceeded in the nominal absence of HCO$_3^-$ and was not affected by disulfonic stilbenes. In contrast, the observed acid extrusion was blocked by NEM and DCCD and depended on the availability of cellular ATP. Because these properties are characteristic of H$^+$-activated ATPases, it is conceivable that a "primary" active H$^+$ pump contributes to pH$_c$ regulation in macrophages.

EXPERIMENTAL PROCEDURES

Materials and Solutions—Minimum essential medium without l-glutamine and L-leucine, heat inactivated fetal bovine serum, RPMI 1640 medium (with L-glutamine, HCO$_3^-$free), Ca$^{2+}$- and Mg$^{2+}$-free Hanks' balanced salt solution, and PBS were from GIBCO. All solutions were found to be endotoxin-free (<0.05 ng/ml) using the Limulus amebocyte lysate assay from Associates of Cape Cod, Wood's Hole, MA. Powdered Brewer's thioglycolate medium was from Difco and heparin sodium (1000 USP units/ml) from Organon Canada. HEPES, antimmun A, 2-deoxy-D-glucose, monensin, NEM, and pro-
benzidine were from Sigma. The acetoxymethylesters of BCECF and indo-1 were obtained from Molecular Probes, Eugene, OR. Nigericin and ionomycin were purchased from Calbiochem. Amiloride and N-(2-methoxy-5-nitrobenzyl)amiloride were the gift of Dr. E. J. Crago, Jr., Merck, Sharp and Dohme Laboratories. DCCD and NMG were from Aldrich. SITS was from Pierce Chemical Co. HEPES-RPMI was prepared by titrating RPMI with 20 mM HEPES-Na to pH 7.35 at 37 °C. Na⁺ solution contained (in mM) 140 NaCl, 5 KCl, 10 glucose, 2 CaCl₂, and 10 HEPES, pH 7.35 at 37 °C. NMG⁺ and K⁺ solutions were prepared by isoosmotic replacement of NaCl by the chloride salts of NMG⁺ and K⁺, respectively, but were otherwise identical. The osmolarity of all media was adjusted to 290 mOsm. Webster mice (Charles River) were injected intraperitoneally with 2 mg of thioglycolate medium. After 5 days, macrophages were harvested by peritoneal lavage with 10 ml of minimal essential medium containing 10% serum and 10 units/ml sodium heparin (7). The cells were washed twice with cold Hanks' solution (5 °C), counted using a Coulter Counter model ZF, and resuspended in HEPES-RPMI containing 10 mM probenecid, in Perkin-Elmer LS-5 or 650-40 fluorescence spectrometers with excitation at 331 nm and emission at 410 nm, respectively. Calibration was done using monensin and nigericin for Na⁺ medium and nigericin alone for K⁺ medium, as described (8). Acid loading was accomplished by one of two methods: 1) adding 1 μM nigericin to 2 × 10⁶ cells suspended in 2 ml of NMG⁺ medium or 2) preincubating 2 × 10⁶ cells in 60 μl of HEPES-RPMI containing 40 mM NH₄Cl at 37 °C for 15 min, followed by sedimentation and resuspension in NH₄⁻free medium. The osmolarity of all media was adjusted to 290 ± 5 mOsm with the major salt. Thiglycolate medium was solubilized in H₂O, autoclaved at 125 °C for 45 min, and stored in the dark at 22 °C until uniformly green.

Cell Isolation and Characterization—6-8-week-old female Swiss Webster mice (Charles River) were incubated in RPMI 2% FCS, 10% FCS, 10 units/ml sodium heparin (7). The cells were washed twice with cold Hanks' solution (5 °C), counted using a Coulter Counter model ZF, and resuspended in HEPES-RPMI at lo'° cells/ml. The proportion of peritoneal cells identified as macrophages was the gift of Dr. E. J. Cragoe, Jr., Merck, Sharp and Dohme Laboratories. DCCD and NMG were from Aldrich. SITS was from Pierce Chemical Co. HEPES-RPMI was prepared by titrating RPMI with 20 mM HEPES-Na to pH 7.35 at 37 °C. Na⁺ solution contained (in mM) 140 NaCl, 5 KCl, 10 glucose, 2 CaCl₂, and 10 HEPES, pH 7.35 at 37 °C. NMG⁺ and K⁺ solutions were prepared by isoosmotic replacement of NaCl by the chloride salts of NMG⁺ and K⁺, respectively, but were otherwise identical. The osmolarity of all media was adjusted to 290 mOsm. Webster mice (Charles River) were injected intraperitoneally with 2 mg of thioglycolate medium. After 5 days, macrophages were harvested by peritoneal lavage with 10 ml of minimal essential medium containing 10% serum and 10 units/ml sodium heparin (7). The cells were washed twice with cold Hanks' solution (5 °C), counted using a Coulter Counter model ZF, and resuspended in HEPES-RPMI containing 10 mM probenecid, in Perkin-Elmer LS-5 or 650-40 fluorescence spectrometers with excitation at 331 nm and emission at 410 nm, respectively. Calibration was done using monensin and nigericin for Na⁺ medium and nigericin alone for K⁺ medium, as described (8). Acid loading was accomplished by one of two methods: 1) adding 1 μM nigericin to 2 × 10⁶ cells suspended in 2 ml of NMG⁺ medium or 2) preincubating 2 × 10⁶ cells in 60 μl of HEPES-RPMI containing 40 mM NH₄Cl at 37 °C for 15 min, followed by sedimentation and resuspension in NH₄⁻free medium; C, cells suspended in K⁺ medium containing 1 mM SITS. Nigericin (1 μM) was added where indicated by arrows. Traces are representative of at least three experiments. Temperature: 37 °C.

RESULTS

Na⁺-independent pH Recovery—To study pH regulation, macrophages were loaded with BCECF and acidified to pH 6.4 using an NH₄Cl pulse. When suspended in Na⁺ solution, acid-loaded cells recovered at an initial rate of 1.20 ± 0.03 pH/min (n = 7) (Fig. 1A). In the presence of 200 μM of the Na⁺/H⁺ antiport inhibitor amiloride, the initial rate of recovery in Na⁺ medium was reduced to 0.48 ± 0.01 pH/min (n = 3). Failure to completely inhibit the pH recovery could be due to incomplete inhibition of Na⁺/H⁺ exchange by amiloride, which is generally competitive with external Na⁺ (2). However, a sizeable fraction of the alkalization persisted even at higher concentrations of amiloride. Recovery in Na⁺ medium containing 400 and 800 μM amiloride was 0.42 ± 0.02 and 0.38 ± 0.02 pH units/min (n = 3 in both), respectively (Fig. 1A). Moreover, a similar fraction of the recovery was resistant to N-(2-methoxy-5-nitrobenzyl)amino amiloride, an amiloride analog known to be over 100-fold more potent than the parent compound (10). In the presence of 12.5 μM N-(2-methoxy-5-nitrobenzyl)amino amiloride, the initial rate of pH recovery in Na⁺ medium was 0.55 ± 0.02 pH/min (n = 3). These results suggest that a sizeable fraction of the pH recovery is mediated by a mechanism different from the Na⁺/H⁺ antiport. Experiments performed in Na⁺-free, K⁺, or NMG⁻ media confirmed this hypothesis (Fig. 1B). In K⁺ medium, the initial rate of recovery from acid loading was 0.60 ± 0.03 pH/min (n = 7). Similar rates were observed in NMG⁻ medium (Fig. 1B). Taken together, the results in Fig. 1, A and B, indicate the existence of a pH regulatory process other than Na⁺/H⁺ exchange.

FIG. 1. Na⁺- and HCO₃⁻-independent cytoplasmic alkalization of acid-loaded macrophages. BCECF-loaded macrophages were preincubated with 40 mM NH₄Cl for 15 min at 37 °C. Traces start upon resuspension of 2 × 10⁶ cells in 2 ml of the indicated NH₄⁻free medium. pH was measured fluorometrically as described under “Experimental Procedures.” A, cells suspended in Na⁺ medium, with or without 800 μM amiloride; B, cells suspended in K⁺ or NMG⁺ medium; C, cells suspended in K⁺ medium containing 1 mM SITS. Nigericin (1 μM) was added where indicated by arrows. Traces are representative of at least three experiments. Temperature: 37 °C.
The persistence of the recovery process in Na\(^+\)-free media also rules out the possibility that it was mediated by Na\(^+\)-dependent Cl\(^-\)/HCO\(_3\) exchange. However, cation-independent Cl\(^-\)/HCO\(_3\) exchange may have been responsible for the alkalinization. This was unlikely under the experimental conditions used here for two reasons: 1) in other nucleated cells, Cl\(^-\)/HCO\(_3\) exchange has been found to be very pHi-sensitive, becoming inactive in acid-loaded cells (11), and 2) all media used in these experiments were nominally HCO\(_3\)-free. Nevertheless, residual HCO\(_3\) present in the media due to equilibrium with atmospheric CO\(_2\) could conceivably support Cl\(^-\)/HCO\(_3\) exchange. To test this possibility, acid-loaded cells were suspended in K\(^+\) medium in the presence of SITS and DIDS, potent inhibitors of Cl\(^-\)/HCO\(_3\) exchange (3). As shown in Fig. 1, the initial rate of pHi recovery was not affected by the presence of 1 mM SITS (0.04 ± 0.02 pH/min; n = 3); similar results were obtained in the presence of 100 μM DIDS (not illustrated). The inability of SITS and DIDS to inhibit the Na\(^+\)-independent pHi recovery ruled out the possibility that it was mediated through Cl\(^-\)/HCO\(_3\) exchange.

The tetracarboxylic form of BCECF, generated by intracellular esterases, is highly water-soluble and therefore expected to remain trapped within the cytoplasm. There have been, however, several recent reports of "leakage" of polyanionic solutes out of the cytoplasmic compartment in macrophages (12, 13). Since the pH of the media used for our experiments was alkaline (7.55) relative to the pHi of acid-loaded cells, it was conceivable that the apparent pHi recovery observed in Na\(^+\)-free media was due to leakage of BCECF out of the cells. Several approaches were taken to rule out this possibility. First, if the apparent pHi recovery were due to leakage of dye into the extracellular medium, subsequent cytoplasmic acidification would have little effect on the fluorescence intensity. In cells suspended in NGM\(^+\) medium, addition of the ionophore nigericin results in sustained cytoplasmic acidification, due to exchange of internal K\(^+\) for extracellular H\(^+\). When 1 μM nigericin was added to BCECF-loaded macrophages in NGM\(^+\) medium following recovery from acid-loading, a substantial and sustained decrease in fluorescence emission signal was recorded (Fig. 1B). This is evidence that BCECF remains in the cytosol after recovery from acid loading and that the apparent pHi recovery is genuine. Second, the anion extrusion mechanism reported by others (12, 13) involves an intermediate state of accumulation in intracellular vesicles. However, when BCECF-loaded murine macrophages were analyzed by fluorescence microscopy, the punctate distribution characteristic of vesicular accumulation (12, 13) was not detected for periods of up to 2 h. Finally, we ruled out leakage of BCECF using probenecid, an inhibitor of organic anion transport. This drug prevents extrusion of fluorescent poly-anions, such as furfuryl-2, into the medium (12, 13). When acid loaded, BCECF-stained macrophages were resuspended in K\(^+\)medium with 1 mM probenecid, the initial rate of recovery averaged 0.47 ± 0.04 pH/min (n = 3), which is not different from the rate in untreated cells. Similar results were obtained when the cells were additionally preincubated for 60 min with 1 mM probenecid (during the periods of BCECF loading and NH\(_4\)Cl preincubation). Taken together, these lines of evidence rule out the possibility that the apparent pHi recovery observed in Na\(^+\)-free media was due to leakage of BCECF into the extracellular medium.

Evidence for a Plasma Membrane Proton Pump—In yeast and fungi, acid extrusion across the plasma membrane is mediated by H\(^+\)-pumping ATPases (14–18). These H\(^+\)-ATPases can be inhibited by NEM in Neurospora (15) and DCCD in yeast and Neurospora (14–16). We considered the possibility that the Na\(^+\)-independent pHi recovery observed in acid-loaded murine macrophages was mediated by ATP-dependent proton extrusion across the plasma membrane. To verify this hypothesis, we tested the effects of NEM and DCCD on the recovery from acid loading. Pretreatment of the macrophages with 1 mM NEM, concomitant with the NH\(_4\)Cl pretreatment, resulted in marked inhibition of pHi recovery. Upon resuspension in K\(^+\) medium (Fig. 2A), the initial rate of recovery was reduced to 0.03 ± 0.01 pH/min (n = 3). Pretreatment with NEM did not affect the extent of acid loading, indicating that cellular viability and membrane integrity were preserved. Subsequent addition of nigericin (1 μM) resulted in a brisk alkalinization due to the dissociation of the transmembrane ΔpH by the ionophore. This observation confirms the intactness of the cells and the sensitivity of BCECF under these conditions. Addition of DCCD (100 μM) to acid-loaded cells suspended in K\(^+\) medium abruptly terminated the pHi recovery and in fact produced a slight acidification (Fig. 2B). As before, addition of nigericin after DCCD induced a rapid alkalinization. The sensitivity of the acid extrusion process to both NEM and DCCD is consistent with the involvement of a H\(^+\)-pumping ATPase.

To further test this hypothesis, we analyzed the ATP dependence of the cytoplasmatic alkalinization process. To deplete intracellular ATP, the cells were preincubated in PBS with 10 mM deoxyglucose and 1 μg/ml antimycin A for 40 min; the NH\(_4\)Cl pulse was applied during the final 15 min of this preincubation. As shown in Fig. 2C, the pHi recovery in K\(^+\) medium was virtually eliminated (0.03 ± 0.01 pH/min; n = 3) in ATP-depleted cells. Nigericin dissipated the intracellular acid load, indicating that cellular integrity was preserved following ATP depletion. In agreement with this finding, cell viability estimated by trypan blue exclusion was found to be consistently 80–90%. Similar viabilities were observed after treating cells with NEM and DCCD.

In mammalian cells, proton-pumping ATPases have been demonstrated in the membranes of several intracellular organelles, including lysosomes, secretory granules, and the cisternae of the Golgi apparatus (17). These ATPases, which normally pump protons from the cytoplasm into the lumen of the organelles, are also sensitive to NEM and DCCD (17). We therefore considered the possibility that the Na\(^+\)-independent cytoplasmatic alkalinization in macrophages could be due to translocation of protons into intracellular organelles, rather than across the plasma membrane. These two alternatives can be resolved by measuring the rate of acid extrusion into the extracellular medium. Translocation of H\(^+\) equivalents across the plasma membrane should increase the rate of external acidification, whereas little change is expected if H\(^+\) are sequestered into organelles. The rate of acid extrusion was estimated by measuring the pH of lightly buffered K\(^+\) solution, titrating with known amounts of KOH and/or HCl. As shown in Table I, acid-loaded macrophages extruded H\(^+\) equivalents into the extracellular medium at a significantly greater rate than did control cells (10.56 ± 0.22 versus 3.68 ± 0.27 nmol/10\(^6\) cells/min, respectively). The appearance of H\(^+\) in the medium can fully account for the simultaneous disappearance of H\(^+\) from the cytoplasm (3.6 nmol/10\(^6\) cells/min). The latter was calculated from the rate of change of pHi and the buffering power (=20 mM/pHi), estimated independently using weak electrolyte pulses (1). The excess acid extrusion associated with intracellular acid loading was markedly inhibited by preincubation with NEM (Table I). These findings suggest that the same process underlies pHi recovery and acid extrusion and that H\(^+\) are being translocated across the plasma membrane. Thus, the data are consistent with the
Proton Pump in Macrophages

Fig. 2. Inhibition of Na⁺-independent cytoplasmic alkalinization of acid-loaded macrophages. BCECF-loaded macrophages were preincubated with 40 mM NH₄Cl for 15 min at 37 °C. pH was measured fluorometrically as described under "Experimental Procedures." Traces start upon resuspension of cells in HCO₃⁻-free K⁺ medium. A, cells were preincubated in the presence (+NEM) or absence (Control) of 1 mM NEM in PBS for 15 min at 37 °C, during the NH₄Cl preincubation step. B, bottom trace: DCCD (100 μM) was added where indicated; top trace: control cells. C, bottom trace: cells were ATP-depleted by preincubation in PBS with 10 mM deoxyglucose and 1 μg/ml antimycin A for 40 min at 37 °C. The NH₄Cl prepulse was applied during the final 15 min of the ATP depletion process. Top trace, control cells. D, cells were preincubated in the presence (+Colchicine) or absence (Control) of 1 μM colchicine in HEPES-RPMI for 15 min at 37 °C, during the NH₄Cl preincubation step. Cells were resuspended in NH₄Cl-free K⁺ medium containing 1 mM colchicine. Nigericin (1 µM) was added where indicated by arrows. Fiml cell viability in A–D was consistently >85%. Traces are representative of at least three experiments. Temperature: 37 °C.

### Table I

**Effect of NEM on Na⁺-independent acid extrusion, by control and acid-loaded macrophages**

The rate of acid extrusion was measured as described under "Experimental Procedures." Upon suspension of macrophages in HEPES-free K⁺ medium, pH was immediately brought to 7.35 using KOH and recording started. The initial rate of acid extrusion was calculated following calibration with known amounts of KOH and HCl. Acid loading was attained by incubating the cells with 40 mM NH₄Cl for 15 min at 37 °C prior to the measurements of acid extrusion. Where indicated, cells were preincubated in PBS with 1 mM NEM for 15 min at 37 °C; in acid-loaded cells this was simultaneous with preincubation in NH₄Cl. The data are means ± S.E. of the number of determinations indicated in parentheses. Temperature: 37 °C.

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<td>Basal</td>
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<td>Acid-loaded</td>
<td>10.56 ± 0.22 (7)</td>
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existence of a proton pump in the plasma membrane of acid-loaded macrophages.

**Possible Role of Ca²⁺-induced Exocytosis**—In animals, functional proton pumps have been demonstrated in the plasma membrane of acid-extruding epithelia, such as the gastric mucosa (18), kidney-collecting tubule (19, 20), and urinary bladder (21, 22). Al-Awqati and his co-workers (19, 22, 23) have presented evidence that in acid-secreting cells of the urinary tract, these proton pumps are inserted into the plasma membrane by exocytosis of small vesicles located under the apical plasma membrane. This process was found to be blocked by colchicine, an inhibitor of microtubule formation. To test whether a similar process is involved in the activation of ATP-dependent H⁺ extrusion in macrophages, the magnitude of the pH recovery was measured in acid-loaded cells treated with 1 mM colchicine. As shown in Fig. 2D, pretreatment for 15 min with this agent was associated with a marked reduction in the rate of Na⁺-independent alkalinization (0.13 ± 0.008 pH/min; n = 6). These data are consistent with a role of microtubules in the activation of H⁺ extrusion in macrophages, although nonspecific effects of colchicine cannot be excluded.

In urinary epithelial cells, insertion of H⁺ pumps into the membrane occurs in response to an increase in [Ca²⁺], which is secondary to intracellular acidification. Conceivably, a similar change in [Ca²⁺], could mediate exocytosis and insertion of pumps into the plasma membrane of macrophages. This possibility was tested by measuring the effect of pH; on [Ca²⁺], using the fluorescent probe indo-1. Cytoplasmic acidification was imposed by adding nigericin (1 µM) to cells suspended in NMG⁺ medium. In contrast to the NH₄⁺ prepulse, which produces only a transient acidification, the combination of nigericin and an outward K⁺ gradient results in a sustained drop in pH; (Fig. 1B). As illustrated in Fig. 3, imposition of cytoplasmic acidification resulted in a significant increase in [Ca²⁺], from a base line of 172.4 ± 8.4 to 298 ± 13.5 nM (n = 8). This increase was not the result of a nonspecific effect of nigericin but was instead attributable to the pH; change, since [Ca²⁺], did not increase significantly when cells were treated with the ionophore in the absence of a K⁺ gradient, i.e. in cells suspended in K⁺ medium. In this medium, addition of nigericin induced a sustained alkalinization, preceded by a small transient acidification (Fig. 1B). The alkalosis is due to the equilibration of the internal and
external pH, catalyzed by nigericin under conditions where \([K^+]_i \approx [K^+]_o\). The initial transient acidification is likely due to cytoplasmic transit of H\(^+\) equivalents released by nigericin from acidic compartments such as secretory granules. Indeed, a similar transient acidification is observed in intact neutrophils but not in degranulated cytoplasts.\(^2\) In conclusion, these results are in keeping with a possible role for \([Ca^{2+}]_i\) in modulating H\(^+\) extrusion, but further studies are required to determine whether the increase in \([Ca^{2+}]_i\) is a necessary signal for insertion of proton pumps into the plasma membrane.

**DISCUSSION**

Taken together, the data presented above suggest that a functional H\(^+\) pump is present in the plasma membrane of murine macrophages. Because the rate of ATP-dependent H\(^+\) extrusion increases in acid-loaded cells, it is likely that the putative H\(^+\) pumps contribute to the maintenance of pHi, homeostasis. This would represent, to our knowledge, the first reported instance of plasmalemmal H\(^+\) pumping in mamma-

to be present in specialized plasma membranes of some acid-secreting epithelia. However, in these cases insertion of the pumps in the membranes is asymmetric, transient, and ostensibly intended for the transepithelial transport of acid equivalents (see Ref. 23 for review).

There exists circumstantial evidence suggesting the presence of H\(^+\) pumps in the plasma membrane of nonepithelial cells. In Ehrlich ascites cells, Heinz et al. (24) detected a hydropolarization upon addition of glucose. Because it correlated with increased acid efflux from the cells, the authors attributed the potential change to activation of an electroneutral H\(^+\) pump. A similar conclusion was reached by Ehrhardt (25), who found a correlation between the transmembrane \(\Delta\psi\) and the membrane potential of cultured hepatocytes. Unfortunately, pHi was not directly measured in these experiments, and a causal relationship between the potential change and the translocation of acid equivalents could not be established.

The presence of H\(^+\) pumps in clathrin-coated vesicles (17, 26, 27) could also be taken as an indication of the existence of pumps in the plasma membrane. However, although the H\(^+\) pumps have been shown to be active in the vesicles, they may not be functional in the plasma membrane. More importantly, clathrin-coated vesicles originate not only from invagination of the plasmalemma, but also by pinching off the trans-cisternae of the Golgi apparatus, and available evidence suggests that a transmembrane \(\Delta\psi\) develops only in the latter (17). Thus, it is unclear whether functional H\(^+\) pumps exist in the vesicles derived from the surface membrane.

Is a plasmalemmal H\(^+\) pumping ATPase suited to regulate pHi? Under the conditions prevailing in the cytoplasm, the energy of hydrolysis of ATP (~58 kJ/mol) greatly exceeds that required to extrude H\(^+\) from the cell against the existing electrochemical gradient. A cytosolic H\(^+\) pump would be thermodynamically competent to counteract the tendency of the cells to become acidic. It is also important to consider that at physiological pHe, hydrolysis of ATP liberates ~0.8 H\(^+\) equivalents into the cytoplasm. This would seemingly negate the net H\(^+\) extruding effect of the pump. However, H\(^+\) consumed during ATP regeneration by aerobic metabolism should offset the H\(^+\) produced during ATP hydrolysis (28). Moreover, with the exception of the gastric mucosa H\(^+\)/K\(^+\) ATPase, the stoichiometry of all other H\(^+\)-pumping ATPases is 2 or 3 H\(^+\) transported per ATP hydrolyzed (see Refs. 23 and 29 for reviews). Therefore, if a similar stoichiometry applied to the macrophage plasmalemmal pump, H\(^+\) extrusion would exceed H\(^+\) generation, even in the absence of ATP resynthesis. Taken together, these considerations indicate that a H\(^+\)-pumping ATPase would be suitable for the regulation of pHi.

In acid-secreting cells of the urinary tract, the H\(^+\) pumps are not constitutively present in the plasma membrane. Instead, they seem to be translocated from an intracellular vesicular pool when acid extrusion is to be initiated (22, 29). This exocytic insertion is triggered by an increase in \([Ca^{2+}]_i\), and is thought to involve the microtubular system (19, 23). A similar mechanism may operate in murine macrophages: cytoplastemic acid loading was found to induce an elevation in \([Ca^{2+}]_i\) (Fig. 3), and the subsequent recovery of pHi was greatly inhibited by colchicine, a microtubule disrupting agent (Fig. 2D). However, a causal relationship between the change in \([Ca^{2+}]_i\) and the stimulation of H\(^+\) extrusion remains to be directly established. Studies analyzing pHi recovery in cells where the mobilization of Ca\(^{2+}\) is precluded are currently in progress. Similarly, the assignment of the effect of colchicine on microtubule disassembly is only tentative and awaits confirmation using other approaches.

In summary, macrophages recover from acid loads by means of Na\(^+\)-dependent and -independent processes. The ATP dependence and pharmacological profile of the latter suggest the operation of H\(^+\) pumps. The substantial pHi recovery displayed by macrophages in the nominal absence of Na\(^+\) and HCO\(_3\) has not been reported in other nonepithelial animal cells, such as fibroblasts (30) and lymphocytes (31). It is conceivable that an additional H\(^+\)-regulatory mechanism was developed by macrophages to ensure their survival in the acidic milieu of tumors and abscesses. The low extracellular pH that prevails in these environments would tend to inhibit Na\(^+\)/H\(^+\) exchange by competition between Na\(^+\) and H\(^+\) at the external transport site (2) and would also reduce the concentration of HCO\(_3\). Under these conditions, proton pumping might be essential for the maintenance of pHi.

**REFERENCES**


\(^2\) S. Grinstein and W. Furuya, unpublished observations.