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Protein kinase C activation accelerates proton extrusion by vacuolar-type H^+-ATPases in murine peritoneal macrophages

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Abstract

The role of protein kinase C in the regulation of vacuolar-type H^+-ATPase (V-ATPase) activity was studied in thioglycolate-elicited mouse peritoneal macrophages. Acid-loaded macrophages suspended in a Na^+- and HCO_3^--free K^+-medium containing Zn^{2+}, a H^+-conductance blocker, exhibited an initial intracellular pH recovery rate of 0.33 ± 0.04 pH/min ($n = 9$). Pretreatment with 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) or mezerein for as little as 3 min induced a marked (82%) increase in the initial pH recovery rate. Stimulation was prevented by the V-ATPase inhibitor, bafilomycin A_1 (200 nM) indicating that the effect of the protein kinase C agonists was via augmentation of proton pump activity. The protein kinase C inhibitor, staurosporine (100 nM) completely blocked the stimulatory effects of TPA and mezerein, suggesting involvement of protein kinase C. In keeping with this notion, the inactive analogue of TPA, 4-phorbol didecanoate did not stimulate recovery from an acid load. Extracellular pH determinations revealed that the observed increase in cytosolic pH recovery rate by the protein kinase C agonists was due to increased extrusion of protons from the cells, likely through V-ATPases located in the plasma membrane. Considered together, these data demonstrate regulation of plasmalemmal V-ATPase-mediated proton extrusion by protein kinase C.

Key words: Vacuolar-type H^+-ATPase; Intracellular pH; Macrophage; Protein kinase C; Phorbol ester

1. Introduction

Maintenance of cytoplasmic pH (pH_i) within the physiological range is crucial to normal mammalian cell function, owing to the narrow pH optima of many intracellular processes. Cells have therefore evolved a number of pH_i regulatory mechanisms designed to counteract deviations in pH_i outside of the normal range. The requirement for strict pH_i regulation is particularly important for cells such as macrophages due to their susceptibility to cytoplasmic acid loading within the inflammatory microenvironment. Both cellular activation with consequent metabolic acid generation and the acidic milieu of inflammation lead to intracellular acid accumulation. In these cells, four mechanisms have been shown to contribute to proton extrusion in response to cytoplasmic acidification: (i) a Na^+/H^+ antiporter [1]; (ii) a Na^+-dependent HCO_3^-/Cl^- exchanger [2]; (iii) a plasmalemmal vacuolar-type H^+-ATPase (V-ATPase) [4–6] and (iv) a proton conductive pathway [7] . Previous studies from our laboratory have identified the importance of V-ATPase-mediated H^+ extrusion in the maintenance of cytoplasmic pH homeostasis and normal cell function [8], particularly under conditions of extracellular acidification.

Cellular activation by bacterial products and other proinflammatory stimuli is frequently mediated by protein kinase C activation. Activation via this signalling pathway has also been shown to generate metabolic acid with resultant cytoplasmic acidification. In peritoneal macrophages, recovery from this acid load is accomplished by the activity of both the Na^+/H^+ exchanger and the plasmalemmal V-ATPase [8]. While the Na^+/H^+ exchanger exhibits increased activity following treatment with agonists of protein kinase C [9], the effect of protein kinase C activation on V-ATPase activity is not known. This information is particularly relevant to the acidic inflammatory milieu, since the Na^+/H^+ exchanger, as well as the other pH_i regulatory mechanisms, exhibit reduced function under these conditions [8]. The purpose of the present studies was to investigate the ability of protein kinase C to regulate V-ATPase-mediated pH_i homeostasis in murine peritoneal macrophages. Thioglycolate-elicited cells were studied to simulate the effect of phorbol esters on cells recruited to sites of local inflammation.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium (with L-glutamine, HCO_3^--free) and Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS) were obtained from Gibco. 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), mezerein, staurosporine, 4-phorbol 12,13 didecanoate (4-PDD), nigericin, 2-[*N*-morpholino]ethane sulfonic acid (MES) and 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) were from Sigma. Powdered brewer's thioglycolate medium was purchased from Difco. The acetoxymethylester of 2,7-biscarboxyethyl-5(6)-carboxy-fluorescein (BCECF) was from Molecular Probes, Eugene, OR. Bafilomycin A_1 was from Kamiya Biomedical Company, Thousand Oaks, CA. K^+ solution was prepared by isoosmotic replacement of NaCl by KCl, but was otherwise identical, and was titrated with potassium hydroxide to the indicated pH at

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37°C. The osmolarity of all media was adjusted to 290 ± 5 mosM with the major salt.

2.2. Cell isolation and characterization

Six-to-eight-week-old female Swiss Webster mice (Charles River Breeding Laboratories, Inc.) were injected intraperitoneally with 2 ml of thioglycolate medium. After 4–5 days, cells were harvested by peritoneal lavage with 10 ml of HBSS. The cells were washed twice with cold HBSS (5°C) and resuspended in HEPES-RPMI at 5×10^6 cells/ml. The proportion of peritoneal cells identified as macrophages by both non-specific esterase staining and Wright's staining was consistently 80–90%. Viability was >95% as assessed by Trypan blue exclusion.

2.3. pH_i measurement and manipulation

pH_i was measured fluorometrically using BCECF as previously described [4]. The cells were loaded by incubation in HEPES-RPMI with 2 μg/ml of the precursor acetoxymethyl ester for 20 min at 37°C. After washing, 1×10^6 cells/ml were used for fluorescence determination in the indicated medium using a Perkin-Elmer LS-50 fluorescence spectrophotometer (Perkin-Elmer Corp., Oceanport, NJ) with excitation at 495 nm and emission at 525 nm using 4- and 6-nm slits, respectively. Calibration was done in K^+-medium using nigericin as earlier described [10]. Acid-loading was accomplished by transfer to NH_4^+-free medium following preincubation of the macrophages in HEPES-RPMI containing 40 mM NH_4Cl for 20 min at 37°C.

2.4. Measurement of acid extrusion

Proton efflux was measured by monitoring the rate of extracellular acidification in a lightly buffered medium using a conventional combination pH electrode. Acid-loaded macrophages (2×10^6/ml) were suspended in a water-jacketed chamber containing Na^+-free K^+-medium with 0.3 mM HEPES [3]. The suspension was magnetically stirred and maintained at 37°C. Calibration was performed at the end of each determination by addition of known amounts of KOH.

3. Results

3.1. Protein kinase C activators stimulate pH_i recovery from an acute acid load

When peritoneal macrophages were acid-loaded for 20 min using the NH_4Cl 'pre-pulse' technique, a pH_i of 6.5 was reproducibly attained. However, after a short delay, an increase in the pH_i was detectable. Medium conditions were manipulated to ensure that the V-ATPase was predominately responsible for this pH_i recovery. First, the cells were suspended in a Na^+- and HCO_3^--free K^+-containing medium (pH 7.3). Further, since a proton conductive pathway has previously been shown to operate under these depolarizing conditions [7], the K^+-medium used in all experiments was supplemented with 100 μM Zn^{2+}. This cation effectively blocks the conductance, minimizing its contribution to the pH_i recovery. Under these conditions, the rate of pH_i recovery was 0.33 ± 0.04 pH/min ($n = 9$). The specific V-ATPase inhibitor, bafilomycin A_1 (200 nM) [11], almost completely prevented recovery (0.04 ± 0.02 pH/min, $n = 4$), indicating that pH_i homeostasis was mediated largely by proton pump activity (Fig. 1A). To investigate the effect of protein kinase C (PKC) activation on V-ATPase-mediated pH_i recovery from an acid load, we used 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) and mezerein. These tumour promoters are known to elicit a rapid and direct activation of PKC in a variety of cells [12]. As demonstrated in Fig. 1B, treatment of macrophages with 200 nM TPA for 3 min prior to resuspension in the recovery medium caused a marked stimulation in the rate of recovery from the acid load. In the TPA-treated cells the initial pH_i recovery rate was 0.60 ± 0.07 pH/min ($n = 9$). Similar increases in the recovery from an acid load were also observed following treatment with 200 nM mezerein, a stimulant of PKC which is structurally unrelated to TPA (Fig. 1C).

As noted above, the recovery medium was designed to specifically study the activity of the V-ATPase i.e. Na^+- and HCO_3^--free K^+-medium containing 100 μM Zn^{2+}. To ensure that the observed increase in the recovery rate after treatment with the phorbol esters was due to stimulation of the V-ATPase, pH_i recovery from acid-loaded macrophages following stimulation with the PKC agonists was determined in the presence and absence of bafilomycin A_1 (Fig. 2). The addition of this antibiotic to cells stimulated with either TPA or mezerein reduced the rate of pH_i recovery to that observed for control cells treated with bafilomycin A_1. Thus, the TPA- or mezerein-induced increase in pH_i recovery could be totally accounted for by an increase in V-ATPase activity.

Having established that both TPA and mezerein increased pH_i recovery by augmenting the activity of bafilomycin-sensitive V-ATPases, we next studied whether this effect was mediated through PKC activation. First, the effect of 4-PDD, an inactive analogue of TPA, was

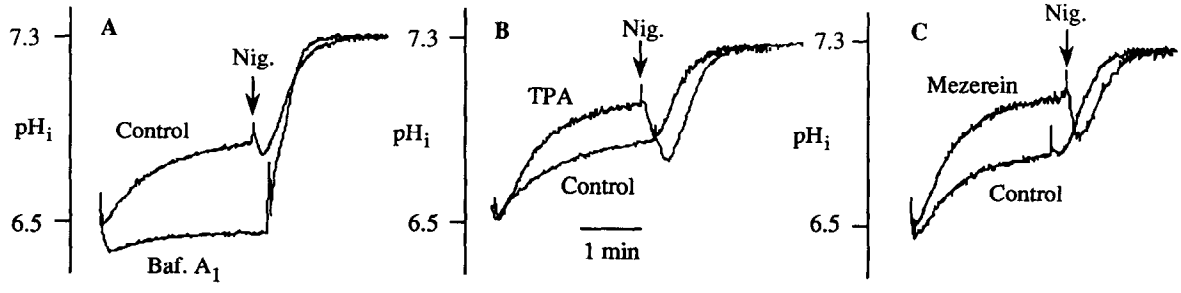

Fig. 1. Effect of PKC agonists on the pH recovery in acid-loaded macrophages Thioglycolate-elicited macrophages, 1×10^6 cells/ml, were simultaneously loaded with 40 mM NH_4Cl and 2 μM BCECF for 20 min at 37°C. Next, the cells were sedimented and resuspended in NH_4Cl and HCO_3^--free K^+-medium containing 100 μM Zn^{2+}. (A) pH recovery of acid-loaded control cells and cells treated with 200 nM bafilomycin A_1. (B) pH recovery of cells treated with 200 nM TPA during the final 3 min of the loading step. (C) pH recovery of cells treated with 200 nM mezerein during the final 3 min of the loading step. Traces are representative of at least three experiments.

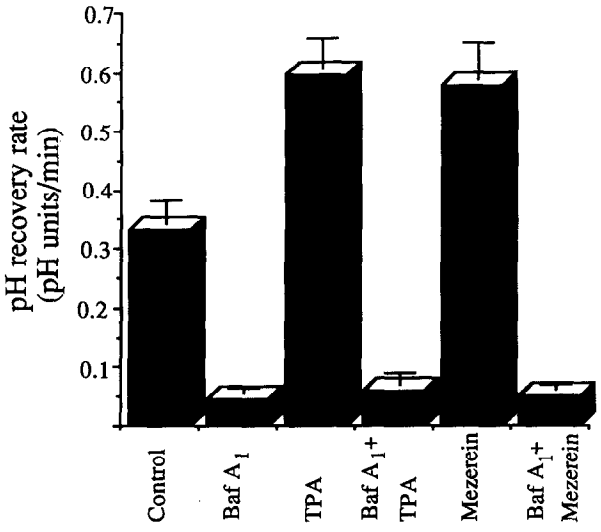

Fig. 2. Evidence that the target for the stimulated increase in the pH recovery rate is a V-ATPase. Thioglycolate-elicited macrophages, 1×10^6 cells/ml, were simultaneously loaded with 40 mM NH_4Cl and 2 μM BCECF for 20 min at 37°C. Next, the cells were pelleted and resuspended in NH_4Cl and HCO_3^--free K^+-medium containing 100 μM Zn^{2+}. The cells were treated during the last 5 min of the loading step with or without 200 nM bafilomycin A_1 (Baf A_1). The effect of bafilomycin A_1 on the recovery induced by 200 nM TPA and 200 nM mezerein are shown in the figure. The results shown are the means ± S.D. of 5 experiments.

examined. As shown in Fig. 3A, this compound had no stimulatory effect on pH_i recovery rate compared to control cells. To further demonstrate that both TPA and mezerein acted through stimulation of PKC, cells were exposed to these compounds following pre-treatment with the PKC inhibitor, staurosporine [13]. Treatment of macrophages for 20 min with 100 nM staurosporine markedly inhibited both the TPA- and mezerein-induced responses (Fig. 3B and C, respectively). Treatment with 100 nM staurosporine did not significantly alter the basal pH_i recovery rate after an acid load in control cells (data not shown).

3.2. *Effect of PKC stimulation on the rate of proton extrusion by acid-loaded macrophages*

V-ATPases have been morphologically and pharmacologically localized in the delimiting membranes of intracellular organelles as well as in the plasmalemmal membrane of phagocytic cells [14–16]. The accelerated pH_i recovery rate observed in response to PKC stimulation might therefore be due to increased pumping of protons into intracellular organelles, rather than across the plasma membrane into the extracellular space. To examine this possibility, proton extrusion into the medium from acid-loaded macrophages was studied as a direct measure of proton translocation across the plasma membrane (Table 1). The proton extrusion rate of acid-loaded but otherwise untreated macrophages was 6.50 ± 0.50 nmol/min/10^6 cells. Pretreatment with TPA (200 nM) for 3 min increased extrusion rate to 13.00 ± 0.81 nmol/min/10^6 cells. A similar increase was observed for mezerein, but not for 4-PDD. Bafilomycin A_1 reduced proton extrusion to comparable low levels indicating that the TPA- and mezerein-induced rises were due to increased V-ATPase-mediated proton extrusion. Finally, staurosporine (100 nM) prevented the TPA and the mezerein effect, confirming that increased proton translocation was due to PKC stimulation.

3.3. *TPA stimulates pH_i recovery in an acidic microenvironment*

To examine the potential in vivo relevance of these findings, the acidic milieu of inflammation was mimicked by permitting acid-loaded control and TPA-treated cells to recover in a low pH extracellular medium. Control cells could still recover from an acid load in acidic medium (pH 6.5). The initial recovery rate for control cells in low pH medium was 0.122 ± 0.01 pH/min and 0.26 ± 0.01 pH/min for cells treated with 200 nM TPA (Fig. 4A). The increase in pH_i was completely blocked by 200 nM bafilomycin A_1 (data not shown). The proton gradient created across the plasma membrane during the recovery phase was rapidly dissipated by subsequent ad-

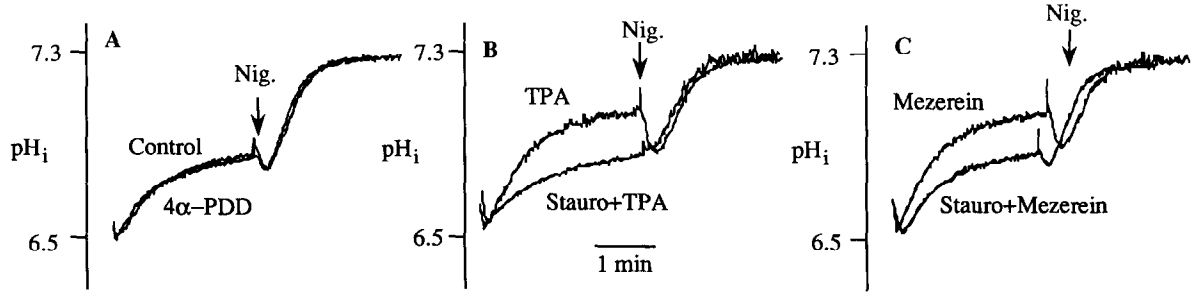

Fig. 3. Evidence for the involvement of PKC in the activation of V-ATPases. Thioglycolate-elicited macrophages, 1×10^6 cells/ml, were simultaneously loaded with 40 mM NH_4Cl and 2 μM BCECF for 20 min at 37°C. Next, the cells were sedimented and resuspended in NH_4Cl and HCO_3^--free K^+-medium containing 100 μM Zn^{2+}. (A) pH_i recovery of acid-loaded control cells and cells treated during the final 3 min of the loading step with 200 nM of 4-PDD; (B) pH_i recovery of cells treated with or without 100 nM staurosporine for 20 min and then with 200 nM TPA during the final 3 min of the loading step; (C) pH_i recovery of cells treated with 100 nM staurosporine for 20 min and then with 200 nM mezerein during the final 3 min of the loading step. Traces are representative of at least three experiments.

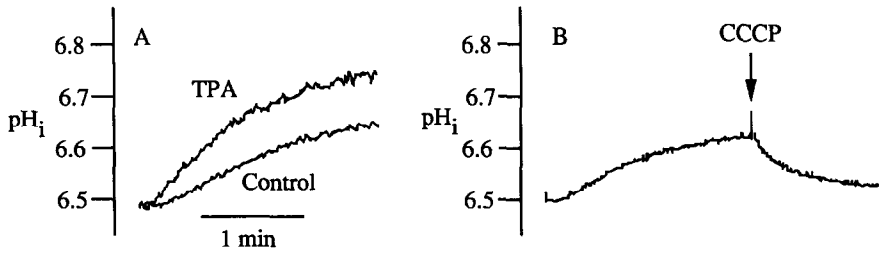

Fig. 4. Cytosolic pH recovery in acidic extracellular medium Thioglycolate-elicited macrophages, 1×10^6 cells/ml, were simultaneously loaded with 40 mM NH_4Cl and 2 μM BCECF for 20 min at 37°C. Next, the cells were sedimented and resuspended in NH_4Cl and HCO_3^--free K^+-medium, pH 6.5. (A) pH recovery of acid-loaded control cells and cells treated during the final 3 min of the loading step with 200 nM of TPA; (B) pH recovery of acid-loaded control cells. Where indicated by arrow, 1 μM CCCP is added to the cell suspension. Traces are representative of at least three experiments.

dition of 1 μM of the protonophore carbonyl cyanide m-chlorophenyl-hydrazone (CCCP) (Fig. 4B), confirming that the recovery involved translocation of H^+ against their electrochemical gradient, consistent with pumping by a V-type ATPase.

4. Discussion

The present studies demonstrate that activation of PKC augments V-ATPase-mediated proton translocation across the plasma membrane of macrophages. Several lines of evidence support this conclusion. First, two structurally distinct agonists of PKC, TPA and mezerein, increase the rate of pH_i recovery from an acute acid load, while the inactive analogue of TPA, 4-PDD, has no effect. Second, the PKC inhibitor, staurosporine, abrogates the stimulatory effects of TPA and mezerein. These data therefore suggest that the effect is mediated via stimulation of PKC. Third, the observation that bafilomycin A_1 completely inhibits the TPA-induced increase in the rate of pH_i recovery indicates that the effect of the phorbol ester was exerted on V-ATPase activity. Finally, direct measurement of proton extrusion reveals a similar bafilomycin-sensitive increase following TPA treatment, a finding consistent with the notion that the effect of PKC stimulation is on V-ATPases located in the plasma membrane.

The cellular mechanism underlying the effect of PKC activation on V-ATPase activity remains to be determined. PKC activation might exert its effect by increasing the number of pumps in the plasma membrane. The rapid time course of the effect makes it unlikely to be mediated through synthesis of new pumps. Rather, exocytic translocation of V-ATPases pre-existing in intracellular organelles may account for the increase. For example, PKC-induced phosphorylation could lead to activation of organellar motility and motor proteins such as kinesins with resultant fusion of intracellular acidic organelles with the plasma membrane [17,18]. The fact that phagocytic cells are laden with granular compartments known to exocytose in response to stimulation makes this possibility attractive. Further, this general mechanism is operative in other cell types as a means of increasing pump activity in response to various stimuli [19]. Alternatively, increased V-ATPase-mediated proton extrusion may be attributed to alterations in function of existing plasmalemmal pumps. Phosphorylation of pump subunits [20] or of recently described activator or inhibitor proteins [21,22] may serve to directly or indirectly modulate V-ATPase function. Further studies are required to distinguish these possibilities.

The possible functional relevance of H^+-pump stimulation was addressed by examining pH_i recovery in acidic medium, thus simulating one aspect of the microenvironment of inflammation. Cells exposed to PKC agonists were able to extrude protons against a concentration gradient and recover their cytosolic pH. These data thus suggest that stimulation of PKC by bacterial peptides or proinflammatory mediators might augment the activity of pH_i regulatory mechanisms and thus contribute to

Table 1
Proton extrusion rate of acid loaded peritoneal macrophages[1]

Treatment	Proton extrusion rate (nmol H^+/min/10^6 cells)
Control (DMSO)	6.50 ± 0.50
TPA (200 nM)	13.00 ± 0.81
Mezerein (200 nM)	12.60 ± 1.88
4-PDD (200 nM)	6.00 ± 0.81
Staurosporine (100 nM) + TPA (200 nM)	5.66 ± 0.47
Staurosporine (100 nM) + Mezerein (200 nM)	6.00 ± 1.26
Bafilomycin A_1 (200 nM)	3.33 ± 0.47
Bafilomycin A_1 (200 nM) + TPA (200 nM)	3.33 ± 0.94
Bafilomycin A_1 (200 nM) + Mezerein (200 nM)	3.33 ± 1.24

[1] Cells were suspended at 2×10^6/ml in lightly buffered K^+-medium and were treated with the agents indicated in the table. After a 20 min ammonium pre-pulse, changes in the extracellular pH were measured with a conventional pH electrode. Cells were treated as indicated with TPA, bafilomycin A_1 or staurosporine during the final 3, 5 or 20 min of the ammonium prepulse, respectively. The results are expressed as the mean nmol H^+ extruded per min per 10^6 cells ± S.D. ($n = 3$).

preservation of immune function by maintenance of pH_i within the physiological range.

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