Regulation of intracellular pH by phospholipase A2 and protein kinase C upon neutrophil adhesion to solid substrata

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Received 4 July 1996

Abstract Adhesion to solid substrata has been shown to increase intracellular pH (pH(i)) of fibroblasts and of other cells (FEBS Lett. (1988) 234, 449–450; Proc. Natl. Acad. Sci. USA (1989) 86, 4525–4529; J. Biol. Chem. (1990) 265, 1327–1332; Exp. Cell Res. (1992) 200, 211–214; FEBS Lett. (1995) 374, 17–20). We have found that the inhibitors of PLA2, 4-bromophenacyl bromide and manoalide, completely blocked the increase of pH(i) and spreading of neutrophils upon adhesion to solid substrata. Inhibition of phospholipase C with neomycin or removal of extracellular Ca^{2+} affects neither neutrophil spreading nor their pH(i). Inhibition of PKC with H-7 or staurosporin increased pH(i). PMA, an activator of PKC, dramatically decreased pH(i) but did not impair the spreading of neutrophils. The effect of arachidonic acid, a product of PLA2 activity, on neutrophil pH(i) and spreading was similar to that of PMA. H-7, an inhibitor of PKC, partially blocked the effect of arachidonic acid (AA) on pH(i). BW755C, an inhibitor of AA metabolism by cyclooxygenase or lipoxygenase, affected neither the pH(i) nor cell spreading. We propose that the increase of pH(i) upon neutrophil adhesion is mediated by PLA2 activity, while PKC decreased pH(i). AA produced by PLA2 activates PKC, thus forming a feedback regulation of pH(i).

Key words: Intracellular pH; Adhesion; Phospholipase A2; Protein kinase C; Arachidonic acid; Neutrophil

1. Introduction

The adhesion of neutrophils to solid substrata modulates the neutrophil sensitivity to stimulation [1] and affects their respiratory burst [2], AA metabolism [3,4], and degranulation [5].

Cell-substratum [6–8] and cell-cell [9,10] adhesive interactions have been shown to cause significant changes of pH(i). We propose that the pH(i) shift serves as a mediator between the adhesive state of the cell and various cellular responses, similar to what has been described for cell activation by growth factors, mitogens, and chemotactic peptides [11].

The increase of pH(i) upon cell stimulation by soluble agents has been shown to be regulated by an Na^+/H^+ antiporter. Na^+/H^+ antiporter activation is a step in the signal transduction pathway, which includes activation of PLC, PKC, and the increase of intracellular concentration of Ca^{2+} [11,12]. Cell adhesive interactions seem to affect pH(i) by a different signal transduction pathway: the increase of pH(i) induced by cell-cell adhesive interactions in fibroblasts can proceed in the presence of inhibitors of PLC and PKC [10].

In this present work we studied the role of PLA2, PLC and PKC in pH(i) regulation during neutrophil adhesion to solid substrata. We also checked the contribution of various H^+-extruding mechanisms to the pH(i) shifts upon adhesion. We measured pH(i) in neutrophils plated onto fibronectin or albumin coated coverslips in the presence of inhibitors of various components of signal transduction pathways, and of cell membrane H^+ extrusion.

2. Materials and methods

2.1. Materials

Bicarbonate-free Hanks' solution, *N*-ethylmaleimide, amiloride, phorbol 12-myristate 13-acetate, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (Cl-NBD), 4-bromophenacyl bromide, neomycin, nigericin, arachidonic acid, and lysophosphatidylcholine were purchased from Sigma. Acetomethyl ester of 2′,7′-bis(2-carboxyethyl)-5,(6)-carboxyfluorescein (BCECF) was obtained from Molecular Probes. HEPES buffer was from Fluka, and manoalide was purchased from Calbiochem.

2.2. Cells

Neutrophils were isolated from freshly drawn donor blood suspended on a bilayer gradient of Ficoll-Paque (1.077 and 1.125) [13]. The washed neutrophils were resuspended in bicarbonate-free Hanks' solution containing 10 mM HEPES, pH 7.35.

Glass coverslips were coated with fibronectin (5 mg/ml) or albumin (200 mg/ml) in 2 h incubation in Hanks' solution at room temperature, and neutrophils (5×105 cells/ml) were plated to the protein coated coverslips in Hanks' solution and incubated for 20 min at 37°C. The cells were adherent and had no contact with neighboring cells. At this concentration the cells adhered as single cells having no contact with neighboring cells.

The effectors neomycin (100 mM), BPB (10 mM), manoalide (6 mM), AA (15 mM), LPC (5 mM), PMA (150 nM), H-7 (20 mM), staurosporin (200 nM), amiloride (10 mM), Cl-NBD (100 mM), NEM (100 mM), and BW755C (20 mM) were added in DMSO to the cells before plating, and respective amounts of DMSO were added to the control cells. The concentration of DMSO did not exceed 1 ml/ml.

The Na^+-free medium used for blocking of Na^+/H^+ antiporter contained 10 mM HEPES, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 0.09% glucose, 5 mM KCl plus 145 mM KCl or NaCl in the control.

2.3. pH measurement

Cells were incubated for 30 min in 5 mM of BCECF before plating and the emission at 520 nm was measured with a microfluorimeter equipped Zeiss microscope at two excitation wavelengths (430 and 490 nm) as described in [14]. Calibration was performed according to [15]. All the data presented are the mean values of pH(i) ± S.E.M. obtained from the measurements of pH(i) in 20–30 cells.

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Abbreviations: PLA2, phospholipase A2; PLC, phospholipase C; PKC, protein kinase C; Cl-NBD, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; NEM, *N*-ethylmaleimide; AA, arachidonic acid; LPC, lysophosphatidylcholine; PMA, phorbol 12-myristate 13-acetate; pH(i), intracellular pH

PII S0014-5793(96)00864-2

3. Results and discussion

Cell adhesion to solid substrata has been shown to increase the pH(i) [6–8]. The pH(i) values of adherent cells varied depending on substrata, duration of adhesive interactions, and density of cell-cell contacts. In our experiments the pH(i) of neutrophils in suspension was 6.85–6.90, similar to that reported earlier [16]. We found that pH(i) of neutrophils adherent to fibronectin coated coverslips increased to 7.30–7.35 (Fig. 1).

What factors induce the increase of pH(i) in neutrophils upon adhesion: attachment to solid substrata per se or specific interactions with extracellular matrix, such as fibronectin? We compared the pH(i) of neutrophils plated to uncoated, as well as to fibronectin or albumin coated coverslips. Similar pH(i) values were observed in neutrophils plated to fibronectin and to albumin coated surfaces (Fig. 1). Thus, it is the attachment of neutrophils to a solid surface, rather than specific interactions with fibronectin, that is responsible for the pH(i) increase. However, for neutrophils adherent to a non-coated glass surface the pH(i) was lower (Fig. 1). When neutrophils were plated onto uncoated glass in the presence of the PKC inhibitors, H-7 or staurosporin, their pH(i) rose to values typical for neutrophils adherent to fibronectin or albumin coated surfaces. These data indicate that the activation of PKC upon neutrophil interaction with a non-coated glass surface may contribute to the lowering pH(i) values.

PKC plays an important role in cell adhesion: the PKC activator PMA has been shown to increase the ability of CHO cells to adhere onto fibronectin but it alters neither the number of cell surface fibronectin receptors nor their affinity [17,18]. PKC inhibitors such a calphostin and sphingosine have been shown to block spreading of CHO and HeLa cells [18,19], in contrast, H-7 or staurosporin does not block this process [19].

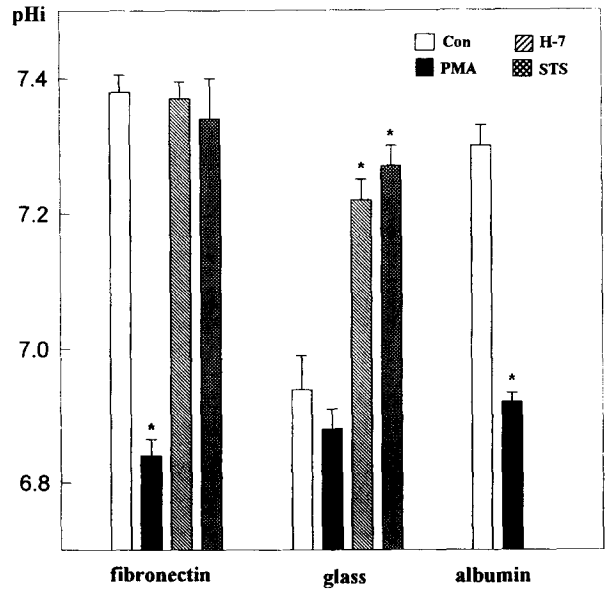

Fig. 1. Effect of PMA, H-7 and staurosporin (STS) on intracellular pH upon neutrophil adhesion to uncoated, fibronectin and albumin coated coverslips. Mean ± S.E.M. $^*P < 0.01$, when compared to the control values at the same substrata.

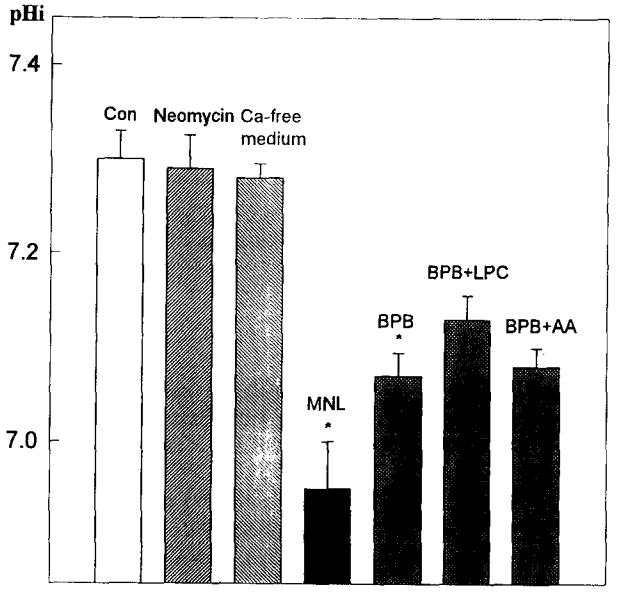

Fig. 2. Effect of neomycin, 4-bromophenacyl bromide, manoalide and Ca-free medium on the intracellular pH of neutrophils upon adhesion to fibronectin coated surface. Mean ± S.E.M. $^*P < 0.01$, when compared to the control value.

We have found that PKC decreases the pH(i) in neutrophils upon adhesion to solid substrata, but does not affect neutrophil spreading. The PKC activator PMA significantly decreased the pH(i) in neutrophils plated onto fibronectin or albumin coated coverslips (Fig. 1). The inhibitors of PKC, H-7 and staurosporin, did not affect pH(i) of neutrophils adherent to fibronectin, but increased the pH(i) of neutrophils adherent to uncoated glass. That is similar to what has been observed with PKC modulation of pH(i) in fibroblasts involved in cell-cell contact interactions [10].

It is known that the activation of PKC increases the pH(i) of neutrophils in suspension [16], and in fibroblasts, in sparse cultures [10]. In contrast, the activation of PKC decreased the pH(i) in adherent neutrophils (Fig. 1) and in fibroblasts in dense cultures, with multiple cell-cell contacts [10]. Similar dual effect of PKC was demonstrated for regulation of intracellular Ca^{2+} [20], and for the release of nitric oxide and prostacyclin [21]. The low pH(i) values resulting from PKC activation in cells having numerous cell-cell [10] or cell-solid substratum contacts (Fig. 1) may inhibit cellular responses to stimulation. That may be one of the mechanisms for contact inhibition of cellular activities and decreased sensitivity to external stimuli in high density adhered cell cultures [1,3,4,20,21]. PKC activation in cells having only a few adhesive contacts increased pH(i) [10,16] and thus facilitated cellular responses to activation.

Intracellular acidification seems to play an important role in apoptosis: decrease of pH(i) is an early event in neutrophils committed to apoptosis by deprivation of granulocyte colony-stimulation factor [22] and in lovastatin-treated HL-60 cells [23]. The PKC activation may play a key role in acidification leading to apoptosis, and it has been shown that the PKC activator PMA induces rapid killing of neutrophils [24]. PKC activation is also reported to accompany compactin (lovastatin analogue)-induced apoptosis [25].

PLA2, another key enzyme of signal transduction, has also been reported to play an important role in cell adhesion. In-

hibitors of PLA2 impair monocyte adhesion and spreading [26], and affect expression of MAC-1 receptors in neutrophils [27].

We have found that the PLA2 inhibitors, bromophenacyl bromide and manoalide, blocked neutrophil spreading and the increase of pH(i) upon adhesion to fibronectin-coated solid substrata (Figs. 2 and 3). The PLA2-mediated increase of pH(i) is independent of extracellular Ca^{2+}, and removing Ca^{2+} from extracellular medium during adhesion affected neither pH(i) (Fig. 2) nor cell spreading. Neomycin, a PLC inhibitor, also had no effect either on the pH(i) (Fig. 1) or on the spreading of neutrophils. Earlier we have shown that the increase in pH(i) induced by cell-cell contact interactions in fibroblasts could be blocked by BPB, but not by either neomycin or Ca^{2+} omission from the extracellular medium [10].

Thus, both PLA2 inhibitors and PKC activator block the adhesion-induced increase of pH(i) in neutrophils. At the same time PLA2 inhibitors completely blocked the spreading of neutrophils, while PMA had no effect (Fig. 3). As a result, similar low pH(i) values were observed in the non-spread

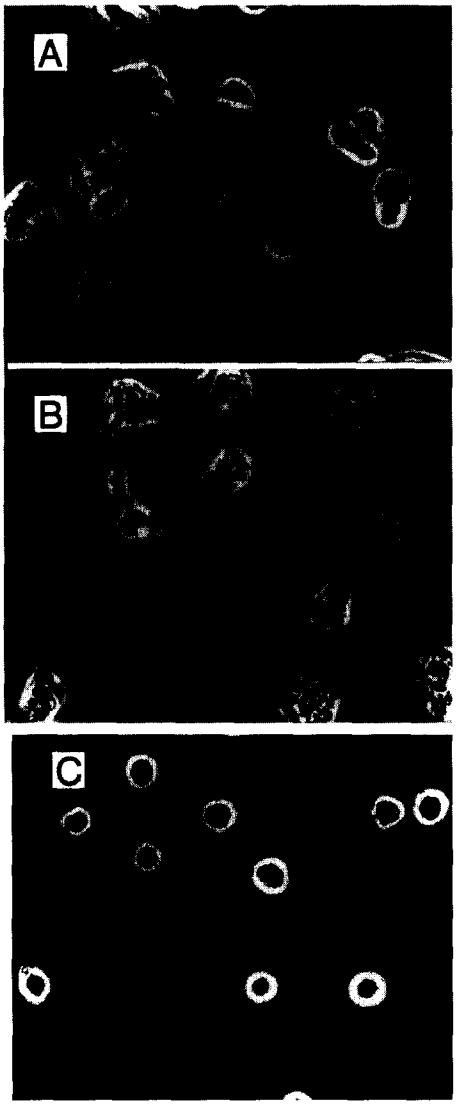

Fig. 3. Intracellular pH and spreading of neutrophils upon adhesion to fibronectin coated coverslips in the presence of PMA and BPB. A: Control cells, pH(i) 7.30 ± 0.03. B: PMA treated cells, pH(i) 6.85 ± 0.02. C: BPB treated cells, pH(i) 7.05 ± 0.02. Mean ± S.E.M.

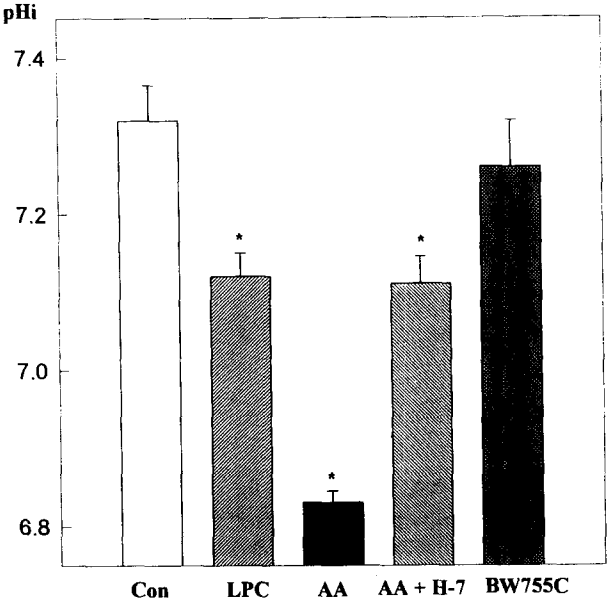

Fig. 4. Effect of lysophosphatidylcholine, arachidonic acid and BW755C on the intracellular pH of neutrophils upon adhesion to fibronectin coated coverslips. Mean ± S.E.M. $^*P < 0.01$, when compared to the control value.

PLA2-inhibited neutrophils and in the well spread PMA-treated cells. Earlier we registered the same low pH(i) values in well spread single fibroblasts and in the non-spread fibroblasts in high cell density cultures [10]. These data demonstrates that cell spreading does not depend on intracellular pH(i).

How does PLA2 affect the change of pH(i) upon adhesion? PLA2 catalyses the hydrolysis of the sn-2 fatty acyl bond of phospholipids to liberate free fatty acids and LPS. Free AA generated by PLA2 provide precursors for eicosanoids while cleavage of phospholipids containing an alkyl ether linkage in the sn-1 position results in the generation of platelet activating factor [28]. Thus, it was reasonable to check whether the products of PLA2 cleavage could affect neutrophil spreading and pH(i) shift.

LPC when added exogenously decreased the pH(i) of neutrophils during adhesion (Fig. 4), but partly restored pH(i) of neutrophils treated with the PLA2 inhibitor BPB (Fig. 2). Because of the toxicity and amphiphilic nature of lysophosphatidylcholine and other lysolipids, it was difficult to find any effect on the adhesion-induced increase of pH(i).

Exogenous AA dramatically decreased the pH(i) of neutrophils adhered to fibronectin coated coverslips (Fig. 4) but AA did not alter the pH(i) of neutrophils adhered in the presence of the PLA2 inhibitor BPB (Fig. 2). AA metabolites did not influence pH(i) regulation: inhibition of the cyclooxygenase and 5-lipoxygenase pathways of AA metabolism by BW755C did not affect pH(i) upon neutrophil adhesion (Fig. 4).

Thus, neither AA nor the products of its metabolism are responsible for the adhesion-induced increase of pH(i). Moreover, it is known that AA can activate PKC [19–21]. The effect of AA on pH(i) and on spreading of neutrophils during adhesion was similar to that of PMA and was partly reversed by PKC inhibition with H-7 (Fig. 4). Thus, AA down-regulates pH(i) upon neutrophil adhesion, probably by activating PKC.

Table 1
Intracellular pH of neutrophils, plated to fibronectin coated surface in the presence of inhibitors of H^+-extruding mechanisms

Active factor	pH(i)
Inhibitors of Na^+/H^+ antiporter	
None	7.38–0.02
Amiloride	7.22–0.02*
Na^+-containing medium	7.11–0.03
Na^+-free medium	6.97–0.03*
Inhibitors of V-type ATPase	
None	7.32–0.02
NEM	6.93–0.03*
NBD-Cl	6.79–0.02*

*$P < 0.01$, when compared to the control value.

The release of AA seems not to be involved in the PLA2-mediated increase by pH(i) of neutrophil adhesion. The functions of PLA2 which are not related to the release of fatty acids have been discussed in the literature [29–31].

Whatever the mechanisms involved in the adhesion-dependent shift of the pH(i) of neutrophils, they are different from those mediating pH(i) shift by soluble factors. The latter is mainly the result of activity of Na^+/H^+ antiporter [11,12]. Our data show that inhibition of Na^+/H^+ antiporter by amiloride only slightly influences the pH(i) of adherent neutrophils (Table 1). V-type ATPase may play a significant role in the adhesion-dependent pH(i) increase: Cl-NBD and NEM, inhibitors of V-type and of other ATPases [32], completely blocked the adhesion-induced increase of pH(i), thus making pH(i) of adherent neutrophils equal to pH(i) of neutrophils in suspension. These agents also fully blocked neutrophil spreading. However the involvement of V-type ATPase needs to be further studied since these agents are not highly specific for this ATPase.

In summary, we have shown that the increase of pH(i) upon neutrophil adhesion is mediated by PLA2 activity, while PKC activity decreases pH(i). AA produced by PLA2 activity could activate PKC, thus forming feedback regulation of pH(i).

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