The requirement of cytosolic phospholipase A₂ for the PMA activation of proton efflux through the N-terminal 230-amino-acid fragment of gp91^{phox}

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The absolute requirement for the 85 kDa cytosolic phospholipase A₂ (cPLA₂) in the PMA stimulation of proton efflux through the NADPH-oxidase-associated proton channel, has previously been demonstrated using a PLB-985 cell line deficient in cPLA₂ (PLB-D). The flux of protons in Chinese-Hamster ovary (CHO) cells that express the N-terminal 230-amino-acid (NT) fragment of gp91^{phox} is activated by arachidonic acid (AA) added externally. To investigate the physiological role of cPLA₂, and the intracellular AA that it releases, in the activation of proton flux through the NT fragment of gp91^{phox}, this fragment was stably expressed in PLB-985 cells (PLB-985 NT) and in PLB-D cells (PLB-D NT). The expression of the NT fragment of gp91^{phox} by itself in PLB-985 did not initiate differentiation and did not alter their

ability to undergo differentiation after the addition of DMSO. Addition of PMA induced a proton efflux from undifferentiated PLB-985 NT cells expressing the NT fragment of gp91^{phox}, which was inhibited by zinc. In contrast, PMA failed to activate proton efflux in undifferentiated PLB-D NT cells, lacking the expression of cPLA₂; however, addition of AA restored the efflux of protons in these cells. These results establish an essential and specific physiological requirement of cPLA₂-generated AA in the activation of proton flux through the NT fragment of gp91^{phox}.

Key words: arachidonic acid, gp91^{phox}, NADPH oxidase, phospholipase A₂, proton channel.

INTRODUCTION

The activation of neutrophils by a number of soluble and insoluble stimuli has been shown to result in the release of arachidonic acid (AA) from cellular phospholipid membranes [1,2]. The release of AA has been associated with the activation of superoxide generation by the NADPH oxidase and the efflux of protons through the associated proton channel [3–6]. The 85 kDa cytosolic phospholipase A₂ (cPLA₂) has been shown to hydrolyse phospholipids containing arachidonate at the *sn*-2 position [7,8]. To examine the role of cPLA₂ in the activation of the NADPH oxidase and proton channel, a PLB-985 cell line, in which the expression of 85 kDa cPLA₂ was suppressed by full-length antisense RNA, PLB-D, was used [9]. The absence of cPLA₂ in PLB-D cells did not alter their ability to differentiate into monocyte- or granulocyte-like cells, nor did it alter the translocation of p47^{phox} and p67^{phox}, the two cytosolic subunits of NADPH oxidase, to the membrane in response to PMA, opsonized zymosan or fMLP (*N*-formylmethionyl-leucylphenylalanine), compared with that observed for PLB-985 cells [9]. Unlike differentiated PLB-985 cells, differentiated PLB-D cells failed to generate superoxide in response to PMA, fMLP or opsonized zymosan. Similarly, PMA failed to activate an efflux of protons from PLB-D cells. However, the generation of superoxide and efflux of protons were both restored by the addition of AA to differentiated PLB-D cells, suggesting an essential role for cPLA₂, and the *in situ* release of AA, in the activation of both the NADPH oxidase and the associated proton channel in phagocyte-like cells [9,10]. Through the use of inhibitors and antisense molecules, various studies have

demonstrated that cPLA₂ is absolutely required for superoxide generation in monocytes [11–13].

The activation of the proton channel in neutrophils [4] and CHO (Chinese-hamster ovary) cells expressing either full-length gp91^{phox} [14] or the N-terminal 230-amino-acid (NT) fragment of gp91^{phox} [15] have all previously been shown to require the external addition of AA. The activation of the proton flux through the NT fragment of gp91^{phox} by intracellular signals known to activate NADPH oxidase has not previously been investigated. In the present study, we have generated two stable cell lines, PLB-985 NT and PLB-D NT, expressing the NT fragment of gp91^{phox}, in order to investigate whether AA released *in situ* by cPLA₂ is also capable of activating proton flux through the NT fragment of gp91^{phox}. A requirement for cPLA₂ in the activation of proton channels may suggest that target sites for AA that are obligatory for activation of the NADPH oxidase are located within this fragment.

MATERIALS AND METHODS

Maintenance of PLB cell lines

The PLB-985 and PLB-D cells were maintained in RPMI 1640 medium with GlutaMAX[™] I, supplemented with 10 % (v/v) foetal calf serum (FCS), 50 units/ml penicillin and 50 µg/ml streptomycin. In addition, 1 mg/ml G418 was present in all PLB-D cell-line cultures [9]. The PLB cell lines were induced to differentiate into granulocyte-like cells by the inclusion of 1.25 % (v/v) DMSO in culture medium for 5 days.

Abbreviations used: AA, arachidonic acid; BCECF-AM, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; CHO, Chinese-hamster ovary; cPLA₂, cytosolic phospholipase A₂; FCS, foetal calf serum; fMLP, *N*-formylmethionyl-leucylphenylalanine; NT fragment, N-terminal 230-amino-acid fragment; PLB-D, PLB-985 cells deficient in cPLA₂; PLB-D NT, PLB-D cells expressing the NT fragment of gp91^{phox}; PLB-985 NT, PLB-985 cells expressing the NT fragment of gp91^{phox}.

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Transfection of PLB-985 and PLB-D cells

PLB-985 or PLB-D cells (10^8 cells/ml) were transfected with 1 μ g of pMEP₄ containing the NT fragment of gp91^{phox} [14] by electroporation (220 V, 960 μ F) to generate PLB-985 NT and PLB-D NT cell lines. The cells were transferred to 20 ml of RPMI 1640, supplemented with 20 % (v/v) FCS, for 48 h. To remove dead cells, the cells were layered upon LymphoprepTM (Nycomed) and centrifuged at 800 *g* for 15 min, at room temperature (22–26 °C). The live cells were recovered from the layer on top of the LymphoprepTM, diluted to 5×10^4 cells/ml in RPMI 1640, supplemented with 10 % (v/v) FCS, 50 units/ml penicillin, 50 μ g/ml streptomycin and 200 μ g/ml Hygromycin B, and aliquots were transferred to 96-well plates at 200 μ l/well. After 14 days, the cells were placed into and maintained in flasks containing RPMI 1640, supplemented with 10 % (v/v) FCS, 50 units/ml penicillin, 50 μ g/ml streptomycin and 200 μ g/ml Hygromycin B. For PLB-D and PLB-D NT cells, the RPMI 1640 medium was additionally supplemented with 1 mg/ml G418 [9].

The induction of expression of NT gp91^{phox}, under the control of the metallothionein-inducible promoter in pMEP₄, was induced by the addition of 10 μ M Cd²⁺ to the culture medium for 16 h (overnight).

Changes in internal pH

PLB-985 NT and PLB-D NT cells were loaded with BCECF-AM [2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein, acetoxymethyl ester] by incubation with 5 μ M BCECF-AM for 10 min at 37 °C [10,16]. The cells were removed from BCECF-AM by centrifugation at 600 *g* for 10 min at room temperature. The BCECF-AM-loaded cells were resuspended at 3×10^6 cells/ml in 140 mM KCl, 5 mM Hepes and 2.7 μ M valinomycin, pH 7.5 (K⁺ medium). All measurements of pH_i were performed at 37 °C in a dual-beam spectrophotometer/fluorimeter, with excitation at 490–455 nm and emission at 520 nm. The efflux of protons was monitored as a rise in pH_i following the addition of 50 nM PMA or 50 nM PMA plus 10 μ M AA [10]. The specificity of the efflux of protons through the NADPH-oxidase-associated proton pathway was assessed as the proton efflux inhibited in the presence of 500 μ M Zn²⁺. The proton efflux through the channel was determined as the change in pH_i following activation, which could be inhibited by zinc calculated as (PMA- or PMA + AA-stimulated Δ pH_i)-(PMA- or PMA + AA-stimulated Δ pH_i in the presence of zinc).

The response of BCECF-AM was calibrated in PLB-985 NT and PLB-D NT cells suspended in K⁺ medium in the presence of 10 μ M nigericin [17,18].

Generation of superoxide

The generation of superoxide was recorded as the reduction of 100 μ M cytochrome *c* (550–540 nm) in a dual-beam spectrophotometer/fluorimeter. The generation of superoxide was monitored following the addition of 50 nM PMA. The activity of the NADPH oxidase was inhibited by the addition of 10 μ M DPI (diphenylene iodinium).

Immunoblot analysis detection of cPLA₂

The expression of cPLA₂ in both PLB-985 NT and PLB-D NT cell lysates separated on SDS/PAGE (7 % gels) was assessed as previously described in [9].

Figure 1 Generation of superoxide by undifferentiated and differentiated PLB-985 NT cells, induced to express the NT fragment of gp91^{phox}

The generation of superoxide was recorded as the reduction of cytochrome *c* (550–540 nm) from undifferentiated PLB-985 NT cells that were incubated overnight (16 h) in the presence of 10 μ M Cd²⁺ to induced to express NT gp91^{phox} (a) and PLB-985 NT cells induced to undergo differentiation by incubation in the presence of 1.25 % (v/v) DMSO for 5 days (b). PMA (50 nM), AA (10 μ M) and DPI (diphenylene iodinium; 10 μ M) were added where indicated.

RESULTS

Generation of superoxide by Cd²⁺-treated PLB-985 NT cells

The cDNA encoding the NT fragment of gp91^{phox} has been previously inserted into the plasmid pMEP₄, downstream of a metallothionein-sensitive promoter [15]. Expression from the cDNA is therefore induced by the presence of 10 μ M Cd²⁺ in the tissue-culture medium. The differentiation of PLB-985 cells into granulocyte-like cells is associated with both an increased expression of the subunits of the NADPH oxidase, including gp91^{phox}, and an increase in the generation of superoxide in response to activators of the NADPH oxidase. Differentiation of PLB-985 cells can be triggered by a number of different compounds. To determine if overnight incubation with Cd²⁺ ions is capable of stimulating the differentiation of PLB-985 NT cells and hence the expression of gp91^{phox}, the ability of undifferentiated PLB-985 NT cells to generate superoxide following incubation (16 h) in the presence of Cd²⁺ was determined. Undifferentiated PLB-985 NT cells failed to generate superoxide in response to both PMA and AA, following overnight incubation in the presence of Cd²⁺ (Figure 1a). Therefore the incubation with Cd²⁺ alone did not trigger the differentiation of PLB-985 NT over the time scale required to induce expression of the NT fragment of gp91^{phox}. However, PLB-985 NT cells grown in the presence of 1.25 % (v/v) DMSO for 5 days to induce differentiation were still capable of generating superoxide in response to PMA (Figure 1b). Therefore the presence of the NT fragment of gp91^{phox} has not disrupted the ability of the PLB-985 NT cells to differentiate.

Figure 2 Zinc-inhibited internal pH change in PLB-985 NT cells

The zinc-inhibited alterations in pH_i were determined as detailed in the Materials and methods section, for differentiated (lines a and b) and undifferentiated (lines c–f) PLB-985 NT (PLB-NT) cells, induced to express the NT fragment of gp91^{phox} by overnight (16 h) incubation in the presence (lines d and f) or absence (lines c and e) of 10 μ M Cd²⁺. PMA (50 nM) and AA (10 μ M) were added where indicated.

Proton efflux in differentiated PLB-985 cells

In the presence of an outward electrochemical gradient, an efflux of protons can be activated by the addition of external AA [4] or as a result of the activation of cPLA₂ by PMA [10]. The NADPH-oxidase-associated proton channel is inhibited by Zn²⁺ [18], therefore the proton efflux specifically through the proton channel can be determined [10]. The efflux of protons through the NADPH-oxidase-associated proton channel (the zinc-sensitive proton efflux) was determined as the proton efflux in the presence of zinc subtracted from proton efflux. The data presented for proton efflux in the present paper are those of the zinc-sensitive proton efflux.

An efflux of protons from the differentiated PLB-985 cells was stimulated following the addition of PMA (Figure 2a) as has previously been reported [10]. The addition of both PMA and AA to differentiated PLB cells showed a slightly larger proton efflux (Figure 2b) than that observed for PMA alone (Figure 2a). Therefore, as previously reported, PMA activates the proton channel in differentiated wild-type PLB-985 cells. This zinc-sensitive proton efflux shown is supported by the full-length gp91^{phox} and the other subunits of the NADPH oxidase expressed in the differentiated PLB-985 cells. The stimulated proton efflux from differentiated PLB-985 cells serves as a control for comparison with PLB-985 NT cell line.

Proton efflux from undifferentiated PLB-985 NT cells

A stable PLB-985 NT cell line was generated to determine whether the activators and intracellular signal pathways previously shown to activate proton efflux in differentiated PLB-985 cells [10] were also capable of activating proton conduction through the NT fragment of gp91^{phox}, expressed in PLB-985 cells. Under the experimental conditions previously described [10], the addition of PMA stimulated a zinc-sensitive proton efflux in undifferentiated PLB-985 NT cells, which had been pre-incubated for 16 h in the presence of Cd²⁺ to express the NT fragment of gp91^{phox} (Figure 2d). Undifferentiated PLB-985 NT cells, which were not pre-incubated with Cd²⁺ to express the NT fragment of gp91^{phox}, did not show any zinc-sensitive proton efflux in response

Figure 3 SDS/PAGE immunoblot analysis of cPLA₂ in lysates from PLB-985 NT and PLB-D NT cells

The arrow indicates the specific cPLA₂ band. Cell lysates of 8×10^6 cells were applied to each lane.

to PMA (Figure 2c). Stimulation of undifferentiated PLB-985 NT cells induced the release of ³H-labelled AA from pre-labelled cells, which was 30 % of that detected in differentiated ones (results not shown), in contrast with our previous study [9], which demonstrates that undifferentiated PLB cells did not release any AA from pre-labelled cells after stimulation. PMA + AA triggered a zinc-sensitive proton efflux from undifferentiated PLB-985 NT cells pre-incubated with Cd²⁺ (Figure 2f), which was higher than that stimulated with PMA alone (Figure 2d), but failed to trigger a response in cells not pre-incubated with Cd²⁺ (Figure 2e). The PMA-activated proton efflux from undifferentiated PLB-985 NT cells was 80 % of that observed for differentiated PLB-985 cells (Figure 2a) above. Therefore proton efflux through the NT fragment of gp91^{phox}, expressed in undifferentiated PLB-985 NT cells, is activated in a similar manner to that of the NADPH-oxidase-associated proton channel in differentiated PLB-985 cells.

Proton efflux from undifferentiated PLB-D NT cells

To investigate the role of the cPLA₂ in the activation of proton efflux through the NT fragment of gp91^{phox}, we generated a stable PLB-D NT cell line through the transfection of PLB-D cells with the cDNA encoding the NT fragment of gp91^{phox}. An immunoblot analysis demonstrating the inhibition of cPLA₂ expression in PLB-D NT cells compared with PLB-985 NT cells is shown in Figure 3.

Figure 4 Zinc-inhibited internal pH change in PLB-D NT cells

The zinc-inhibited proton efflux from differentiated (lines a and b) and undifferentiated (lines c–f) PLB-D NT cells, incubated for 16 h with $10 \mu\text{M}$ Cd^{2+} to induce the expression of the NT fragment of gp91^{phox} (lines d and f) or incubated in the absence of Cd^{2+} and therefore not expressing the NT fragment of gp91^{phox} (lines c and e). Internal pH changes in response to 50 nM PMA (lines a, c and d) or a combination of 50 nM PMA plus $10 \mu\text{M}$ AA (lines b, e and f) are shown.

Under the same conditions as those used for investigating the proton efflux from undifferentiated PLB-D NT cells, PMA failed to stimulate an efflux of protons from differentiated PLB-D cells (Figure 4a). The efflux of protons from differentiated PLB-D cells was only observed following the addition of PMA + AA (Figure 4b). Therefore the differentiated PLB-D cells retain their phenotype, that the PMA activation of proton efflux is dependent upon the expression of cPLA₂ [10].

PMA failed to stimulate an efflux of protons from either undifferentiated PLB-D NT cells (Figure 4c) or undifferentiated PLB-D NT cells pre-incubated with Cd^{2+} (Figure 4d). However, addition of PMA + AA restored an efflux of protons in undifferentiated PLB-D NT cells that had been pre-incubated with Cd^{2+} (Figure 4f). The addition of PMA + AA did not activate an efflux of protons in undifferentiated PLB-D NT cells which had not been pre-incubated with Cd^{2+} to induce expression of the NT fragment of gp91^{phox} (Figure 4e). Since PMA was capable of activating proton efflux from PLB-985 NT cells treated with Cd^{2+} (Figure 2d), the observed failure to activate proton efflux in PLB-D NT cells strongly suggests that the 85 kDa cPLA₂ is required for PMA-activation of proton efflux through the NT fragment of gp91^{phox}. Therefore the 85 kDa cPLA₂ is required for the activation of proton conduction through the NT fragment of gp91^{phox}, expressed in undifferentiated PLB-985 cells. The ability of AA to trigger proton efflux in undifferentiated PLB-D NT cells expressing the NT fragment of gp91^{phox} is in keeping with the previously reported observations that cPLA₂ is required to release AA and that AA activates the opening of the proton conductance pathway, through the NT fragment of gp91^{phox} [10].

In conclusion, the present study demonstrates that the expression of the NT fragment of gp91^{phox} in undifferentiated PLB-985 NT cells can support a zinc-sensitive efflux of protons stimulated by PMA. This proton efflux could not be stimulated in PLB-D NT cells expressing the NT fragment of gp91^{phox}, but lacking cPLA₂. However, addition of AA to the cells stimulated with PMA restored their ability to support proton efflux. Thus these results suggest an essential requirement of cPLA₂-generated AA for the stimulated proton efflux through

the NT fragment of gp91^{phox} expressed in undifferentiated PLB-985 NT cells. It has been reported previously that the zinc-sensitive efflux of protons from differentiated PLB-985 cells is activated by PMA in a cPLA₂-dependent manner [10]. The similarities in the characteristics of the activation of the proton efflux through the NT fragment of gp91^{phox} and the NADPH-oxidase-associated proton channel provide supporting evidence for the hypothesis that gp91^{phox} functions as a proton channel [14]. The role of gp91^{phox} as the phagocytic proton channel is currently an issue of debate. One of us (L. M. Henderson, with R. W. Meech) previously demonstrated that gp91^{phox} functions as a proton channel [19]; however, DeCoursey et al. [20] hold a contrasting view. This topic is the subject of a recent series of short review articles [19–22].

DISCUSSION

We have previously reported that the NADPH-oxidase-associated proton channel of neutrophils [4], proton conduction through gp91^{phox} expressed in CHO cells [14] and the NT fragment of gp91^{phox} expressed in CHO cells [15] are all activated following the external addition of AA. In the present study, we demonstrate that the N-terminal fragment of gp91^{phox} can also be activated by PMA in a more physiological system. As has previously been proposed for the PMA activation of differentiated PLB-985 cells [9,10], the present results suggest that cPLA₂ is required for activation of the proton efflux through the NT fragment of gp91^{phox} and that the mechanism by which AA opens the channel is located within these 230 amino acids. As the generation of superoxide by the NADPH oxidase [5,9,23] and the opening of the NADPH-oxidase-associated proton channel are both activated by PMA in a cPLA₂-dependent manner, it is possible that there is a common mechanism for the activation and maintenance of both. Although AA, released by cPLA₂, is the substrate for eicosanoid synthesis by cyclo-oxygenase, lipoxygenase and cytochrome P450, AA itself, and not its metabolites, activates the proton channel, since undifferentiated PLB cells release AA after stimulation, but have no capacity for eicosanoid formation (results

not shown), as opposed to differentiated ones, in accordance with undifferentiated HL-60 cells [24,25]. In addition, the activation of the proton channel and the NADPH oxidase by AA have both been reported to be unaltered by the presence of cyclo-oxygenase and lipoxygenase inhibitors. Therefore the opening of the proton channel and the activation of superoxide generation are not mediated via metabolites of these enzymes [2,6]. Recent results (R. Levy, unpublished work) show co-localization and association of cPLA₂ and NADPH oxidase in the plasma membranes after activation, which enables the regulation of the oxidase. The localization of cPLA₂ after activation of PLB cells expressing the NT fragment of gp91^{phox} is not as yet known and is under current investigation. The levels of AA release detected in these cells raise the possibility that these cells underwent some degree of differentiation and induction of oxidase components, which is not sufficient for superoxide production (Figure 1), but is sufficient for mediation of cPLA₂ to the membranes after activation. The spectral properties of the cytochrome *b*₅₅₈ haems have been reported to change from a low-spin hexa-co-ordinated haem iron to a high-spin penta-co-ordinated state, following the addition of AA [26]. Therefore an AA-induced alteration in the conformation of gp91^{phox} could account for the shift in the spectral properties of cytochrome *b*₅₅₈ and result in the activation of both the NADPH oxidase and its associated proton channel.

In conclusion, we have shown that the proton channel formed by the NT fragment of gp91^{phox} can be activated in PLB-985 cells in a similar manner to that previously described for the NADPH oxidase associated proton channel. Moreover, the model of differentiated PLB-D cells lacking any cPLA₂ expression offers a unique tool for exploring the physiological role of AA released by cPLA₂ as the activator of the proton channel *in vivo* and for the role of gp91^{phox} as the NADPH-oxidase-associated proton channel.

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