Protein kinase C- β contributes to NADPH oxidase activation in neutrophils

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We have analysed the involvement of the β isotype of the protein kinase C (PKC) family in the activation of NADPH oxidase in primary neutrophils. Using immunofluorescence and cell fractionation, PKC- β is shown to be recruited to the plasma membrane upon stimulation with phorbol ester and to the phagosomal membrane upon phagocytosis of IgG-coated particles (Fc γ -receptor stimulus). The time course of recruitment is similar to that of NADPH oxidase activation by these stimuli. The PKC- β specific inhibitor 379196 inhibits the response to PMA as well as to IgG-coated bacteria. Partial inhibition occurs

between 10 and 100 nM of inhibitor, the concentration at which PKC- β , but not other PKC isotypes, is targeted. Neutrophils isolated from a mouse that lacks PKC- β also showed an inhibition of NADPH oxidase activation by PMA and IgG-coated particles. The level of inhibition is comparable to that achieved with 379196 in human neutrophils. Thus the PKC- β isotype mediates activation of NADPH oxidase by PMA and by stimulation of Fc γ receptors in neutrophils.

Key words: Fc γ receptor, knockout, respiratory burst.

INTRODUCTION

NADPH oxidase is the major enzyme system responsible for oxygen-dependent microbial inactivation by professional phagocytes such as polymorphonuclear leucocytes (neutrophils) [1]. The enzyme is normally dormant but becomes active after stimulation of receptors such as Fc γ receptors, which are occupied during phagocytic uptake of the opsonized pathogen [2]. Activation of the NADPH oxidase can also be provoked directly by activation of the lipid-dependent serine/threonine kinase, protein kinase C (PKC), using phorbol esters [3,4]. This suggests that PKC can be a major mediator in the activation of the NADPH oxidase.

PKC is not a single enzyme but comprises a family of related isotypes [5]. Ten PKC isotypes have been described so far, which fall into three categories based on primary structure and biochemical properties: PKC- α , β , β , β and γ (classical PKCs), PKC- δ , ϵ , η and θ (novel) and PKC- ζ and ι (atypical). We and others have shown the presence of at least PKC- β , β and δ in neutrophils [6]. Using recently developed selective PKC inhibitors of the indolocarbazole and bisindolylmaleimide class, evidence has been provided for an involvement of these PKC isotypes in the stimulation of the NADPH oxidase although the contribution of the individual PKC isotypes is not known [7–10].

Two tools have recently been generated which allow for a further definition of PKC isotypes relevant to NADPH oxidase activation. Firstly, a specific PKC- β inhibitor has been developed which enables pharmacological interference with this PKC isotype in the human system [11,12]. Second, Leitges et al. targeted the gene for PKC- β in the mouse, resulting in a mouse which lacks PKC- β and provides a very specific model with which to analyse the consequences of the loss of this kinase [13]. In the present paper we show that PKC- β is activated under conditions of activation of NADPH oxidase. Furthermore, we have used the specific inhibitor and knockout mice to study the involvement of PKC- β in the activation of neutrophil NADPH oxidase. We conclude that PKC- β contributes to the activation

of NADPH oxidase in response to PMA and to Fc γ receptor activation.

EXPERIMENTAL

Materials

Antibodies to PKC- β and β were obtained from Santa Cruz. These antibodies reacted with a single band by Western blot analysis of neutrophil lysates. The PKC- β inhibitor, 379196, is an analogue of the previously described LY333531. 379196 was a gift of Dr James R. Gillig and Dr Kirk D. Ways (Eli Lilly Research Laboratories, Indianapolis, IN, U.S.A.).

Neutrophil isolation

Human neutrophils were routinely isolated from buffy coats provided by the North London Blood Transfusion Services. Buffy coats were diluted in 0.9% (w/v) NaCl and allowed to sediment in the presence of heparin (5 units/ml) and 1% dextran (final concentrations). A leucocyte-rich fraction was collected and centrifuged over a 20% volume Ficoll-Paque cushion at 1000 *g* for 10 min. The pellet was subjected to hypotonic lysis in water for 10 s, after which the suspension was adjusted to 0.9% (w/v) NaCl. Cells were then collected by centrifugation at 500 *g* for 5 min and resuspended in buffers, as specified below. To isolate neutrophils from mice, an intraperitoneal injection of 0.5 ml thioglycollate was given, mice were killed after 16 h by a lethal CO₂ dose and the peritoneal cavities were lavaged with 10 ml of sterile RPMI. Neutrophils were then collected by centrifugation and resuspended in RPMI.

Subcellular fractionation

Cells were resuspended in buffer A [10 mM sodium phosphate (pH 7.4), 140 mM NaCl, 10 mM KCl], preincubated at 37 °C and stimulated as indicated in the Figure legends. Cells were then

Abbreviation used: PKC, protein kinase C.

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immediately diluted in 10 vol of ice-cold buffer A, collected by centrifugation, and resuspended in 3 ml buffer B [10 mM Pipes (pH 7.0) 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl_2] containing 1 mM benzamidine, 25 mM NaF, 10 mM β -glycerophosphate, 2 $\mu\text{g}/\text{ml}$ aprotinin, 100 μM Tos-Lys- CH_2Cl , 1 μM pepstatin, 50 $\mu\text{g}/\text{ml}$ PMSF, 1 $\mu\text{g}/\text{ml}$ di-isopropyl phosphorofluoridate. The cell suspension was homogenized in a Dounce homogenizer by 20 strokes, sonicated for 5 s at 4 °C and centrifuged at 1000 g for 20 min at 4 °C. The supernatant was loaded onto a discontinuous gradient of 50 %, 35 % and 20 % (w/w) sucrose in buffer B, supplemented with inhibitors as above, and the gradient was centrifuged for 2 h at 160 000 g at 4 °C in a Beckman SW41 rotor. A 750 μl portion of the upper phase (cytosol) was collected, to which 250 μl of 4 $\times$ buffer C was added [where buffer C is 62.5 mM Tris/HCl (pH 6.8), 2 % SDS, 2.5 % glycerol, 1 mM β -mercaptoethanol, 0.025 % (w/v) Bromophenol Blue]. The 20/35 % interphase (plasma membrane) was collected, washed twice in ice cold buffer A, and resuspended in 200 μl of buffer C.

Cells stimulated with latex particles were fractionated in the same way except that 15 % sucrose, instead of 20 %, was used as the top layer. Latex particles were isolated from the 0/15 % interphase, washed 3 times in buffer A, and resuspended in buffer C.

Immunofluorescence

Cells were resuspended in buffer A, preincubated at 37 °C, and stimulated as indicated in the Figure legends. A 50 μl aliquot of cells were dropped into 0.5 ml of buffer A on a coverslip and allowed to settle for 5 min at 22 °C. Cells were then washed 3 times in buffer A, fixated for 60 min at 22 °C in buffer A, containing 3.0 % paraformaldehyde and 0.1 % glutaraldehyde, washed 4 times in buffer A, then permeabilized for 15 min at 22 °C in 0.5 % Triton/buffer A, and washed a further 4 times in buffer A. After blocking 2 times for 15 min in 1 mg/ml sodium borohydride/buffer A, and washing again 4 times in buffer A, cells were blocked for 2 h at 22 °C in 5 % goat serum/buffer A. Subsequently, cells were incubated for 48 h at 4 °C in the primary antibody (1:50 in 5 % goat serum/buffer A), followed by a 1 h incubation at 22 °C, and 6 washes in buffer A. After a 1 h incubation at 22 °C with the secondary antibody (1:200 in 5 % goat serum/buffer A), cells were washed 6 times in buffer A and 2 times in water. Coverslips were then blotted dry and sealed in Mowiol Mount, as described before [14]. Analysis of the cells was performed on a Leica Confocal Microscope.

NADPH oxidase assay

NADPH oxidase activity was measured using an oxygen electrode (Rank Brothers, Cambridge, U.K.). The electrode was calibrated with sodium dithionite, taking the oxygen concentration in 1 ml of water at 37 °C as 230 nmol. A 1 ml portion of neutrophils (5×10^7 cells/ml of RPMI) were preincubated for 1 min at 37 °C in the electrode chamber and stimulated as indicated in the Figure legends. To test the effect of inhibitors, cells were preincubated for 15 min at 20 °C in buffer A containing inhibitors at concentrations as indicated in Figure legends, before being placed in the electrode chamber.

RESULTS AND DISCUSSION

We confirmed the presence of PKC- β_I and β_{II} in neutrophils. In order to investigate the response of these PKC isotypes to stimulation of the cells, we performed cell fractionation and immunofluorescence assays. In non-treated cells both PKC- β_I

Figure 1 Response of PKC- β isotypes to phorbol esters

Neutrophils were stimulated with 1 $\mu\text{g}/\text{ml}$ PMA for the indicated lengths of time, and plasma membrane (A) and cytoplasmic fractions (B) were isolated by sucrose-gradient centrifugation, as described in the Experimental section. A 10 μg aliquot of protein from each fraction was analysed by SDS/9% PAGE and Western blotting using PKC- β_I (○) and β_{II} (●) specific antibodies. Immunoreactivity was visualized by ECL[®] detection (Amersham International) and quantified using NIH image software. PKC- β_I (C and E) and PKC- β_{II} (D and F) localization in control (C and D) and 1 $\mu\text{g}/\text{ml}$ PMA-stimulated (E and F) neutrophils. Cells were processed as described in the Experimental section and analysed by confocal immunofluorescence microscopy using a Cy5-coupled goat anti-rabbit secondary antibody. An inverted image is shown with PKC staining in black. Filled arrow, PKC- β_I hot spot and filamentous stain; open arrow, PKC- β membrane localization. The PKC- β_I hot spot and phorbol ester stimulated-PKC- β_I and β_{II} membrane translocation were observed in a large number of independent experiments.

and β_{II} are predominantly present in the cytosolic fraction (Figure 1). A diffuse staining was observed for both PKC isotypes (Figures 1C and 1D) indicating cytoplasmic localization, however, in addition, PKC- β_I staining was characterized by a 'hot spot' on the nucleus, from which it showed extensions into a filamentous compartment (Figure 1C). This staining is reminiscent of that observed by Kiley and Parker [15] in non-differentiated U937 cells in culture (note that the nomenclature for the PKC- β splice variants in this reference is different from the one used here). PKC- β_I and β_{II} are recruited to the plasma membrane in response to the phorbol ester, PMA (Figure 1). Recruitment to the membrane was time-dependent (Figures 1A and 1B) and occurred at 0.5 min incubation with PMA and was maximal at 2 min. Similarly, stimulation of the cells with IgG-coated latex particles led to a time-dependent recruitment of

Figure 2 Response of PKC- β isotypes to IgG-coated latex particles

(A) Cells were incubated with IgG-coated latex particles for the indicated lengths of time and fractionated as described in the Experimental section. Latex particles were washed extensively and analysed by SDS/9% PAGE and Western blotting for PKC- β_1 (○) and β_{II} (●), as described in Figure 1(A). The zero time point represents particles that were added after sonication of non-treated cells and were afterwards recovered in the same way as the phagocytosed particles. A repeat experiment showed a similar transient time-dependent phagosome-association of PKC- β_1 and β_{II} with levels of both PKC isotypes peaking at 8 min. (B) Immunofluorescence image of phagocytosed latex particles and PKC- β_1 . Particles were coated with human and mouse IgG and visualized with Cy2-coupled goat anti-mouse antibody (green). PKC- β_1 staining was visualized using Cy5-coupled goat anti-rabbit antibody (red). The same pattern was observed in multiple cells.

PKC- β_1 and β_{II} to the site of particle intake in the cell (Figures 2A and 2B). PKC- β isotypes were not recruited to the plasma membrane fraction after Fc γ -receptor activation, suggesting that recruitment occurs specifically at the site of phagocytic intake (Figure 2B). The time dependence of the redistribution of PKC- β_1 and β_{II} is similar to that of the activation of the NADPH oxidase in neutrophils (results not shown). Our observation that PKC- β is recruited to the phagosomal membrane upon Fc γ -receptor activation differs somewhat from the results of Sergeant and McPhail, who showed recruitment of PKC- β to the total plasma membrane fraction during phagocytosis [16]. These differences may be due to the fact that we used IgG-opsonized latex particles of approx. $0.82\ \mu\text{m}$ as stimulus, whereas Sergeant and McPhail used serum-opsonized zymosan particles. Most importantly, during phagocytosis PKC- β is present at the NADPH oxidase activation site in the cell, where it is perfectly situated to undertake this role. Similarly, PKC- β is present in the plasma membrane upon phorbol ester treatment. Since redistribution of PKC is a measure of its activation, we suggest that PKC- β_1 and β_{II} are active under these conditions. Thus PKC- β_1

Figure 3 Inhibition of NADPH oxidase by 379196

The rate of oxygen consumption was measured in response to $0.1\ \mu\text{g/ml}$ PMA (A) or IgG-opsonized *S. aureus* bacteria (B) after 15 min pre-incubation in the presence of the indicated concentrations of 379196. Absolute levels of oxygen consumption in the absence of inhibitor were $7 \pm 2\ \text{nmol/min/10}^7$ cells (A) and $20 \pm 4\ \text{nmol/min/10}^7$ cells (B). The final concentration of DMSO in these assays was 1%. ($n = 7$; means $\pm$ S.E.M.)

and β_{II} may play a role in the activation of the oxidase in neutrophils.

In order to test whether PKC- β activation is important for the regulation of NADPH oxidase, we measured the effect of the recently described PKC- β specific antagonist, 379196. The reported EC_{50} of this inhibitor for PKC- β inhibition *in vitro* is $30\ \text{nM}$, whereas other PKC isotypes are inhibited at concentrations of $600\ \text{nM}$ [12]. At concentrations above micromolar range other kinases may be targeted. Ishii et al. reported that the related compound, LY333531, inhibited PKC- β auto-phosphorylation in intact cells at the EC_{50} , suggesting that *in vitro* and *in vivo* inhibition by this class of compounds are comparable [11]. PMA-induced activation of NADPH oxidase is inhibited by 379196 over a large concentration range from $10\ \text{nM}$ to $10\ \mu\text{M}$ (Figure 3A). At $100\ \text{nM}$ the PMA response is inhibited by 55%, indicating that PKC- β contributes to approx. 55% of the activation. At $1\ \mu\text{M}$ the oxidase is inhibited by a further 30–40%. This is most likely due to inhibition of PKC- δ , which we and others observed to be present in neutrophils [6]. NADPH oxidase, activated by IgG-

Figure 4 Characterization of neutrophils from PKC- β $-/-$ mice

(A) FACS analysis of cell populations from PKC- β $-/-$, $+/-$ and $+/+$ litter mates obtained from the peritoneal cavity 16 h after injection with 0.5 ml of 3% thioglycollate. FACS was performed using F4/80 (Serotec, Kidlington, Oxford, U.K.) and Gr-1 (BD Pharmingen, Cowley, Oxford, U.K.) antibodies. The analysis was done blind on 20 animals with the genotype being assessed after the analysis ($+/+$, $n = 2$; $+/-$, $n = 12$; $-/-$, $n = 5$). (B) PKC- δ levels in the cells obtained in (A). A 10 μ l portion of cell homogenate of each mouse (10^6 cell equivalents) was analysed by SDS/PAGE and Western blotting using a PKC- δ antibody, and PKC- δ levels were determined as described in Figure 1(A). (C) Levels of p47^{phox} (open bars) and p67^{phox} (filled bars) in neutrophils from heterozygous and PKC- β -deficient litter mates. Neutrophils from 3 animals were pooled and samples each containing 10^6 cell equivalents were analysed by SDS gel electrophoresis and Western blotting as described in Figure 1(A) ($n = 6$; means $\pm$ S.E.M.).

opsonized *Staphylococcus aureus*, was inhibited by 45% in the range at which PKC- β is targeted; full inhibition was only observed at 10 μ M (Figure 3B). The fact that inhibition at 1 μ M did not differ from that at 100 nM suggests that PKC- β contributes to NADPH oxidase activation through this receptor and that no other PKC isotypes are involved. However, a non-PKC

Figure 5 NADPH oxidase activity in neutrophils from PKC- β $-/-$ mice

(A) NADPH oxidase activation in response to 0.1 μ g/ml PMA in neutrophils from wild-type, heterozygous and PKC- β -deficient litter mates. Neutrophils from 3 animals were pooled and oxidase activity was measured on 6×10^6 cells ($n = 3$; means $\pm$ S.E.M.). (B) NADPH oxidase activation in neutrophils from wild-type (light bars) and PKC- β -deficient (dark bars) mice in response to 0.1 μ g/ml PMA or IgG-coated latex particles. Neutrophils were pooled and NADPH oxidase was measured on 5×10^6 cells. The average of three independent experiments is shown ($n = 3$; means $\pm$ S.E.M.).

component is involved since full inhibition occurs at concentrations beyond the EC₅₀ for PKC. In this respect it is of interest that other kinases have been suggested to activate NADPH oxidase, including p21-activated protein kinase, phosphatidic acid-regulated kinases and MAP kinases (mitogen-activated protein kinases) [17–19]. Figure 3 also reveals a stimulatory effect of 379196 on oxygen consumption in the nanomolar concentration range. This may represent an action of this inhibitor on components other than PKC- β , since PKC- β is not targeted at these concentrations. Staurosporine, a related PKC inhibitor, has similar stimulatory effects on NADPH oxidase activity at concentrations below the effective inhibitory range for PKC isotypes [20].

Thus pharmacological intervention suggests that PKC- β isotypes may contribute to activation of the oxidase. To obtain more direct evidence for this mechanism we investigated NADPH oxidase activation in a mouse model system in which PKC- β had been deleted by targeted gene deletion [13]. Neutrophils were isolated from the peritoneal cavity of PKC- β -deficient mice and from heterozygous and wild-type litter mates after induction of sterile peritonitis using thioglycollate. FACS analysis, using the macrophage marker F4/80 and the mature neutrophil marker Gr-1, revealed that wild-type, heterozygous and homozygous PKC- β -deficient mice contained identical percentages of neutro-

phils in the peritoneal lavages (Figure 4A). Thus based on the Gr-1 marker, the three cell populations contain similar numbers of mature neutrophils. No differences were observed between the populations in the levels of PKC- δ , the other major PKC-isotype in neutrophils (Figure 4B). NADPH oxidase activation in these cells was measured in an oxygen-consumption assay using an oxygen electrode. PKC- β -deficient neutrophils show a 50% reduction of the oxidase response to PMA compared to cells isolated from wild-type or heterozygous animals (Figure 5A). This is not due to changes in NADPH oxidase components since the levels of p47^{phox} and p67^{phox} are the same in PKC- β -deficient neutrophils and control cells from heterozygous mice (Figure 4C). Oxygen consumption, in response to IgG-coated latex particles, was reduced to the same extent as the response to PMA in neutrophils isolated from PKC- β -deficient mice (Figure 5B). Thus PKC- β is involved in the activation of NADPH oxidase by PMA and Fc γ -receptor stimuli.

Although PKC has been implicated in the activation of NADPH oxidase in neutrophils, the evidence has largely been based on biochemical correlates and pharmacological intervention using inhibitors that are not isotype selective. Hence the contribution of the individual PKC isotypes to oxidase activation has been difficult to assess. In the present study we have addressed this using PKC- β -deficient mice and a PKC- β -specific inhibitor. We show that PKC- β contributes to approx. 50% of the stimulation induced by PMA and by IgG-coated particles. Interestingly, Korchak et al. reported a similar degree of inhibition of NADPH oxidase in HL60 cells in response to treatment with antisense oligonucleotides to PKC- β [21]. Our evidence indicates that PKC- β is involved in the activation of NADPH oxidase in primary neutrophils; however, it is clear that it is not the only component. A pathway is still operating in PKC- β -deficient mice which is sufficient to activate NADPH oxidase in the absence of PKC- β . The nature of this pathway is at present unclear and it appears dependent on the stimulus used. The PMA stimulus recruits PKC- δ and the combined PKC- β and δ recruitment appears to account for 90% of the oxidase activation. However, our pharmacological data suggest that PKC- δ is not involved in Fc γ -receptor-mediated activation. This needs to be confirmed in a knockout model. The Fc γ -receptor stimulus may involve another kinase, since at high concentrations of kinase inhibitor NADPH oxidase activation is fully inhibited.

Thus the NADPH oxidase appears to be activated in parallel by a number of kinases, and different stimuli recruit different subsets of kinases for activation of the enzyme.

This work was supported by the Wellcome Trust and the Chronic Granulomatous Disease Trust.

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