Involvement of protein kinase D in Fcγ-receptor activation of the NADPH oxidase in neutrophils

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Protein kinases involved in the activation of the NADPH oxidase by Fcγ receptors in neutrophils were studied. Of three different protein kinase C (PKC) inhibitors, Gö 6976 inhibited the NADPH oxidase completely, whereas bisindolylmaleimide I and Ro 31-8220 caused a 70–80 % inhibition. Thus a Gö 6976-sensitive, bisindolylmaleimide I/Ro 31-8220-insensitive component contributes to NADPH oxidase activation induced by Fcγ receptors. Down-regulation of PKC isotypes resulted in inhibition of Fcγ-receptor-activated NADPH oxidase, but a down-regulation-insensitive component was still present. This component was sensitive to Gö 6976, but insensitive to Ro 31-8220. It has been shown previously that protein kinase D/PKC-μ (PKD) shows this same pharmacology *in vitro*. We show that PKD is present in neutrophils and that, in contrast with PKC isotypes, PKD is not down-regulated. Therefore PKD may participate in NADPH oxidase activation. To obtain direct evidence for this we adopted an antisense approach. Antisense PKD inhibited NADPH oxidase induced by Fcγ-receptor stimulation by 50 % and the Ro 31-8220-insensitive component in the activation was inhibited by antisense PKD. *In vitro* kinase assays showed that PKD is activated by presenting IgG-opsonized particles to neutrophils. Furthermore, PKD localizes to the area of particle intake in the cell and phosphorylates two of the three cytosolic components of the NADPH oxidase, p40^{phox} and p47^{phox}. Taken together, these data indicate that Fcγ receptors engage PKD in the regulation of the NADPH oxidase.

Key words: antisense, kinase inhibitors, phagocytosis, protein kinase C, signal transduction.

INTRODUCTION

Polymorphonuclear leucocytes or 'neutrophils' constitute an important component of the white-blood-cell mass and are integrated in the body's inflammatory response to microbial invasion. Microbial inactivation by neutrophils involves phagocytosis of the pathogen and release of toxic superoxide radicals into the phagosome [1]. Superoxide is generated by the NADPH oxidase enzyme complex consisting of an electron transport chain in the membrane, which is activated by an interaction with a complex of cytosolic proteins [1]. Under normal conditions the enzyme is dormant. Receptor systems that have been shown to activate the oxidase include those for the chemotactic peptide formyl-Met-Leu-Phe ('fMLP') and Fcγ receptors, the latter being occupied during phagocytic uptake of the opsonized pathogen [2,3].

Several kinases have been proposed to play a role in the activation of the NADPH oxidase in neutrophils. p21^{rac}-activated kinase is activated under conditions of oxidase activation and phosphorylates p47^{phox}, one of the components of the NADPH oxidase [4]. In addition several phosphatidic acid-activated kinases have been shown to be involved in oxidase activation [5,6]. A major contributor to the activation is the lipid-dependent serine/threonine kinase protein kinase C (PKC), as suggested by the following evidence: (1) activation of the NADPH oxidase can be provoked by activation of PKC using phorbol esters [7–10]; (2) PKC phosphorylates critical components of the NADPH oxidase enzyme system [11–16]; (3) pharmacological evidence is consistent with PKC being involved in the activation [17–21]; (4) reconstitution experiments using membrane and cytosolic fractions indicate that PKC can activate the NADPH oxidase [22,23]; and (5) PKC-β antisense oligonucleotides inhibit the NADPH oxidase in HL60 cells [24].

PKC is not a single enzyme, but comprises a family of related isotypes [25–29]. Ten PKC isotypes have been described so far, which fall into three categories on the basis of primary structure and biochemical properties. PKC-α, -$\beta_{\rm I}$, -$\beta_{\rm II}$ and -γ (classical PKCs), PKC-δ, -ϵ, -η and -θ (novel PKCs) and PKC-ζ and -ι (atypical PKCs). All require phospholipids for activity. Furthermore the classical PKCs are stimulated by Ca^{2+} and diacylglycerol (DAG), whereas the novel PKCs respond to DAG alone, and the atypical PKCs do not respond to either Ca^{2+} or DAG [25]. Recently, a fourth group of PKC isotypes has been identified consisting of PKC-μ and -ν [30–32]. PKC-μ is the human homologue of mouse protein kinase D (PKD). PKD/PKC-μ (and by inference PKC-ν) is included in the PKC family on the basis of its regulation by DAG/phorbol ester, however it differs from other PKC family members in its catalytic domain, substrate profile and sensitivity to kinase inhibitors [33–37]. For this reason, some investigators argue that this fourth PKC subfamily represents a distinct family of kinases [38].

We are investigating the regulation of the NADPH oxidase by PKC isotypes. In a recent study [39], we employed mice genetically targeted for the PKC-β isotype and showed that PKC-β is involved in activation of the NADPH oxidase by Fcγ receptors. It also emerged that a non-PKC-β kinase is involved in the activation. The present study resulted from the observation that different classes of PKC inhibitors affect the NADPH oxidase in different ways. We hypothesized that these were a reflection of an involvement of PKD/PKC-μ (hereafter referred

Abbreviations used: BIM I, bisindolylmaleimide I; F-actin, filamentous actin; DAG, diacylglycerol; PH, pleckstrin homology; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; PKD, protein kinase D; TLCK, tosyl-lysylchloromethane.

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to as PKD) in the activation process. So far, the presence or absence of PKD in neutrophils has not been determined. We show that PKD is present in neutrophils, is activated by Fcγ-receptor stimulation and recruited to the area of particle intake. Furthermore, we provide direct evidence for the regulation of the NADPH oxidase by this kinase using antisense oligonucleotide approaches and show that components of the NADPH oxidase are substrates for PKD.

MATERIALS AND METHODS

Neutrophil isolation

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.

Staphylococcus aureus opsonization and labelling

An overnight culture of *S. aureus* in 200 ml of EZMix™ Luria–Bertani broth (Sigma, Poole, Dorset, U.K.) was incubated at 62 °C for 15 min and centrifuged at 1000 ***g*** for 25 min at 20 °C. The resulting cell pellet was resuspended in 6 ml of 50 mM Tris/HCl (pH 8.8) containing 50 mg/ml (final concentration) soluble human IgG (Instituto Grifol, Barcelona, Spain) and incubated at 37 °C for 2 h. Cells were then collected by centrifugation, washed in buffer A [10 mM NaH_2PO_4, 140 mM NaCl and 10 mM KCl (pH 7.4)], and resuspended in 20 ml of RPMI (Gibco, Paisley, Renfrewshire, Scotland, U.K.) at a concentration of 3×10^{10} cells/ml. To prepare ^{3}H-labelled *S. aureus*, cells were grown in 10 ml of Luria–Bertani broth, as above, in the presence of 250 μCi of D-[6-^{3}H]glucose (Amersham Biosciences, Little Chalfont, Bucks., U.K.). Opsonization was carried out as for non-labelled *S. aureus*, and cells were resuspended in RPMI at a final concentration of 1×10^{10} cells/ml.

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) was preincubated for 2 min at 37 °C in the electrode chamber and stimulated as indicated in the text and Figure legends. To test the effect of inhibitors, cells were preincubated for 15 min at 20 °C in RPMI containing inhibitors at concentrations as indicated in the text and Figure legends, before being placed in the electrode chamber. Details of down-regulation and antisense experiments are given in the Figure legends.

Phagocytosis assay

Phagocytosis was measured as described previously [40]. Cells (1×10^8) were placed in RPMI in a stirring chamber at 37 °C for 5 min and stimulated by adding 500 μl of ^{3}H-labelled IgG-opsonized *S. aureus*. A portion (100 μl) was taken after 2 min and added to 1 ml of ice-cold RPMI media containing 1 mM *N*-ethylmaleimide and 100 μg/ml lysostaphin. Samples were then incubated at 37 °C for 15 min, placed on ice and centrifuged at 5000 ***g*** for 4 min. A portion (500 μl) of the supernatant was mixed with Ultra Gold™ (Packard, Groningen, The Netherlands) and counted in a liquid-scintillation counter.

Antisense experiments

Phosphorothioated PKD sense (GCGATGAGCGCCCCTC-CGGTC) and antisense (GACCGGAGGGGCGCTCATC-GC) oligonucleotides were obtained from Alta Bioscience (Birmingham, U.K.). In a BLAST search, no similarity was found between these oligonucleotides and any human sequence other than PKD. Neutrophils were isolated as described above and 1×10^7 cells were incubated in 200 μl of RPMI in the presence of 10 μM oligonucleotide for 16 h at 4 °C. Cells were then diluted to 1 ml in RPMI and assayed for NADPH oxidase activity or phagocytosis. Samples for Western blotting were prepared by resuspending neutrophils in ice-cold buffer B [10 mM Pipes (pH 7.4), 100 mM KCl, 5 mM NaCl, 3.5 mM $MgCl_2$, 50 mM NaF, 10 mM benzamidine, 10 mM β-glycerophosphate, 2 μg/ml aprotinin, 1 μM pepstatin, 100 μM tosyl-lysylchloromethane (TLCK) and 100 μM leupeptin], sonicating for 10 s at maximal setting in a Soniprep 150 sonicator (MSE, Crawley, Surrey, U.K.), and homogenizing by 50 up-and-down strokes in a Dounce homogenizer (pestle A). Subsequently, a post-nuclear supernatant was prepared by centrifuging at 1000 ***g*** for 10 min at 4 °C. The post-nuclear supernatant was set to buffer C [1 × buffer C: 62.5 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 2.5 % (v/v) glycerol, 1 mM 2-mercaptoethanol and 0.025 % (w/v) Bromophenol Blue] and incubated for 10 min at 95 °C, after which the sample was analysed by SDS/PAGE.

PKD immunocomplex kinase assays and phosphorylation of recombinant *phox* proteins

Neutrophils were incubated in RPMI and stimulated with IgG-opsonized *S. aureus*. Immediately after stimulation, the cells were diluted in an ice-cold buffer A, collected by centrifugation, resuspended in buffer D [50 mM Tris/HCl (pH 7.5), 1 % (v/v) Triton X-100, 10 μg/ml aprotinin, 0.2 mM PMSF, 1 mM diisopropylfluorophosphate, 1 μg/ml pepstatin, 10 μg/ml TLCK and 'complete' inhibitors (Boehringer Mannheim) as specified] and incubated for 30 min at 4 °C. The cell lysate was cleared by centrifugation at 16000 ***g*** for 15 min. The supernatant was removed and incubated for 2 h with a PKD antiserum (SC-935; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) at a 1:100 dilution, followed by incubation with Protein A–Sepharose (Sigma) for 1 h. Immunoprecipitates were collected by centrifugation and pellets were washed three times in buffer D and three times in buffer E [30 mM Tris/HCl (pH 7.5) and 10 mM $MgCl_2$]. Pellets were resuspended in 20 μl of buffer E and assayed for kinase activity. Autokinase activity was measured by incubating the resuspended pellet with 100 μM ATP and 10 μCi of [γ-^{32}P]ATP for 10 min at 37 °C. The reaction was set to buffer C by addition of 4 × buffer C, the immunocomplexes were incubated for 10 min at 95 °C and analysed by SDS/PAGE [9 % (w/v) gel] and autoradiography. Substrate kinase activity was measured by incubating the resuspended pellet with 100 μM ATP, 4 μCi of [γ-^{32}P]ATP and 10 μg of syntide (Sigma) for 20 min at 37 °C. The reaction mixture was spotted on to Whatman P81 paper, the filter papers were washed in 30 % (v/v) acetic acid and ^{32}P incorporation into the peptide was determined.

For phosphorylation of recombinant *phox* proteins (see [41]), 55 pmol of PKD (Calbiochem, Nottingham, U.K.) was incubated in 30 μl of a solution containing 50 mM Hepes (pH 7.5), 0.25 mM

EDTA, 12.5 mM $MgCl_2$, 0.75 mM $CaCl_2$, 50 μg of phosphatidylserine, 50 ng of PMA, 100 μM ATP and 4 μCi of [γ-^{32}P]ATP with 1 μg of recombinant $p40^{phox}$, $p47^{phox}$ or $p67^{phox}$ (a gift from Dr Frans Wientjes and Dr Dongmin Shao, Centre for Molecular Medicine, University College London, London, U.K. [42]) for 15 min at 37 °C. The reaction was stopped by the addition of 10 μl of buffer C, and 20 μl of the mixture was analysed by SDS/PAGE followed by autoradiography.

Immunofluorescence

Immunofluorescence was essentially performed as described previously [39a], with the following modifications. Cells were plated on to glass or fibronectin/collagen-coated coverslips for 1 h followed by stimulation as indicated. Cells were washed and fixed in buffer A containing 4% paraformaldehyde and 0.1% glutaraldehyde for 20 min. In some cases, the cells were stimulated and fixed in solution and then placed on to the coverslips. After four washes with buffer A, cells were permeabilized for 15 min in 0.5% Triton X-100/buffer A, washed four times with buffer A and blocked twice for 15 min in 1 mg/ml sodium borohydride/buffer A and 2 h at 22 °C with 5% (v/v) goat serum/buffer A. Cells were incubated for 48 h with primary antibody [1:50 dilution in 5% (v/v) goat serum/buffer A] at 4 °C and 1 h at 23 °C. After six washes, incubation with specific FITC- or tetramethylrhodamine β-isothiocyanate ('TRITC')-labelled secondary antibody [1:200 dilution in 5% (v/v) goat serum/buffer A] was performed for 1 h at 23 °C, followed by four washes with buffer A. After four final washes with buffer A, coverslips were mounted in Mowiol Mount plus 0.1% *p*-phenylenediamine and visualized on a Leica DMRBE confocal microscope (Milton Keynes, U.K.) using the Leica TCS NT software (version 1.6.587).

Other procedures

SDS/PAGE was performed using the standard Laemmli method [43]. For Western analysis, proteins were separated by SDS/PAGE and transferred on to nitrocellulose as described previously [44]. Polyclonal antibodies against PKC-β_I (SC-209), PKC-β_{II} (SC-210), PKC-δ (SC-937) and PKD (SC-935 and SC-936) were obtained from Santa Cruz and, where indicated, the PKD antigenic peptide (SC-935p, Santa Cruz) was used in preabsorption experiments. Monoclonal anti-(PKD) antibody (#34-290) was from Calbiochem. Blots were processed as described previously [41].

RESULTS

Identification of a Gö 6976-sensitive, Ro 31-8220/bisindolylmaleimide I (BIM I)-insensitive kinase involved in Fcγ-receptor-mediated NADPH oxidase activation but not phagocytosis

The NADPH oxidase of neutrophils is the main non-mitochondrial oxygen-consuming enzyme system in neutrophils. To assay NADPH oxidase activation, we measured the rate of oxygen consumption by neutrophils in response to IgG-opsonized *S. aureus* particles (Fcγ-receptor stimulus), or in response to the phorbol ester PMA. Prior to stimulation, the baseline rate of oxygen consumption was allowed to stabilize at 0 nmol/min. Stimulation of 5×10^7 neutrophils with 3×10^9 IgG-opsonized *S. aureus* particles changed the rate to 18 ± 2 nmol/min per 10^7 cells (mean $\pm$ S.E.M.; $n = 30$), whereas addition of buffer did not have an effect (0.3 ± 0.1 nmol/min per 10^7 cells, $n = 20$).

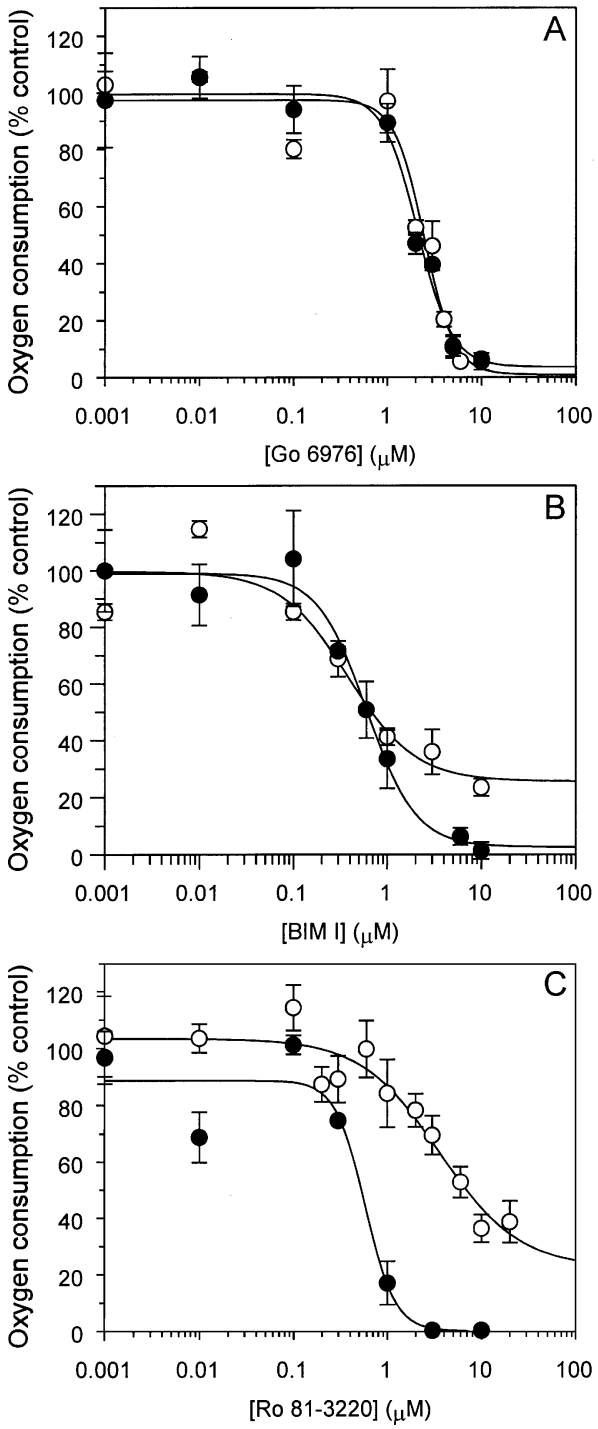

Figure 1 Effect of PKC inhibitors on oxygen consumption

Neutrophils (5×10^7 cells) were incubated with different concentrations of Gö 6976 (**A**), BIM I (**B**) or Ro 31-8220 (**C**) and stimulated either with IgG-opsonized *S. aureus* (○) or 1 μg/ml PMA (●). Oxygen consumption was measured as described in the Materials and methods section. Results are expressed as the percentage of control activity (means $\pm$ S.E.M.) obtained from incubation in the absence of inhibitors (*S. aureus* stimulation: 18 ± 2 nmol/min per 10^7 cells, $n = 30$; PMA stimulation: 26 ± 2 nmol/min per 10^7 cells, $n = 20$). Rates of oxygen consumption in the absence of a stimulus (0.3 ± 0.1 nmol/min per 10^7 cells, $n = 20$) were not affected by the inhibitors. Curve fitting was performed using GraFit software (Microsoft). Each curve fit was on the basis of 50 or more data points.

The phorbol ester PMA, at a concentration of 0.1 μg/ml, changed the rate of oxygen consumption to 26 ± 2 nmol/min per 10^7 cells ($n = 20$).

Table 1 Phagocytosis of IgG-opsonized *S. aureus*

Neutrophils were incubated with ^{3}H-labelled IgG-opsonized *S. aureus* at 37 °C, after which uptake of bacteria was measured as described in the Materials and methods section. Data are expressed as the percentage phagocytosis, which represents the fraction of ingested bacteria relative to the total number of presented bacteria. This was calculated by subtracting the amount of non-ingested *S. aureus* from the total amount of ^{3}H-labelled *S. aureus* presented to the cells. For inhibitor studies, 5×10^7 cells were preincubated for 15 min in the absence (−) or presence of 10 μM Gö 6976 or Ro 31-8220, after which phagocytosis was measured. Values represent the means ± S.E.M. of six observations. In the down-regulation study, 5×10^7 cells were pre-incubated at 37 °C in the absence (− PMA) or presence (+ PMA) of 0.1 μg/ml PMA for 4 h, after which phagocytosis was measured. Values represent the means ± S.E.M. of 10 observations. In the antisense study, 1×10^7 cells were incubated for 16 h at 4 °C in the absence (− oligo) or presence of 10 μM sense (+ sense PKD) or antisense (+ antisense PKD) PKD oligonucleotide, after which phagocytosis was measured. Values represent the means ± S.E.M. of six observations.

Treatment	Phagocytosis (%)
−	68 ± 4
Gö 6976	72 ± 3
Ro 31-8220	72 ± 1
− PMA	69 ± 1
+ PMA	64 ± 3
− oligo*	38 ± 6
+ sense PKD*	37 ± 3
+ antisense PKD*	45 ± 5

* A different cell number was used in the antisense experiments.

Three different PKC inhibitors were tested for their ability to inhibit NADPH oxidase activated by IgG-opsonized *S. aureus*, and a comparison was made with NADPH oxidase activated by PMA. The indolocarbazole Gö 6976, an inhibitor quoted to be specific for Ca^{2+}-dependent PKC isotypes [45], inhibited NADPH oxidase activated by both stimuli with comparable efficacy ($IC_{50} = 2.4 \pm 0.41$ μM and 2.1 ± 0.27 μM for *S. aureus* and PMA respectively; error represents the standard error of the Fit obtained using the GraFit Software) (Figure 1A). Full inhibition was observed at high concentrations of Gö 6976. Bisindolylmaleimide I (BIM I), which inhibits all PKC isotypes [46], also inhibited NADPH oxidase activation by both stimuli ($IC_{50} = 380 \pm 24$ nM and 602 ± 92 nM for *S. aureus* and PMA respectively) (Figure 1B). Interestingly, PMA-activated NADPH oxidase was completely inhibited at high BIM I concentrations, whereas the activation induced by IgG-opsonized *S. aureus* was inhibited by only 70 % (Figure 1B). The inhibitor Ro 31-8220, a related bisindolylmaleimide compound, also completely inhibited the PMA-stimulated activity and partially inhibited the Fcγ-receptor-stimulated activity (Figure 1C). The IC_{50} values were 580 ± 22 nM and 3.6 ± 2.6 μM respectively. None of the inhibitors affected the rate of oxygen consumption in the absence of a stimulus.

The inhibition of NADPH oxidase activity by PKC inhibitors could be at the level of the oxidase itself or through an effect on particle intake. In order to differentiate between these possibilities, we measured the effects of the three inhibitors on phagocytosis of ^{3}H-labelled IgG-opsonized *S. aureus*. None of the three inhibitors affected phagocytosis, even at the 10 μM concentration that resulted in profound inhibition of the NADPH oxidase activity (Table 1). The lack of inhibition was not due to the inability to measure phagocytosis in our assay, since the phosphoinositide 3-kinase (PI 3-kinase) inhibitor wortmannin, which is known to inhibit phagocytosis [47,48], inhibited uptake of IgG-opsonized *S. aureus*.

Down-regulation of PKC unmasks the Gö 6976-sensitive/Ro 31 8220-insensitive NADPH oxidase activation component

Down-regulation of PKC occurs after prolonged treatment with phorbol ester. Figure 2(A) shows that incubation of neutrophils at 37 °C for 4 h in the presence of the phorbol ester PMA resulted in depletion of PKC-β_I, -β_{II} and -δ (the isotypes we observed to be present in neutrophils). This confirms that, under these conditions, down-regulation of PKC isotypes occurred in our system. Concomitant with the down-regulation of PKC isotypes, NADPH oxidase activation in response to acute PMA treatment was completely depleted (Figure 2B). Cells preincubated for 4 h in the absence of PMA, in which PKC down-regulation had not occurred, retained their response to the acute PMA stimulus (Figure 2B). The loss of NADPH oxidase activation in PMA-pretreated cells was not due to an effect of the pretreatment on the basal rates of oxygen consumption, which for cells preincubated with PMA was 0.1 ± 0.1 nmol/min per 10^7

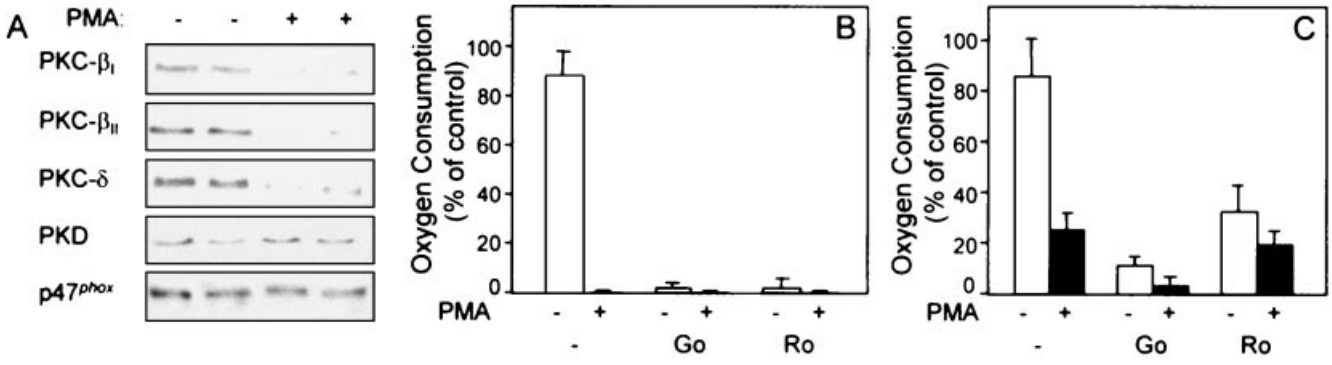

Figure 2 Effect of PKC down-regulation on oxygen consumption

Neutrophils (5×10^7 cells) were preincubated in the absence (−) or presence (+) of 0.1 μg/ml PMA for 4 h at 37 °C. (**A**) After 4 h, cells were extracted in buffer C containing 2 μg/ml aprotinin, 1 μM pepstatin, 100 μM TLCK and 100 μM leupeptin, and analysed by SDS/PAGE and Western blotting for the presence of PKC-β_I, PKC-β_{II}, PKC-δ, PKD or p47^{phox}. A representative example of three independent experiments is shown. (**B** and **C**) After 4 h, the oxygen consumption was measured in response to PMA (**B**) or IgG-opsonized *S. aureus* (**C**) in the absence or in the presence of 10 μM of Gö 6976 (Go) or Ro 30-8220 (Ro) as indicated. Oxygen consumption is expressed as the percentage of the activity at the start of the 4 h incubation (PMA stimulation: 26 ± 5 nmol/min per 10^7 cells, $n = 11$; IgG-opsonized *S. aureus* stimulation: 18 ± 3 nmol/min per 10^7 cells, $n = 15$). Results in (**B**) and (**C**) represent means ± S.E.M. of 11–15 observations (− conditions) or six observations (Go and Ro conditions), obtained in at least three independent experiments.

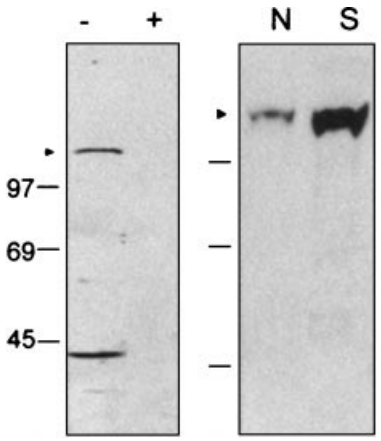

Figure 3 Identification of PKD in neutrophils

Western blots of neutrophil extracts were incubated with the antibody SC-935 in the absence (right panel and — lane, left panel) or presence (+) of the appropriate antigenic peptide SC-935p. A complete neutrophil extract (N; prepared as in Figure 2A) was used. The position of the 110 kDa-immunoreactive PKD band (arrowheads) is shown. The position of molecular-mass markers (in kDa) is indicated on the left. N, neutrophil extract; S, PKD standard (539671; Calbiochem). The 110 kDa-competed band was observed in four experiments, using this or other batches of antibody.

cells ($n = 10$) and for cells preincubated without PMA 0.2 ± 0.1 nmol/min per 10^7 cells ($n = 10$). The 4 h preincubation period itself did not change the inhibition characteristics of the oxygen consumption; PMA-induced oxygen consumption, observed in cells preincubated for 4 h in the absence of PMA, was completely inhibited by both Gö 6976 and Ro 31-8220, as observed previously in cells that had not been preincubated (compare Figure 2B with Figure 1).

Oxygen consumption induced by IgG-opsonized *S. aureus* was also reduced in PMA-pretreated neutrophils when compared with the response in cells incubated for 4 h in the absence of PMA (Figure 2C). Crucially, the inhibition of the response in PKC down-regulated cells was not complete, but a residual NADPH oxidase activation component was still present. This residual activity was inhibited by Gö 6976, but not by Ro 31-8220 (Figure 2C). No effect of PKC down-regulation for 4 h was observed on the phagocytosis of ^{3}H-labelled IgG-opsonized *S. aureus* under these conditions (Table 1).

It has been reported recently that the kinase PKD is inhibited by Gö 6976, but not by Ro 31-8220 [35,49,50]. This pharmacology is very similar to the one above for activation of NADPH oxidase by IgG-opsonized *S. aureus* and for the down-regulation-resistant component of the oxidase. This suggests that PKD may represent this component. We investigated if PKD is present in neutrophils. Figure 3 shows a Western blot of neutrophil extracts probed with a PKD antibody recognizing the C-terminus of PKD (SC-935; Santa Cruz Biotechnology). An immunoreactive band of approx. 110 kDa, the appropriate molecular mass for PKD, was detected and was competed with antigenic peptide. A band of approx. 45 kDa was also detected in some cases. This may represent a PKD breakdown product, since it was not observed on all occasions. The 110 kDa-immunoreactive band co-migrated with a PKD standard on SDS/PAGE, suggesting that it indeed represented PKD (Figure 3). The 110 kDa-immunoreactive band was also detected using two additional antibodies, a polyclonal, which recognizes PKD at the C-terminus (SC-936; Santa Cruz) and a monoclonal which recognizes PKD at the N-terminus (#34-290; Calbiochem) (results not shown). Altogether, these data indicate that PKD is present in neutrophils. Using the antibody SC-935 it was observed that, unlike PKC isotypes, PKD was not down-regulated with 4 h of PMA treatment (Figure 2A). Therefore PKD may represent the down-regulation resistant, Gö 6976-sensitive/Ro 31-8220-insensitive activity.

Inhibition of NADPH oxidase activation by antisense PKD

Recent reports [51–53] show that proteins can be efficiently depleted from primary neutrophils using antisense oligonucleotides. This approach has been used to establish the involvement of signal transduction intermediates in neutrophil function [51–53]. We employed antisense phosphorothioated oligonucleotides to obtain direct evidence for the regulation of NADPH oxidase by PKD, and made use of the fact that neutrophils retain their oxidative burst when incubated in RPMI, even when incubated for prolonged lengths of time. Typically, 1×10^7 cells stimulated with 3×10^9 IgG-opsonized *S. aureus* particles showed a rate of oxygen consumption of 32 ± 5 nmol/min ($n = 9$). After incubation for 16 h, the rate of oxygen consumption was 26 ± 4 nmol/min ($n = 15$). A preliminary experiment indicated that, over this period, neutrophils were able to incorporate ^{35}S-labelled methionine, suggesting they retain a capacity to turnover protein (results not shown). Neutrophils were incubated for 16 h with antisense or sense PKD oligonucleotides, after which oxygen consumption was measured. Oxygen consumption in response to Fcγ-receptor activation was reduced by 51% in cells treated with antisense oligonucleotide when compared with sense-treated cells (Figure 4A). No significant difference was observed between sense-treated cells (22 ± 3 nmol/min per 10^7 cells, $n = 15$) and non-treated cells (26 ± 4 nmol/min per 10^7 cells, $n = 15$). The basal rate of oxygen consumption (response to 'stimulation' with buffer) was 0.5 ± 0.2 nmol/min per 10^7 cells ($n = 12$), and no differences were observed between sense-treated, antisense-treated or non-treated cells. Western-blot analysis, using the two different batches of PKD antibody, showed that the levels of PKD were reduced in cells treated with antisense oligonucleotides (Figure 4C), whereas no effect was observed on PKC isotype levels (Figure 4C).

A number of reports suggest that PKD is activated by PKC isotypes, as reflected by PKD autophosphorylation or syntide phosphorylation in *in vitro* immunoprecipitate kinase assays [50,54,55]. We therefore examined the effect of antisense PKD oligonucleotides on phorbol ester-activated NADPH oxidase. No effect of antisense oligonucleotides was observed on oxygen consumption induced by 0.1 μg/ml PMA (Figure 4B).

These results show that PKD participates in the activation of the NADPH oxidase in response to Fcγ-receptor stimulation of neutrophils. Next, we investigated if PKD represented the Ro 31-8220-insensitive component in the activation, as suggested above. Ro 31-8220 partially inhibited NADPH oxidase activation by Fcγ receptor in neutrophils incubated for 16 h at 4 °C, as shown above with fresh neutrophils (compare Figure 4A with Figure 1). The Ro 31-8220-insensitive component was completely inhibited by PKD antisense oligonucleotides, but not by the sense oligonucleotides (Figure 4A). No effect of the inhibitor was observed on basal rates of oxygen consumption in the absence of a stimulus, independent of the presence of the oligonucleotide.

The stimulation of the NADPH oxidase by PKD was not caused by an effect of this kinase on phagocytosis of the IgG-opsonized bacterial particles used to activate it. Antisense oligonucleotides did not inhibit phagocytosis of ^{3}H-labelled particles, in agreement with the fact that the inhibitor Gö 6976

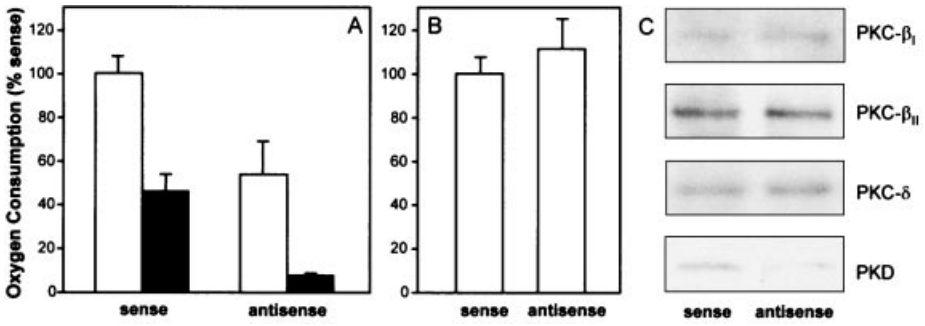

Figure 4 Inhibition of NADPH oxidase activation by antisense PKD

Neutrophils were incubated in the presence of 10 μM sense or antisense PKD oligonucleotide for 16 h at 4 °C, after which oxygen consumption in response to IgG-opsonized *S. aureus* (**A**) and 0.1 μg/ml PMA (**B**) was measured, and PKD and PKC isotype content of neutrophil extracts (**C**) was determined. In (**A**), cells were pretreated for 15 min in the absence ($n = 12$; white bars) or presence ($n = 6$; black bars) of 10 μM Ro 31-8220 prior to the oxygen consumption assay. (**A** and **B**) Results are expressed as percentage of activity (means $\pm$ S.E.M.) measured in the presence of the sense oligonucleotide (IgG-opsonized *S. aureus*: 22 ± 3 nmol/min per 10^7 cells, $n = 12$; PMA: 25 ± 7 nmol/min per 10^7 cells, $n = 7$). (**C**) Neutrophil extracts were prepared as in Figures 2(A) and 3, and PKD and PKC isotype content was assessed by Western blotting following treatment with sense and antisense oligonucleotides. Panels shown are representative of three observations.

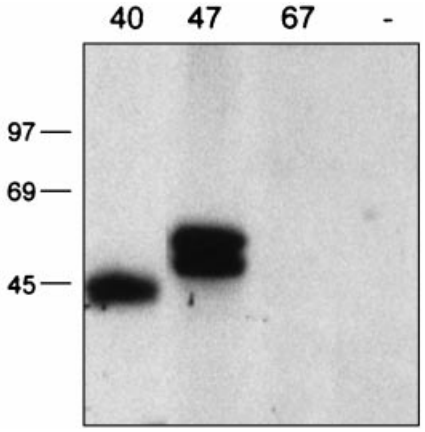

Figure 5 Phosphorylation of cytosolic NADPH oxidase components by PKD

p40^{phox} (40), p47^{phox} (47) and p67^{phox} (67) (1 μg of each) or no substrate (—) were incubated for 5 min in the presence of 55 pmol of PKD, after which the reaction was stopped and analysed by SDS/PAGE [12.5% (w/v) gels]. An autoradiograph of the gel is shown, with the position of molecular-mass markers (in kDa) indicated on the left. The result shown is representative of five (p40^{phox} and p47^{phox}) or three (p67^{phox}) observations.

did not inhibit phagocytosis (Table 1). Since phagocytosis is the same in control, sense- and antisense-treated cells, the effect of the antisense oligonucleotides on NADPH oxidase appears specific and not related to an effect on cell integrity or cell survival. Thus PKD is involved in the activation of the NADPH oxidase by Fcγ receptors.

Phosphorylation of NADPH oxidase components by PKD

The cytosolic components of the NADPH oxidase, p47^{phox}, p40^{phox} and p67^{phox}, are substrates for a number of kinases, and phosphorylation is associated with activation of the oxidase [11–16]. To investigate the mechanism by which PKD could activate the NADPH oxidase, we analysed if p47^{phox}, p67^{phox} and p40^{phox} were direct substrates of PKD. A portion (1 μg) of each of these proteins was incubated with PKD, purified as recombinant enzyme from insect cells, under conditions as described. Figure 5 shows that p47^{phox} as well as p40^{phox} were phosphorylated by PKD, whereas p67^{phox} was not phosphorylated. Thus, of the three cytosolic components, two are phosphorylated by PKD and possible targets for PKD in neutrophils.

PKD responses to Fcγ-receptor stimulation during phagocytosis

The above data suggest that the Fcγ receptor engages PKD to regulate the NADPH oxidase. In order to obtain evidence that PKD is regulated by Fcγ-receptor stimulation, we measured the response of PKD to Fcγ-receptor activation in immunocomplex kinase assays. Cell extracts were obtained from control or activated cells and we confirmed that equal amounts of PKD were present. Cell extracts were then subjected to immunoprecipitation using the SC-935 antibody and PKD kinase activity was measured employing syntide, a peptide that is phosphorylated preferentially by PKD. Syntide kinase activity in the PKD immunoprecipitate increased from 0.53 ± 0.06 to 1.53 ± 0.45 pmol/min per 10^7 cells (mean $\pm$ S.E.M.; $n = 7$, $P < 0.05$), corresponding to an Fcγ-receptor-induced PKD activity of 0.99 ± 0.43 pmol/min per 10^7 cells.

These data suggest that PKD is activated by occupation of the Fcγ receptor. We investigated this further by examining the localization of PKD by immunofluorescence microscopy. Cells were placed on a coverslip and stimulated for either 30 s or 2 min with IgG-coated *S. aureus* particles, after which the cells were fixed and processed. In control cells, PKD was present throughout the cell, but not in the nucleus, which was devoid of PKD staining (Figure 6A). Upon stimulation with IgG-coated particles, PKD immunoreactivity was observed to localize to vesicular structures, representing the 'phagosome', the internalized structure which contains the ingested particles after phagocytosis (Figure 6A). To verify the specificity of the immunoreaction, the incubation was performed in the presence of antigenic peptide and no immunoreactivity was observed in the presence of this peptide. To confirm that PKD localized to the area of particle intake, we performed co-localization experiments. Firstly, particles presented to neutrophils were co-opsonized with rhodamine-labelled mouse IgG in addition to non-labelled human IgG. Ingestion of the particle can be followed by inspecting rhodamine fluorescence and, as shown in

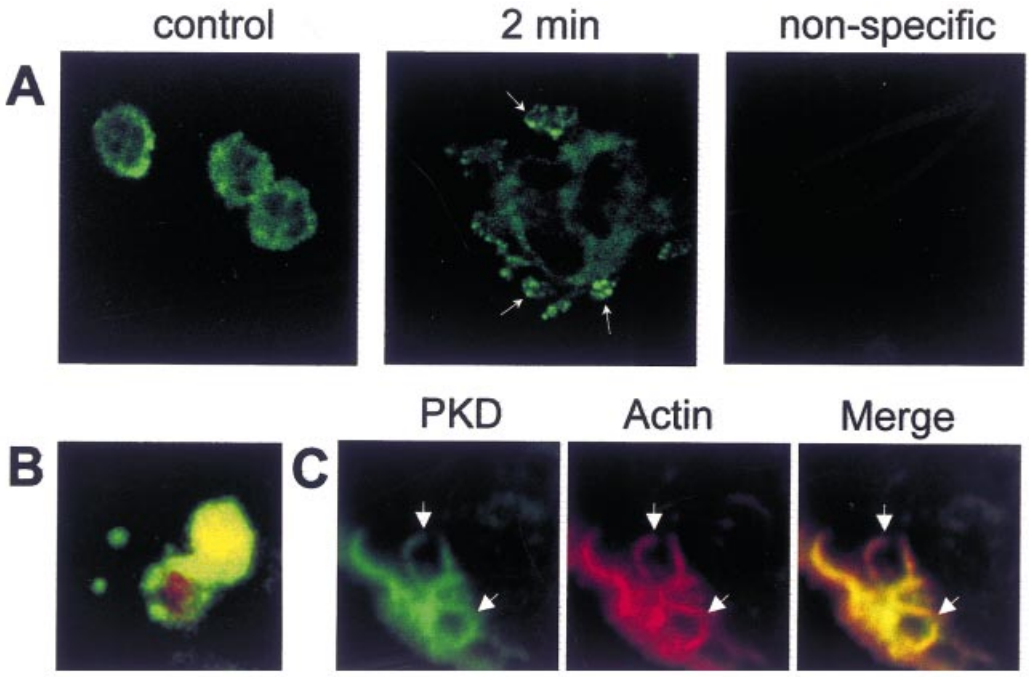

Figure 6 Localization of PKD in neutrophils

(**A**) Neutrophils were plated on to fibronectin/collagen-coated glass and incubated in the absence (control) or presence of IgG-opsonized *S. aureus* for 2 min. Cells were fixed and the localization of PKD was determined by immunofluorescence microscopy as described in the Materials and methods section. PKD immunoreactivity is shown in green. Arrowheads show the area of particle intake. Incubation of cells with PKD antibody preabsorbed with the peptide used to generate the serum (non-specific), but otherwise incubated as the control, is also shown. The results shown are representative of 40 observations (**B**) Neutrophils were treated and processed as in (**A**), except that *S. aureus* particles had been co-opsonized with human IgG and rhodamine-labelled mouse IgG. The panel shows the area of particle intake of the cell. The labelled particle is in red and PKD in green. The result shown is representative of 16 observations. (**C**) Neutrophils were treated as above, except that rhodamine-labelled phalloidin was added for 20 min during the secondary antibody incubation to detect F-actin (actin). The area of particle intake is shown, with F-actin in red and PKD in green. Arrows indicate the F-actin ring and PKD ring surrounding the particle. The results shown are representative of 16 observations.

Figure 6(B), the ingested particles were surrounded by a ring of PKD staining, suggesting PKD became recruited to the phagosomal membrane. Secondly, we employed the fact that phagocytosed particles are surrounded by a filamentous actin (F-actin) ring. This F-actin ring was clearly visible by rhodamine–phalloidin staining of the phagocytosing cell and, as shown in Figure 6(C), PKD co-localized with this F-actin ring. Thus, on the basis of these criteria, PKD localizes to the phagosomal membrane during phagocytosis and, as such, is in the right compartment to execute its effect on the NADPH oxidase.

DISCUSSION

In the present study, we have identified a component in the activation of the NADPH oxidase in neutrophils that is sensitive to the indolocarbazole Gö 6976, but not to the two bisindolylmaleimides BIM I and Ro 31-8220. This activation component was also present in neutrophils that had been depleted of their PKC content by pretreatment with phorbol ester. None of the PKC isotypes fulfil this pharmacology, since the classical PKCs are sensitive to all these treatments, whereas the novel PKCs are sensitive to down-regulation and bisindolylmaleimides. The atypical PKCs are sensitive to bisindolylmaleimides. Therefore the component is not one of the classical, novel or atypical PKC isotypes. We argued that the component may be PKD, since this kinase has been shown to have these pharmacological characteristics *in vitro* [35] and it is not down-regulated by long-term phorbol ester treatment [56]. That PKD is involved in the NADPH oxidase activation is shown convincingly by the use of PKD antisense oligonucleotides. The use of antisense oligonucleotides also indicated that PKD indeed represented the Ro 31-8220-insensitive component in the activation of the NADPH oxidase. By the same evidence, PKD is not involved in PMA-induced NADPH oxidase activation. PKD can phosphorylate components of the NADPH oxidase providing a mechanism for its action in NADPH oxidase activation. Furthermore, PKD is activated by stimulation of Fcγ receptor in neutrophils and localizes to the phagosomal membrane upon phagocytosis of particles presented to the cells.

The present results allow an evaluation of the relative contributions of PKC isotypes and PKD to NADPH oxidase activation; however, it should be noted that the Western blotting methods employed may not accurately reflect the extent of the depletion of individual components and alternative explanations may be put forward on this basis. The contribution of PKD to NADPH oxidase activation is approx. 50% on the basis of the level of inhibition by the PKD antisense oligonucleotides. A comparison with the pharmacological data suggests that not all PKD is 'Ro 31-8220-insensitive', since this component only represents 15–20% of the activation level. Thus, in neutrophils, Fcγ-receptor-stimulated PKD activation is partly via PKC (Ro 31-8220-sensitive). Regulation of PKD by PKC is compatible with evidence from the literature suggesting that this takes place in many different cell systems [50,54,55,57]. Rozengurt and co-workers [58,59] have provided evidence for a regulatory phosphorylation of PKD by PKC and, in addition, interactions

with PKC isotypes may be involved in its regulation. However, inhibition of PKD by Ro 31-8220, implying regulation by PKC, is in apparent contrast with the lack of an effect of PKD antisense oligonucleotides on phorbol ester-stimulated NADPH oxidase. It appears then that phorbol esters largely activate the oxidase directly through PKC, perhaps recruiting levels of PKC that are not normally recruited by Fcγ-receptor activation, thereby overwhelming any contribution of PKD.

Of interest is the NADPH oxidase activation component that is not sensitive to Ro 31-8220. Clearly this is regulated by PKD, since it is inhibited by the PKD antisense oligonucleotide. Exactly which mechanisms constitute the PKC-independent activation remains to be established. PKD contains a pleckstrin homology (PH) domain in its regulatory domain, which may represent a regulatory element. Mutation or deletion of the PH domain results in increased constitutive activity, suggesting that it may be important for allosteric regulation of the kinase [60]. In B-cells, the PH domain serves as a protein interaction domain, mediating the interaction with the tyrosine kinase Bruton's tyrosine kinase ('BTK') [61]. It is also a target for G-protein $\beta\gamma$ subunits and, upon interaction, PKD becomes activated [62]. However, at present no precedent exists in the literature for coupling of Fc receptors to heterotrimeric G-proteins. Generally, PH domains are targets for lipids, such as phosphatidylinositol 3,4,5-trisphosphate [63]. Since PI 3-kinase activation occurs after Fcγ-receptor activation, this pathway may participate in the regulation of PKD. However, data obtained from studies of a different tyrosine kinase receptor, the platelet-derived growth factor ('PDGF') receptor, suggest that activation of PKD occurs independently of PI 3-kinase [55].

PKD could activate the NADPH oxidase through phosphorylation of its cytosolic components $p47^{phox}$ and/or $p40^{phox}$.The consequences of these phosphorylations at the molecular level remain to be established. Localization of PKD inside the cell indicates that PKD is in a position to carry out these phosphorylations, since it associates with the phagosomal membrane during phagocytosis of opsonized particles.

Using PKC-β knockout mice and a PKC-β inhibitor, we have shown [39] that PKC-β participates in the activation of the oxidase, but that a PKC-β-independent component is also involved. It is attractive to speculate that this component may be PKD. The PKC-independent regulation of PKD suggests that parallel activation of these kinases may be required for full activation of the oxidase. It should be taken into account that our studies employed IgG-opsonized bacterial particles, which, in principle, only engage the Fcγ receptor on the cell surface. Oxidase activation by IgG-opsonized particles could be considered suboptimal when compared with activation by serum-opsonized particles [64]. It is not inconceivable that other kinases are recruited in response to the latter stimulus. It is of interest in this respect that other kinases have been implicated in the activation of the oxidase, including $p21^{rac}$-activated kinase and mitogen-activated protein kinases ('MAPK') [4]. Clearly, the exact coupling of these different kinase systems to the different classes of receptor implicated in the activation of the NADPH oxidase requires further examination.

In summary, our evidence shows that PKD is activated by Fcγ-receptor stimulation in neutrophils. Activation of PKD contributes to activation of the NADPH oxidase.

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