Lipid rafts determine efficiency of NADPH oxidase activation in neutrophils

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Abstract **We have investigated the contribution of lipid rafts to activation of the NADPH oxidase enzyme system in neutrophils. Membrane-bound NADPH oxidase subunits are present in the lipid raft compartment of neutrophils. Cytosolic NADPH oxidase components are mainly absent from but are recruited to rafts upon Fcγ receptor activation. In parallel, protein kinase C isotypes are recruited to the rafts. Kinetic analysis of NADPH oxidase activation revealed that rafts determine the onset but not the maximal rate of enzyme activity. Thus lipid rafts serve to physically juxtapose the NADPH oxidase effector, protein kinase C and Fcγ receptor, resulting in efficient coupling.**

Key words: Raft; NADPH oxidase; Neutrophil; Protein kinase C; Fcγ receptor; Methyl-β-cyclodextrin

1. Introduction

Polymorphonuclear leukocytes or 'neutrophils' constitute an important component of the white blood cell mass and are essential in the body's response to microbial invasion. Microbial inactivation by neutrophils involves phagocytosis of the pathogen and release of toxic superoxide radicals into the enclosed phagosome [1]. Superoxide is generated by the NADPH oxidase enzyme complex which under normal conditions consists of a dormant flavocytochrome in the membrane. The components of the NADPH oxidase complex have been identified as the membrane-bound gp91^{phox} and p22^{phox} (which form the flavocytochrome), the cytosolic p40^{phox}, p47^{phox} and p67^{phox}, and the small GTP-binding protein *rac*. It is now accepted that interaction of cytosolic *phox* proteins with the membrane-bound components is a major step in the activation of NADPH oxidase. This interaction occurs upon phosphorylation of oxidase components, with phosphorylation of p47^{phox} by protein kinase C (PKC) and its subsequent association with the other oxidase components, as a major activating step.

Various receptor systems have been shown to activate the oxidase including Fcγ receptors which are occupied during phagocytic uptake of the opsonised pathogen [2]. Activation in response to presentation of IgG-opsonised particles to the cell is very rapid suggesting a high efficiency of coupling of the Fcγ receptor to the oxidase. Two types of Fcγ receptor are expressed on neutrophils [3,4]. FcγRIIA (CD32) is a 40-kDa transmembrane-spanning molecule with a cytoplasmic tail that allows its interaction with intracellular signal transduction systems, whereas FcγRIIIB is a heavily glycosylated molecule of 50–70 kDa, embedded into the outer leaflet of the plasma membrane via a glycosylphosphatidylinositol (GPI) anchor. The number of molecules of FcγRIIIB on the plasma membrane is approximately 10–15-fold greater than that of FcγRIIA suggesting it would be the major receptor for IgG-opsonised particles on these cells [3]. A characteristic of GPI-anchored receptors is their association with cholesterol-rich membrane subdomains, 'lipid rafts', with a density different from the bulk of the plasma membrane [5,6]. The presence of a GPI anchor suggests that the FcγRIIIB receptor localises to lipid rafts and that lipid rafts may participate in its signal transduction mechanism. It has been proposed that lipid rafts mediate the juxtaposition of FcγRIIIB and FcγRIIA so that the generation of an intracellular response occurs through the latter receptor [7,8].

A recent proteomics study suggested the presence of lipid rafts in neutrophils and identified some of the components of these rafts, which included cytoskeletal proteins and several membrane proteins including Fc receptor proteins [9]. This confirms the general notion that these lipid compartments function as physical platforms for signal integration at the plasma membrane. We hypothesised that the oxidase itself localises to the raft compartment so that efficient receptor coupling can occur. Here we show that components of the NADPH oxidase do indeed localise to these lipid microdomains. PKC, a known signal transduction component of Fcγ receptor-mediated NADPH oxidase activation, becomes localised to the raft compartment upon cell stimulation. Furthermore, kinetic analysis of the oxidase response showed that depletion of the rafts delays the onset of NADPH oxidase activation without affecting the maximal rate of enzyme activity. Our data indicate that lipid rafts mediate the efficiency of oxidase coupling to the receptor and provide a first example of a complete signal transduction chain being assembled at this physical platform.

2. Materials and methods

2.1. 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 IU/ml) and 1% dextran (final concentrations). A leukocyte-

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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 H_2O for 10 s after which the suspension was adjusted to 0.9% NaCl (w/v). Cells were then collected by centrifugation at 500×*g* for 5 min and resuspended in buffers as specified below.

2.2. Raft isolation

Neutrophils were incubated at 4°C in buffer A (20 mM HEPES pH 7.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) for 30 min, homogenised by 20 up and down strokes in a Dounce homogeniser after which 80% sucrose stock in buffer A was added to the cell lysate to give a final concentration of 40% sucrose. The mixture was then layered *under* a sucrose step gradient (30% and 5% in buffer A) and centrifuged at 250 000×*g* for 2.5 h. Detergent-insoluble, cholesterol-rich membrane fractions, representing membrane rafts, will float to the interphase between 5% sucrose and 30% sucrose layers whilst solubilised proteins or cytoskeleton-associated, detergent-insoluble proteins remain at the bottom of the gradient. After spinning, the gradient was fractionated from top to bottom in 10 fractions.

2.3. Staphylococcus aureus *opsonisation*

An overnight culture of *S. aureus* in 200 ml EZMix™ LB Broth (Sigma) was incubated at 62°C for 15 min and centrifuged at 2500 rpm for 25 min at 20°C. The resulting cell pellet was resuspended in 6 ml 50 mM Tris–HCl pH 8.8 containing 50 mg/ml (final concentration) of soluble human IgG (Instituto Grifol, Barcelona, Spain) and incubated at 37°C for 2 h. Cells were then collected by centrifugation, washed in 10 mM NaH_2PO_4, 140 mM NaCl, 10 mM KCl, pH 7.4, and resuspended in 20 ml RPMI (Gibco, UK) at a concentration of 3×10^{10} cells/ml.

2.4. NADPH oxidase assay

NADPH oxidase activity was measured using an oxygen electrode (Rank Brothers, Cambridge, UK) [10]. The electrode was calibrated with sodium dithionite taking the amount of oxygen in 1 ml of water at 37°C as 230 nmol. One ml of neutrophils (5×10^7 cells/ml 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.

2.5. Other procedures

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using the standard Laemmli method [11]. For Western analysis, proteins were separated by SDS–PAGE and transferred onto nitrocellulose as described [12]. Antibodies were obtained from Santa Cruz ($PKC\beta_I$: SC-209; $PKC\beta_{II}$: SC-210; PKCδ: SC-937) and TransLab (flotillin, rabbit anti-human). Anti-human $gp91^{phox}$ and $p22^{phox}$ monoclonal antibodies (mAb49 and mAb448 respectively) were gifts from Prof. D. Roos (Amsterdam). Polyclonal antibodies to individual NADPH oxidase components were generated as described. Blots were processed as described [12].

3. Results and discussion

3.1. NADPH oxidase components localise to neutrophil lipid rafts

Components of the NADPH oxidase may physically localise to lipid rafts (cholesterol-rich, detergent-insoluble membrane compartments) which as such would form a platform for coupling to the activating receptor. In order to investigate this, we isolated lipid rafts from neutrophils and measured the presence of NADPH oxidase components by Western blotting. First we confirmed the presence of lipid rafts in neutrophils by analysing the behaviour of known raft markers in sucrose gradients of cell extracts from normal and raft-depleted cells. Fig. 1A shows that a low density Triton X-100-insoluble fraction can be obtained from neutrophils by sucrose gradient fractionation. The raft marker flotillin was associated with this fraction indicating that neutrophils indeed contain lipid rafts and that these are characterised by the marker flotillin. A second marker, caveolin, was absent from

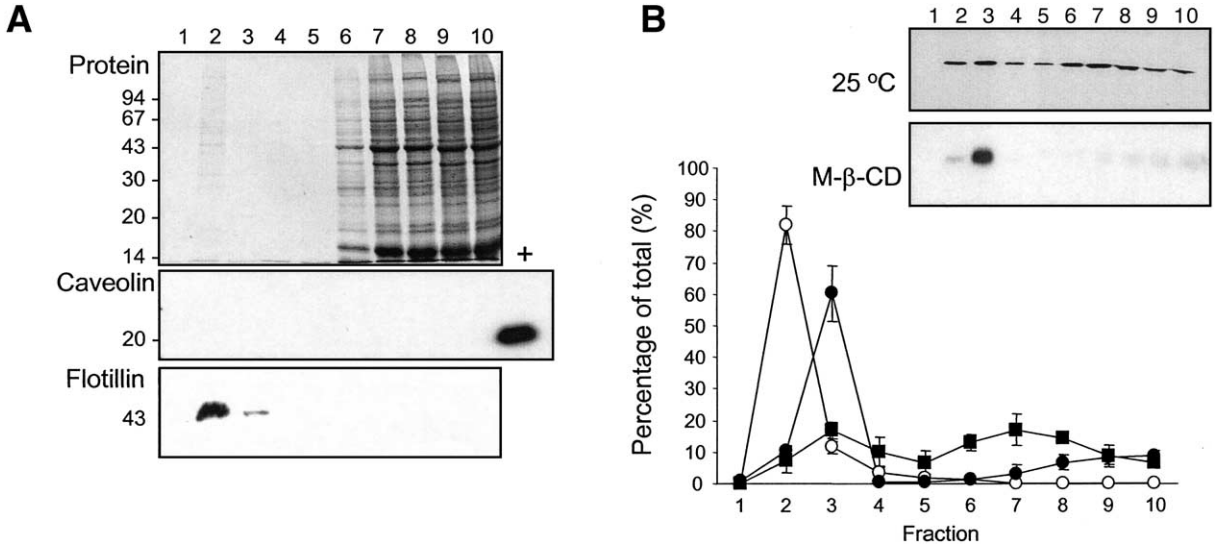

Fig. 1. Isolation and characterisation of neutrophil rafts. A: Neutrophils were homogenised at 4°C in 20 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, the homogenate was loaded onto a 5% and 30% discontinuous sucrose density gradient and centrifuged as described in Section 2. Top to bottom fractions were collected. Ten μl of each fraction was analysed by SDS–PAGE and the resulting gel was stained with Coomassie blue R-250 (upper panel), electroblotted and probed with anti-human caveolin antibody (middle panel) or anti-human flotillin monoclonal antibody (bottom panel). Five μg of total human endothelial lysate was used as positive control in the caveolin blot (middle panel lane labelled +). B: Neutrophils were fractionated as described in A except that the procedure was carried out at 25°C or at 4°C but after pretreatment of the cells with M-β-CD for 30 min. Ten μl of each fraction was resolved by SDS–PAGE, electroblotted and probed with anti-human flotillin antibody (upper panels). The distribution of flotillin was quantified from the Western blot signals using the Scion Image analysis software program (Scion Corp.; http://www.scioncorp.com). Open circles: control cells (from A); filled circles: after M-β-CD; filled squares: 25°C extraction. Data represent mean and standard error of three independent experiments.

neutrophils. In keeping with existing biochemical evidence, the integrity of neutrophil rafts critically depended upon extraction at 4°C (Fig. 1B) and changed when the cholesterol-sequestering agent methyl-β-cyclodextrin (M-β-CD) was employed (Fig. 1B). As can be seen in Fig. 1, a degree of integrity appears to be maintained even after M-β-CD treatment since a flotillin-rich light fraction can still be isolated from neutrophils. The exact nature of this fraction is unclear; however, similar cholesterol-depleted detergent-insoluble light membrane fractions have been observed in other systems. In rat adipocytes cholesterol depletion has been linked to a loss of insulin receptor signal transduction, even though a cholesterol-depleted floating fraction could still be observed [13].

Next we investigated if components of the NADPH oxidase localise to lipid rafts. The fractionation of gp91^{phox} and p22^{phox} in sucrose gradients was analysed by Western blotting using polyclonal (PEP37 for gp91^{phox} and MC2 for p22^{phox}), and monoclonal (mAb48 for gp91^{phox} and mAb449 for p22^{phox}) antibodies. In resting neutrophils, a proportion of gp91^{phox} and p22^{phox} was present in the raft fraction (Fig. 2A). Performing this experiment at 25°C, or preincubating the cells with M-β-CD, resulted in a loss of association of gp91^{phox} with this fraction confirming that gp91^{phox} localises to the lipid rafts (Fig. 2A). Quantitative densitometry of the immunoblots showed that 10 ± 1% (n = 3) of total gp91^{phox} present in the cells was present in fraction 2 and 20 ± 1% (n = 3) of total p22^{phox} (Fig. 2A). These data suggest that in resting neutrophils, the core flavocytochrome of the NADPH oxidase is present in the raft compartment of the plasma membrane. Compartmentalisation of the NADPH oxidase is consistent with data by Wientjes et al. who showed a clustered appearance of the flavocytochrome in immunoelectron microscopy studies [14].

We also assessed the distribution of the cytosolic components of the NADPH oxidase, p40^{phox}, p47^{phox} and p67^{phox}. Very low amounts of p40^{phox} (3 ± 1% (n = 3)) and p67^{phox} (5 ± 1% (n = 3)) were present in membrane rafts (Fig. 2B), however, p47^{phox} was never detected, even after overexposure of the Western blots.

3.2. Redistribution of NADPH oxidase components upon Fcγ receptor activation

Upon activation of the cells with IgG-opsonised *S. aureus* particles, the levels of NADPH oxidase components in lipid rafts increased (Figs. 2C and 3). The increase was most pronounced for p47^{phox} which was absent in rafts from control cells but was recruited to the rafts upon receptor activation. The increase in p47^{phox} was around 40-fold, given that it is below the detection threshold in rafts isolated from non-stimulated cells. Of the other components, the gp91^{phox} content in lipid rafts doubled after activation whilst the amount of p67^{phox} increased five-fold and the amount of p40^{phox} three-fold. It is well established that the association of p47^{phox} with the membrane-bound *phox* proteins is a rate-limiting determinant in NADPH oxidase activation. Consequently, membrane translocation of p47^{phox} is tightly correlated with NADPH oxidase activation. The recruitment of p47^{phox} to the raft fraction is therefore highly indicative of activation of the NADPH oxidase in this fraction. Association of p47^{phox} with the flavocytochrome may stabilise the entire complex, thus explaining the increased recovery of gp91^{phox} in the raft fraction.

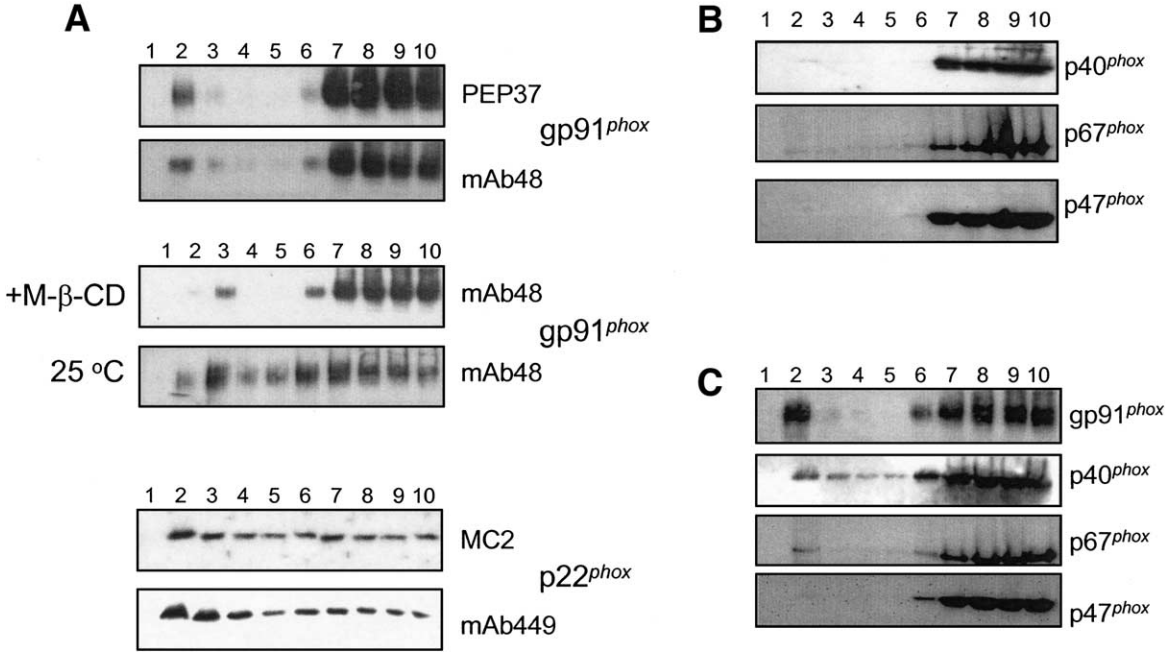

Fig. 2. Distribution of NADPH oxidase components. Neutrophils were homogenised at 4°C in 20 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and the homogenate was centrifuged in a 5% and 30% discontinuous sucrose density gradient. Fractions from top to bottom were collected. 10 μl of each fraction was analysed by SDS–PAGE. Proteins were electroblotted and probed with antibodies of NADPH oxidase components. A, upper panels: Blots were analysed using anti-human gp91^{phox} polyclonal (PEP37) and monoclonal (mAb48) antibodies (n = 3); middle panels: neutrophils pretreated with 10 mM M-β-CD or lysed with Triton X-100 at 25°C were fractionated and fractions were analysed using mAb48 antibodies (n = 2); lower panels: blots were probed with anti-human p22^{phox} polyclonal (MC2) and monoclonal (mAb449) antibodies (n = 2). B: Cytosolic components. Blots were probed with polyclonal antibodies to p40^{phox}, p47^{phox} and p67^{phox}. C: Recruitment of NADPH oxidase components to rafts upon IgG-opsonised *S. aureus* stimulation for 2 min (n = 4).

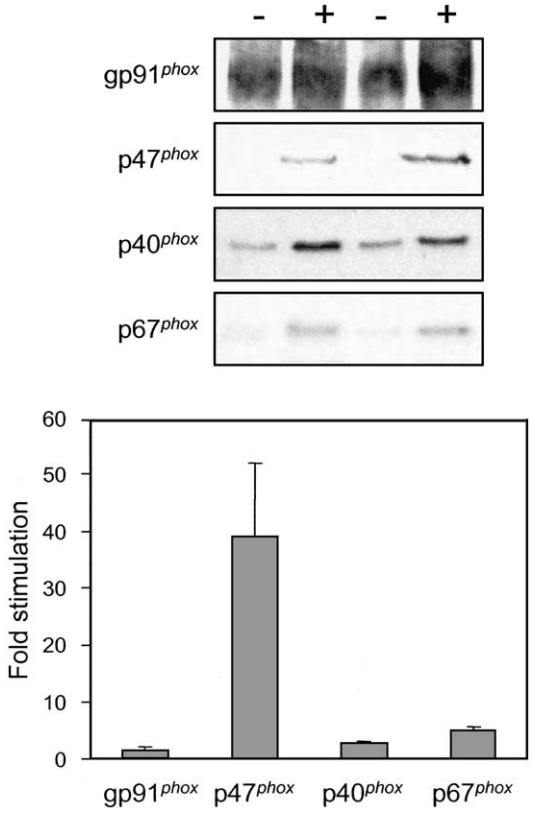

Fig. 3. Recruitment of NADPH oxidase components to lipid rafts. Neutrophils were stimulated with IgG-opsonised *S. aureus* particles or left untreated. A lipid raft fraction ('fraction 2') was obtained from the cells as described in the legend to Fig. 2 and analysed by SDS–PAGE and Western blotting using antibodies specific for each of the NADPH oxidase components. − indicates control cells, + indicates stimulated cells. Rafts isolated from two separate (control and stimulated) batches of cells were analysed. Bottom panel: quantification of the immunoreactive bands shown in the top panels. The presence of NADPH oxidase components in lipid rafts was analysed using the Scion image program (Scion Corp.; http://www.scioncorp.com) and the increase in immunoreactive signal was expressed in the bottom graph for each component. Data represent averages and spread of the two independent experiments shown in the top panels.

3.3. PKC is recruited to lipid rafts upon cell activation

Activation of the NADPH oxidase is thought to involve signalling through the PKC signal transduction pathway. PKC phosphorylates p47^{phox} which results in a conformational change that allows its interaction with the flavocytochrome. Neutrophils are known to contain several PKC isotypes [10,15] and we examined the presence of these in lipid rafts during Fcγ receptor-mediated activation of the NADPH oxidase. Fig. 4 shows that rafts prepared from control non-stimulated neutrophils did not contain PKCδ. Activation by IgG-opsonised *S. aureus* particles resulted in the recruitment of PKCδ to the raft fraction. PKCβ isotypes were not present in rafts prepared from control cells and were not recruited to this compartment upon cell activation (data not shown). Our earlier observations showed that PKCδ down-regulation results in reduction of NADPH oxidase activation by Fcγ receptors [10] and that PKCδ is recruited to the area of phagocytosis of the IgG-opsonised particle [16]. Thus PKCδ mediates the initial raft-dependent coupling of Fcγ receptors to the NADPH oxidase.

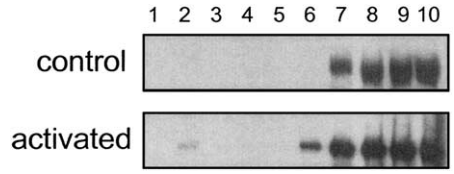

Fig. 4. PKCδ recruitment to rafts upon IgG-opsonised *S. aureus* stimulation. Neutrophils were homogenised in 20 mM HEPES, pH 7.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and the homogenate centrifuged in a 5% and 30% discontinuous sucrose density gradient. Fractions from top to bottom were collected and 10 μl of each fraction was analysed by SDS–PAGE. Proteins were transferred to nitrocellulose and the filter was probed with anti-human PKCδ antibodies ($n = 2$).

3.4. NADPH oxidase activation is affected by M-β-CD and filipin

Previous studies have shown that lipid rafts do not participate in the activation of NADPH oxidase in neutrophils in response to serum-opsonised particles (combined activation of FcγRIII, FcγRII and complement receptor 3) [8]. Nevertheless, our observations that NADPH oxidase components are localised in lipid rafts and that PKCδ is recruited to this compartment upon stimulation suggest that rafts may play a role in the activation process. The available study leaves open the possibility that lipid rafts affect the efficiency of coupling of the oxidase to the receptor, rather than the maximal rate of enzyme activity as was measured. In order to characterise the involvement of lipid rafts in NADPH oxidase activation in more detail, we measured the kinetics of NADPH oxidase

Table 1
Oxygen consumption by neutrophils stimulated with IgG-opsonised *S. aureus* particles

Pretreatment	Stimulus (particles per cell)	Onset time (min)	Time to max (min)	Max rate (nmol/min)
–	100	0.40 ± 0.01	1.30 ± 0.14	420 ± 10
M-β-CD	100	0.40 ± 0.01	1.30 ± 0.12	420 ± 10
–	50	0.53 ± 0.06	1.83 ± 0.06	307 ± 32
M-β-CD	50	1.67 ± 0.06	3.00 ± 0.15	311 ± 35
–	25	0.70 ± 0.10	2.40 ± 0.20	160 ± 10
M-β-CD	25	2.00 ± 0.17	4.00 ± 0.30	158 ± 12
–	50	0.50 ± 0.05	1.49 ± 0.04	308 ± 19
Filipin	50	1.18 ± 0.03	2.27 ± 0.09	307 ± 13

Neutrophils were pretreated with 10 mM of M-β-CD, 1 μg/ml filipin or left untreated before stimulation with IgG-opsonised *S. aureus* particles. The kinetics of oxygen consumption was recorded as a reflection of NADPH oxidase activation (Fig. 5). The delay to the initiation of the response (onset time), the time to reach maximum rate (time to max) and the maximum rate (max rate) of oxygen consumption were measured. Data are the mean ± S.E.M. of three observations. The M-β-CD and filipin experiments were performed on two different batches of neutrophils, each of which has its own non-treated control. Filipin effects were only measured for the 50 particles per cell stimulation condition.

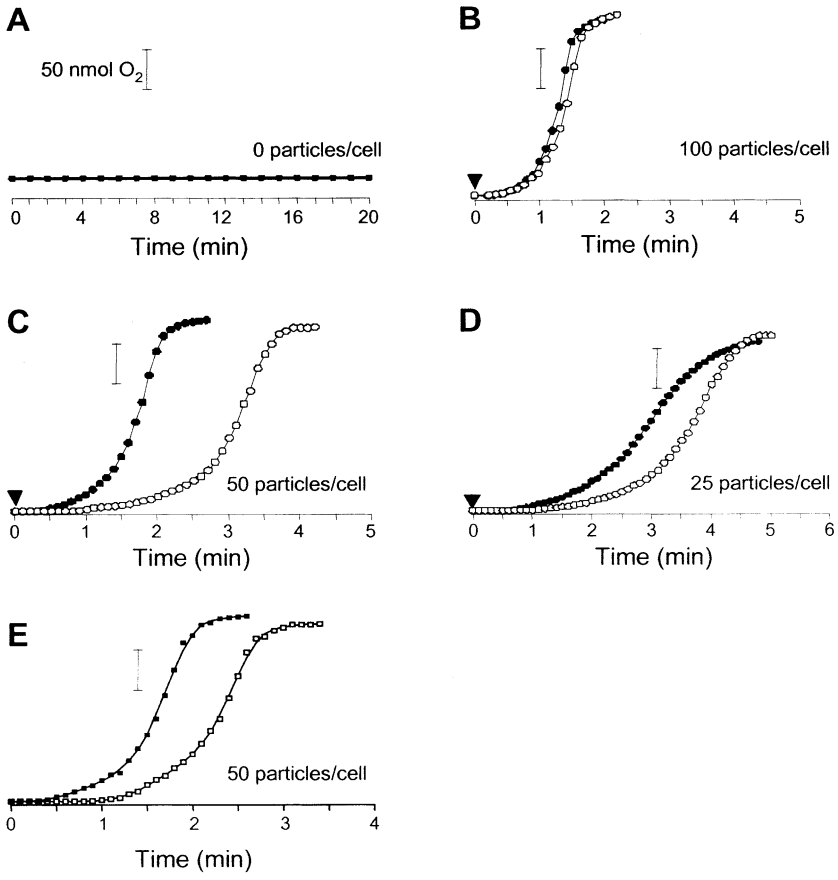

Fig. 5. Effects of M-β-CD and filipin on NADPH oxidase activation. A–D: Traces of oxygen consumption measured in neutrophils treated with 10 mM of M-β-CD (open circles) or in untreated neutrophils (filled circles). A: No stimulus. B: After stimulation with 100 IgG-opsonised *S. aureus* particles per cell. C: After stimulation with 50 IgG-opsonised *S. aureus* particles per cell. D: After stimulation with 25 IgG-opsonised *S. aureus* particles per cell. E: Traces as in C except that cells were preincubated for 30 min with 1 μg/ml filipin instead of M-β-CD. Representative traces of three independent observations are shown. A quantitative analysis of these traces is presented in Table I. Scale bars represent 50 nmol O_2.

activation upon Fcγ receptor stimulation at different stimulus intensities, under normal conditions and under raft depletion. Neutrophils were pretreated with M-β-CD for 30 min and NADPH oxidase responses to Fcγ receptor stimulation were measured by oxygen consumption. As shown in Fig. 5 and Table 1, M-β-CD did not activate NADPH oxidase itself and in keeping with the earlier study, the rate of NADPH oxidase activity was not affected by the presence of M-β-CD. However, M-β-CD significantly delayed activation of the NADPH oxidase. This delaying effect of M-β-CD was observed when lower intensity stimuli were employed (25 or 50 particles/cell) and did not occur at high stimulus intensity (100 particles/cell). Thus activation of the NADPH oxidase effector system at the raft microdomain is specifically associated with the efficient initiation of the response at low stimulus intensity. To further substantiate our observations we employed a second raft-disrupting agent, filipin, and tested its effect on activation of the NADPH oxidase in response to application of IgG-opsonised particles. In the same way as with M-β-CD, pretreatment of neutrophils with filipin did not affect the maximal rate of NADPH oxidase activation, but reduced the onset of the NADPH oxidase response and the time interval required to reach maximal rate after the application of the stimulus (Fig. 5E, Table 1). Taken together, the data indicate that lipid rafts are involved in the activation of the NADPH oxidase by Fcγ receptors. The contribution of lipid rafts to oxidase activation is subtle; under conditions of low receptor occupancy, the coupling process is improved by the physical association of the receptor and the effector in lipid rafts whereas under conditions of high receptor occupancy, no such mechanisms are required to still allow efficient receptor–effector interactions.

The NADPH oxidase enzyme system provides an ideal model to study receptor response coupling since it is activated acutely and its activity can be followed in time. Proper evaluation of the involvement of lipid rafts in its activation by Fcγ receptors has only been possible by a full kinetic analysis. Using such analysis we have shown that lipid rafts allow the Fcγ receptor, PKCδ and effector system to become juxtaposed resulting in efficient initiation of the response. We previously determined that inhibition of PKCβ and protein kinase D results in a reduced rate of NADPH oxidase activity [10,15]. It appears then that individual signal transduction intermediates fulfil a distinct requirement for oxidase activation, i.e. initial coupling through PKCδ and full activation through PKCβ and protein kinase D.

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