Volume 274, number 1,2, 93-95 FEBS 09075 November 1990

Protein kinase C controls Fcγ receptor-mediated endocytosis in human neutrophils

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Received 24 July 1990; revised version received 17 September 1990

The aim of this study is to clarify which signaling mechanism operates in Fcγ receptor-mediated endocytosis in human neutrophils. Endocytosis of immune complexes was inhibited by antibodies directed to cell membrane phospholipase C (PLC) and A_2 (PLA_2) (maximal inhibition obtained was 57% and 28%, respectively), being almost abolished by these antibodies if used in combination (up to 91% inhibition). The protein kinase C (PKC) activator, phorbol 12,13-dibutyrate, reversed this inhibitory effect. Four different PKC inhibitors (H-7, palmitoylcarnitine, sphingosine, and tamoxifen) produced a dose-dependent inhibition of endocytosis, up to over 80% in each case. H-8 (1–10 μM) which inhibits cyclic nucleotide protein kinases but not PKC had no effect upon endocytosis. It is concluded that Fcγ receptor-induced activation of PLC and PLA_2 triggers endocytosis by activation of PKC.

Protein kinase C; Endocytosis; Fcγ receptor; Phospholipase C; Phospholipase A_2; Neutrophil activation

1. INTRODUCTION

Neutrophil activation via the FcγR is a major event in host organism defense. The intracellular signals generated, which eventually produce a combination of two responses (receptor-mediated endo/phagocytosis and the so-called 'respiratory burst' with superoxide production), are not elucidated and seem rather complex and intertwined.

Several recent lines of evidence point to the multifunctional enzyme PKC, originally discovered by Nishizuka [1,2], as possibly involved in FcγR-mediated signaling [3–5], with proposed roles in triggering phagocytosis [6–8] and/or superoxide production [9–11]. However, PKC role is still unclear, the main two unresolved issues being (i) how important PKC activity is and (ii) which is(are) the pathway(s) linking the FcγR to PKC activation.

The pesent study addresses these issues in the case of FcγR-triggered endocytosis in human neutrophils showing that (i) PKC activation is crucial to triggering endocytosis and (ii) there are (at least) two different pathways of PKC activation starting from the FcγR, through activation of cell-membrane PLC and PLA_2.

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Abbreviations: FcγR, Fcγ receptor; PKC, protein kinase C; PLC, phospholipase C; PLA_2, phospholipase A_2; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; H-8, *N*-2-(methylamino)ethyl-5-isoquinolinesulfonamide

2. MATERIALS AND METHODS

2.1. *Cells*

Human neutrophils were isolated as follows: after separating monocytes and lymphocytes out of fresh blood samples from healthy volunteers (anti-HIV and HBsAg negative) as previously described [12], the resulting pellet was gently mixed with a double volume of PBS containing 0.625% polyvinylacetate and 0.2% gelatin (freshly prepared). After 75 min incubation (37°C, 100% humidity, 10% CO_2) in vertical cylinders, polymorphonuclear cells were harvested from the erythrocyte-free supernatant and washed three times.

2.2. *Immune complexes*

Two different radiolabeled immune complexes were prepared in vitro (1 h at 37°C incubation of antigen and antibody in PBS containing 0.2% gelatin; antigen at 5 μg/ml), using [^{125}I]BSA with rabbit IgG anti-BSA and [^{125}I]IgM (human, affinity-purified, polyclonal) with rabbit IgG anti-IgM. Radioactive labeling of the antigen was done according to Regoeczi [13] to yield a specific activity of $3.5\text{–}4.5 \times 10^8$ cpm/mg protein.

2.3. *Anti-phospholipase antibodies*

Monospecific anti-phospholipase C and A_2 antibodies ($F(ab')_2$ fragment of the IgG molecule) were prepared as previously described [12,14]. The antibodies dose-dependently inhibit the respective enzymes in living cells [12,14–17].

2.4. *Endocytosis assay*

Cells ($0.2\text{–}1.0 \times 10^7$/ml) were incubated in culture medium (RPMI 1640, Flow Laboratories) containing various concentrations of PKC inhibitors or of anti-phospholipase antibodies, for 15 min or 1 h, respectively. After adding the immune complexes (at a final 3×10^5 cpm/ml) for 15 min at 37°C and washing, cells were resuspended for 10 min in 20 mM HCl (pH 1.7) to dissociate membrane-bound immune complexes, which were not internalised. After two washings, cellular radioactivity was measured. All operations after the incubation with immune complexes were performed at 0°C. Cells not exposed to drugs and incubated with the radiolabeled antigens instead of the immune complexes were used as a control for nonspecific inter-

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00145793/90/$3.50

nalisation and the counts were subtracted from all other readings. Cells incubated with the immune complexes but not exposed to drugs were used as an endocytosis control and results were expressed as percentage referred to this control taken as 100%.

3. RESULTS

Neutrophil endocytosis of our in vitro-prepared IgG-coated immune complexes normally occurred at a high rate: at 15 min actually all the cell-bound radiolabeled ligand was internalised, leaving less than 5% exposed on the cell surface. Both types of immune complexes were used in all experiments and no significant differences were noted.

The internalisation rate was significantly reduced by the four different PKC inhibitors: sphingosine, palmitoylcarnitine, tamoxifen and H-7 (Fig. 1). At the concentrations used, the first two drugs did affect neither receptor-ligand binding nor cell viability. Tamoxifen showed toxic effects above 200 μM (viability 50% only at 250 μM tamoxifen) and H-7 appeared to interfere with binding to the FcγR above 15 μM (total bound radioactivity about 50% of control at 25 μM H-7).

Anti-PLC and anti-PLA_2 antibodies also inhibited endocytosis, having a powerful additive inhibitory effect if used in combination (Fig. 2A). This inhibition was reversed by the PKC activator phorbol 12,13-dibutyrate (100 nM) (Fig. 2B). Non-immune IgG, used in similar concentrations, as a specificity control for the antibodies, had no effect upon endocytosis.

H-8 did not show any effect upon endocytosis at concentrations up to 10 μM at which it inhibits cyclic nucleotide-dependent protein kinases but not PKC.

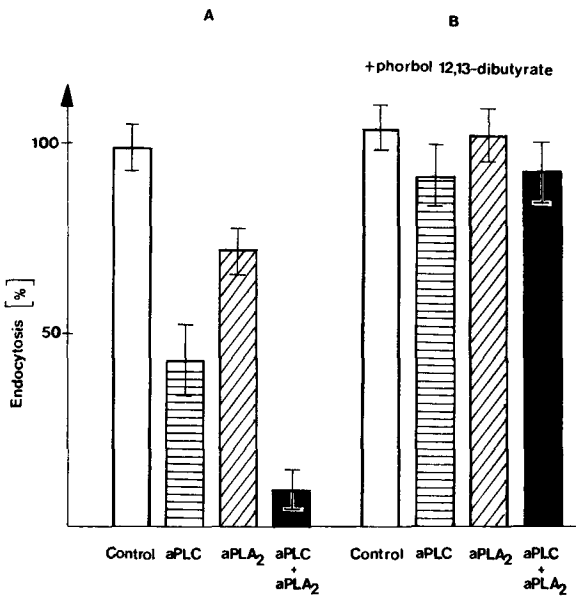

Fig. 2. (A) Maximal inhibition of endocytosis obtained with anti-PLC and anti-PLA_2 antibodies used alone or in combination. (B) Reversal of the antibody-induced inhibition by 100 nM phorbol 12,13-dibutyrate. Blank bars are specificity controls using non-immune IgG instead of the antibodies. Maximal inhibition was determined at excess antibody concentration (100 $\mu g/10^6$ cells). Means ± SD of 4 separate experiments.

4. DISCUSSION

The dose-dependent, up to over 80% inhibition of endocytosis by four different drugs which share only the quality of being PKC inhibitors [18–21], strongly

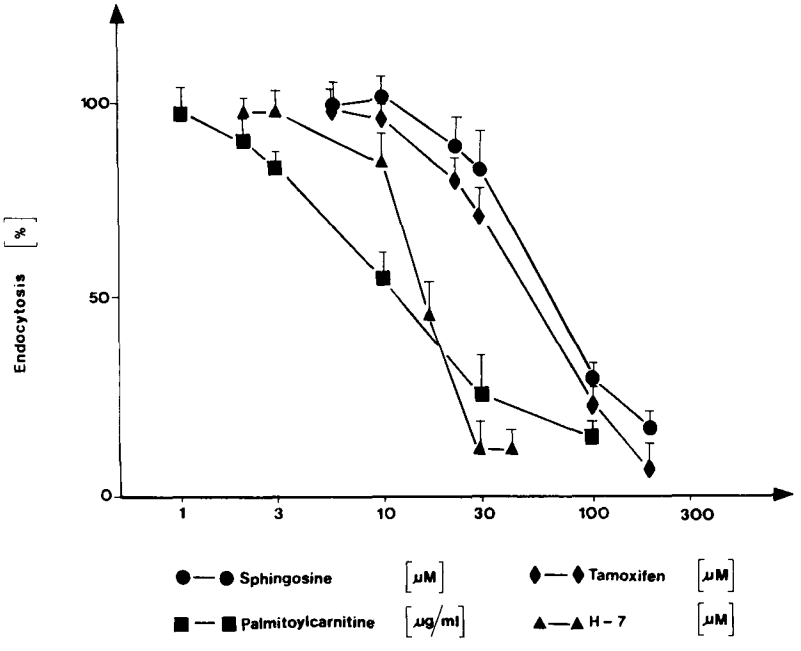

Fig. 1. Inhibition of endocytosis induced by the PKC inhibitors H-7, tamoxifen, palmitoylcarnitine and sphingosine. Means ± SD of 6 separate experiments.

suggests that PKC activation is a necessary event in triggering FcγR-mediated endocytosis in human neutrophils. Similar experiments indicate that PKC may as well be involved in other receptor-mediated endocytosis like enveloped virus internalization (S.N. Constantinescu, C. Cernescu, F. Baltă and L.M. Popescu 'Protein kinase C and viral infectivity', submitted).

PLC-type hydrolysis and phosphoinositides as well as activation of PLA_2 were shown to be triggered by the neutrophil FcγR [3,8]. Both could lead to PKC activation [2,22]. Our results with specific anti-PLC and anti-PLA_2 antibodies, which bind to and inhibit the cell-membrane enzymes [12,14–17], suggest an important cooperative involvement of PLC and PLA_2 activity in triggering endocytosis. The endocytosis inhibition induced by PLC and PLA_2 blockade is probably due to the failure of subsequent PKC activation because this inhibition is reversed if the assay medium contains the direct PKC activator phorbol 12,13-dibutyrate. PLC-dependent inositolphosphate-induced calcium transients [22] seem to play no significant role in this case of receptor-mediated endocytosis, although neutrophil FcγR-mediated *phago*cytosis was shown to have some calcium requirements [10].

In order to test any possible contribution from cyclic nucleotide signaling in triggering endocytosis, we used 1-10 μM H-8, selectively inhibiting cAMP- and cGMP-dependent protein kinases (K_i for A kinase, G kinase and PKC of 1.2, 0.48 and 15 μM, respectively [19]). Apparently, the lack of effect upon endocytosis shows that cyclic nucleotides are not positively involved in this type of FcγR signaling; moreover, cAMP-dependent protein kinase may negatively modulate FcγR-mediated phagocytosis [9].

In conclusion, our results suggest that PKC activation plays the major role in controlling FcγR-mediated endocytosis in human neutrophils. Two synergistic pathways appear to link the FcγR to PKC, through activation of membrane PLC and PLA_2, respectively.

Acknowledgements: L.M.P. thanks Prof. H. Hidaka, Nagoya University, for the generous gift of H-7 and H-8. We also thank M. Dorobantu for technical assistance and Dr A.C. Bancu for useful advice.

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