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The effect of inhibition of both diacylglycerol metabolism and phospholipase A_2 activity on superoxide generation by human neutrophils

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A 'cocktail' consisting of an inhibitor of diacylglycerol kinase (R59022, 10 μM), an inhibitor of diacylglycerol lipase (RHC80267, 10 μM), and an inhibitor of phospholipase A_2 (either 100 μM indomethacin, or 100 μM sodium meclofenamate) markedly enhanced superoxide production by human neutrophils stimulated with post-receptor stimuli, fluoride and γ-hexachlorocyclohexane. On the other hand, the response to the C3b/Fc receptor stimulus, opsonized zymosan, was marginally decreased whilst that to the Fc receptor stimulus, aggregated IgG, was virtually unaffected. Since the inhibitors used are deemed to inhibit the main routes of arachidonate production, these results call into question the role of arachidonate in the transduction of O_2^- generation by post-receptor stimuli, but support a role for arachidonate in receptor-mediated transduction.

Neutrophil; Superoxide; Diglyceride kinase; Diglyceride lipase; Arachidonate; Protein kinase C

1. INTRODUCTION

The pathways involved in the signal-transduction of the respiratory burst in the neutrophil are far from clear. It has been proposed that stimulus-activation coupling in many cell types involves the breakdown of phosphatidylinositol bisphosphate (PIP_2) to give inositol trisphosphate (which increases intracellular Ca^{2+}) and diacylglycerol (DAG) (which activates protein kinase C) and that the two pathways function synergistically [1,2]. Evicence has been put forward which suggests that synergistic interaction between these two pathways could participate in signal transduction for the neutrophil respiratory burst [3-6]. However there is controversy over whether protein kinase C really is implicated in receptor-mediated O_2^- production: there are some data indicating that it is not involved [7,8] and some that it is [9,10]. There is evidence that oleoyl acetylglycerol (OAG) (and by implication, DAG) can invoke transduction mechanisms for the oxidative burst which do not involve protein kinase C [11] and other evidence for a receptor-mediated burst which is PIP_2 independent but which does involve protein kinase C [12,13]. In this latter case it could be of importance that DAG can be derived from phosphatidylcholine [14].

Several observations suggest that arachidonate or its metabolites could be involved in stimulus-activation coupling for the respiratory burst. Thus exogenous arachidonate can activate NADPH-oxidase in intact neutrophils [15] and in a cell-free preparation of neutrophil cytosol and membrane components (discussed in [16]). However, whether endogenously released arachidonate is implicated

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Abbreviations: O_2^-, superoxide; DAG, diacylglycerol; Hepes, 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid; PIP_2, phosphatidylinositol bisphosphate; γ-HCCH, γ-hexachlorocyclohexane; PA, phosphatidic acid

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in neutrophil transduction mechanisms is still an open question, though participation of arachidonate has been suggested [11] and lipoxins derived from arachidonate are reported to activate protein kinase C [17].

The main potential sources of arachidonate are deemed to be from phosphatidylcholine, phosphatidylethanolamine or phosphatidic acid (PA) by phospholipase A_2 action, or from the phosphoinositides by sequential action of phospholipase C and DAG lipase (see [18] for review). It is possible to inhibit all these pathways simultaneously. DAG lipase can be inhibited by RHC80267 [19] and the generation of PA from DAG by DAG kinase can be inhibited by R59022 [20]. Indeed we recently reported results with these two compounds on the neutrophil respiratory burst [21], results which have since been confirmed [22,23]. It has also been demonstrated that neutrophil phospholipase A_2 can be inhibited by indomethacin [24] and by meclofenamate [25]. A combination of either of these latter two compounds with the inhibitor of DAG lipase plus the inhibitor of DAG kinase should prevent release of arachidonate from all the sources specified above.

In the present study we have examined the effect of this inhibitory cocktail on superoxide release produced by both receptor and non-receptor stimuli, in order to obtain some information as to whether endogenous arachidonate is involved in the transduction of the oxidative burst. The receptor stimuli used were opsonized zymosan and aggregated IgG, and the post-receptor stimuli were fluoride [26], which acts, at least in part, via the G-protein coupled to phospholipase C [27] and γ-hexachlorocyclohexane (γ-HCCH). This latter agent is known to activate the oxidative burst [28]. It can cause PIP_2 breakdown but there is evidence that its action involves perturbation of membrane phospholipids [29], rendering them more accessible for degradation. Its mechanism of action thus implies the potential mobilization of arachidonate from several phospholipid sources. In this connection it is significant that lipocortin, which quite clearly can prevent the release of arachidonate and its metabolites [30], has not only been shown to inhibit phospholipase A_2 by sequestering its phospholipid substrates [31] but to manifest calcium-dependent binding to phosphatidylserine liposomes [32].

2. MATERIALS AND METHODS

Blood was collected from human volunteers by venipuncture and neutrophils prepared by Ficoll-Isopaque separation as described [21]. The cells were subsequently suspended in the appropriate buffer. For opsonized zymosan, IgG and γ-HCCH, a Ca^{2+}-free Tyrode solution was employed, containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/ml glucose, 1 mg/ml bovine serum albumin and buffered with 20 mM (Hepes) at pH 7.4. A modified Dulbecco's phosphate buffered saline (PBS) was used for all fluoride experiments: a Ca^{2+} and Mg^{2+} free solution containing 136.9 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 in which the concentration of NaCl was reduced so that the final salt concentration was physiological after the addition of NaF.

Zymosan was opsonized with autologous serum as previously described [21] and suspended in Ca^{2+}-free Tyrode solution to give a final concentration of 1 mg/ml. IgG (human) was suspended at 2.5 mg/ml in Ca^{2+}-free Tyrode solution. Heat-aggregated IgG was produced by heating the IgG for 15 min at 63°C.

The neutrophils suspended at 4×10^6 cells/ml were equilibrated for 20 min at 37°C, the appropriate concentration of drug then added and allowed a further 20 min preincubation. Into the assay tubes was dispensed 1 mg ferricytochrome *c* (horse heart type III), plus either the particular stimulus at the appropriate dilution, or Tyrode, and also either 75 units superoxide dismutase or Tyrode. The final Ca^{2+} concentration was 3 mM, except in fluoride experiments where it was 0.31 mM, to avoid precipitation of CaF_2.

The reaction was initiated by the addition of 2×10^6 cells to the assay tubes, and incubation continued at 37°C for 100 min for IgG stimulus, or 30 min for all other stimuli, the reaction being stopped by the addition of 1 mM *N*-ethylmaleimide. Following centrifugation at 1400 × *g* for 10 min at 4°C, the absorbance of the supernatant was read at 550 nm in a Beckman DU-50 spectrophotometer. The amount of O_2^- produced was calculated by dividing the difference in absorbance of the samples, with and without superoxide dismutase, by the extinction coefficient for the change between ferricytochrome *c* and ferrocytochrome *c* ($E_{550} = 15.5\ mM^{-1} \cdot cm^{-1}$) and the resulting value converted to nmol O_2^- produced. Results are expressed as % mean maximum O_2^- produced by the stimulus alone.

R59022 was obtained from Janssen Pharmaceutical (England), sodium meclofenamate from Parke Davis (England), all other reagents were from Sigma and RHC80267-2 was a generous gift from Dr A. Khandwala of Revlon Health Care Group (USA).

3. RESULTS

A combination of the DAG kinase inhibitor, R59022 at 10 μM, the DAG lipase inhibitor, RHC80267 at 10 μM, and indomethacin at 100 μM, markedly shifted the concentration-response curve to fluoride to the left. The mean maximum was also increased with the drug combination (fig.1A). Similar results were obtained when meclofenamate

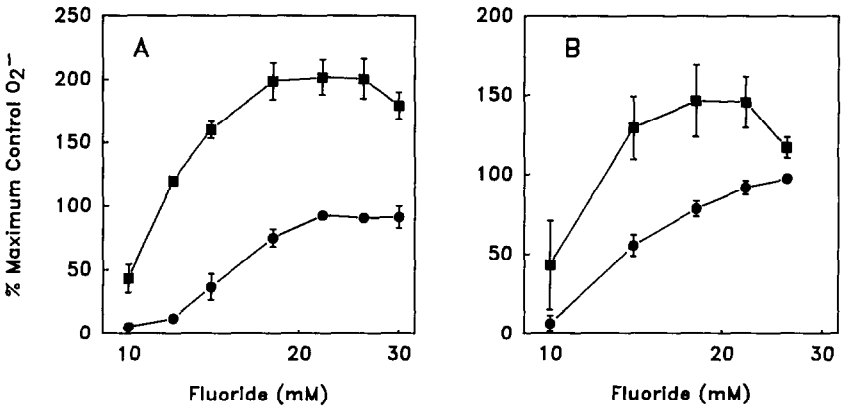

Fig.1. Potentiation of fluoride-induced O_2^- generation by a combination of inhibitors of arachidonate production. Results are expressed as % mean maximum O_2^- with fluoride alone (15.5 nmol per 10^6 cells in A; 20.7 nmol per 10^6 cells in B). Fluoride concentration is expressed on a log scale. (●) Fluoride alone; (■) with a combination of the DAG kinase inhibitor, R59022 (10 μM), the DAG lipase inhibitor, RHC80267 (10 μM), and a phospholipase inhibitor which was indomethacin (100 μM) in A ($n=5$) and meclofenamate (100 μM) in B ($n=3$). All data points represent mean ± SE.

was substituted for indomethacin (fig.1B). The same two cocktails of reagents caused marked left-shifts in the concentration-response curves to γ-HCCH. The meclofenamate-containing cocktail also increased the mean maximum response (fig.2A,B).

When used with receptor stimuli, the combination of drugs RHC80267, R59022 and sodium meclofenamate produced the opposite effect. In 4 experiments there was always a consistent right shift in the concentration-response curve to opsonized zymosan (fig.3). All data points in figs.1,2 and 3 represent mean ± SE; at some points the errors are too small to appear. The meclofenamate-containing cocktail also inhibited IgG-stimulated O_2^- release by 26 ± 11.8% (mean ± SE, $n=3$) but had no significant effect on heat aggregated IgG-mediated superoxide release (6 ± 7.9% inhibition, $n=3$).

In 7-experiments sodium meclofenamate (100

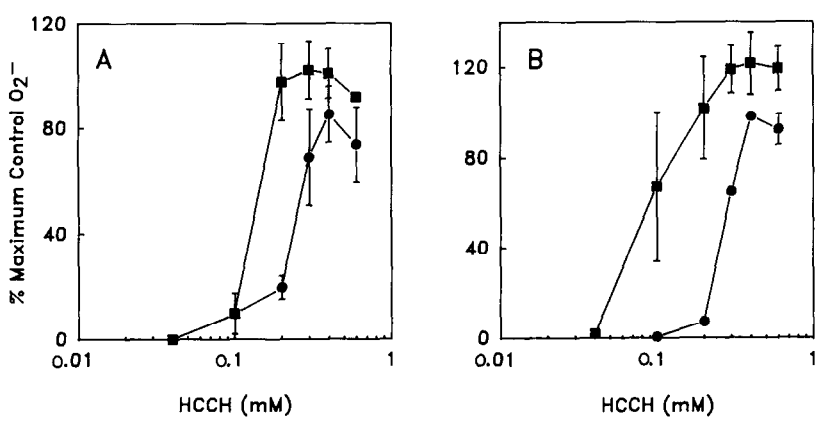

Fig.2. Potentiation of γ-HCCH-induced O_2^- generation by a combination of inhibitors of arachidonate production. Results are expressed as % mean maximum O_2^- produced by γ-HCCH alone (27.4 nmol per 10^6 cells in A; 29.2 nmol per 10^6 cells in B). (●) γ-HCCH alone; (■) with a combination of the DAG kinase inhibitor, R59022 (10 μM), the DAG lipase inhibitor, RHC80267 (10 μM), and a phospholipase inhibitor which was indomethacin (100 μM) in A ($n=3$) and meclofenamate (100 μM) in B ($n=3$). All data points represent mean ± SE.

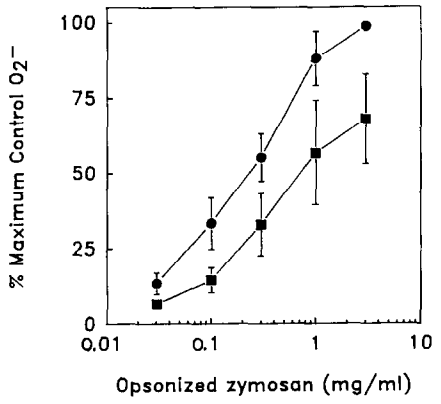

Fig.3. The effect of a combination of inhibitors of arachidonate production on opsonized zymosan-stimulated O_2^- production. Results are expressed as per cent of the maximum O_2^- produced by opsonized zymosan alone (26.8 nmol per 10^6 cells). (●) Opsonized zymosan alone; (■) with a combination of the DAG kinase inhibitor, R59022 (10 μM), the DAG lipase inhibitor, RHC80267 (10 μM), and sodium meclofenamate (100 μM). All data points represent mean ± SE ($n=4$).

μM) used on its own with these receptor stimulants did not decrease superoxide production (not shown) and we have previously reported that when R59022 and RHC80267 were used on their own the former enhanced and the latter had no effect on receptor-mediated superoxide production [21].

4. DISCUSSION

Indomethacin and meclofenamate were described quite independently by two separate groups as being inhibitors of phospholipase A_2 [24,25]. In the present study, remarkably similar results were obtained with post-receptor stimuli when either of these was added to the combination of R59022 plus RHC80267. In experiments with receptor stimuli, only meclofenamate was used in the cocktail since, in a rigorous experimental study, indomethacin has been described as competitively inhibiting the binding and neutrophil activation of some receptor ligands [33,34].

In considering the problem of signal transduction for the respiratory burst in the neutrophil, a role for arachidonate seems to be implied in both the study whose data indicate that respiratory burst transduction can involve PIP_2 turnover but not protein kinase C activity [11], and the study whose data indicate that it can involve protein kinase C activity but not PIP_2 turnover [12,13]. Certainly exogenous arachidonate can activate NADPH oxidase [15,16]. The role of endogenous arachidonate in the transduction process is difficult to investigate rigorously. Techniques involving the use of radioactive arachidonate have several serious drawbacks (discussed in [18]). One major drawback is that several distinct pools of arachidonate exist and there may be continuous acylation and reacylation of the phospholipids in these pools, even in a 'resting' cell. Another is that reacylation of the key phospholipids which are transiently deacylated during cell activation may confuse the results, especially if cell activation is measured over periods up to 10 min or more. We have therefore chosen to address the question by measuring the effect of a mixture of agents which have been shown to inhibit the main pathways involved in arachidonate release. The results show that not only did the inhibitors fail to decrease O_2^- generation by stimuli acting at non-receptor sites, but they actually increased it. However the cocktail of inhibitors marginally decreased receptor-stimulated O_2^- production. An interpretation of these results must take cognisance of the possibility that indomethacin, in addition to its known effects on cyclooxygenase and phospholipase A_2, may also be an inhibitor of DAG metabolism [35]. Taking this possibility into account (and with the caveat that the inhibitors used could have actions other than the ones specified), one explanation for the increased O_2^- generation seen when these agents were used with post-receptor stimuli is that, since they could have inhibited DAG metabolism and increased DAG levels, the resultant increased protein kinase C activation could have more than compensated for the elimination of the putative arachidonate pathway(s). If this explanation is correct, it emphasizes the importance of the protein kinase C pathway in the oxidative burst.

There are several possible explanations for the fact that the mixture of inhibitors had a different effect on the receptor-mediated response from that seen with post-receptor stimuli, i.e. a marginal depression instead of enhancement. One is that the marked protein kinase C activation brought about by increased DAG levels could have had a negative feedback effect either by phosphorylation of the receptor, as happens with α_1-adrenergic receptors [36] or by block of the coupling of the receptor to the G-protein(s) [37]. Another possible explana-

tion is that receptor-mediated generation of an endogenous inhibitor of protein kinase C such as sphingosine could have modulated the response [38]. However, a further possible explanation, and one that merits consideration, is that the oxidative burst which follows receptor-stimulation might, in addition to PIP_2 degradation, require the activation of another pathway (one which involves arachidonate) perhaps through phospholipase A_2 coupled to the receptor [39], the coupling possibly being by the G-protein reported to be involved with the PIP_2-independent pathway [13]. If arachidonate generation were a necessary element in receptor-mediated O_2^- release, inhibition of such generation might well give the results obtained in the present paper, since even very potent activation of the protein kinase C pathway would not then compensate for the elimination of this putative arachidonate pathway. A receptor-coupled arachidonate pathway could explain the results previously reported [12], i.e. that pretreatment of neutrophils with PMA resulted in virtual elimination of the subsequent FMLP-induced PIP_2 turnover but increased the concomitant O_2^- production. Support for the possibility that an arachidonate pathway could be implicated in the receptor-mediated oxidative burst has been provided by a recent report that lipocortin, known to inhibit the release of arachidonate and its metabolites, decreases receptor-mediated but not PMA-mediated H_2O_2 production [40].

Clearly the transduction pathways for the neutrophil respiratory burst are extremely complex and await clarification.

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