Superoxide generation by either 1-oleoyl-2-acetylglycerol or A23187 in human neutrophils is enhanced by indomethacin

M. Maureen Dale and Adrienne Penfield

Department of Pharmacology, University College London, Gower Street, London WC1E 6BT, England

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Indomethacin at a concentration (10^{-4} M) which depressed the effect on O_2^- generation by fMet-Leu-Phe, markedly enhanced O_2^- generation by both 1-oleoyl-2-acetylglycerol and the calcium ionophore, A23187. These results are explicable in terms of the hypothesis that synergism between cytosolic calcium and protein kinase C is involved in signal transduction for the respiratory burst in the human neutrophil.

Neutrophil *Superoxide* *Phorbol ester* *Indomethacin* *Calcium*

1. INTRODUCTION

When neutrophils are exposed to certain stimuli, their O_2 uptake increases (the 'respiratory burst') and large amounts of superoxide ($O_2^{\cdot-}$) are generated [1]. This reaction leads to the formation of other toxic oxygen products which are important in the killing of microbes (review [1]) and which may well be implicated in tissue damage in complex-mediated disease [2]. However, as emphasized in a recent review [3], the events involved in signal transduction for the respiratory burst are not yet fully understood.

Nishizuka has recently proposed that two events – generation of the protein kinase C activator, DAG, and an increase in cytosolic calcium – may be involved in stimulus-activation coupling in various cell types and that the two pathways may function synergistically [4]. Recently we reported synergism between the synthetic diacylglycerol, OAG, and the calcium ionophore, A23187, in superoxide generation in the neutrophil [5]. Similar studies in which the tumor promoter, PMA, was used as the protein kinase C activator, had been previously reported by us [6] and by others [7,8]. We have also recently reported [9] that prostaglandins of the E series, which are believed to stimulate adenyl cyclase, had an unexpected enhancing effect on OAG-induced superoxide generation, whereas they decreased fMet-Leu-Phe-induced $O_2^{\cdot-}$ production and had no effect on $O_2^{\cdot-}$ production by PMA or A23187.

Here we report that indomethacin, one of the main non-steroidal anti-inflammatory drugs, also has unexpected enhancing actions on $O_2^{\cdot-}$ generation. This drug has completely different effects on $O_2^{\cdot-}$ production depending on the method of stimulation used. Thus indomethacin has a consistent and marked inhibitory effect, if $O_2^{\cdot-}$ is generated as a result of receptor-activation with fMet-Leu-Phe, whereas it has a marked enhancing effect if $O_2^{\cdot-}$ is generated by direct activation of either of the two pathways specified above. We suggest that these results can be explained on the basis of inhibition by indomethacin of the metabolism of OAG and DAG by diacylglycerol lipase.

Abbreviations: OAG, 1-oleoyl-2-acetylglycerol; DAG, diacylglycerol; PMA, phorbol myristate acetate; PI, phosphatidylinositol

2. MATERIALS AND METHODS

Neutrophils were collected from human volunteers by venipuncture, prepared by Ficoll-

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Isopaque separation as described [6] and suspended in calcium-free Tyrode solution containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/ml glucose and 1 mg/ml bovine serum albumin.

The cells were equilibrated for 20 min at 37°C; to those cells which were to be stimulated with either fMet-Leu-Phe or A23187, cytochalasin B (10 μg/ml) was then added. After a further 10 min incubation, 2.5×10^6 cells were dispensed into tubes containing 1 mg ferricytochrome *c* (horse heart type III, Sigma), 10^{-4} M indomethacin and either Tyrode solution or 75 units superoxide dismutase (bovine blood, Sigma). Following a 20 min incubation, appropriate dilutions of OAG, A23187 or fMet-Leu-Phe were added to the cells. The final calcium concentration in all samples was 3 mM. After 30 min incubation at 37°C, the reaction was stopped by the addition of 1 mM *N*-ethylmaleimide (Sigma). Following centrifugation at 1400 × *g* for 10 min at 4°C, the absorbance of the supernatant was read at 550 nm in a Perkin Elmer SP-1800 spectrophotometer. The amount of $O_2^{\cdot-}$ 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_{550nm} = 15.5 $mM^{-1} \cdot cm^{-1}$) and the resulting value multiplied by 2000 to convert it to nmol $O_2^{\cdot-}$ per 5×10^6 neutrophils.

The effect of indomethacin on the $O_2^{\cdot-}$ response to a particular stimulant was calculated by taking the difference between $O_2^{\cdot-}$ production with and without indomethacin and expressing this difference as a percentage of the maximum amount of $O_2^{\cdot-}$ generated by that particular stimulant.

3. RESULTS

In 6 experiments, 10^{-4} M indomethacin consistently decreased fMet-Leu-Phe-induced $O_2^{\cdot-}$ generation, resulting in a markedly depressed dose-response curve (figs 1 and 2). A lower concentration of indomethacin, 10^{-6} M, was without effect.

In experiments with OAG, concentrations of $1.1–2.3 \times 10^{-4}$ M gave maximum $O_2^{\cdot-}$ release. In 8 experiments the mean maximum release with OAG alone was 46.89 (± 6.4) nmol $O_2^{\cdot-}$ per 5×10^6 cells. In all these experiments 10^{-4} M indomethacin increased the $O_2^{\cdot-}$ generation produced by submaximal concentrations of OAG and in 5 of the 8 experiments the maximal response was also increased. The increase of the OAG response by indomethacin, calculated as a percentage of the maximum response obtained with OAG is given in fig.1. In two experiments with OAG, several concentrations of indomethacin were used and the ef-

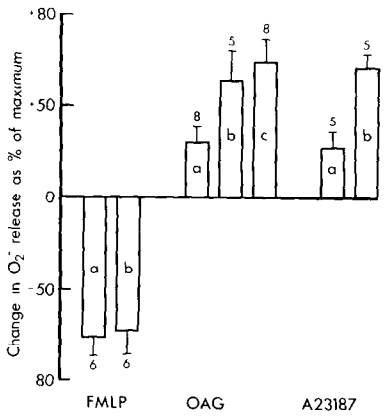

Fig.1. The effect of indomethacin (10^{-4} M) on $O_2^{\cdot-}$ generation with fMet-Leu-Phe, OAG and A23187. The results are given as the change in $O_2^{\cdot-}$ production with indomethacin, expressed as a percentage of the maximum $O_2^{\cdot-}$ obtained with each stimulant alone. The mean maximum response in nmol $O_2^{\cdot-}$ per 5×10^6 cells was 25.7 (SE 1.9), 46.87 (SE 6.4) and 17.6 (SE 3.2), with fMet-Leu-Phe, OAG and A23187, respectively. The number of experiments is given in brackets. For fMet-Leu-Phe: a = 5×10^{-8} M, b = 5×10^{-7} M. For OAG: a = 4.5×10^{-5} M, b = 6.8×10^{-5} M, c = 1.1×10^{-4} M. For A23187: a = 10^{-5} M, b = 5×10^{-5} M.

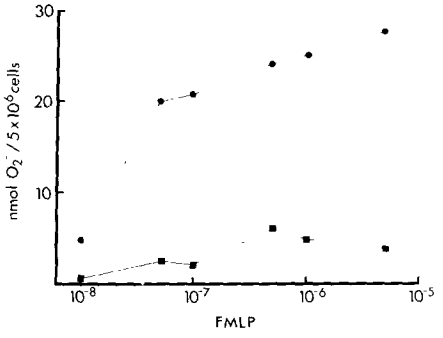

Fig.2. The effect of indomethacin (10^{-4} M) on $O_2^{\cdot-}$ generation by fMet-Leu-Phe. fMet-Leu-Phe alone (●), with indomethacin (■).

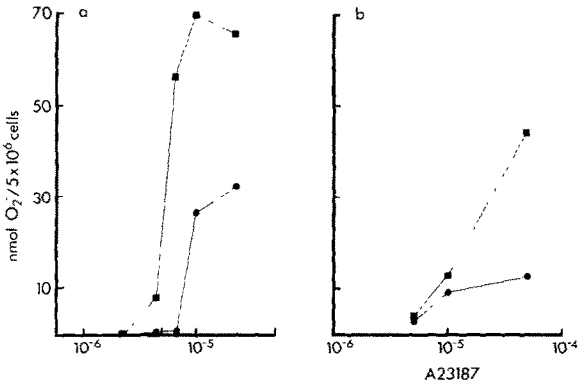

Fig.3. (a) The effect of indomethacin, 10^{-4}M, on $O_2^{\cdot-}$ generation with OAG. OAG alone (●), OAG in the presence of indomethacin (■). (b) The effect of indomethacin (10^{-4} M) on superoxide generation with A23187. A23187 alone (●), A23187 with indomethacin (■).

fect of the indomethacin was shown to be concentration-dependent, increasing over the concentration range 10^{-6}–10^{-4} M (not shown). Because normalization of measurements does not give a clear picture of the raw data, the results of an actual experiment are given in fig.3a. In two experiments, bromophenacyl bromide, 10^{-5} M, and mepacrine, 10^{-4} M (concentrations which inhibit the response to fMet-Leu-Phe), had no effect on the $O_2^{\cdot-}$ generation by OAG (not shown).

The calcium ionophore, on its own, is a very poor stimulant of $O_2^{\cdot-}$ generation, only moderate effects being produced, even by high concentrations. In 5 experiments the maximum release with this agent was obtained with a concentration of 5×10^{-5} M, the mean amount of $O_2^{\cdot-}$ generated being only 17.6 (± 3.2) nmol per 5×10^6 cells. In all these experiments, 10^{-4} M indomethacin increased the $O_2^{\cdot-}$ generation produced by high concentrations of A23187. The potentiation of the response to A23187, given as a percentage of the maximum response, is shown in fig.1, and the results of an actual experiment in fig.3b.

4. DISCUSSION

Indomethacin clearly has several different actions on the neutrophil. In low concentrations it inhibits the cyclo-oxygenase which converts arachidonate into cyclic endoperoxides [10]. In higher concentrations it inhibits phospholipase A_2 [11] especially if the calcium concentration is low [12,13].

Previous studies have reported inhibitory effects of indomethacin on neutrophil $O_2^{\cdot-}$ generation with fMet-Leu-Phe [14,15]. Here, indomethacin decreased the response to fMet-Leu-Phe but caused an unexpected, marked enhancement of the response to the protein kinase C activator, OAG, and to the calcium ionophore A23187. The most likely reason for the effect on OAG is that indomethacin inhibits the enzymes which metabolize OAG. It has been shown that OAG is metabolized rapidly in situ, presumably by the enzymes which metabolize endogenous DAG, such as DAG kinase [4]. DAG may also be deacetylated by a diglyceride lipase [16]. Indomethacin has been shown to be a potent inhibitor in human platelets of diglyceride lipase but not diglyceride kinase [17]. It may well be having a similar effect on this enzyme in neutrophils. We recently reported marked enhancement of OAG-mediated generation of $O_2^{\cdot-}$ in human neutrophils by PGE_1 and PGE_2 and suggested that this effect of these prostaglandins could be due to an inhibition of DAG kinase, although an alternative explanation of the effect seen with the prostaglandins could be inhibition of DAG lipase. However, there is clearly a difference in the action of the prostaglandins and indomethacin, since, although both enhance the effect of OAG and decrease the effect of fMet-Leu-Phe on $O_2^{\cdot-}$ generation, they have different effects on 'the calcium pathway'. PGE_2 does not modify A23187-mediated $O_2^{\cdot-}$ generation, whereas indomethacin markedly increases it. It has already been established that a rise in intracellular Ca^{2+} per se is not a sufficient stimulus for $O_2^{\cdot-}$ generation [18] and that the additional stimulus is probably protein kinase C activation [5–8]. How is it that, in the presence of indomethacin, a rise in cytosolic Ca^{2+} caused by A23187, now appears to be a very efficient stimulus for $O_2^{\cdot-}$ generation? An explanation of this effect of indomethacin based on the current model of the signal-transduction mechanism in cells such as the neutrophil [4,19,20] would be that the following sequence of events occurs: (1) the increased concentration of cytosolic Ca^{2+}, produced by A23187, stimulates generation

of DAG by calcium-dependent enzymes, such as the phospholipase C responsible for the breakdown of PI [20,21]; (2) the DAG concentration rises because indomethacin inhibits its degradation by inhibiting diglyceride lipase (and possibly diglyceride kinase); (3) subsequent synergism between the DAG pathway and the Ca^{2+} pathway results in $O_2^{\cdot-}$ gneration.

A direct effect of the rise in cytosolic calcium on protein kinase C may participate in the response but is clearly not the main basis for it, because such a rise, in the absence of indomethacin, results in only a modest production of $O_2^{\cdot-}$.

If the mechanism of action of indomethacin in increasing $O_2^{\cdot-}$ production by OAG and A23187 is as suggested here it is apparently not mediated by adenyl cyclase, since indomethacin in the concentrations used, does not increase cyclic AMP [22]. Furthermore, it is unlikely that the well documented effect of indomethacin on phospholipase A_2 [11–13] is implicated in the action on OAG and A23187, since other phospholipase A_2 inhibitors, such as bromophenacyl bromide and mepacrine (review [23]), were entirely without effect on OAG-induced $O_2^{\cdot-}$ generation.

Why do the E-type prostaglandins not have this effect on A23187-induced $O_2^{\cdot-}$ generation, since, like indomethacin, they too enhance superoxide production by OAG? It could be a question of the degree of inhibition of OAG degradation caused by each agent. Another possibility is that the prostaglandins, by increasing cAMP, could have some inhibitory effects on phospholipase C and thus on the generation of DAG, as has been proposed for both platelets [24] and neutrophils [25]. This would mean that, in the presence of the E-type prostaglandins, an A23187-mediated rise in cytosolic calcium would not result in an increase in DAG and thus would not result in increased $O_2^{\cdot-}$ production.

Indomethacin probably has multiple effects on fMet-Leu-Phe-induced $O_2^{\cdot-}$ generation. For instance, it could decrease the affinity of the receptor or it could inhibit polyphosphoinositide turnover by inhibiting phospholipase C and/or DAG kinase. The effect of indomethacin found in this study, may explain the reported increase in $O_2^{\cdot-}$ generation obtained with indomethacin in zymosan-stimulated neutrophils [26].

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