Br. J. Pharmac. (1987), **92**, 63–68

Comparison of the effects of indomethacin, RHC80267 and R59022 on superoxide production by 1,oleoyl-2,acetyl glycerol and A23187 in human neutrophils

M. Maureen Dale & Adrienne Penfield

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

1 Indomethacin (10^{-4} M) causes marked augmentation of O_2^- release from human neutrophils when these are stimulated by either 1,oleoyl-2,acetylglycerol or the divalent cation ionophore, A23187, the concentration-response curve for each agent being shifted to the left and the maximum response to each increased.

2 The diacylglycerol kinase inhibitor, R59022 (10^{-5} M) has effects very similar to those of indomethacin on both the 1,oleoyl-2,acetylglycerol-induced and the A23187-induced concentration-response curves for O_2^- generation.

3 The diacylglycerol lipase inhibitor, RHC80267 (10^{-5} M) on the other hand, has a similar effect to indomethacin on 1,oleoyl-2,acetylglycerol-induced O_2^- generation but, unlike indomethacin, has no effect on A23187-induced O_2^- generation.

4 Comparison of the effects of these three agents provides a clue to the locus of the action of indomethacin in increasing superoxide release, suggesting that it may act as a diacylglycerol kinase inhibitor. A component of diacylglycerol lipase inhibition may also be present. It is suggested that these results could have relevance for the use of indomethacin as an anti-inflammatory agent in chronic rheumatoid diseases.

Introduction

When neutrophils are exposed to certain stimuli their O_2 uptake increases ('the respiratory burst') and large amounts of superoxide (O_2^-) and other toxic oxygen products are generated (Badwey & Karnovsky, 1980). These products are believed to be important in the killing of micro-organisms (reviewed by Badwey & Karnovsky, 1980), and there is increasing evidence that they are implicated in the tissue damage of complex-mediated disease (Johnston & Lehmeyer, 1976; Tate & Repine, 1984). Recent evidence suggests that O_2^- produced by neutrophils may be a key factor in the actual aetiology of some complex-mediated diseases such as rheumatoid arthritis.

Thus, Lunec *et al.* (1985) have shown that O_2^- released from neutrophils can modify IgG molecules causing them to aggregate. It was demonstrated that the aggregates so formed were themselves able to stimulate O_2^- production from neutrophils, thus providing the basis for a self-replicating mechanism. Lunec *et al.* reported that the aggregates of modified IgG had a characteristic autofluorescence, and that identical aggregates were found in fresh rheumatoid synovial fluid. They have proposed that the modified IgG molecules form part of a self-replicating mechanism for the production of tissue-damaging and IgG-modifying oxygen radicals, and could thus be the basis of the self-perpetuating reactions believed to underlie rheumatoid arthritis.

We have recently found that indomethacin, an anti-inflammatory drug used extensively in the treatment of rheumatoid arthritis, increased O_2^- production from human neutrophils, when used in concentrations of 10^{-6}–10^{-4} M (Dale & Penfield, 1985). Our findings accord with those of Gay *et al.* (1984, 1985) who used human neutrophils stimulated by opsonized zymosan, and with those of Bromberg & Pick (1983), who used activated macrophages. We have since found that some other non-steroidal anti-inflammatory drugs (NSAIDS) have effects similar to indomethacin (Dale & Penfield, unpublished results) and that the same drugs increase O_2^- production by aggregated IgG and opsonized zymosan (Dale & Muid, unpublished results). Opsonized zymosan and aggregated IgG stimulate neutrophils by acting on Fc and C3b recep-

tors. Since ligands which stimulate phagocytes by acting on these receptors are present in rheumatoid joints (i.e. immune complexes and complement components), it is clearly possible that indomethacin and the other drugs investigated, when used to provide temporary relief of signs and symptoms, may in fact be exacerbating the progress of the underlying autoimmune/inflammatory condition. It seemed to us important to investigate the mechanism of the observed effect of these agents not only because of the potential clinical significance of the findings but also in order to further the understanding of the pharmacological actions of these agents. In order to study the mechanisms involved we needed to examine the effect of the drug on post-receptor transduction events for the respiratory burst.

Nishizuka (1984) had proposed that two transduction events, generation of the protein kinase C activator, diacylglycerol (DAG) and an increase in cytosolic calcium, were involved in post-receptor stimulus-transduction coupling in various cell types, and that the two pathways functioned synergistically. Both events result from hydrolysis of phosphatidylinositol bisphosphate, which gives rise both to DAG and to inositol trisphosphate, the latter being able to mobilize intracellular calcium (reviewed by Berridge & Irvine, 1984).

Recently we reported that synergism of the two pathways occurred in O_2^- generation in human neutrophils, using the DAG analogue, 1,oleoyl-2,acetylglycerol (OAG) to stimulate protein kinase C and the calcium ionophore, A23187, to raise cytosolic calcium (Penfield & Dale, 1984). Similar studies in which the tumour promoter phorbol myristate acetate, was used as the protein kinase C activator, had previously been described by us and by others (Dale & Penfield, 1984; Robinson *et al.*, 1984; Di Virgilio *et al.*, 1984). Thus the significance of both pathways in the transduction of the respiratory burst has been well established. In addition it has been found that indomethacin exacerbates O_2^- generation by both OAG and A23187, shifting the concentration-response curve to the left for each substance, and frequently increasing the maximum response as well (Dale & Penfield, 1985). On the basis of these results we proposed that indomethacin, in addition to inhibiting cyclo-oxygenase, also had an effect on DAG metabolism, inhibiting either DAG kinase or DAG lipase or both (Dale & Penfield, 1985). Rittenhouse-Simmons (1980) had provided evidence that indomethacin was, in fact, an effective inhibitor of human platelet DAG metabolism over the range of concentrations which we had found to exacerbate O_2^- generation. In the present study we have sought to throw light on the mechanism of indomethacin's effect on O_2^- generation by comparing it with RHC80267, a specific DAG lipase inhibitor (Sutherland & Amin, 1982), and with R59022, a specific DAG kinase inhibitor (de Chaffoy de Courcelles *et al.*, 1985). The effect of indomethacin had been found to be dose-related over the concentration range 10^{-6}–10^{-4} M (Penfield & Dale, 1985) but in the present study only 10^{-4} M (which is not in fact the maximally effective concentration) was used, since in order to compare the effect of indomethacin with other agents it was important to ensure that as pronounced an indomethacin response as possible was obtained.

Methods

Neutrophils were collected from human volunteers by venipuncture, prepared by Ficoll-Isopaque separation as previously described (Penfield & Dale, 1984) and suspended in calcium-free Tyrode solution containing NaCl 137 mM, KCl 2.7 mM, $MgCl_2$ 1 mM, glucose 1 mg ml^{-1} and bovine serum albumin 1 mg ml^{-1}. The cells were equilibrated for 20 min at 37°C; to those cells which were to be stimulated with A23187, cytochalasin B (5 μg ml^{-1}) was added. After a further 5 min incubation, 2.5×10^6 cells were dispensed into tubes containing 1 mg ferricytochrome C (horse heart type III, Sigma); either indomethacin (10^{-4} M) or one of the enzyme inhibitors (RHC80267 10^{-5} M, or R59022 10^{-5} M, Tyrode solution being added to control tubes) and either Tyrode solution or 75 units superoxide dismutase (bovine blood, Sigma). The final calcium concentration in all samples was 3 mM. Following a 20 min incubation, appropriate dilutions of OAG (Sigma) or A23187 (Sigma) were added to the cells.

After a 30 min incubation period with stimulus, at 37°C, the reaction was stopped by the addition of N-ethyl maleimide (1 mM, Sigma). Following centrifugation at 1400 *g* for 10 min at 4°C, the absorbance of the supernatant was read at 550 nm in a Pye Unicam SP-1800 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 ($E_{550nm} = 15.5\,mM^{-1}\,cm^{-1}$) and the resulting value converted to nmol O_2^- per 5×10^6 neutrophils.

The results from each separate experiment were normalized, expressing each value for O_2^- production as a percentage of the maximum response obtained in that particular experiment. In this form, the means, with standard errors, from several experiments were calculated and graphically displayed, as shown below.

R59022 (6-[2]-4-[(4-fluorophenyl) phenylmethylene-1-piperidinyl]-7 methyl-5H-thiazolo-[3,2-a] pyrimidin-5-one) was purchased from Janssen Pharmaceuticals Ltd and RHC80267 (1,6-di (O-(carbamoyl) cyclohexanone oxime) hexane) was a generous gift from Dr Tai of the Division of Medicinal Chemistry & Pharmacology, College of Pharmacy,

Lexington, Kentucky, U.S.A. and from Dr Sutherland of the Revlon Health Care Group, NY, U.S.A.

Results

This series of experiments was carried out over a relatively long period of time, utilizing several different batches of OAG, between which we have found there to be a certain amount of variability. In addition, the OAG concentration-response curve for O_2^- generation from human neutrophils varies to a greater or lesser extent between individuals. Consequently, the control concentration-response curves for OAG presented here (Figure 1a, b, c,) are not identical. However, in spite of this quantitative fluctuation, the results to be described occurred completely consistently and, furthermore, the relative effects of the agents in question were found to be remarkably consistent when tested within the same experiment.

In 11 experiments in which the effects of the DAG lipase inhibitor, RHC80267 (10^{-5} M) were tested on OAG-mediated O_2^- generation, this agent behaved in a similar way to indomethacin, 10^{-4} M, i.e. it increased the amount of O_2^- generated (Figure 1a, b). As with indomethacin, the effect was particularly marked at submaximal concentrations of OAG.

In 5 experiments, the DAG kinase inhibitor, R59022 (10^{-5} M) also increased OAG-induced O_2^- generation. With this agent also, the effect was particularly marked at submaximal concentrations (Figure 1c). When indomethacin, RHC80267 and R59022 were tested concomitantly on OAG-induced O_2^- generation in neutrophils from the same individual, a very close parallelism in the actions of the three agents was observed (results not shown).

In 2 experiments, the effect on the response to a low, threshold concentration of OAG, of adding indomethacin at the same time as R59022 or RHC80267, was investigated (Table 1). In both experiments the results of using both putative inhibitors together was considerably greater than the results using either alone. When indomethacin (10^{-4} M) was used together with RHC80267 (10^{-5} M) the results were, in general, additive. When indomethacin, 10^{-4} M, was used

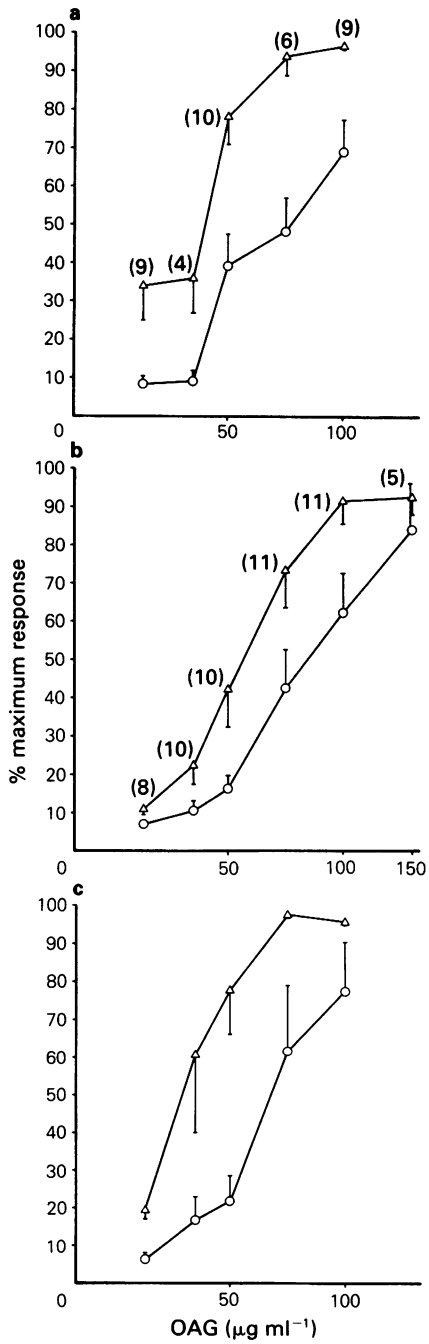

Figure 1 (a) The effect of indomethacin (10^{-4} M) on O_2^- generation with 1-oleoyl-2,acetylglycerol (OAG); OAG alone (○); OAG in the presence of indomethacin (△). (b) The effect of RHC80267 (10^{-5} M) on O_2^- generation with OAG; OAG alone (○); OAG in the presence of RHC80267 (△). (c) The effect of R59022 (10^{-5} M) on O_2^- generation with OAG: OAG alone (○); OAG in the presence of R59022 (△). The vertical lines represent standard errors. Number of observations shown in parentheses in (1a) and (b); $n = 5$ in (c). The mean maximum O_2 release was 64.8 nmol O_2 per 5×10^{-6} neutrophils (s.e.mean 0.7).

Table 1 The effects of indomethacin (Indo) (10^{-4} M) RHC 80267 (10^{-5} M) R59022 (10^{-5} M) and combinations of indomethacin with RHC80267 and R59022, respectively, on O_2^- production by a threshold dose (30 μg ml^{-1}) of 1-oleoyl-2,acetylglycerol (OAG)

Stimulus	*Expt. 1.*	*Expt. 2*	*Mean* ± range
OAG (30 μg ml^{-1})	4.1	10.8	7.5 ± 3.4
OAG + Indo (10^{-4} M)	9.1	14.7	11.9 ± 2.8
OAG + RHC80267 (10^{-5} M)	5.4	13.8	9.6 ± 4.2
OAG + Indo + RHC80267	12.6	34.6	23.6 ± 11
OAG + R59022 (10^{-5} M)	15.7	24.6	20.2 ± 4.5
OAG + Indo + R59022	78.3	97.2	87.8 ± 9.4

The results have been expressed as a percentage of the maximum value for O_2^- release obtained in each experiment.

together with R59022, 10^{-5} M, the results were greater than additive. These latter results suggest that the two agents may be acting at different sites on the DAG kinase. However, to establish whether this is so the kinetics of the system would obviously have to be studied in detail at the enzymic level.

When the three agents were tested on O_2^- generation by A23187, which by itself is a poor stimulus, a different profile of activity was observed. R59022 (10^{-5} M) in 6 experiments, markedly increased A23187-induced O_2^- generation (Figure 2b). Indomethacin (10^{-4} M) in 6 experiments had a similar effect (Figure 2a). However, in 11 experiments, RHC80267 (10^{-5} M) had no consistent effect on the A23187 response (Figure 2c).

Discussion

The role of DAG and protein kinase C in signal transduction for the respiratory burst was called into question recently when it was found that some putative inhibitors of protein kinase C, such as Cl, and H7, did not in fact inhibit O_2^- release by agents which acted on receptors, such as fMet-Leu-Phe or C5a

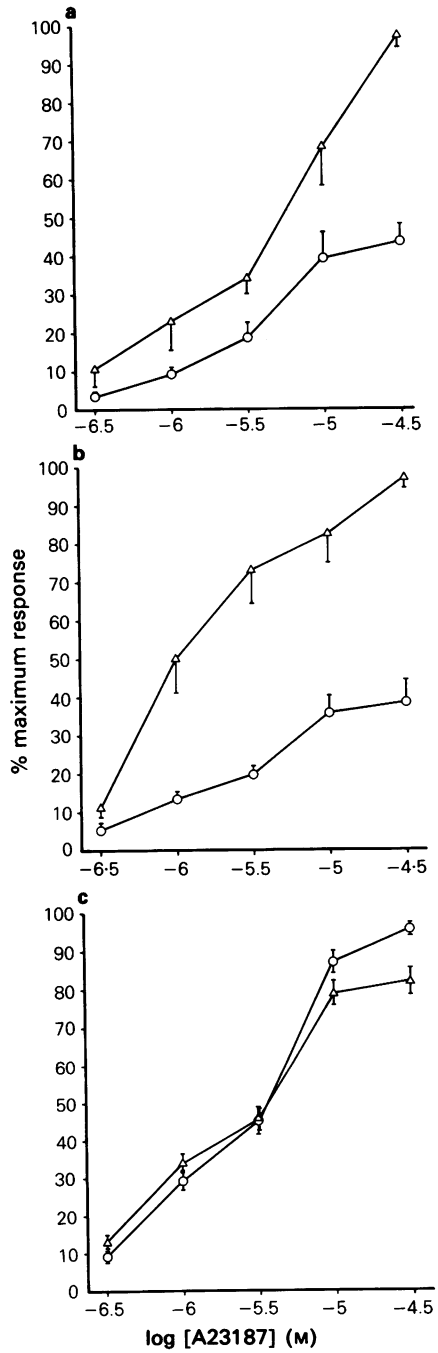

Figure 2 (a) The effect of indomethacin (10^{-4} M) on O_2^- generation with A23187: A23187 alone (○): A23187 in the presence of indomethacin (△). (b) The effect of R59022 (10^{-5} M) on O_2^- generation with A232187: A23187 alone (○): A23187 in the presence of R59022 (△). (c) The effect of RHC80267 (10^{-5} M) on O_2^- generation with A23187: A23187 alone (○); A23187 in the presence of RHC80267 (△). The vertical lines represent standard errors; $n = 6$ in (a) and (b); $n = 11$ in (c). The mean maximum O_2^- release was 32.3 nmol O_2^- per 5×10^6 neutrophils.

(Gerard *et al.*, 1986; Wright & Hoffman, 1986). However, the inhibitors which were used are known not to be specific for protein kinase C. Cl affects both protein kinase C and the cyclic AMP-dependent kinase, the K_i for the former enzyme being ~20 μM and for the latter, ~3 μM (Gerard *et al.*, 1986) and the respective K_i values for H7 are 6 μM and 3 μM (Hidaka *et al.*, 1984). The cyclic AMP-dependent kinase is known to inhibit neutrophil responses (Fantone & Kinnes, 1983). Results with agents like Cl and H7, which have similar actions on both stimulatory and inhibitory pathways of stimulus/response coupling, must obviously be interpreted with caution. In any event, more recently, sphingoid long-chain bases, which have been shown to be effective inhibitors of protein kinase C *in vitro* (Hannun *et al.*, 1986) have been reported to be very effective inhibitors of the respiratory burst in neutrophils stimulated with agents acting on receptors such as fMet-Leu-Phe and opsonized zymosan (Wilson *et al.*, 1986). This is strong evidence that the protein kinase C pathway is in fact involved in signal transduction for O_2^- generation.

We had originally put forward a hypothesis, based on Nishizuka's 1984 model of signal transduction, to explain the increase by indomethacin, of O_2^- release (Dale & Penfield, 1985). We suggested that indomethacin had an inhibitory effect on DAG kinase of DAG lipase or both. DAG analogues such as OAG are metabolized rapidly by the enzymes which metabolize DAG (Rittenhouse-Simmons, 1980; Nishizuka, 1984; de Chaffoy de Courcelles *et al.*, 1985). Inhibition of these enzymes would lead to an increased concentration of DAG or OAG in the membrane and thus more activation of protein kinase C and more O_2^- production.

The increase in O_2^- generation by A23187 could also be explained by our hypothesis, as follows: (1) The increased cytosolic calcium produced by A23187 stimulates generation of DAG by the calcium-dependent phospholipase C responsible for breakdown of phosphatidylinositol. (This enzyme was described by Cockcroft *et al.*, 1980). (2) The DAG concentration rises because its metabolism in inhibited. (3) Subsequent synergism between the DAG pathway and the calcium pathway (Penfield & Dale, 1984) results in an increase in O_2^- generation over and above that produced by A23187 alone.

Indomethacin and R59022 have very similar profiles of action in that each increases O_2^- generation by both OAG and A23187. RHC80267, on the other hand, does not have a similar profile to indomethacin in that it increases O_2^- generation by OAG but not by A23187. Clearly, exogenous OAG is metabolized by both the DAG kinase and DAG lipase, as shown by the present results, whereas endogenous DAG appears to be metabolized mainly by the kinase. This latter suggestion is supported by the fact that the O_2^- generation that occurs after receptor stimulation with FMLP, aggregated IgG or opsonized zymosan, and which results in endogenous DAG formation, is potentiated by R59022 but not by RHC80267 (Muid *et al.*, 1987).

R59022 is reported to be a specific inhibitor of DAG kinase (de Chaffoy de Courcelles *et al.*, 1985). It inhibits the DAG kinase of both human red blood cell membranes and intact human platelets. The IC_{50} for inhibition of DAG kinase in human platelets is 3.8×10^{-6} M, and 80% inhibition of the enzyme is seen with 10^{-5} M, the concentration used here. If R59022 is acting in the same way in human neutrophils as in human platelets and red cells, then the similarity of its action to that of indomethacin suggests that indomethacin causes enhancement of O_2^- production mainly by an action on DAG kinase, though a small component of action on DAG lipase cannot be discounted.

Further studies of the effects of indomethacin and the two inhibitors on the purified isolated enzymes would be of interest and are under way, though such studies with isolated enzymes are only of value in determining a locus of action if the agents concerned can be demonstrated to have effects in the intact cells, as shown here.

This work was funded by the Arthritis and Rheumatism Council. We wish to thank Mr R.E. Muid and Mrs J. Mancini for technical assistance. We wish to thank Dr Tai of the Division of Medicinal Chemistry & Pharmacology, College of Pharmacy, Lexington, Kentucky, U.S.A. and also Dr Sutherland of Revlon Health Care Group, N.Y., U.S.A., for the gift of RHC80267.

References

BADWEY, J.A. & KARNOVSKY, M.L. (1980). Active oxygen species and the functions of phagocytic leukocytes. *A. Rev. Biochem.*, **49**, 695–726.

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315–321.

BROMBERG, Y. & PICK, E. (1983). Unsaturated fatty acids as second messengers of superoxide generation by macrophages. *Cell. Immunol.*, **79**, 240–252.

COCKCROFT, S., BENNETT, J.P. & GOMPERTS, B.D. (1980). Stimulus-secretion coupling in rabbit neutrophils is not mediated by phosphatidylinositol breakdown. *Nature*, **288**, 275–277.

DE CHAFFOY DE COURCELLES, D., ROEVENS, P. & VAN BELLE, H. (1985). R59022, a diacylglycerol kinase inhibitor: its effect on diaclyglycerol and thrombin-induced C kinase activation in the intact platelet. *J. biol. Chem.*, **260**, 15762–15770.

DALE, M.M. & PENFIELD, A. (1984). Synergism between phorbol ester and A23187 in superoxide production by neutrophils. *FEBS lett.*, **175,** 170–172.

DALE, M.M. & PENFIELD, A. (1985). Superoxide generation by either 1-oleoyl-2-acetylglycerol or A23187 in human neutrophils is enhanced by indomethacin. *FEBS lett.*, **185,** 213–217.

FANTONE, J.C., MARASCO, W.A., ELGAS, L.J. & WARD, P.A. (1984). Stimulus specificity of prostaglandin inhibition of rabbit polymorphonuclear leukocyte lysosomal enzyme release and superoxide anion production. *Am. J. Path.*, **115,** 9–16.

GAY, J.C., LUKENS, J.N. & ENGLISH, D.K. (1984). Differential inhibition of neutrophil superoxide generation by nonsteroidal antiinflammatory drugs. *Inflamm.*, **8,** 209–222.

GAY, J.C., ENGLISH, D. & LUKENS, J.N. (1985). Stimulation of neutrophil oxidative metabolism by indomethacin. *Agents & Actions*, **16,** 336–341.

GERARD, C., McPHAIL, L., MARFAT, A., STIMLER-GERARD, N.P., BASS, D.A. & McCALL, C.E. (1986). Role of protein kinases in stimulation of human polymorphonuclear leukocyte oxidative metabolism by various agonists. *J. clin. Invest.*, **71,** 61–65.

HANNUN, Y.A., LOOMIS, C.R., MERRILL, A.H., Jr. & BELL, R.M. (1986). Sphingosine inhibition of protein kinase C activity and of phorbol dibutyrate binding in vitro and in human platelets. *J. biol. Chem.*, **261,** 12604–12609.

HIDAKA, H., INAGAKI, M., KAWAMOTO, S. & SASAKI, Y. (1984). Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. *Biochem.*, **23,** 5036–5041.

JOHNSTON, R.B. & LEHMEYER, J. (1976). Elaboration of toxic oxygen by-products by neutrophils in a model of immune complex disease. *J. clin. Invest.*, **57,** 836–841.

LUNEC, J., BLAKE, D.R., McCLEARY, S.J., BRAILSFORD, S. & BACON, P.A. (1985). Self-perpetuating mechanisms of immunoglobulin G aggregation in rheumatoid inflammation. *J. clin. Invest.*, **76,** 2084–2090.

MUID, R.E., PENFIELD, A. & DALE, M.M. (1987). The diacylglycerol kinase inhibitor, R59022, enhances the superoxide generation from human neutrophils induced by stimulation of fMet-Leu-Phe, IgG and C3b receptors. *Biochem. biophys. Res. Commun.*, **143,** 630–637.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature*, **308,** 693–698.

PENFIELD, A. & DALE, M.M. (1984). Synergism between A23187 and 1-oleoyl-2-acetylglycerol in superoxide production by human neutrophils. *Biochem. biophys. Res. Commun.*, **125,** 332–336.

RITTENHOUSE-SIMMONS, S. (1980). Indomethacin-induced accumulation of diglyceride in activated human platelets: the role of diglyceride lipase. *J. biol. Chem.*, **255,** 2259–2262.

ROBINSON, J.M., BADWEY, J.A., KARNOVSKY, M.L. & KARNOVSKY, M.J. (1984). Superoxide release by neutrophils: synergistic effects of a phorbol ester and a calcium ionophore. *Biochem. biophys. Res. Commun.*, **122,** 734–739.

SUTHERLAND, C.A. & AMIN, D. (1982). Relative activities of rat and dog platelet phospholipase A_2 and diglyceride lipase: selective inhibition of diglyceride lipase by RHC80267. *J. biol. Chem.*, **257,** 14006–14010.

TATE, R.M. & REPINE, J.E. (1984). Phagocytes, oxygen radicals and lung injury. In *Free Radicals in Biology*, Vol. VI. ed. Pryer, W.A. pp. 199–209. New York: Academic Press.

DI VIRGILIO, F., LEW, D.P. & POZZAN, T. (1984). Protein kinase C activation of physiological processes in human neutrophils at vanishingly small cytosolic calcium levels. *Nature*, **310,** 691–693.

WILSON, E., OLCOTT, M.C., BELL, R.M., MERRILL, A.H., Jr. & LAMBETH, J.D. (1986). Inhibition of the oxidative burst in human neutrophils by sphingoid long-chain bases. *J. biol. Chem.*, **261,** 12616–12623.

WRIGHT, C.D. & HOFFMAN, M.D. (1986). The protein kinase C inhibitors H-7 and H-9 fail to inhibit human neutrophil activation. *Biochem. biophys. Res. Commun.*, **135,** 749–755.

(Received November 28, 1986.
Revised May 11, 1987.
Accepted May 22, 1987.)