Biochemical Pharmacology, Vol. 36, No. 20, pp. 3459–3462, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

DIACYLGLYCEROL KINASE INHIBITOR, R59022, POTENTIATES NEUTROPHIL OXIDASE ACTIVATION BY Ca^{2+}-DEPENDENT STIMULI

EVIDENCE FOR TWO SEPARATE BUT CONVERGENT PATHWAYS

ERYL COOKE, FUTWAN A. AL-MOHANNA and MAURICE B. HALLETT*
University Department of Surgery, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

(*Received* 16 *March* 1987; *accepted* 8 *July* 1987)

Abstract—An inhibitor of diacylglycerol kinase, R59022, enhanced activation of the neutrophil oxidase stimulated by the Ca^{2+}-ionophore, A23187 (1 μM), and by *N*-formyl-methionyl leucyl-phenylalanine (1 μM). The enhancement was reversed by two inhibitors of c-kinase, retinal (10 μM), and gossypol (20 μM). Activation by phorbol-myristyl-acetate and unopsonised latex beads were not enhanced. It was concluded that the chemotactic peptide generated diacylglycerol, but that maximum activation of c-kinase by this route was not achievable. The role of diacylglycerol in activation by beads remained unclear.

Activation of the neutrophil oxidase plays an important role in combating infection and may also play a role in mediating tissue damage in inflammatory diseases. The intracellular mechanisms that couple stimulation to this response remain unestablished. However, two routes have been indentified, one dependent upon a rise in intracellular Ca^{2+} [1, 2] which can result in extracellular production of oxygen metabolites [3], and another which is independent of a such a rise [1] and occurs during phagocytosis [3]. It was also clear that other intracellular messengers were generated which act with intracellular Ca^{2+} [1, 2]. Although the nature of these messengers is not clear, one possiblity was diacylglycerol. It has been suggested that diacylglycerol produced during the breakdown of phosphatidyl inositol would enable activation of protein kinase C at resting cytoplasmic Ca^{2+} concentrations [4]. Although direct evidence has been difficult to obtain, an agent, R59022†, has been recently reported which may be of use in testing this hypothesis.

R59022 inhibits diacylglycerol kinase, an enzyme which reduces the concentration of diacylglycerol and is associated with the neutrophil plasma membrane [5]. In platelets, R59022 has been shown to elevate diacylglycerol concentrations following stimulation and also to increase phosphorylation of a 40 k protein [6]. Since inhibitors of cyclic AMP phosphodiesterase have proved useful in evaluating the role of cyclic AMP, the possibility exists that R59022 may also be useful in an analogous way, for examining the second messenger role of diacylglycerol. The aim of the work presented in this paper, therefore, was to use this agent to test the possibility that diacylglycerol had a second messenger role in neutrophil oxidase activation.

* To whom correspondence should be addressed.

† Abreviations used: PMA, phorbol 12-myristate 13-acetate; f-mlp, *N*-formyl-methionyl-leucyl-phenylalanine; R59022, 6-(2-(4-((4-fluorophenyl)phenylmethylene)-1-piperidinyl-7-methyl 5 *H*-thiazolo-(3,2-a) pyrimidin-5-one.

MATERIALS AND METHODS

Neutrophils were prepared from the peritoneal exudate of rats 14–18 hr after intraperitoneal injection with sodium caseinate, as previously described [7]. The cells were suspended in Hepes-buffered Krebs medium, containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 25 mM Hepes, 0.1% bovine serum albumin and the pH adjusted to 7.4 with NaOH. Luminol-dependent chemiluminescence was monitored using a purpose-built photon-counting luminometer [8]. Oxygen consumption was measured using a Clark-type electrode (Rank Bros., Bottisham, U.K.). N-fmlp, PMA and cytochalasin B were purchased from Sigma Chemical Company; latex beads (dia. 1 μm) from Polyscience (Warrington, PA); and R59022 from Janssen Life Products (Wantage, U.K.). Gossypol acetic acid was kindly given by Dr G. M. H. Waites, World Health Organisation, Geneva, Switzerland. The soluble agents were dissolved in DMSO and added to cell suspensions to give a final DMSO concentration of less than 0.3%.

RESULTS

Effect of R59022 on oxidase activation

Pre-incubation with R59022 (0.1–20 μM) had no significant effect on the resting level of luminol-dependent chemiluminescence. However, under these conditions the response to an optimal concentration of f-mlp, 1 μM, was enhanced by up to 4-fold (Fig. 1a). At 20 μM R59022 the fold enhancement was 3.4 ± 0.4 (N = 3). Stimulation by the cal-

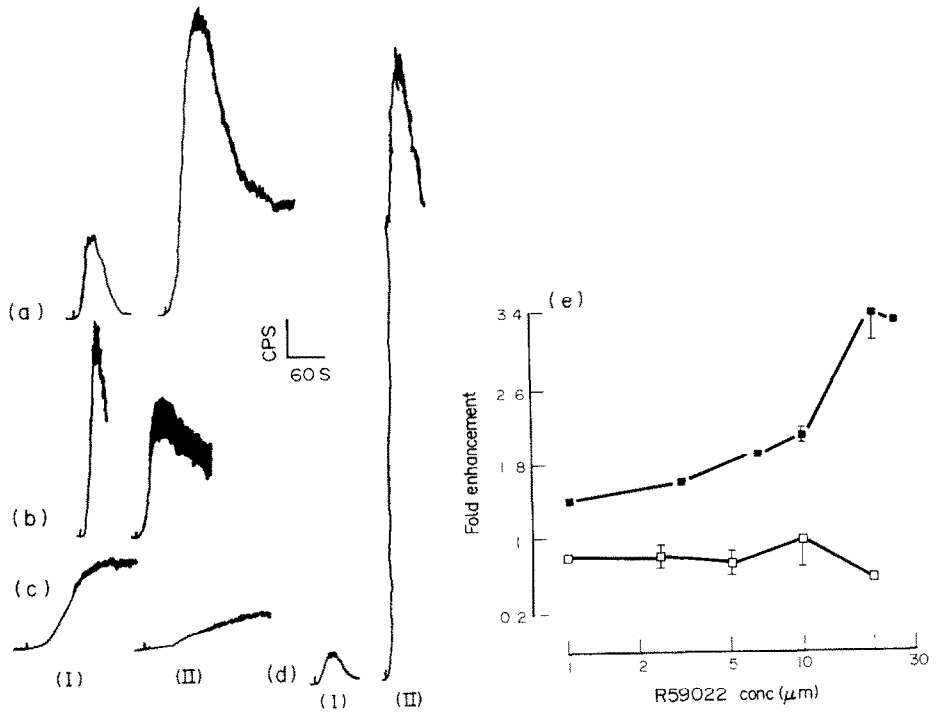

Fig. 1. The effect of R59022 on luminol-dependent chemiluminescence from rat neutrophils (5.10^6 cells) stimulated by (a) f-mlp, 1 μM, (b) latex beads (dia. 1 μm), 5.10^9/ml, (c) PMA, 0.1 μg/ml and (d) A23187, 1 μM, added at the point indicated on the trace. In each pair of traces (i) represents the response of untreated cells and (ii) represents the response from cells pre-incubated for 5 min at 37° with R59022, 20 μM. Luminescence was recorded as counts per second (cps), the vertical axis of the calibration bar representing 4.10^3 cps. Each trace was representative of at least 3 separate determinations. The graph (e) shows the fold enhancement with preincubation with R59022 at the concentration shown with the stimulus being latex beads, 5.10^9/ml (□) and f-mlp, 1 μM(■). The bars show the standard error where larger than the symbol.

cium ionophore, A23187, was also enhanced, 20 μM R59022 producing approximately 20-fold enhancement (Fig. 1d). In contrast, responses induced by PMA and unopsonised latex beads (dia. 1 μm) were not enhanced, and at 20 μM R59022 inhibition was observed (Fig. 1b and c). It was noted that a characteristic of the responses to both f-mlp and latex beads following pre-treatment with R59022 was a reduction in the rate at which the chemiluminescence returned to baseline (see Fig. 1a and b). The concentration of R59022 required for 50% maximum enhancement of the response to f-mlp was approximately 15 μM (Fig. 1e).

Enhancement by R59022 was via c-kinase activation

Several possibilities existed for the mechanism of enhancement by R59022, namely (i) that the enhancement was an artifact caused by inteference to the chemiluminescence assay; (ii) that the enhancement was due an effect on the cytoskeleton, producing a "cytochalasin-B type" effect (cytochalasin B produces an enhancement of oxidase activation induced by f-mlp and A23187, but not by PMA or latex beads [9, 10]); (iii) that the enhancement was the result of elevated diacylglycerol causing increased activation of c-kinase.

The first possibility would seem unlikely because of the differential effect of the agent on various stimuli. Furthermore, the enhancement was also observed when oxidase activity was determined by the measurement of oxygen consumption (Fig. 2). Since the enhancement observed with A23187 was not as great as observed with chemiluminescence, the possibility exists that part of the enhancement of this latter response may reflect secretion of peroxidase. The second possibility was also excluded by the demonstration that the enhancement could be produced in cells pre-treated with cytochalasin B (Fig. 3d). The third possibility, was tested by the use of inhibitors of c-kinase, retinal [11] and gossypol [12]. Although retinal has previously been shown not to inhibit activation by f-mlp [13], it did, however, prevent the enhancement due to R59022 (10 μM), reducing the enhancement from 4.0- to 1.6-fold (Fig. 3b). However, the presence of retinal increased the response slightly and consequently may have contributed to the subsequent inhibition of enhancement by R59022. Gossypol, which did not increase the response, did however prevent the enhancement induced by R59022 (Fig. 3c). These results are therefore consistent with the enhancement being mediated by c-kinase activation.

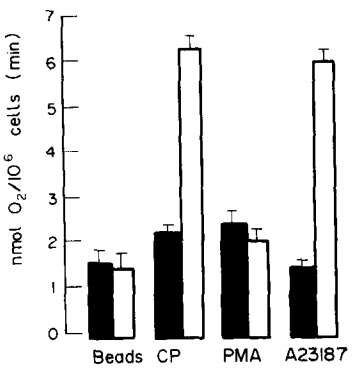

Fig. 2. Effect of R59022 on oxygen consumption by rat neutrophils. The oxygen consumption rates in response to latex beads (4×10^9 beads/ml)—beads; chemotactic peptide (1 μM)—CP; phorbol myristate acetate (0.1 μg/ml)—PMA; and ionophore A23187 (1 μM) are shown in the absence (open bars) and presence (closed bars) of R59022 (20 μM). The error bars show the standard error for three determinations.

DISCUSSION

The results presented here demonstrate that the inhibitor of diacylglycerol kinase, R59022, produced a marked enhancement of the oxidase activation simulated by two intracellular Ca^{2+}-dependent stimuli, f-mlp and A23187. The enhancement was overcome by inhibitors of c-kinase, suggesting that c-kinase activity was crucial for this effect. Since the response to PMA, which causes direct activation of c-kinase, was not enhanced, it was concluded that enhancement resulted from a step prior to the activation of this enzyme. This was therefore consistent with the enhancement produced by R59022 being mediated by an elevation in intracellular diacylglycerol of the appropriate fatty acid composition and at a site appropriate for activation of c-kinase. It was not possible to demonstrate directly an increase in total cellular diacylglycerol content, using thin layer chromatography. However, no attempt was made to measure individual diacylglycerols of particular fatty-acid composition.

Since inhibitors of c-kinase have not been found to inhibit neutrophil activation by f-mlp [13–17], it has therefore been concluded that activation by this stimulus was not mediated by c-kinase. The demonstration here of an enhancement which can be inhibited by these agents therefore leads to three conclusions. (i) Stimulation by f-mlp generated non-Ca^{2+} intracellular messengers, including c-kinase-activating diacylglycerol. (ii) The concentration of diacylglycerol generated, even at the optimal receptor occupancy, was insufficient to maximally activate c-kinase (if at all) at the existing intracellular Ca^{2+} concentration (approx. 0.6 μM). (iii) Activation of the c-kinase route or the c-kinase independent route both lead to activation of the oxidase. It is interesting to note that the activity of the isolated oxidase can be enhanced by calmodulin [18] and that c-kinase can phosphorylate and activate the oxidase *in vitro* [19].

Similar conclusions were drawn for stimulation by A23187, namely that diacylglycerol was generated at a concentration insufficient to activate c-kinase. The massive enhancement (20-fold) observed (Fig. 1d) may reflect the high intracellular Ca^{2+} concentration (approx. 10 μM) existing under these conditions.

The lack of enhancement of response to unopsonised latex beads emphasises that the intracellular mechanism for activation by this stimulus is different from that for f-mlp. Whereas stimulation by f-mlp is totally dependent on a rise in intracellular Ca^{2+}

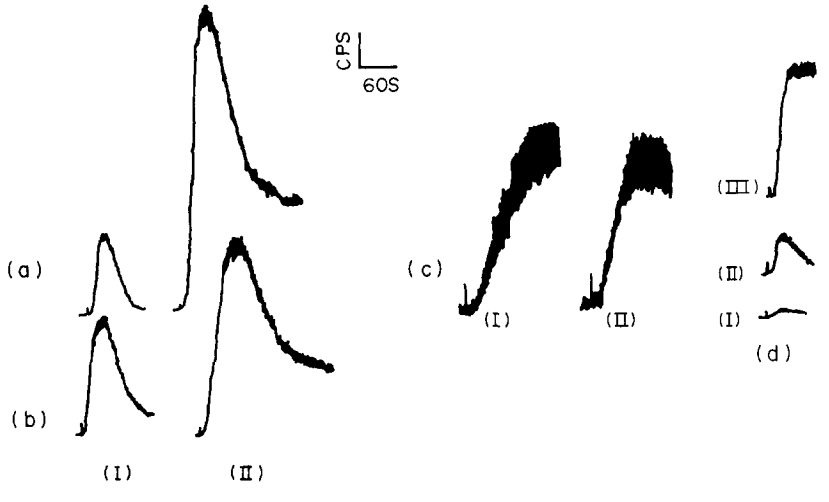

Fig. 3. The effect of c-kinase inhibitors and cytochalasin B on the enhancement due to R59022.
In the pairs of traces a, b and c, the chemiluminescence responses to f-mlp (1 μM) are compared in (i) with the responses from cells pretreated with R59022 (20 μM), (ii). The pairs (a) represent untreated cells, (b) cells treated with retinal, 10 μM, (c) cells treated with gossypol, 20 μM. In the group of traces (d) the lower trace represents the response from untreated cells to f-mlp, the middle trace in the presence of cytochalasin B (5 μg/ml), and the upper trace in the presence of both cytochalasin B and R59022, 20 μM. The luminescence calibration bar represents 4.10^3 cps for (a) and (b), 4.10^2 cps for (c) and 4.10^4 cps for traces (d). Traces shown were representative of at least 3 separate experiments.

[1, 20], activation by unopsonised latex beads [1] and particles opsonised with C3bi [21] does not depend on such a rise. It has previously been demonstrated that activation by latex beads was mediated by c-kinase and it was suggested therefore that diacylglycerol may play a role [13]. The demonstration here that no enhancement was observed with an agent expected to elevate the concentration of this putative messenger therefore leads to two possibilities; (i) diacylglycerol played no part in signal transduction by this route, or (ii) diacylglycerol did play a part but the concentration generated was sufficiently high to maximally activate c-kinase at the existing Ca^{2+} concentration (approx. 0.1 μM). Since a membrane-bound intracellular messenger such as diacyglycerol would be restricted to the phagosomal membrane, this latter possibility cannot be excluded. Furthermore, such a messenger would provide a mechanism for activating the oxidase only in the phagosomes and not elsewhere in the cell.

The results presented suggest that when elevated, diacylglycerol can activate c-kinase, which in turn can trigger the neutrophil oxidase. However, no clear evidence was provided that this process occurred during stimulation by physiological routes. The possibility therefore remains that second messengers other than Ca^{2+} and diacylglycerol also operate in the neutrophil.

Acknowledgements—We are grateful to the MRC for financial support, Mr D. Grimshaw for performing TLC, and Dr Waites, WHO, for kindly supplying gossypol. F.A.A. holds a MOD (Saudi) Scholarship.

REFERENCES

1. A. K. Campbell and M. B. Hallett, *J. Physiol. (Lond.)* **338**, 537 (1983).
2. T. Pozzan, D. P. Lew, C. B. Wollheim and R. Y. Tsien, *Science* **221**, 1413 (1983).
3. M. B. Hallett and A. K. Campbell, *Biochem. J.* **216**, 459 (1983).
4. Y. Nishizuka, *Nature, Lond.* **308**, 693 (1984).
5. S. Cockcroft, J. M. Baldwin and D. Allan, *Biochem. J.* **221**, 477 (1984).
6. D. de Chaffoy de Courcelles, P. Roevens and H. Van Belle, *J. biol. Chem.* **260**, 15762 (1985).
7. M. B. Hallett, J. P. Luzio and A. K. Campbell, *Immunology* **44**, 569 (1981).
8. A. K. Campbell, M. B. Hallett and I. Weeks, *Meth. Biochem. Anal.* **31**, 317 (1985).
9. E. Cooke, F. A. Al-Mohanna and M. B. Hallett, *Biochem. Soc. Trans.* **13**, 1173 (1986).
10. F. A. Al-Mohanna and M. B. Hallett, *Biochim. biophys. Acta* **927**, 366 (1987).
11. S. M. Taffet, A. R. C. Greenfield and M. K. Haddox, *Biochem. biophys. Res. Commun.* **114**, 1194 (1983).
12. K. Kimura, K. Sakurado and N. Katoh, *Biochim. biophys. Acta* **839**, 276 (1985).
13. E. Cooke and M. B. Hallett, *Biochem. J.* **232**, 323 (1985).
14. P. H. Naccache, T. F. P. Molski and R. I. Sha'afi, *FEBS Lett.* **193**, 227 (1985).
15. C. Gerard, L. C. McPhail, A. Marjal, N. P. Stimler-Gerard, D. A. Bass and C. E. McCall, *J. clin. Invest.* **77**, 61 (1986).
16. R. I. Sha'afi, T. F. P. Molski, C-K. Huang and P. H. Naccache, *Biochem. biophys. Res. Commun.* **137**, 50 (1986).
17. C. D. Wright and M. D. Hoffman, *Biochem. biophys. Res. Commun.* **135** 749 (1986).
18. H. P. Jones, G. Ghai, W. F. Petrone and J. McCord, *Biochim. biophys. Acta* **714**, 152 (1982).
19. J. A. Cox, A. Y. Jeng, N. A. Sharkey, P. M. Blumberg and A. I. Tauber, *J. clin. Invest.* **76**, 1932 (1986).
20. D. P. Lew, C. B. Wollheim, F. A. Waldrogel and T. Pozzan, *J. Cell Biol.* **99**, 1212 (1984).
21. D. P. Lew, T. Andersson, J. Hed, F. DiVirgilio, T. Pozzan and O. Stendahl, *Nature, Lond.* **315**, 509 (1985).