Vol. 125, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 30, 1984 Pages 332-336

SYNERGISM BETWEEN A23187 AND 1-OLEOYL-2-ACETYL-GLYCEROL IN SUPEROXIDE PRODUCTION BY HUMAN NEUTROPHILS

Adrienne Penfield and M. Maureen Dale

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

Received October 8, 1984

Concentrations of the calcium ionophore A23187 and 1-oleoyl-2-acetyl-glycerol, which on their own are minimally effective in stimulating superoxide release from human neutrophils, show marked mutual potentiation when given simultaneously. The potentiating effect of the diacylglycerol can be shown to be dose-related. These results support the hypothesis that synergism between cytosolic calcium and protein kinase C is involved in signal transduction for the respiratory burst in the human neutrophil.

Neutrophils, when exposed to certain stimuli, manifest an increased O_2 uptake (the 'respiratory burst') and generate large amounts of superoxide (O_2^-)[1] and hydrogen peroxide. This reaction forms the basis for the production of toxic oxygen products which are important for effective microbial killing by the cell (reviewed in 1) and are also thought to be implicated in the tissue damage of complex-mediated disease (2). However, as is emphasized in a recent review (3) the events involved in the transduction mechanisms of the respiratory burst are not yet fully understood.

Nishizuka has recently proposed that two events - an increase in diacylglycerol (which activates protein kinase C) and an increase in cytosolic calcium - may be involved in stimulus-secretion coupling in various cell types, and that the two pathways may function synergistically (4). Recently we reported synergism between the tumor promoter, PMA[1], and a calcium ionophore in activation of the respiratory burst in the neutrophil (5) and similar results have been described independently by others (6,7). In the present study we report that low concentrations of the ionophore A23187 and 1-oleoyl-2-acetylglycerol,

[1] The abbreviations used are: O_2^-, superoxide; OAG, 1-oleoyl-2-acetyl-glycerol; and PMA, 4β-phorbol 12-myristate 13-acetate.

0006-291X/84 $1.50

(OAG), which, given separately, have little effect, result in substantial superoxide production when given simultaneously.

MATERIALS AND METHODS

The methods used were as previously described (5). Neutrophils, collected from human volunteers by venipuncture, were prepared by Ficoll-Isopaque separation and suspended in calcium-free Tyrode solution containing NaCl (137mM), KCl (2.7mM), $MgCl_2$ (1mM), glucose (1mg ml^{-1}) and bovine serum albumin (1mg ml^{-1}). After equilibration for 20 minutes at 37°C, 2.5 x 10^6 cells, in a volume of 500μl, were dispensed into 2.5ml tubes (NA2S, Sterilin, England) to which had been added 1mg ferricytochrome C (horse heart type III, Sigma Chemical Co.), appropriate dilutions of A23187 (Sigma Chemical CO.), OAG and either Tyrode solution or superoxide dismutase (75 units, bovine blood, Sigma Chemical Co.) to a final volume of 875μl. The final calcium concentration in all samples was 3mM. After incubation (30 minutes at 37°C) the reaction was stopped by the addition of 500μl N-ethyl maleimide (1mM, Sigma Chemical Co.). The amount of superoxide produced was measured by the superoxide dismutase-inhibitable reduction of ferricytochrome C at 550nm using a Perkin-Elmer SP-1800 spectrophotometer (5). OAG was synthesized by Dr. A. Watts, Biochemistry Department, University of Oxford, England.

RESULTS

The possibility of potentiation of A23187 by OAG, and *vice versa*, was tested by measuring the effect of one agent at the lower end of the dose-response curve of the other. Attention was confined to this part of the dose-response relationship because, as has been stressed (4), high concentrations of each agent may cause cell stimulation non-specifically.

The mean percentage potentiation of the response to A23187 by OAG is shown in figure 1a and the converse in 1b. Since the expression of the mean results of several experiments as percentage potentiation does not necessarily give a clear idea of the actual data, the full results of two representative experiments are also given - the effect of two, low concentrations of OAG on the A23187 response in figure 1c and the effect of a low concentration of A23187 on the OAG response in 1d.

OAG, 4.5 x 10^{-5}M resulted in maximal or very substantial O_2^- production in most subjects and it was only possible to obtain a measure of potentiation at this concentration in one subject in whom the response to this concentration was clearly submaximal.

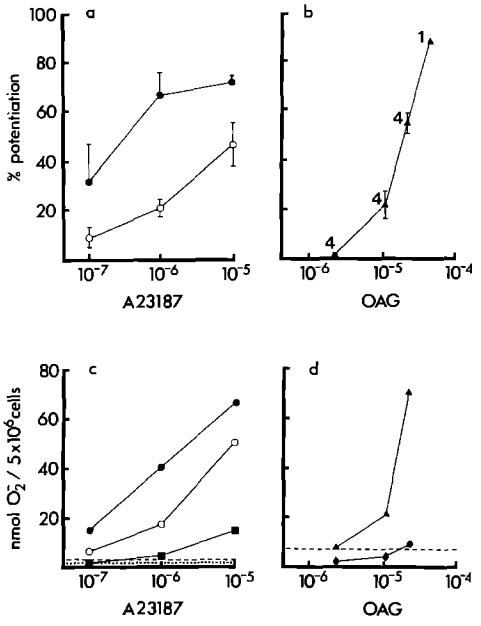

Figure 1. Synergism between A23187 and OAG in superoxide production.
a) Potentiation of the response to A23187 in the presence of OAG 1.1×10^{-5}M (○) or 2.3×10^{-5}M (●), expressed as a percentage of the maximum response obtained. The points represent the mean values and the bars, standard errors. The mean amount of O_2^- produced with the highest concentration of ionophore + OAG was 60(s.e. 5.3)nmoles per 5×10^6 cells (n=3).
b) Potentiation of the response to OAG in the presence of A23187 10^{-6}M (▲). The points represent the mean values and the bars, standard errors. The mean amount of O_2^- produced with the highest concentration of OAG + ionophore was 66(s.e. 0.9)nmoles per 5×10^6 cells. The number of experiments is given beside each point.
c) Results of an individual experiment showing the effect of OAG on the dose-response curve of O_2^- production with A23187.
A23187 alone (■); A23187 with OAG 1.1×10^{-5}M (○); A23187 with OAG 2.3×10^{-5}M (●). The dotted line gives O_2^- production with OAG, 1.1×10^{-5}M, given alone; the dashed line gives the O_2^- production with OAG 2.3×10^{-5}M, given alone.
d) Results of an individual experiment showing the effect of A23187 on the dose-response curve of O_2^- production with OAG.
OAG alone (◆); OAG with 10^{-6}M A23187 (▲). The dashed line gives O_2^- production with A23187 10^{-6}M by itself.

DISCUSSION

It seems clear that synergism between protein kinase C and calcium in signal transduction occurs in several different cell types. In addition to the examples cited in the recent review (4), such synergism has also been reported in amylase secretion by pancreatic acini (8) and in activation of hepatocyte glycogen phosphorylase (9), as well as in the activation of the respiratory burst

(5,6,7). In most of the studies specified, the phorbol ester tumor promoter, PMA, has been used to activate protein kinase C. PMA has been shown to activate protein kinase C directly (10) and is believed to mimic the action of the naturally occurring diacylglycerol which is produced, when cells are stimulated, as a result of the breakdown of inositol phospholipids (11,12). However, unlike diacylglycerol, PMA is metabolized slowly and remains in cell membranes for prolonged periods of time (4). It may also have additional actions e.g. on protein alkylation (13) or phospholipase A_2 (14) or on the relationship between surface receptors and adenyl cyclase (15) in various cell types. The action of OAG, a synthetic diacylglycerol, is also similar to that of the diacylglycerol produced in cells after stimulation, as shown by experiments with platelets. However, OAG is rapidly metabolized *in situ* (reviewed in 4),and is not reported to have other actions, such as tumor promotion. Thus the use of OAG, rather than PMA, as a protein kinase C activator can be considered to represent more closely the events occurring naturally in living cells. OAG has been shown to stimulate exocytosis in neutrophils (16) and to stimulate superoxide production (17).

In a recent noteworthy study of neutrophil activation, it was reported that high concentrations of PMA (20nM) could induce an increase in oxygen consumption even when the cytosolic calcium was 10-20 times below the resting level (9). It was suggested that activation of protein kinase C might be sufficient, on its own, to induce NADPH-oxidase activation without the participation of the 'calcium pathway'. This could imply that synergism of the two pathways is not essential in stimulation of the respiratory burst in the neutrophil. However, as has been stressed above, a high concentration of PMA may possibly have other actions besides stimulation of protein kinase C and its effect may not reflect accurately the events which happen in the neutrophil under physiological conditions. In the present experiments OAG rather than PMA was used as the protein kinase C activator. The results indicate that not only does OAG potentiate the action of A23187, but that an increase in cytosolic calcium produced by a low concentration of A23187 can convert a subthreshold or minimal effect of OAG

into a substantial response. This provides further support for the hypothesis that both pathways may be involved, physiologically, in stimulus-activation coupling for the respiratory burst in the neutrophil.

ACKNOWLEDGEMENTS

We are very grateful to Dr. A. Watts of the Biochemistry Department, Oxford University, for the gift of OAG. We thank Mr. R.E. Mould for technical help. This work was supported by grants from the Emily le Rossignol Fund and the Arthritis and Rheumatism Fund, Great Britain.

REFERENCES

1. Badwey, J.A., and Karnovsky, M.L. (1980) Annu. Rev. Biochem. 49, 695-726.
2. Johnston, R.B., and Lehmeyer, J. (1976) J. Clin. Invest. 57, 836-841.
3. Babior, B.M. (1984) J. Clin. Invest. 73, 599-601.
4. Nishizuka, Y. (1984) Nature (Lond.) 308, 693-698.
5. Dale, M.M., and Penfield, A. (1984) FEBS Lett. (In press)
6. Robinson, J.M., Badwey, J.A., Karnovsky, M.L., and Karnovsky, M.J. (1984) Biochem. Biophys. Res. Commun. 122, 734-739.
7. Di Virgilio, F., Lew, D.P., and Pozzan, T. (1984) Nature (Lond.) 310, 691-693.
8. de Pont, J.J.H.H.M., and Fleuren-Jakobs, A.M.M. (1984) FEBS Lett. 170, 64-68.
9. Fain, J.N., Li, S-Y., Litosch, I., and Wallace, M. (1984) Biochem. Biophys. Res. Commun. 119, 88-94.
10. Castagna, M., Takai, Y., Kaibuchi, K., Sauo, K., Kikkawa, U., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
11. Michell, R.H. (1975) Biochem. Biophys. Acta 415, 81-147.
12. Berridge, M.J. (1981) J. Molec. cell. Endocrinol. 24, 115-140.
13. Mueller, G.C., and Weltz, P.W. (1982) In Carcinogenesis 7, Hecker, E., Fusenig, N.E., Kunz, W., Marks, F., and Thielman, H.W. (eds.) Raven Press, N.Y. 499-511.
14. Levine, L.,and Hassad, A. (1977) Biochem. Biophys. Res. Commun. 79, 477-480.
15. Belman, S., and Garte, S.J. (1982) In Carcinogenesis 7, Hecker, E., Fusenig, N.E., Kunz, W., Marks, F., and Thielman, H.W. (eds.) Raven Press, N.Y. 561-563.
16. Kajikawa, N., Kaibuchi, K., Matsubara, Y., Kikkawa, U., Takai, Y., Nishizuka, Y., Itoh, K., and Tomioka, C. (1983) Biochem. Biophys. Res. Commun. 116, 743-750.
17. Fujita, I., Irita, K., Takeshige, K., and Minakami, S. (1984) Biochem. Biophys. Res. Commun. 120, 318-324.