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REACTIVE OXYGEN PRODUCTION, ARACHIDONATE METABOLISM AND CYCLIC AMP IN MACROPHAGES

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Summary: Chemiluminescence was used as an indicator of the production of reactive oxygen species by thioglycollate-elicited rat peritoneal macrophages stimulated by A23187. This action of the ionophore was inhibited by bromophenacyl bromide and nordihydroguaiaretic acid, inhibitors of the phospholipase A_2 and lipoxygenase enzymes, respectively. The cyclo-oxygenase inhibitors, indomethacin and aspirin, did not diminish the light output. Preincubation of the cells with the 8-bromo- or dibutyryl analogues of cyclic AMP or with the cyclic AMP-phosphodiesterase inhibitors theophylline and RO-20-1724, or with PGE_2, inhibited the A23187-evoked chemiluminescence. The results suggest that the lipoxygenase pathway of arachidonic acid metabolism may make a significant contribution to reactive oxygen production. This process may be modulated, and its duration limited, by cyclic AMP-mediated actions of prostaglandins, which are products of the cyclo-oxygenation of arachidonate.

Upon recognition of a stimulus, macrophages frequently respond with increased oxygen uptake and activation of the hexose mono-phosphate shunt (1). The "extra" oxygen consumed is utilised in mitochondrial respiration and in the non-mitochondrial formation of reactive oxygen species (2). It has been demonstrated that this respiratory burst, with its accompanying generation of reactive oxygen species, is essential for the destruction of ingested micro-organisms (3,4). The presence of reactive oxygen metabolites can be inferred from the light emitted (chemiluminescence) during their reaction with the easily-oxidizable substance luminol (5-amino-2,3-dihydro-1,4-phthalazinedione)(5).

Mouse peritoneal macrophages respond to the particulate stimulus zymosan with a burst of luminol-dependent chemiluminescence (CL)(6), approximately 65% of which is glucose-dependent and Ca^{++}-independent, whilst the remaining portion is Ca^{++}-dependent and glucose-independent. The latter component can

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Abbreviations: CL: chemiluminescence. H_2O_2: hydrogen peroxide. NDGA: nordihydroguaiaretic acid. TG: thioglycollate broth.

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be mimicked by the addition of the ionophore A23187 (6). This paper shows that the Ca^{++}-dependent CL response evoked by A23187 is apparently linked to the metabolism of arachidonic acid. As the ionophore also stimulates the production of prostaglandins and cyclic AMP (7,8), the possible relationship between these parameters and the generation of CL is examined.

Materials and Methods. A23187 was from Calbiochem (La Jolla Calif., USA). Verapamil was from Knoll AG (Sydney, Australia). All other reagents were purchased from Sigma (St. Louis, MO, USA). Rat peritoneal macrophages, elicited with Brewer's thioglycollate medium, were prepared as described previously (9). CL was measured in the presence of luminol in a Packard liquid scintillation counter set in the 'off-coincidence' mode (10). Preliminary experiments indicated that none of the inhibitors used (except NDGA) could quench the light emitted from the chemical reaction between H_2O_2 and luminol. Therefore, provided that the reaction between luminol and oxygen metabolites other than H_2O_2 is similarly unaffected, any reduction in A23187-initiated CL is likely to be due to the direct action of the inhibitor on the cells. NDGA, dissolved in dimethylformamide (final concentration of solvent = 0.01%), caused about 15% inhibition of the light emitted by the H_2O_2-luminol reaction. This inhibition, which may be ascribed to the anti-oxidant properties of NDGA (11), was taken into account in calculating the results (Table 1). Where applicable, the results were compared by the Wilcoxon rank sum test.

Results and Discussion: On addition of A23187 (6.7μM) to TG-elicited rat peritoneal macrophages, there was a brief lag period before the onset of light emission. The CL response, which reached its peak within 4-5 min, was relatively short-lived, declining to its basal level after 10-15 min (Fig. 1). Qualitatively similar profiles were observed with resident and BCG-elicited macrophages; the magnitude of light emission was greater in TG- and BCG-elicited cells than in resident peritoneal macrophages (L.K. Lim, unpublished observations).

The CL response to A23187 was abolished completely when the macrophages were pretreated with the calcium channel blocker verapamil (12) or incubated in a Ca^{++}-depleted medium (Table 1). This suggests that production of the 'light-emitting' species may be dependent upon A23187-promoted trans-membrane Ca^{++} fluxes. Ca^{++} may be involved in a calmodulin-associated event since trifluoperazine (50μM), a "blocker" of calmodulin-stimulated reactions, completely inhibited light output (data not shown). CL was also reduced totally in the presence of the phospholipase A_2 inhibitor BPB (13), indicating that the elevation of free arachidonate may be directly or indirectly involved in activated oxygen formation (Table 1). The most significant step may be the lipoxygenation of arachidonic acid since NDGA, which inhibits this process (13), diminished the light output; indomethacin and aspirin, which inhibit

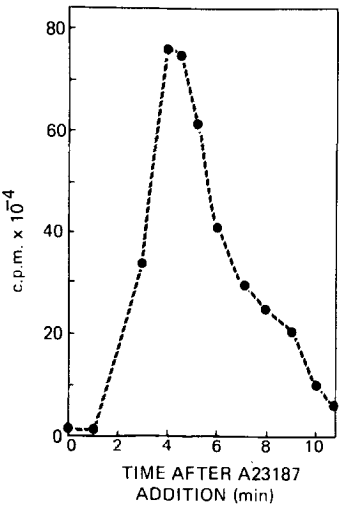

Fig. 1. A typical profile of the chemiluminescent response in A23187-treated, TG-elicited rat peritoneal macrophages. The rat peritoneal macrophages (1 x 10^6 cells) were preincubated with 50µl of luminol (final concentration 40µM) for 10 min at 37°C in a total volume of 2.5 ml phosphate-buffered saline. A23187 (6.7µM) was added at zero time and the counts were recorded continuously for 10 min. Chemiluminescence is expressed as cpm x $10^{-4}/10^6$ cells.

cyclo-oxygenase (14), had no significant effect on CL (Table 1). The latter result was somewhat unexpected since the conversion of PGG_2 to PGH_2 is accompanied by the release of an oxygen-centred radical (15). The lipoxygenation steps most likely to be responsible for generating the 'light-emitting' species are those involving the conversion of arachidonic acid to

Table 1: Effects of inhibitors on chemiluminescence induced by A23187 in rat peritoneal macrophages.

Conditions	% chemiluminescence
A23187 (6.7µM)	100
A23187 + verapamil (500µM)	2.3 ± 0.1
A23187 + Ca^{++}-free medium	1.6 ± 0.1
A23187 + BPB (50µM)	0.2 ± 0.1
A23187 + NDGA (2.5µM)	7.8 ± 0.7
A23187 + indom (20µM)	95.8 ± 3.6
A23187 + aspirin (50µM)	110.3 ± 5.5

Experiments were performed as described in Fig. 1. Inhibitors were added 30 min before the addition of A23187. In some experiments, the cells were incubated in a Ca^{++}-free medium. Values represent the means ± S.E.M. from 3-6 experiments. BPB = bromo-phenacyl bromide; NDGA = nordihydroguaiaretic acid; indom = indomethacin. Chemiluminescence is expressed as a % of the peak cpm of the A23187-stimulated macrophages (8 x 10^5 cpm/10^6 cells).

hydroxy-eicosatetraenoic acid (10), which include the intermediate formation of a reactive hydroperoxide (hydroperoxy-eicosatetraenoic acid). The present studies do not preclude a significant contribution to the CL from the NAD(P)H oxidase which is largely responsible for the glucose-dependent CL seen after zymosan addition to mouse peritoneal macrophages (6). It should be noted, however, that the A23187-treated macrophages used in the present studies were incubated in a glucose-free medium. Should an NAD(P)H oxidase be involved, a lipoxygenase product must presumably be associated with its activation since BPB and NDGA were such potent inhibitors of A23187-induced CL (Table 1). There have been no reports of such a relationship in macrophages.

Both superoxide dismutase and catalase caused a marked reduction in CL (Table 2), suggesting that the superoxide anion ($O_2^{\cdot-}$) and H_2O_2 are major luminol-reactive products. However, when A23187-treated macrophages were assayed for these two species, no significant alteration in the level of H_2O_2 was found and only a small increase in superoxide anion was detected (data not shown). These observations can be interpreted to mean that hydroxyl radicals formed during the interaction between superoxide anion and H_2O_2 may be the intermediate CL-inducing species. This reaction occurs rapidly when catalyzed by a trace metal, such as Fe^{3+}, present in the medium (Fenton reaction). This would explain why superoxide dismutase and catalase are inhibitory and would account for the very small amount of superoxide anion and H_2O_2 detected in the medium. Desferrioxamine, a chelator of Fe^{3+}, has recently been reported to be a scavenger of hydroxyl radicals (16). Although this compound inhibited the

Table 2. Effects of superoxide dismutase and catalase on the chemiluminescent response to A23187 in rat peritoneal macrophages.

Conditions	% chemiluminescence
A23187 (6.7μM)	100
A23187 + SOD (175 units)	39.6 ± 0.2
A23187 + SOD (350 units)	27.1 ± 0.3
A23187 + SOD (700 units)	22.1 ± 0.1
A23187 + SOD (1400 units)	18.0 ± 0.2
A23187 + SOD (2800 units)	12.8 ± 0.2
A23187 + catalase (16625 units)	50.1 ± 0.4
A23187 + catalase (33250 units)	31.4 ± 0.4
A23187 + catalase (66500 units)	16.8 ± 0.2

Experiments were performed as described in Table 1. Values represent the means ± S.E.M. from 3-5 experiments. SOD = superoxide dismutase. Chemiluminescence is expressed as a % of the peak cpm of the A23187-stimulated macrophages (8×10^5 cpm/10^6 cells).

A23187-stimulated CL response (data not shown), it strongly quenched the light emitted from the reaction between H_2O_2 and luminol even at nanomolar concentrations. Thus, it was not possible to discriminate between a direct action of desferrioxamine on the cells and its quenching action.

We have shown previously (8) that the newly-synthesized prostaglandins in A23187-treated macrophages, acting through cyclic AMP, have a negative feedback effect on their own production. The target is a step prior to the transformation of arachidonic acid (8). If a portion of the CL is associated with the lipoxygenation of arachidonate, it should also be subjected to feedback inhibition. Indeed, elevation of intracellular cyclic AMP, through exogenously added 8-bromo- or dibutyryl cyclic AMP analogues or through the action of PGE_2 on adenylate cyclase, reduced the CL response significantly (Table 3). Another adenylate cyclase agonist, cholera toxin, could not be used as it strongly quenched the CL from the H_2O_2-luminol reaction. The cyclic AMP phosphodiesterase inhibitors, theophylline and RO-20-1724 (17,18) would be expected to maintain the augmented level of cyclic AMP induced by A23187 (8) and these two agents inhibited the induction of CL by the ionophore (Table 3).

Lipoxygenase products are of significance in regulating the functions of immune cells (19,20) and participating in inflammatory reactions (21,22). The present results and those presented elsewhere (9,10) suggest that an

Table 3. Efects of PGE_2, cyclic AMP analogues and cyclic AMP-PDE inhibitors on the chemiluminescent response to A23187 in rat peritoneal macrophages.

Conditions	% chemiluminescence
A23187 (6.7μM)	100
A23187 + 8-br-cyclic AMP (400μM)	69.8 ± 7.9
A23187 + 8-br-cyclic AMP (800μM)	53.4 ± 2.3
A23187 + 8-br-cyclic AMP (1.2mM)	37.2 ± 2.0
A23187 + dibutyryl cyclic AMP (400μM)	79.1 ± 8.1
A23187 + dibutyryl cyclic AMP (800μM)	58.5 ± 4.3
A23187 + dibutyryl cyclic AMP (1.2mM)	20.6 ± 2.4
A23187 + theophylline (1mM)	60.3 ± 3.3
A23187 + RO-20-1724 (1mM)	37.1 ± 10.8
A23187 + PGE_2 (10nM)	90.6 ± 2.1
A23187 + PGE_2 (100nM)	79.8 ± 4.2
A23187 + PGE_2 (1μM)	68.6 ± 5.2
A23187 + PGE_2 (10μM)	52.5 ± 9.5

Experiments were performed as described in Table 1. Values represent the means ± S.E.M. from 3-5 experiments. Chemiluminescence is expressed as a % of the peak cpm of the A23187-stimulated macrophages (8×10^5 cpm/10^6 cells).

additional functional role for the lipoxygenase pathway may be its capacity to generate oxygen metabolites.

The production of these toxic species, which contribute to the 'killing' capacity of phagocytes (3,4), may be modulated by events occurring on the other branch of the arachidonic acid cascade. Newly-synthesized prostaglandins, acting via the intermediary action of cyclic AMP and cyclic AMP-dependent protein kinase (Hunt, N.H. and Lim, L.K. unpublished observations), exert a feedback inhibition on a step(s) prior to the transformation of arachidonic acid (8). Thus, a soluble stimulus, A23187, triggers both CL and prostaglandin production in macrophages via a common mechanism which enlarges the free arachidonic acid pool. The duration of these responses is limited by the regulatory processes that are initiated by the prostaglandin products (8). It is suggested that these phenomena are relevant to the inflammation process, but this awaits confirmation in a system in vivo.

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