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CYCLIC NUCLEOTIDE METABOLISM AND REACTIVE OXYGEN PRODUCTION By MACROPHAGES

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Summary: The production of reactive oxygen species by elicited rat peritoneal macrophages was assessed by *in vitro* measurement of chemiluminescence in the presence of luminol. The divalent ion ionophore A23187 stimulated the production of reactive oxygen species. This action was inhibited by monobutyryl and dibutyryl derivatives of cyclic AMP but was not affected by derivates of cyclic GMP. Cyclic AMP and cyclic GMP concentrations increased rapidly in macrophages exposed to A23187 or zymosan. Indomethacin (20 μmol/l) inhibited the increase in cyclic AMP concentration but not the increase in cyclic GMP concentration. Neither A23187 nor zymosan stimulated adenylate cyclase activity in broken cell preparations of macrophages. The observations are consistent with the hypothesis that PGE produced by macrophages after phagocytotic stimuli may inhibit certain macrophage functions and perform a regulatory role in these cells. This action of PGE may be mediated by cyclic AMP.

The production of reactive oxygen species by phagocytes results in the generation of chemiluminescence (1-5). This can be detected in the presence of the readily oxidizable substance luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) (1). Since the ability of macrophages to kill bacteria may be dependent upon the production of reactive oxygen species, chemiluminescence may be an effective indicator of the microbicidal capacity of these cells (3).

Addition of the yeast cell wall extract zymosan to mouse peritoneal macrophages produces a sustained burst in the

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chemiluminescence of luminol, which has two components (6, 7). Approximately 65% of the chemiluminescence is glucose-dependent and calcium-independent; the mechanism by which reactive oxygen species are produced is known to be related to the metabolism of glucose (6, 7). The remaining component is calcium-dependent and glucose-independent; a similar, but more short-lived, burst of chemiluminescence can be induced with the bivalent metal ion ionophore A23187 (6-8). PGE_2 and cyclic AMP, which are both produced by macrophages in response to phagocytic stimuli or A23187 addition (7,9,10), inhibit a number of macrophage functions, including phagocytosis (11), tumoricidal activity (12), release of lysosomal enzymes (13), response to migration inhibitory factor (14) and the production of plasminogen activator (15). In view of these inhibitory effects, it was of interest to study the possible interrelationships between cyclic nucleotide metabolism and the intensity of the chemiluminescent response to phagocytic and non-phagocytic stimuli in peritoneal macrophages.

Materials and Methods. Zymosan, lot No. 1212, was from ICN Pharmaceuticals, Cleveland, Ohio. A23187 was kindly provided by Dr. R.L. Hamill (Eli Lilly). PGE_1 and PGE_2 were gifts from Dr. J. Pike (Upjohn Co., Kalamazoo, Mich.). Indomethacin and all nucleotides were from Sigma. Male DA rats were injected with 10ml of Brewers' thioglycollate medium. Three days later macrophages were harvested by peritoneal lavage. Mouse peritoneal macrophages were used in the experiments reported in Table 1. The cells were washed once and resuspended in Eagles MEM (16), pH 7.2, buffered with 20 mmol/l HEPES and containing 5 mmol/l glucose. In some experiments macrophages were purified by adherence to plastic Petri dishes (17). The responses to various agents were identical in purified and unpurified populations. The cells were preincubated in glass vials for 1 hr at 37°C. Nucleotides and indomethacin (where present) were added 30 min prior to the addition of A23187 (6.7 μmol/l) or zymosan (20 particles/cell). Chemiluminescence was measured in the presence of luminol in a Packard liquid scintillation counter set in the "off coincidence" mode (18). For assay of cyclic AMP and cyclic

Abbreviation: PGE_2, prostaglandin E_2.

Table 1: Effect of exogenous nucleotides upon the chemiluminescence engendered by the ionophore A23187 in mouse peritoneal macrophages.

Macrophages were preincubated for 30 min with nucleotides before addition of A23187. Chemiluminescence in controls (A23187 only) was 985 ± 132 x 10^3 counts per 5 min.

Values are mean ± S.E.M. Number of determinations indicated in parentheses.

[a]Dibutyryl adenosine 3',5'-monophosphate.

[b]Monobutyryl adenosine 3',5'-monophosphate.

Treatment	(mmol/l)	Chemiluminescence as % control
Control		100.0 (14)
dbcAMP[a]	0.4	61.2 ± 3.3 (7)
dbcAMP	0.8	36.3 ± 2.8 (7)
mbcAMP[b]	0.4	60.0 (2)
mbcAMP	0.8	28.8 ± 2.7 (4)
ADP	0.8	77.4 ± 1.2 (3)
ATP	0.8	22.8 ± 0.8 (3)

GMP, cells were boiled for 2min to inactivate the enzymes of cyclic nucleotide metabolism. The supernatant was assayed by the method of Brooker (19). The specificity of the assays, and the criteria used to validate their application to this system, have been detailed elsewhere (20). For assays of adenylate cyclase activity, cells were homogenized by 6 strokes of a Dounce homogenizer (B pestle) and assayed according to Salomon et al. (21), slightly modified (22).

Results and Discussion. Addition of either zymosan or A23187 to rodent macrophages produces a large increase in the chemiluminescence of luminol (6-8). The effect of A23187 was inhibited by butyryl derivatives of cyclic AMP (Table 1) and also by PGs of the E series (6-8). ATP and ADP also inhibited the chemiluminescence induced by A23187 (Table 1); the former nucleotide has been shown to inhibit zymosan-induced chemiluminescence in rabbit alveolar macrophages (23). Although the mechanism has not been investigated further it is known that exogenous ATP can influence intracellular events, for example DNA synthesis in thymocytes

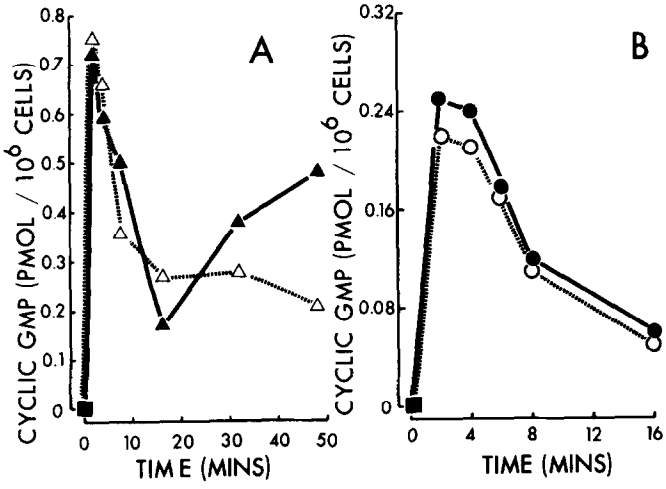

Fig. 1. Effect of (A) zymosan and (B) A23187 upon macrophage cyclic GMP content. Macrophages were preincubated for 30 min in buffer. Indomethacin (final concentration 20 μmol/l) was then added to half of the incubations (indicated by open symbols, broken lines) and the cells were preincubated for a further 30 min. A23187 (final concentration 6.7 μmol/l) or zymosan (20 particles/cell) were added at 0. At various times subsequently, aliquots of cells were withdrawn and boiled for 2 min. After centrifugation to remove debris, the supernatant was stored at -20°C until assayed for cyclic GMP. The data is expressed as the change in macrophage cyclic GMP content after zymosan or A23187. The basal (unstimulated) cyclic GMP concentration was 0.27 ± 0.02 (mean ± S.E.M., n=35) and did not change significantly during the experiments. The points are means from 3-5 experiments. Zymosan (A) and A23187 (B) produced significant ($P < 0.05$) changes in cyclic GMP concentration at all points. Indomethacin did not affect the changes in cyclic GMP concentration.

(24). The following agents (at 0.8 mmol/l) had no significant influence on chemiluminescence induced by A23187 : 3'-AMP, 5'-AMP, cyclic AMP, adenosine, sodium butyrate, GMP, GDP, GTP, monobutyryl cyclic GMP and dibutyryl cyclic GMP.

Since butyryl derivates of cyclic AMP inhibited chemiluminescence, intracellular levels of cyclic AMP and cyclic GMP were measured at various times after addition of zymosan or A23187. Cyclic GMP levels increased significantly in both cases (Fig. 1). It has been suggested (25) that guanylate cyclase may be activated by hydroxyl radicals generated as a result of the interaction of appropriate agonists with responsive cells. It

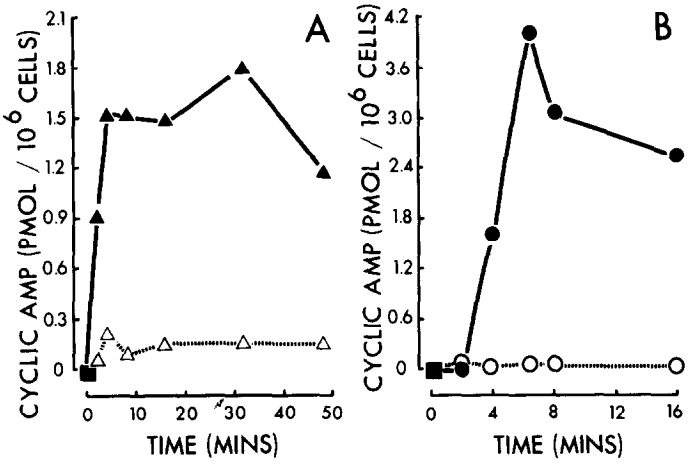

Fig. 2. Effect of (A) zymosan and (B) A23187 upon macrophage cyclic AMP content. The experiments were performed as described in Figure 1. The data is expressed as the change in macrophage cyclic AMP content after zymosan or A23187. The basal (unstimulated) cyclic AMP concentration was 0.80 ± 0.03 pmol/10^6 cells (mean ± S.E.M., n=52) and did not change significantly during the course of the experiments. The points are means from 3-5 experiments. Zymosan (A) produced significant ($P < 0.05$) changes in cyclic AMP concentration at all points. A23187 (B) produced significant changes at 4,6,8 and 16 min. Indomethacin abolished the increases in cyclic AMP.

is possible that macrophage guanylate cyclase is activated by hydroxyl radicals generated in response to zymosan and A23187. Since indomethacin did not inhibit the observed increase in cyclic GMP concentration (Fig. 1) it is probable that the metabolism of arachidonic acid via the cyclooxygenase pathway is not related to the observed changes in cyclic GMP metabolism.]2-Hydroperoxyeicosatetraenoic acid, an intermediate in the lipoxygenase pathway, stimulates cyclic GMP production in neutrophils (26). Derivatives of cyclic GMP stimulate the release of lysosomal enzymes from neutrophils (27) but, as noted above, they did not affect A23187-induced chemiluminescence in macrophages. Synthetic derivates of cyclic GMP do not modify bactericidal activity in human monocytes (28).

Table 2: Effects of agents upon cyclic AMP production in intact and homogenized macrophages.

Intact macrophages were incubated for 10 min at 37°C in the presence of 0.5 mmol/l 3-isobutyl-1-methylxanthine (an inhibitor of cyclic AMP phosphodiesterase activity). Adenylate cyclase activity was measured in cell homogenates.
Significance of difference from control was assessed by Student's t test. Values of P >0.05 were considered not significant (N.S.). Values are mean ± S.E.M. from 3 determinations. [a] Zymosan concentration was 1 mg/ml.

Treatment	Concentration (mol/l)	Cyclic AMP (pmol/ 10^6 intact cells)	P	Adenylate cyclase activity (pmol cyclic AMP/mg protein/12 min)	P
Control	-	10 ± 1	-	412 ± 45	-
PGE_2	10^{-4}	250 ± 15	<0.001	1775 ±122	<0.001
Isoprenaline	10^{-4}	48 ± 1	<0.001	738 ± 64	<0.01
A23187	10^{-5}	-	-	408 ± 33	N.S.
Zymosan[a]	-	-	-	323 ± 46	N.S.
Glucagon	10^{-6}	11 ± 1	N.S.	403 ± 45	N.S.
Parathyroid hormone	10^{-6}	11 ± 1	N.S.	409 ± 73	N.S.
Salmon calcitonin	10^{-6}	9 ± 1	N.S.	455 ± 92	N.S.
NaF	10^{-2}	-	-	1972 ± 48	<0.001

Cyclic AMP levels in the macrophages increased rapidly after the addition of zymosan or A23187 (Fig. 2). At concentrations which were optimal for the generation of chemiluminescence, A23187 produced a larger increase in cyclic AMP than did zymosan. Indomethacin (20μmol/l) completely blocked the increases in cyclic AMP observed with both agents (Fig. 2), indicating that the metabolism of arachidonic acid via the cyclooxygenase-dependent pathway might be related to the changes in cyclic nucleotide metabolism observed in macrophages. Neither zymosan nor A23187 stimulated adenylate cyclase activity in broken cell preparations (Table 2). Among potential agonists tested, PGE_2 and isoprenaline were the only ones which increased cyclic AMP formation in intact and broken cell preparations (Table 2). These observations are consistent with the hypothesis that zymosan and A23187 increase macrophage cyclic AMP content indirectly, probably via the production of PGE_2 (8, 9).

Arachidonic acid engenders chemiluminescence in macrophages which have been preincubated with zymosan (7,29,30). This and other evidence presented elsewhere (7,29,30) suggests that the chemiluminescence induced by A23187 is a consequence of the metabolism of arachidonic acid via the lipoxygenase pathway. PGE_2 inhibits A23187-induced chemiluminescence (8) but does not affect that induced by arachidonic acid (7,29,30). Therefore the site of inhibition by PGE_2 is likely to be the phospholipase enzyme which liberates endogenous arachidonic acid from phospholipid. The present observations suggest that the action of endogenous PGE_2 is mediated via the production of cyclic AMP. Phospholipase in platelets is inhibited by cyclic AMP (31,32) although, to date, this has not been reported in macrophages.

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