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CYCLIC AMP AND THE REGULATION OF PROSTAGLANDIN PRODUCTION BY MACROPHAGES

L.K. Lim, N.H. Hunt+, R.D. Eichner++ and M.J. Weidemann*

Departments of Experimental Pathology and Immunology++,
John Curtin School of Medical Research
*Department of Biochemistry, Faculty of Science,
Australian National University, P.O. Box 334,
Canberra, A.C.T. 2600, Australia

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Summary: The ionophore A23187 (6.7μM) increased the rates of formation of prostaglandins and cyclic AMP in suspensions of thioglycollate-elicited rat peritoneal macrophages. Both effects were inhibited by the calmodulin blocker trifluoperazine (50μM) and the calcium channel blocker verapamil (500μM). Inhibitors of phospholipase A_2 and cyclo-oxygenase also blocked both actions of A23187. The stimulated prostaglandin formation was markedly reduced when the cells were preincubated with 8-bromo-cyclic AMP (1mM), dibutyryl cyclic AMP (1mM) or cholera toxin (500ng/ml). Addition of exogenous arachidonic acid (30μM) alleviated this inhibition. We propose that the effect of A23187 on macrophages includes a 'self-limiting' mechanism whereby newly-synthesized prostaglandins can inhibit, via cyclic AMP, a step(s) prior to the transformation of arachidonic acid and thus modulate their own production.

Prostaglandins act as local regulators of the activities of immune cells (1). Several macrophage functions, including tumouricidal activity (2) and chemiluminescence (3), are sensitive to inhibition by PGE_2. Since PGE_2 stimulates cyclic AMP formation in macrophages (4,5,6), the nucleotide may mediate some of the inhibitory effects of the prostanoid (2,3,7). Furthermore, the formation of cyclic AMP in A23187-treated rat peritoneal macrophages is inhibited by indomethacin, which suggests that PGs are involved in this process (4,6,8). The activation of adenylate cyclase by an endogenous metabolite of arachidonic acid, probably PGE_2 and/or PGI_2, seems to be involved in increasing the cyclic AMP content after A23187 addition to macrophages (6). This paper: (i) provides further evidence that the newly-synthesized PGs in A23187-treated rat peritoneal macrophages directly stimulate the formation of cyclic AMP; and (ii) demonstrates that PGs may regulate their own production through cyclic AMP-dependent inhibition of a step prior to arachidonic acid transformation.

+ To whom correspondence and reprint requests should be addressed.
Abbreviations: PG, prostaglandin; BPB, bromo-phenacyl bromide; TFP, trifluoperazine; BCG, Bacillus Calmette-Guérin.

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Materials and Methods: A23187 was from Calbiochem (La Jolla CA, USA). Trifluoperazine and verapamil were from Smith, Kline and French (Sydney, Australia) and Knoll AG (Sydney, Australia), respectively. All other reagents were purchased from Sigma (St. Louis, MO). Cyclic AMP (8) and PGs (9) were assayed by radioimmunoassay. The radioligands were purchased from Amersham, U.K. The anti-PGE and anti-PGF antisera were gifts of Mr. J. Ip, Miles Laboratory (Melbourne, Australia) and anti-6-keto-$PGF_{1\alpha}$ was kindly provided by Dr L. Best (Free University of Brussels, Belgium). Rat peritoneal macrophages, elicited with Brewer's thioglycollate medium, were prepared as described previously (10). Where applicable, results were compared using the Wilcoxon rank sum test.

Results and Discussion: Addition of A23187 to the elicited macrophage suspensions caused a rapid increase in the amount of 6-keto-$PGF_{1\alpha}$ and PGE ($P<0.001$) within 1 min, followed by a plateau after 2-3 min (Fig. 1). A similar increase in the level of PGF was also detected (data not shown). Although qualitatively similar profiles were observed with resident and BCG-elicited macrophages, the resident population produced larger amounts of PGs (L.K. Lim, unpublished observations). A comparable phenomenon has been reported by others (11,12).

The ionophore also stimulated a parallel increase in the concentration of cyclic AMP (4,6). This action of the ionophore was inhibited by indomethacin,

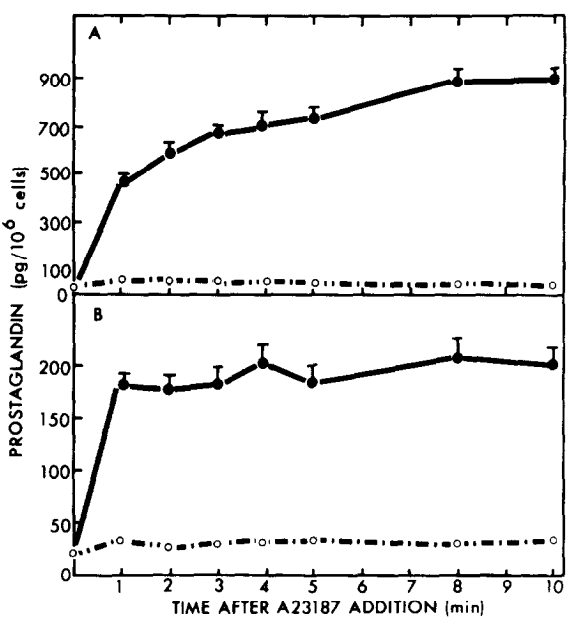

Fig. 1: Effect of A23187 upon the concentration of PGs in rat thioglycollate-elicited peritoneal macrophages. Cells were treated with A23187 (final concentration 6.7μM). Samples were taken at designated times, acidified with 1.33M formic acid to pH 3.5 and snap-frozen. After thawing, the supernates were assayed for 6-keto-$PGF_{1\alpha}$ (panel A) and PGE (panel B). Values represent the means from 4-6 experiments, each assayed in duplicate. Unless otherwise shown, the S.E.M. were within 10% of the mean value (σ) control; (●) A23187.

Table 1

Designation	PGE ($pg/10^6$ cells)	cyclic AMP ($pmol/10^6$ cells)
control	3.5±0.1	0.8±0.1
A23187 (6.7μM)	185.2±5.6	5.2±0.2
A23187 + BPB (50μM)	8.9±0.1	0.9±0.1
A23187 + hydrocortisone (1mM)	16.4±0.2	1.4±0.1
A23187 + aspirin (50μM)	19.2±0.1	1.4±0.1
A23187 + indomethacin (20μM)	13.8±0.2	1.2±0.1
A23187 + Ca^{++}-free medium	18.2±0.1	1.5±0.2
A23187 + verapamil (500μM)	14.4±0.2	1.2±0.1
A23187 + TFP (50μM)	30.4±0.2	1.7±0.1

Effects of various inhibitors on the concentrations of PGE and cyclic AMP in A23187-treated, rat peritoneal macrophages. Rat peritoneal macrophages were preincubated either with or without the inhibitors (final concentrations shown) for 30 min prior to the addition of A23187. At various times (up to 10 min), samples were withdrawn for PGE (terminated by acidification to pH 3.5 with 1.33M formic acid and snap-freezing) or cyclic AMP (terminated by boiling for 2 min) measurements. After thawing, all samples were cleared of cell debris by centrifugation and the PG or cyclic AMP concentrations were determined by radioimmunoassay. Values represent the means ± S.E.M. of the peak PG or cyclic AMP concentration, from 4-10 experiments, each assayed in duplicate.

suggesting that PGs may function as intermediaries in the stimulatory effect of A23187 (4,6). We have extended considerably these original observations with indomethacin by using a larger range of inhibitors. Disruption of arachidonic acid metabolism, using either the phospholipase A_2 inhibitors BPB (13) and hydrocortisone (14) or the cyclo-oxygenase inhibitors indomethacin and aspirin (15), blocked A23187-stimulated PG formation (Table 1). The reduced PG formation observed when the cells were incubated in a Ca^{++}-free medium or after preincubation with verapamil (16) suggests that the action of the ionophore may be dependent upon an influx of exogenous Ca^{++} ions which then participates in a calmodulin-dependent activation of arachidonic acid metabolism, as suggested by the inhibitory effect (Table 1) of the drug TFP (17). By analogy with events in platelets (18), calmodulin may be involved in the activation of phospholipase A_2. Each inhibitor used affected PG and cyclic AMP formation similarly (Table 1), consistent with the hypothesis (4,6,8) that PGs are responsible for the action of A23187 in stimulating cyclic AMP production.

The plateau observed in the profile of PG production (Fig. 1) may reflect a rapid 'switch-off' of prostanoid synthesis, since these metabolites are not

Table 2

Designation	PGE ($pg/10^6$ cells)	cyclic AMP ($pmol/10^6$ cells
control	2.3±0.1	1.2±0.1
A23187 (6.7μM)	289.5±15.4	6.3±0.1
A23187 + 8-bromo-cyclic AMP (1mM)	114.8±9.6	N.D.
A23187 + dibutyryl cyclic AMP (1mM)	121.7±10.4	N.D.
A23187 + cholera toxin (500ng/ml)	68.3±2.1	29.3±1.8

Effect of an elevated cyclic AMP concentration on the production of PGE in A23187-treated rat peritoneal macrophages. Rat peritoneal macrophages were preincubated either with or without the cyclic AMP analogues or cholera toxin (final concentration as shown) for 30 min prior to the addition of A23187. At various times (up to 10 min), samples were withdrawn for PGE or cyclic AMP measurements and assayed as described in Table 1. Values represent the means ± S.E.M. of the peak PG and cyclic AMP concentration, from 4-8 experiments, each assayed in duplicate. N.D. = not determined.

known to be degraded by the cells. In view of the inhibition of many macrophage functions by cyclic AMP (1,2,8), the possibility exists that this nucleotide might also contribute to the cessation of prostanoid synthesis. Elevation of intracellular cyclic AMP concentration, either through addition of the 8-bromo- and dibutyryl analogues, or endogenously through the action of cholera toxin on adenylate cyclase (19), significantly reduced the ability of the macrophages to produce PGs in response to A23187 (Table 2). Cholera toxin produced a marked stimulation in the cyclic AMP concentration that was sustained throughout the incubation period (Table 2).

The target of this negative action of cyclic AMP appears to be an event occurring prior to the transformation of arachidonic acid, since exogenous arachidonic acid alleviated the inhibition of PG formation in 8-bromo-cyclic AMP and A23187-treated macrophages (Table 3). The increase in PG production from arachidonic acid added in the absence of A23187 was not inhibited by 8-bromo-cyclic AMP (data not shown). As expected, since both A23187 and the exogenous arachidonic acid stimulate PG formation through the same biosynthetic pathway, their actions were additive (Table 3). It should be noted that the amount of PGE in these samples could not be estimated accurately because of a strong cross-reaction (≃ 25%) between exogenously added arachidonic acid and the anti-PGE antibody used in the radioimmunoassay. A much smaller (≃ 5%) degree of cross-reaction was observed with the

Table 3

Designation	PGF ($pg/10^6$ cells)	6-keto-$PGF_{1\alpha}$ ($pg/10^6$ cells)
control	4.9±1.3	12.1±0.1
A23187 (6.7μM)	230.1±30.4	396.9±26.4
Arachidonic acid (30μM)	155.4±13.1	183.7±23.1
A23187 + arachidonic acid	428.2±49.4	554.4±58.3
A23187 + 8-bromo-cyclic AMP (1mM)	28.8±9.5	39.6±1.6
A23187 + 8-bromo-cyclic AMP + arachidonic acid	167.1±13.3	178.6±12.9

Effects of arachidonic acid and 8-bromo-cyclic AMP on the production of PGF and 6-keto-$PGF_{1\alpha}$ in A23187-treated rat peritoneal macrophages. Rat peritoneal macrophages were pretreated with or without 8-bromo-cyclic AMP (1mM) for 30 min. Some incubations were subsequently treated for a further 5 min with 30μM arachidonic acid prior to the addition of A23187. Samples were taken (up to 10 min) and assayed as described in Table 1. Values represent the means ± S.E.M. of the PG concentration at 10 min from 4 experiments, each assayed in duplicate.

antibodies to PGF and 6-keto-$PGF_{1\alpha}$. This cross-reactivity was taken into account in making the calculations presented in Table 3.

Considered together, the results suggest that, in macrophages, A23187 action leads to an increase in the level of endogenous free arachidonic acid. This action probably involves activation of a phospholipase and simultaneous inhibition of acyl transferase (20). Cyclo-oxygenation of arachidonic acid results in the formation of PGs. These PGs, probably PGE_2 and/or PGI_2, activate adenylate cyclase, leading to a rise in intracellular cyclic AMP (6). PGs, acting via the cyclic AMP thus formed, may thereby limit their own production. The cyclic AMP-mediated 'self-limiting' mechanism is probably directed at a step prior to the metabolic conversion of arachidonic acid, for example, the phospholipase or acyl transferase enzymes, and may involve the action of a cyclic AMP-dependent protein kinase (21). The series of events postulated here (Fig. 2), if correct, would explain the rapid increase in PG production, its equally rapid cessation after A23187 addition (Fig. 1) and the concurrent limitation of the formation of lipoxygenase products and cellular processes associated with these metabolites that have, in fact, been observed (10,22).

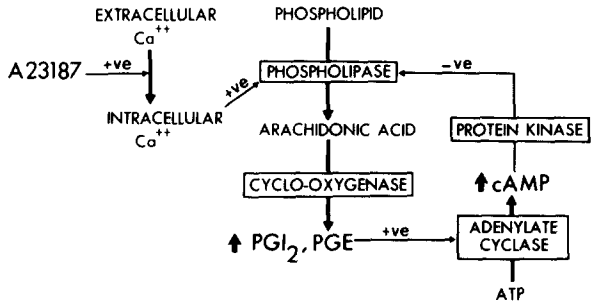

Fig. 2: 'self-limiting' mechanism of prostaglandin synthesis in A23187-treated, rat peritoneal macrophages. The Ca^{++} influx promoted by A23187, results in activation of a phospholipase. The activated enzyme deacylates phospholipids and raises the endogenous pool of free arachidonic acid. (A23187 may also inhibit the activity of acyl transferase and prevent reacylation of the arachidonate). Transformation of arachidonic acid via the cyclo-oxygenase pathway yields PGs (E_2 and/or I_2) which activate adenylate cyclase, ultimately enhancing the intracellular level of cyclic AMP. The cyclic nucleotide, possibly acting through cyclic AMP-dependent protein kinase, inhibits further PG formation. The site of action of cyclic AMP-dependent protein kinase is probably at an event(s) occurring prior to arachidonic acid transformation.

It is well established that PGs of the E-series profoundly influence the activities of other leucocytes (1) and are important inflammatory mediators (23). Furthermore, macrophages are probably the major PG-producing cells at sites of inflammation (24). It is clear, therefore, that the self-regulation of PG production by macrophages has a number of implications for the limitation of the inflammatory process. It is possible that biological roles for leukotriene B_4 and formylated peptides from bacterial cell walls may include the initiation of the 'self-limiting' mechanism; both agents have been demonstrated to promote Ca^{++} influx into cells in a manner analogous to A23187 (25).

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REFERENCES

1. Goodwin, J.S. and Webb, D.R. (1980). Clin. Immunol. Immunopathol. 15, 106-122.
2. Schultz, R.M., Stoychkov, J.N., Pavlidis, N., Chirigos, M.A. and Olkowski, S.L. (1979). J. Reticuloendothelial Soc. 26, 93-102.
3. Weidemann, M.J., Peskar, B.A., Wrogemann, K., Rietschel, E.Th., Staudinger, H. and Fischer, H. (1978). FEBS lett. 89, 136-140.
4. Gemsa, D., Seitz, M., Kramer, W., Grimm, W., Till, G. and Resch, K. (1979). Exp. Cell. Res. 118, 55-62.
5. Remold-O'Donnell, E. and Alpert, H.R. (1979). Cell. Immunol. 45, 221-229.

6. Lim, L.K., Hunt, N.H., Evans, T. and Weidemann, M.J. (1981). Biochem. Biophys. Res. Commun. 103, 745-750.
7. Oropeza-Rendon, R.L., Bauer, H.C. and Fischer, H. (1980). J. Immunopharmac. 2, 133-147.
8. Hunt, N.H., Smith, B. and Pembrey, R. (1980). Clin. Sci. Mol. Med. 58, 463-467.
9. Jaffe, B.M. and Behrman, H.R. (1974). Methods ofHormonal Radio-immunoassay, pp. 19-36, Academic Press, New York.
10. Smith, R.L., Hunt, N.H., Merritt, J.E., Evans, T. and Weidemann, M.J. (1980). Biochem. Biophys. Res. Commun. 96, 1079-1087.
11. Bonney, R.J., Wightman, P., Davies, P., Sadowski, S.J., Kuehl, F.A. and Humes, J.L. (1978). Biochem. J. 176, 433-442.
12. Humes, J.L., Burger, S., Galvage, M., Kuehl, F.A., Wightman, P.D., Dahlgren, M.E., Davies, P. and Bonney, R.J. (1980). J. Immunol. 124, 2110-2116.
13. Volwerk, J.J., Peiterson, W.A. and DeHaas, G.H. (1974). Biochem. 13, 1446-1454.
14. Hong, S.C.L. and Levine, L. (1976). Proc. Natl. Acad. Sci. USA 72, 2994-2998.
15. Vane, J.R. (1971). Nature 231, 232-235.
16. Deleers, M., Mahy, M. and Malaisse, W.J. (1982). Eur. J. Pharmacol. 83, 231-224.
17. Levin, R.M. and Weiss, B. (1977). Molec. Pharmacol. 13, 690-697.
18. Wong, P.Y.-K. and Cheung, W.Y. (1979). Biochem. Biophys. Res. Commun. 90, 473-480.
19. Johnson, G.L., Kaslow, H.R. and Bourne, H.R. (1978). J. Biol. Chem. 253, 7120-7123.
20. Kroner, E.E., Peskar, B.A., Fischer, H. and Ferber, E. (1981). J. Biol. Chem. 256, 3690-3697.
21. Hunt, N.H., Lim, L.K., Eichner, R., Buffinton, G. and Weidemann, M.J. (1983). Manuscript in preparation.
22. Lim, L.K., Hunt, N.H. and Weidemann, M.J. (1983). Manuscript in preparation.
23. Bonta, I.L. and Parnham, M.J. (1980). Trends in Pharmacol. Sci. 347-349.
24. Morley, J., Bray, M.A., Jones, R.W., Nugteren, D.H. and Van Dorp, D.A. (1979). Prostaglandins 17, 729-736.
25. Serhan, C., Radin, A., Smolen, J.G., Korchat, H., Samuelsson, B. and Weissman, G. (1982). Biochem. Biophys. Res. Commun. 107, 1006-1012.