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Studies on the production of endogenous pyrogen by rabbit monocytes

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(Date)

STUDIES ON THE PRODUCTION OF ENDOGENOUS PYROGEN BY RABBIT MONOCYTES

by

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B.A., Yale College, 1980

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of the School of Medicine, Yale University
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ABSTRACT

The studies reported herein were undertaken to investigate the role of calcium and cyclic nucleotides as second messengers in the stimulation of endogenous pyrogen production from rabbit monocytes by bacterial endotoxin.

We found that monocytes stimulated with endotoxin produced endogenous pyrogen, even under conditions of high or low extracellular calcium concentrations. Maximal production occurred when the calcium concentration was in the near-physiological range. Prolonged incubation of cells with a calcium chelator prevented subsequent activation with endotoxin, an effect which was rapidly reversible by readdition of calcium, but not other cations. Addition of small amounts of lanthanum, a blocker of membrane calcium channels, to cells prevented this reversal by subsequent calcium addition. New cell separation techniques were developed to obtain monocyte-enriched samples for study of calcium fluxes. Incorporation of a calcium ionophore into the cell membrane resulted in calcium influx, but did not stimulate pyrogen production. Further, no measurable influx or efflux of calcium occurred during stimulation of mononuclear cells with endotoxin. These observations suggest that a slowly-exchangeable calcium pool was

necessary for the production of endogenous pyrogen, but that a rise in intracellular calcium by itself was not a necessary or sufficient stimulus. Thus, calcium cannot be considered a second messenger in this system.

Incubation of cells with agents shown to increase cyclic 3', 5' AMP or cyclic 3', 5' GMP levels in monocytes similarly did not stimulate pyrogen production or modulate its production during endotoxin stimulation. Therefore, cyclic nucleotides do not act as second messengers in the derepression of the endogenous pyrogen genome either.

Given the widespread production of endogenous pyrogen by nearly all vertebrates and the major role that the endogenous pyrogen/Interleukin-1 molecule plays in controlling the immune response, further work should be undertaken to define the mechanism for its production more clearly. Suggestions for such future areas of investigation are discussed.

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Dr. Kathleen Gekowski and John Donaldson made our laboratory a stimulating and enjoyable place in which to work.

To my parents,

Claire B. and Myer O. Sigal,

who have supported me in this,

as in all my endeavors

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INTRODUCTION

Ancient physicians recognized a close association between inflammation and elevated body temperature. So regular was this association that Celsus, a physician of the early Roman Empire, considered heat a cardinal sign of inflammation, along with its sister features of swelling, pain, and redness.

The nature of the link between fever and inflammation, however, was hidden for many centuries by the Greek doctrine developed by Empedocles in the fifth century B.C. of four fundamental elements and their corresponding forms in the body, the humors. Humoral theory maintained that health depended upon a proper balance of blood, phlegm, black bile, and yellow bile in the body, and that fever indicated an excess of yellow bile, the physiological equivalent of fire. This Hippocratic tradition was continued by Galen who developed a full system of anatomy and physiology based upon it in the second century A.D. These concepts withstood all challenges until Harvey's demonstration of a closed circulatory system with rapid mixing of blood from all parts of the body made them untenable in the seventeenth century. Freed from the bias of this long-standing dogma, physicians again began to look for the cause of fevers, and their early

hypotheses relied upon the heat generated by friction and fermentation (reviewed in 1).

The first experimental attempts to explain the link between inflammation and fever, however, were reported independently in the nineteenth century by Weber (2) and Billroth (3) who showed that animals injected with pus from draining wounds became febrile. They did not assess the potential pyrogenicity of the agents responsible for pus formation, however. Welch later postulated that exogenous agents induced fever indirectly through the generation of a product by host leukocytes (4). In 1943 Menkin presented the first experimental evidence for such a product (5), a euglobulin he named pyrexin. He later crystallized this agent and defined its biological properties (6-8), but other investigators have shown that its characteristics were almost identical to those of bacterial endotoxin (9). This suggests that pyrexin may have been a modified form of endotoxin or that it relied upon contamination with endotoxin for its biological activity.

Beeson reported the isolation of a pyrogen from polymorphonuclear leukocytes (PMN's) in 1948 (10). In later work with Bennett he showed that the biological properties of this leukocytic pyrogen (LP) differed from those of pyrexin and endotoxin (9, 11). Subsequently, Atkins and Wood demonstrated that rabbits made febrile by injection with typhoid vaccine had a transferable circulating pyrogen

which appeared in their bloodstream after clearance of the injected vaccine. The characteristics of this endogenous pyrogen (EP) were similar to those described by Bennett and Beeson for LP (12, 13).

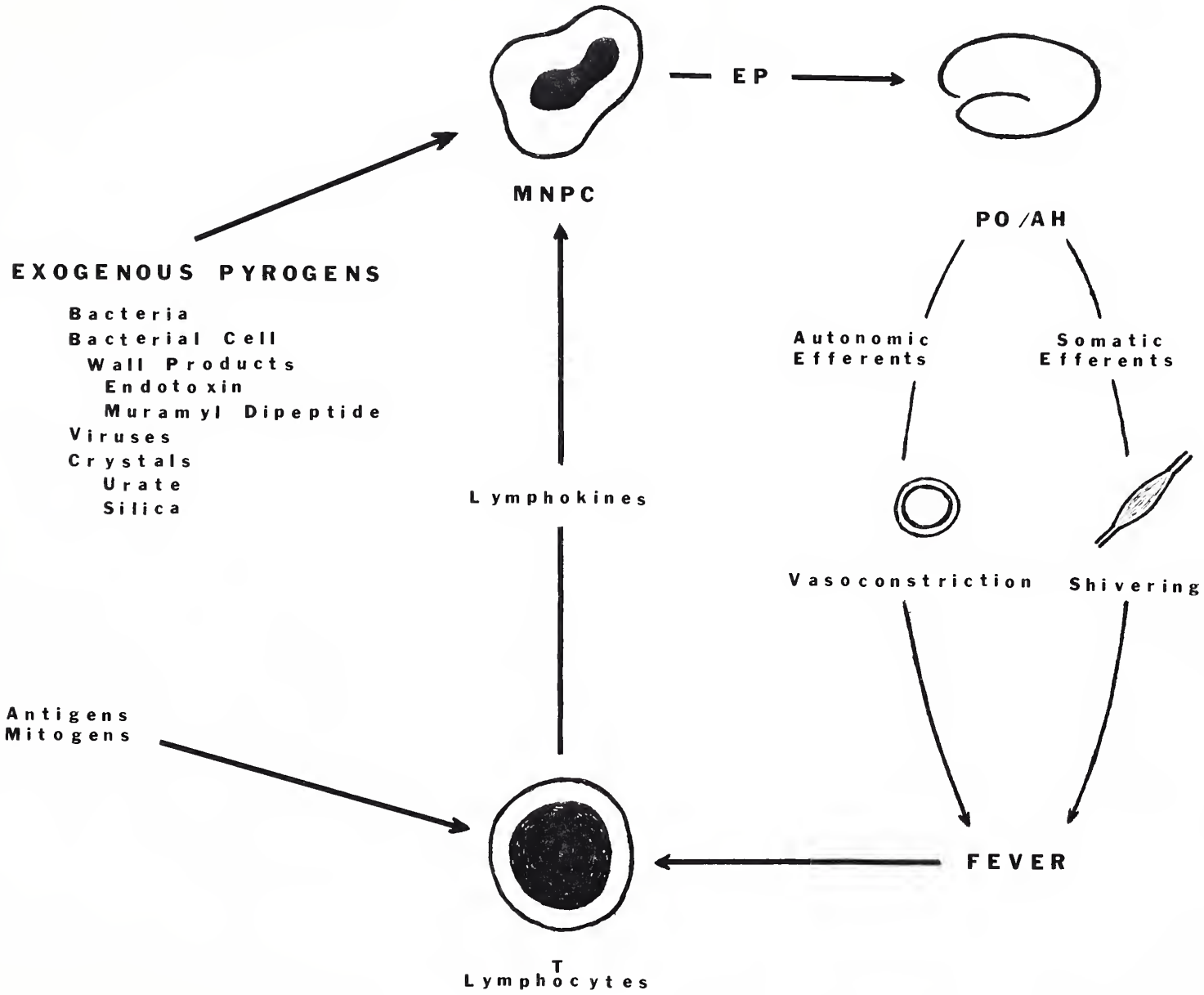
While early work indicated that PMN's produced EP, in 1967 human blood monocytes (14), rabbit peritoneal macrophages (15), and rabbit alveolar macrophages (16) were all shown to be potent sources of EP. Other mononuclear phagocytic cells (MNPC's), including human synovial exudate macrophages (17) and rabbit Kupffer cells (18, 19), produce EP, and there is recent evidence that keratinocytes do so, as well (20). With the development of efficient cell separation techniques it has been possible to show that PMN's do not, in fact, secrete EP, and it is likely that all EP production attributed to them in prior work was due to the presence of small numbers of MNPC's "contaminating" the culture conditions (21).

The chemical nature of EP has been investigated extensively over the past thirty years, and the results of these studies have been reviewed in detail by Dinarello and Wolff (22). EP is a small protein or polypeptide with a molecular weight of 15,000 daltons. A 40,000 dalton pyrogen has also been described which may be a trimer of the smaller protein. Pyrogenicity is destroyed by heat or protease treatment, but it resists extraction with organic solvents. The portion of EP responsible for its pyrogenicity seems to

be common to several species, as EP from many species will produce a febrile response in others. Small species-defined differences exist, however, and have allowed the preparation of rabbit antibodies to human EP (23) and of goat antibodies to rabbit EP (24). Considerable evidence indicates that EP is identical to another MNPC product which stimulates T lymphocyte proliferation and activity, lymphocyte activating factor (LAF) (24, 25). These molecules are collectively referred to as Interleukin-1 (Il-1).

What is known about the pathogenesis of fever in homeotherms is summarized in Figure 1. Unstimulated cells do not release EP in culture, and no preformed EP can be demonstrated within them when they are physically disrupted (26, 27). Agents shown to stimulate EP release from MNPC's in vitro include heat-killed bacteria (16, 28), products of bacterial cell walls (29-32), viruses (33), crystals of urate and silica (34), and lymphokines released from T lymphocytes activated by antigens (35, 36) or mitogens (37). Circulating EP acts upon the body's thermoregulatory center located in the preoptic regions of the anterior hypothalamus (PO/AH) to increase body temperature by both reducing heat loss and increasing heat production (38). There is recent evidence that hyperthermia augments the proliferation of activated T lymphocytes, as well, thus making the effects of EP (or Il-1) on immunoregulation even more potent (39). Poikilotherms also produce EP, and they react to its presence by seeking a warmer ambient environment (40).

Figure 1: The Pathogenesis of Fever in Homeotherms

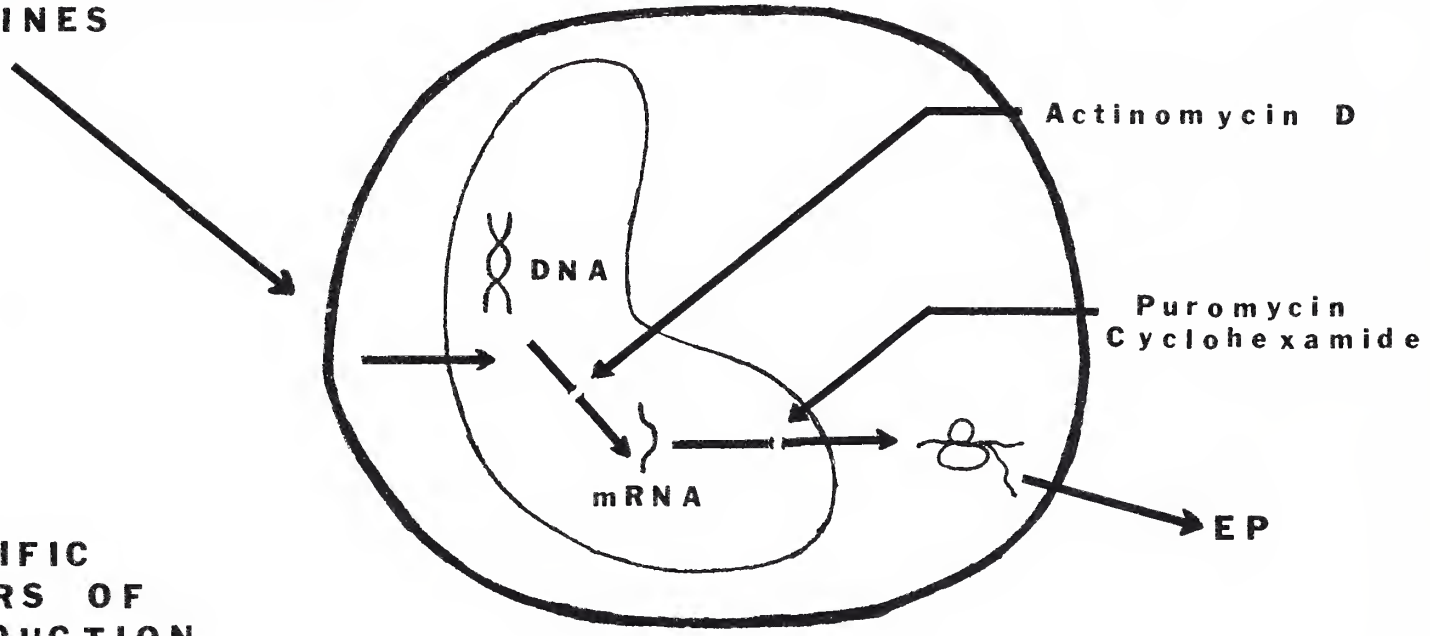

EP
MNPC
EXOGENOUS PYROGENS
Bacteria
Bacterial Cell
Wall Products
Endotoxin
Muramyl Dipeptide
Viruses
Crystals
Urate
Silica
Lymphokines
PO/AH
Autonomic Efferents
Somatic Efferents
Vasoconstriction
Shivering
FEVER
Antigens
Mitogens
T
Lymphocytes

Inhibitors of DNA transcription, such as Actinomycin D, and inhibitors of RNA translation, such as puromycin and cyclohexamide, prevent EP release when added to MNPC's within two hours after addition of known stimuli (41, 42). As further evidence that EP release requires de novo synthesis, MNPC's incubated with stimuli in the presence of radioactively-labeled amino acids release EP which carries the label (43).

Together these observations suggest that EP is an inducible protein, and that its production is repressed in the unstimulated MNPC. The mechanism by which known stimuli of EP production cause derepression of the EP genome, with subsequent transcription into mRNA and translation into protein, remains unknown. Figure 2 summarizes the available information concerning the mechanism involved. Phagocytosis does not appear to be necessary to the process, since incubation of MNPC's with cytochalasin B (44) or colchicine (45) in quantities sufficient to prevent phagocytosis does not prevent EP production. While corticosteroids inhibit EP production in vitro, specific cyclooxygenase inhibitors do not (46). Consistent with these findings, recent evidence suggests that lipoxygenase inhibitors prevent EP production (47). Thus, prostaglandins (PG's) do not appear to be important in generating EP, but leukotrienes (LT's) may play a role. There is no evidence that exogenous LT's are a sufficient stimulus to generate EP production, however.

Figure 2: The Mechanism of Endogenous Pyrogen Production

EXOGENOUS PYROGENS
LYMPHOKINES



NON-SPECIFIC
BLOCKERS OF
EP PRODUCTION

Metabolic Inhibitors
Corticosteroids
Lipoxygenase Inhibitors

Wood and his colleagues reported several years ago that rabbit peritoneal exudate (PE) cells released EP "spontaneously" when incubated in saline, but were inactive if incubated in plasma. By altering the composition of the incubation medium used they showed that the presence of potassium (K^+) or calcium (Ca^{2+}) in the extracellular fluid had an inhibitory effect on EP release (48-50). On the basis of cell-associated ion measurements they postulated that PE cells released EP as a response to K^+ depletion. Thus, replenishing the cells with extracellular K^+ would remove the stimulus to release EP. In further support of this hypothesis they reported that incubating cells with ouabain, an inhibitor of the sodium-potassium pump, allowed continued EP release in the presence of extracellular K^+, presumably by preventing cellular uptake of the added K^+. They proposed that Ca^{2+}, on the other hand, exerted its inhibitory effect on EP release by altering the cell membrane in such a way as to make the cell unresponsive to K^+ depletion (50).

While this work presents an interesting hypothesis for the intracellular mechanism of EP production, the experimental system imposes several limitations upon its usefulness, some of which were acknowledged by the authors. First, study of the release of EP from cells which were already producing it cannot be used to elucidate the mechanism by which EP production is induced. In this

system, for example, EP release did not require protein synthesis (49). Second, at the time of those experiments PMN's were thought to be the primary source of EP. Consequently, the PE cells used were primarily PMN's, and measurements of cell-associated ions were, therefore, largely influenced by the ion content of the PMN's present. MNPC's, the cells actually responsible for EP production, were present in smaller and variable amounts. This may explain the authors' inability to show any direct correlation between cellular Na^+ or K^+ levels and EP production. Third, more recent attempts to recreate this system of "spontaneously" active PE cells have been unsuccessful, making further research on the system difficult. It is possible that the unidentified macromolecular activator of cells they described in exudate fluid was endotoxin (50). Due to the design of their system, it is not possible to exclude contamination of the ouabain used with endotoxin, as well. Fourth, *in vitro* incubation of stimulated blood leukocytes in the presence of physiological levels of Ca^{2+} and K^+ does not prevent EP release (51).

White and Petersdorf attempted to extend these findings using an *in vivo* system (52). They found that rabbits made acutely or chronically hypokalemic produced more EP in response to a standard dose of heat-killed bacteria than did normal rabbits. Hyperkalemia with concurrent hypercalcemia,

however, did not reduce EP production from control levels. Unfortunately, the complexity of an *in vivo* system makes it difficult to change a single physical parameter without producing countless other changes designed to maintain a homeostatic balance. It is clear, however, that any inhibitory effect of Ca^{2+} or K^{+} on EP production was a relative one, not an absolute one as Wood's work suggested.

In more recent years research in many diverse biological systems has shown an almost universal role for Ca^{2+}, alone or in conjunction with cyclic nucleotides, as a second messenger for stimulus-secretion coupling (53). While there have been attempts to establish such a role for second messengers in the activation of MNPC's, the evidence has been fragmentary and sometimes contradictory.

Several investigators have used the ionophore A23187 as a tool to investigate the importance of Ca^{2+} in MNPC activation. There have been reports that A23187 does (54) and does not (55) stimulate lysosomal enzyme secretion. In another study alveolar macrophages incubated with A23187 demonstrated an increased rate of intracellular bacteriocidal activity and a rise in intracellular acid phosphatase when magnesium (Mg^{2+}) was included in the incubation medium. No such effect was seen with Ca^{2+}, however (56).

Adenosine 3', 5' cyclic monophosphate (cAMP) has been reported to inhibit macrophage migration (57), to decrease

secretion of plasminogen activator by mouse PE cells (58) and a macrophage-like tumor cell line (J774.2) (59), and to decrease the secretion of lysosomal enzymes by mouse PE cells (60). Others have shown that guanosine 3', 5' cyclic monophosphate (cGMP) either decreases (61) or increases (62) human monocyte chemotaxis, but does not affect the response of macrophages to a lymphokine, macrophage inhibitory factor (MIF) (57). There is a brief report that isobutyl-methyl-xanthine (IBMX), a phosphodiesterase inhibitor which should increase endogenous levels of both cAMP and cGMP, does not stimulate or modulate EP production by human monocytes (63). However, no attempt has been made to manipulate selectively the cAMP or cGMP concentration in MNPC's to assess its role in EP production.

Given the diverse nature of known stimulators of EP production, ranging from inorganic urate crystals to biological products of stimulated lymphocytes, it seems reasonable that some common pathway exists within the MNPC which is responsible for derepression of the EP genome. The studies reported herein were undertaken to investigate the role of Ca^{2+} and/or cyclic nucleotides as second messengers in this process.

MATERIALS AND METHODS

General

All glassware was sterilized by dry heat at 180°C for two hours to inactivate any pyrogenic contaminants. All reagents were demonstrated to be pyrogen-free by their inability to stimulate EP production from buffy coat monocytes *in vitro*. Samples from all blood and incubation supernatants were found to be sterile by culture for at least three days at 37°C in thioglycollate broth. Except as noted, all media and reagents were stored at 2°C.

Incubation Media

Most incubations were performed in Hank's balanced salt solution (HBSS) prepared from a 10X concentrate without calcium, magnesium, or bicarbonate (GIBCO). Some experiments were carried out in Eagle's minimum essential medium (MEM) which contained calcium and magnesium (Flow Labs). Both media were supplemented with 1.96 g/l sodium bicarbonate, 50,000 u/l penicillin, and 50,000 ug/l streptomycin base.

Reagents

Boivin extracted endotoxin from E. coli 0127.B8 (LPS, Difco Laboratories) was prepared as a stock solution of 5 mg/ml in sterile saline (Abbott Laboratories) and diluted to a working solution of 1 ng/ml in saline (for MEM incubations) or in HBSS before each use.

$CaCl_2.2H_2O$, $MgCl_2.6H_2O$ (Certified Grade, Fisher Scientific), $LaCl_3.7H_2O$ (Aldrich Chemicals), and ethylene-glycol-bis-(B-amino-ethyl ether) N,N' tetra-acetic acid (EGTA, Sigma Chemical Co.) were sterilized by dry heat at 180°C for two hours before use. Solutions of 100 mM $CaCl_2$, 100 mM $MgCl_2$, and 75 mM $LaCl_3$ were prepared in sterile water (Abbott Laboratories). 20 mM EGTA was prepared in HBSS, using 5.6% sodium bicarbonate (w/w) to adjust the pH to 7.25. The calcium binding capacity of EGTA, as measured by its competitive ability to bind radioactive Ca^{2+}, was not altered by the sterilization process. Ethylenediaminetetra-acetic acid, disodium (EDTA, Disodium Edetate Injection, Riker Labs) was diluted to 100 mM in sterile water.

Heparin (Sodium Injection, Eli Lilly and Co.) 1000 u/ml and Polymyxin B (PMB, Aerosporin, Burroughs Wellcome Co.) 50,000 u/ml were prepared in sterile saline. Epinephrine (EPI, Adrenalin, Parke-Davis) and propranolol hydrochloride (PROP, Inderal Injectable, Ayerst Labs) were stored as 1 mg/ml solutions in sterile water. N^6, O^2 - dibutyryl adenosine 3', 5' - cyclic monophosphate monosodium salt (db-

cAMP, Sigma Chemical Co.) 1 mg/ml and Staphylococcal enterotoxin B (SE, Sigma Chemical Co.) 1 mg/ml were prepared in HBSS. Ionophore A23187 (Calbiochem) 2.0 mM and Prostaglandin E_1 (PGE_1, prost-13-en-1-oic acid, 11, 15 dihydroxy-9-oxo-[11a, 13E, 15S], Sigma Chemical Co.) 1 mg/ml were prepared in 95% ethanol and stored at 0°C.

Preparation and Incubation of Cells for EP Production

Blood was collected into heparinized glass syringes from female 5-6 pound New Zealand White rabbits by cardiac puncture after anesthesia with intravenous sodium pentobarbital (75 mg in 1.5 ml) (Nembutal, Sodium Solution, Abbott Laboratories). In each experiment, blood from at least two donors was mixed and centrifuged at 1000 X g for fifteen minutes. The buffy coats were aspirated, resuspended in saline (for experiments in MEM) or HBSS, and washed twice. Total leukocytes were counted twice by hemocytometer, and differential counts were made on 200 consecutive cells from Wright-Giemsa stained smears (mean %'s ± S.E. : lymphocytes 28.8 ± 3.0, granulocytes 61.6 ± 3.2, monocytes 9.6 ± 0.4). Since monocytes are the only important source of EP in blood cells (21), the washed cells were divided into aliquots, each of which contained two to four doses of 2 X 10^6 monocytes mixed with other leukocytes. A standard pyrogenic dose of 100 pg/ml LPS was used in all experiments to stimulate EP production.

In some experiments, to test the effect of extracellular cations upon EP production, the incubation medium was altered by addition of $CaCl_2$, $MgCl_2$, $LaCl_3$, EGTA, or EDTA. In other experiments, cells were preincubated with EGTA for up to three hours before the addition of LPS and/or cations. Some of these EGTA-preincubated cells were incubated further with added cations for up to three hours more before the addition of LPS.

Ionophore A23187, db-cAMP, EPI, PROP, PGE_1, and SE were incubated with cells alone, in combination, or with the addition of a standard dose of LPS to test their ability to stimulate EP production. In some experiments, PMB (200 u/ml) was added to cell incubations to ascertain whether EP production was due to LPS contamination of potential activators (64).

In one set of experiments (Ca^{2+} pulse), cells suspended in HBSS were incubated with A23187 for five minutes to allow incorporation of the ionophore into the cell membranes. $CaCl_2$ and EGTA were then added to achieve a buffered free Ca^{2+} concentration, and incubation was continued for fifteen minutes. More EGTA was added before beginning the final incubation. This protocol allowed a transient (fifteen minute) pulse of ionized Ca^{2+} into the cells without requiring them to maintain such a high level of internal free Ca^{2+} over the extended incubation period.

After addition of all reagents, volumes were standardized so that each dose of 2 X 10^6 monocytes was suspended in a total volume of 2.5 ml. The cells were then incubated (5% CO_2, 37°C) for 20 hours. The suspensions were cleared by centrifugation for ten minutes at 1000 X g, and the supernatants aspirated and assayed for EP. Cell viability, as measured by the ability of the pelleted cells to exclude eosin dye, was 75-90% in all experiments and was not reduced by any of the experimental agents.

Assay of EP

The EP content of supernatants was measured by intravenous injection into rabbits. The maximum elevation of temperature above baseline (fever) evoked within one hour, between 0.4 and 0.8°C, has been shown to be linearly related to the dosage of EP injected (65). The techniques used to record temperatures were those described by Atkins and Heijn (66), except that rectal temperatures were recorded continuously with a paper recorder (Rustrak TSC, Norwood, MA).

In most experiments rabbits received a dose of each supernatant in random order. Supernatants were stored at 2°C, but were allowed to warm to room temperature before injection. This enabled us to administer injections from a single experiment over a two to four day period without appreciable loss of pyrogenicity in the supernatants.

$^{45}Ca^{2+}$ Tracer Studies

Cell Preparation

In order to measure cell-associated $^{45}Ca^{2+}$ after addition of ionophore A23187 or of LPS, relatively pure samples of monocytes were prepared using density gradients of Percoll or mixtures of Ficoll and Hypaque.

Percoll separation technique. A two-step centrifugation procedure, based upon the one developed by Pertoft, et al., (67) for isolating human blood monocytes, was used. Nine volumes of autoclaved commercial Percoll, a colloidal suspension of polyvinylpyrrolidone-coated silica particles averaging 15 - 30 nm in diameter (Pharmacia, Inc.), was added to one volume of 10X concentrate HBSS to make a stock solution of isotonic Percoll (SIP) with density 1.124 g/ml. Heparinized rabbit blood was collected in the manner described above, and 28 ml were layered over 16 ml of 60% (v/v) SIP in sterile saline, density 1.076 g/ml, and centrifuged for thirty minutes at 800 X g at room temperature. The interphase band, containing primarily mononuclear cells, was aspirated from each tube. The cells from two tubes were combined, suspended in a total volume of 28 ml in sterile saline, and layered over 12 ml of 55% (v/v) SIP in sterile saline, density 1.070 g/ml. The cells were centrifuged for sixty minutes at 800 X g at 4°C, and the interphase bands were removed. It should be noted that no such interphase bands formed with the 50% (v/v) SIP solution used by Pertoft and colleagues for human cell separations.

Rather, all cells pelleted to the bottom following the second centrifugation. After washing the isolated cells twice in MEM, total and differential leukocyte counts were made (differential count mean %'s ± S.E.: lymphocytes 14.0 ± 4.4, granulocytes 2.5 ± 0.5, monocytes 78.5 ± 4.6).

Ficoll-Hypaque separation technique. Despite the fact that the Percoll used was not contaminated with LPS, as measured by the Limulus Amebocyte Assay (68), cells isolated with Percoll produced EP without further stimulation. Therefore, a second cell separation technique was developed using density gradients of Ficoll 400, a synthetic polymer of sucrose and epichlorohydrin (Pharmacia, Inc.), and Hypaque, 3, 5 - diacetamido - 2, 4, 6 - triiodobenzoic acid (Hypaque Sodium 50%, Winthrop Laboratories Div., Sterling Drug, Inc.), in the place of Percoll. A stock solution of isotonic Ficoll-Hypaque (SFH), density 1.076 g/ml, was made by adding one volume of Hypaque to four volumes of a 7.9% (w/w) aqueous solution of Ficoll 400 which had been passed through a Nalgene filter (Nalgene Labware, Nalge Co.). From this stock solution three additional solutions were prepared and tested against each other for their suitability as separation media. Solution A was made by adding one volume of sterile saline to thirty seven volumes SFH; solution B by adding one volume saline to eighteen volumes SFH; and solution C by adding one volume saline to twelve volumes SFH. These solutions had densities of 1.074, 1.072, and 1.070 g/ml, respectively.

Heparinized rabbit blood was obtained in the usual fashion, and diluted with an equal volume of sterile saline. 28 ml of the diluted blood were layered over 16 ml SFH and centrifuged for thirty minutes at room temperature at a force of 800 X g. The mononuclear cells present in the interphase layer were collected, and total and differential leukocyte counts were made. The cells from two tubes were resuspended in a total volume of 28 ml with sterile saline, layered over 12 ml of solution A, B, or C, and centrifuged for sixty minutes at 4°C with a force of 800 X g. The interphase cells were collected, and total and differential leukocyte counts were again performed. The results, presented in Table 1, showed that solution C provided the purest preparation of monocytes, though with some sacrifice of yield. It was used, therefore, for subsequent experiments requiring monocyte-enriched samples.

Ionophore Study

Cells purified by Percoll separation were suspended in MEM in a concentration of 1.0 X 10^6 monocytes/ml. 0.8 ml aliquots of this suspension were incubated in a 37°C water bath with 0.1 ml of $^{45}CaCl_2$ (86 mg Ca/ml, 1-9 mCi/ml, Radioactive Center, Amersham, England) that had been diluted 1:100 with MEM, and 0.2 ml of a 2 mM solution of ionophore A23187 or of MEM (control). At timed intervals, 50 ul aliquots were removed and passed through a 0.45 micron pore

TABLE 1

Cell Separations on Ficoll-Hypaque Gradients

Sample	Total WBC's (X 10^8)[+]	Differential Counts % PMN's	% Lymph's	% MNPC's	Total MNPC's (X 10^8)	MNPC Yield (%)
Whole Blood	8.468 (.002)	37 (3)	50 (6)	14 (4)	1.2 (.3)	100
First Spin	0.96 (.02)	3 (2)	56 (4)	41 (2)	0.39 (.02)	33 (8)
Second Spin (Soln A)	0.124 (.005)	---	25 (2)	75 (2)	0.093 (.004)	23 (6)
Second Spin (Soln B)	0.058 (.002)	0.7 (.6)	15 (4)	84 (4)	0.049 (.002)	12 (3)
Second Spin (Soln C)	0.067 (.002)	1	6 (1)	93 (1)	0.062 (.002)	16 (4)

[+]Values in parentheses denote standard errors of the mean.

size filter (Type HA, Millipore Corp.). Each filter was immediately washed twice with 1 ml aliquots of ice cold MEM, and then placed in a scintillation vial with 4 ml of Hydrofluor (National Diagnostics). Total counts were measured by resuspending 50 ul of the unfiltered incubation medium in 4 ml of Hydrofluor. Each sample was counted for one minute on a scintillation counter (Model LS 3133P, Beckman Instruments).

LPS Study

Cells separated on Ficoll-Hypaque gradients were suspended in MEM in a concentration of 2.3 X 10^6 monocytes/ml. 0.9 ml aliquots were incubated in a 37°C water bath with 0.05 ml of the $^{45}CaCl_2$ solution described above. After two hours, 100 pg LPS and/or 500 u PMB were added in 0.1 ml MEM. 50 ul aliquots were removed at timed intervals and filtered, washed, and assayed, as above.

Calculation of Free Calcium Concentration [Ca^{2+}]

When Ca^{2+} and chelators were mixed in solution, the free calcium concentration [Ca^{2+}] was calculated using the method of O'Sullivan to determine the "apparent" stability constants of chelate complexes at pH 7.30, the initial incubation pH, from the published "absolute" values of the constants (69). The calculated values for the stability constants were 3.98 X 10^7 M^{-1} for Ca-EDTA complexes, and 1.896 X 10^7 M^{-1} for Ca-EGTA complexes.

RESULTS

Effects of Free Calcium on EP Production

Buffy coat cells were incubated with LPS in HBSS with or without added $CaCl_2$. LPS stimulated EP production in all incubations, with Ca^{2+} concentrations up to 2.0 mM (Table 2). The greatest response occurred with 0.5 mM $CaCl_2$ in the incubation medium, but the responses in media containing 1.0 mM or no added $CaCl_2$ were not significantly lower, as measured by Student's t test for independent samples. Incubation with 2.0 mM $CaCl_2$ added did significantly diminish the response seen following incubation with lower Ca^{2+} concentrations, as measured by Student's t test for paired samples.

When other cells were incubated with LPS in an incubation medium prepared by diluting MEM 1:2 with sterile saline, EP was likewise produced despite the addition of $CaCl_2$ or EDTA to alter the $[Ca^{2+}]$ (Table 3). The maximum EP production occurred with a calculated $[Ca^{2+}]$ of 0.4 mM, and it was significantly increased over production in media with higher or lower calculated concentrations of free Ca^{2+}.

TABLE 2

Effect of Extracellular Calcium on EP Production in HBSS

Mean fevers produced by supernatants from 2 X 10^6 monocytes incubated with or without LPS in HBSS with various concentrations of $CaCl_2$.

LPS (pg/ml)	[$CaCl_2$] (mM)	Fever (S.E.) (°C)	Number of Recipients
0	2.0	0.0	2
100	0	0.675 (.063)$^{+}$	4
100	0.5	0.900 (.087)	3
100	1.0	0.788 (.097)$^{\circ}$	4
100	2.0	0.363 (.080)$^{+,\circ}$	4

$^{+}$ P<0.025 by paired t test
$^{\circ}$ P<0.05 by paired t test

TABLE 3

Effect of Extracellular Calcium on EP Production in MEM

Mean fevers produced by supernatants from 2 X 10^6 monocytes incubated with 100 pg/ml LPS in MEM-saline solutions with various concentrations of EDTA and Ca^{2+} added.

[EDTA] (mM)	[EDTA]/ $[Ca^{2+}]^+$	Calculated Free Ca^{2+} Concentration (mM)	Fever (S.E.) (°C)$^-$
0	0	1.9°	0.767 (.081)$^=$
0.50	0.56	0.4	1.25 (.04)$^{='('++}$
1.00	1.11	2.3 X 10^{-4}	0.650 (.065)$^($
2.00	2.22	2.1 X 10^{-5}	0.738 (.115)$^{++}$

$^+$ Based on a total calcium concentration of 1.8 mM in MEM, and a resulting total calcium concentration of 0.9 mM in our incubation medium which was MEM diluted 1:2 with saline

° 1.0 mM $CaCl_2$ was added to this solution.

$^-$ There were four recipients for each supernatant.

$^=$ $P<0.025$ by paired t test

$^{('++}$ $P<0.005$ by paired t test

Effects of EGTA Preincubation on Subsequent EP Production

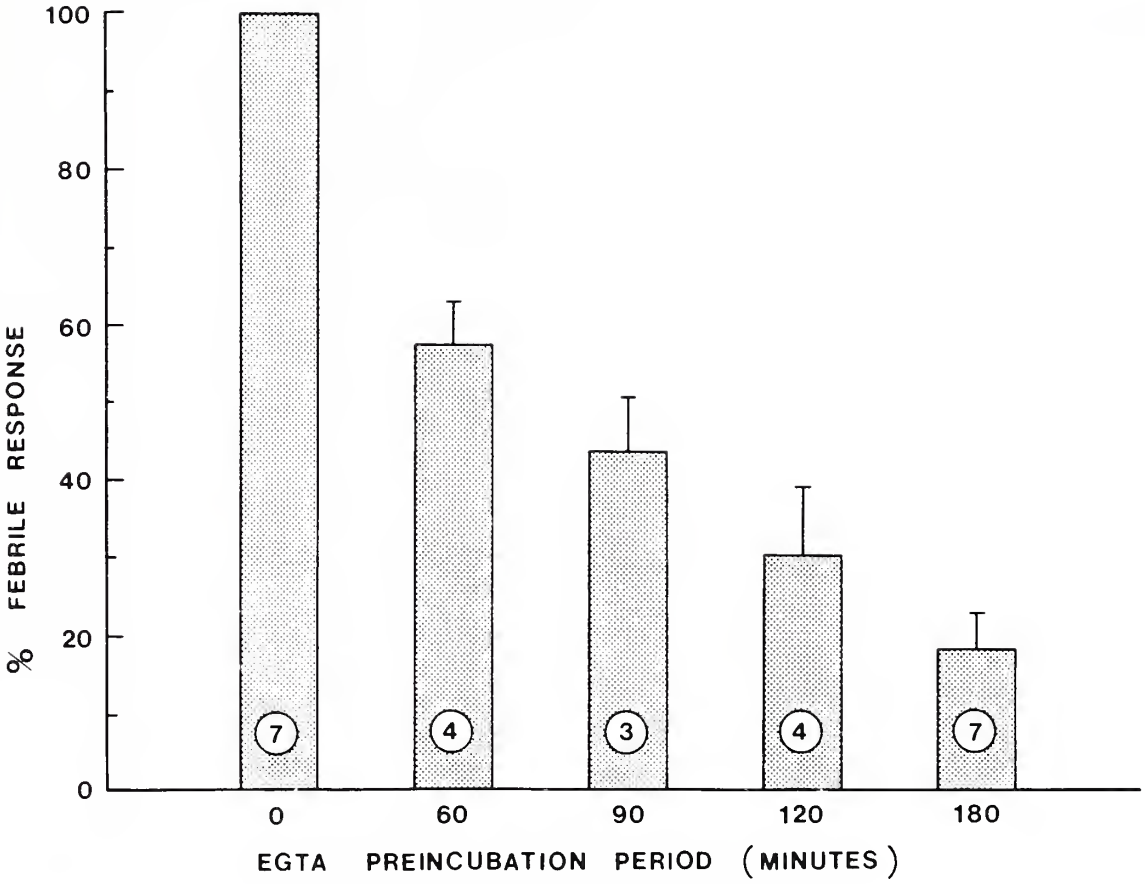
Cells were preincubated with 5 mM EGTA for 0 - 3 hours before the addition of LPS. Twenty hour supernatants were assayed for EP, and the mean fevers of EGTA-preincubated cells were expressed as percentages of the responses from cells activated with LPS without preincubation, after control responses (from cells incubated alone) were subtracted from both (Figure 3). The mean fever for cells not preincubated with EGTA was 0.80 ± 0.05°C (S.E.). Preincubation with EGTA resulted in substantial inhibition of LPS-stimulated EP production, and the degree of inhibition increased with prolongation of the preincubation period. Cells preincubated for three hours with EGTA released only 19% of the EP released by cells that had not been pretreated (mean fever 0.23 ± 0.04°C (S.E.)).

Reversibility of EGTA Effects

Inhibition of LPS-induced EP production by cells preincubated for three hours with 5 mM EGTA was partially reversed when 5 mM $CaCl_2$ was added to the incubation medium simultaneously with LPS (Figure 4). EP production under these conditions was 53% of the amount produced by cells not preincubated with EGTA. Lesser amounts of $CaCl_2$ were not sufficient to restore EP production (Table 4). A second preincubation of cells with $CaCl_2$ added to the incubation medium for up to three hours after the initial treatment

Figure 3: Effects of EGTA Preincubation on EP Production

Mean fevers produced by supernatants from 2 X 10^6 monocytes incubated for twenty hours with 100 pg/ml LPS after preincubation of cells with 5 mM EGTA for 0-180 minutes, expressed as a percentage of the response seen when cells were not preincubated with EGTA. Numbers of recipients for each supernatant are given on each bar, and standard errors are shown.


100
80
60
40
20
0
% FEBRILE RESPONSE
7
4
3
4
7
0
60
90
120
180
EGTA PREINCUBATION PERIOD (MINUTES)

with EGTA before adding LPS did not affect the ultimate release of EP compared to that seen when $CaCl_2$ and LPS were added together (Table 5).

Substitution of 5 mM $MgCl_2$ or $LaCl_3$ for $CaCl_2$ did not restore EP production following EGTA preincubation (Figure 5). Further, incubation of cells with 0.1 mM $MgCl_2$ or $LaCl_3$ for three hours after the initial treatment with EGTA prevented EP production when LPS and 5 mM $CaCl_2$ were subsequently added.

Effects of Ionophore A23187 on EP Production

Cells were incubated for twenty hours with 0.20 uM A23187 in HBSS to which 25 uM $CaCl_2$ or $MgCl_2$ or both had been added. Under these conditions the ionophore did not stimulate EP production (Table 6). Cells incubated with both 0.20 uM A23187 and 100 pg/ml LPS released EP, though in amounts that were significantly lower than those produced in the absence of A23187 ($P<0.05$ by Student's t test for paired samples).

In another set of experiments, we attempted to produce a pulse of intracellular calcium influx by incubating cells for five minutes in HBSS with 0.20 or 2.00 uM A23187, followed by the addition of Ca^{2+} and EGTA to produce buffered solutions with $[Ca^{2+}]$ of 0.37 or 2.3 uM. Incubations were continued for fifteen minutes, followed by the addition of either more EGTA in HBSS to reduce the $[Ca^{2+}]$ to 0.02 uM or more HBSS alone to keep the $[Ca^{2+}]$

Figure 4: Reversibility of EGTA Effects with Calcium

Mean fevers produced by supernatants from 2 X 10^6 monocytes preincubated with 5 mM EGTA for three hours before the addition of LPS (100 pg/ml) alone or with $CaCl_2$, expressed as a percentage of the response when cells were not pretreated with EGTA. Numbers of recipients for each supernatant are given on each bar, and standard errors are shown.

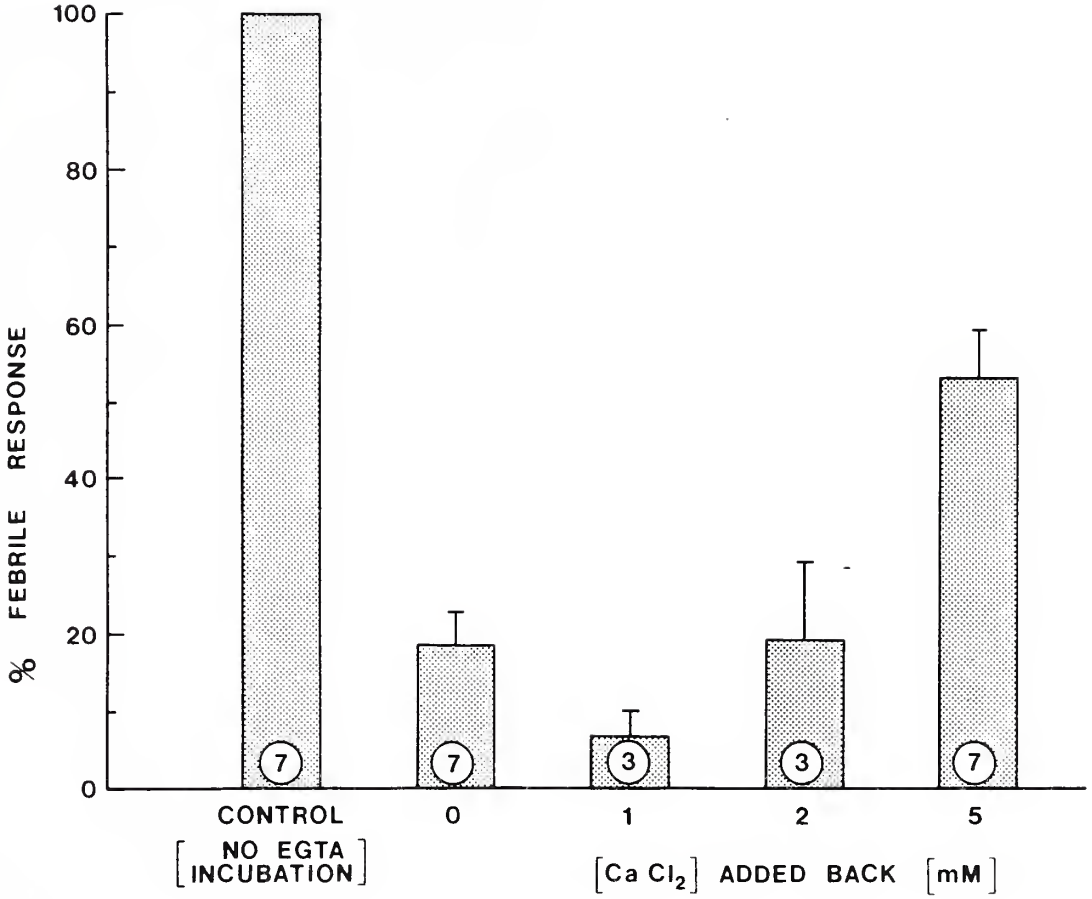

100
80
60
40
20
0
% FEBRILE RESPONSE
7
7
3
3
7
CONTROL
[NO EGTA INCUBATION]
0
1
2
5
[Ca Cl2] ADDED BACK [mM]

TABLE 4

Calcium Concentration Required for Reversal of EGTA Effects

Mean fevers produced by supernatants from 2 X 10^6 monocytes preincubated for three hours with 5 mM EGTA before the addition of 0.25 ng LPS and varying concentrations of $CaCl_2$. There were three recipients for each supernatant.

[$CaCl_2$] Added (mM)	Fever (S.E.) (°C)
0	0.28 (0.02)
1	0.17 (0.03)
2	0.23 (0.08)
5	0.45 (0.09)

TABLE 5

Time Course of Calcium Effects

Mean fevers produced by supernatants from 2 X 10^6 monocytes preincubated for three hours with 5 mM EGTA, and then incubated with 5 mM $CaCl_2$ for varying lengths of time before the addition of 0.25 ng LPS. There were four recipients for each supernatant.

Time Incubated with $CaCl_2$ before LPS Addition (Minutes)	Fever (S.E.) (°C)
0	0.45 (0.08)
60	0.48 (0.08)
120	0.40 (0.04)
180	0.48 (0.07)

Figure 5: Specificity of the Calcium Effect

Mean fevers produced by supernatants from 2 X 10^6 monocytes preincubated with 5 mM EGTA for three hours before the addition of 100 pg/ml LPS with or without the addition of $CaCl_2$, $MgCl_2$, or $LaCl_3$ (5 mM). To two aliquots of cells, 0.1 mM $MgCl_2$ or $LaCl_3$ was added after the EGTA preincubation, and cells were incubated for an additional three hours before the addition of LPS and 5 mM $CaCl_2$. Numbers of recipients for each supernatant are given on each bar, and standard errors are shown.

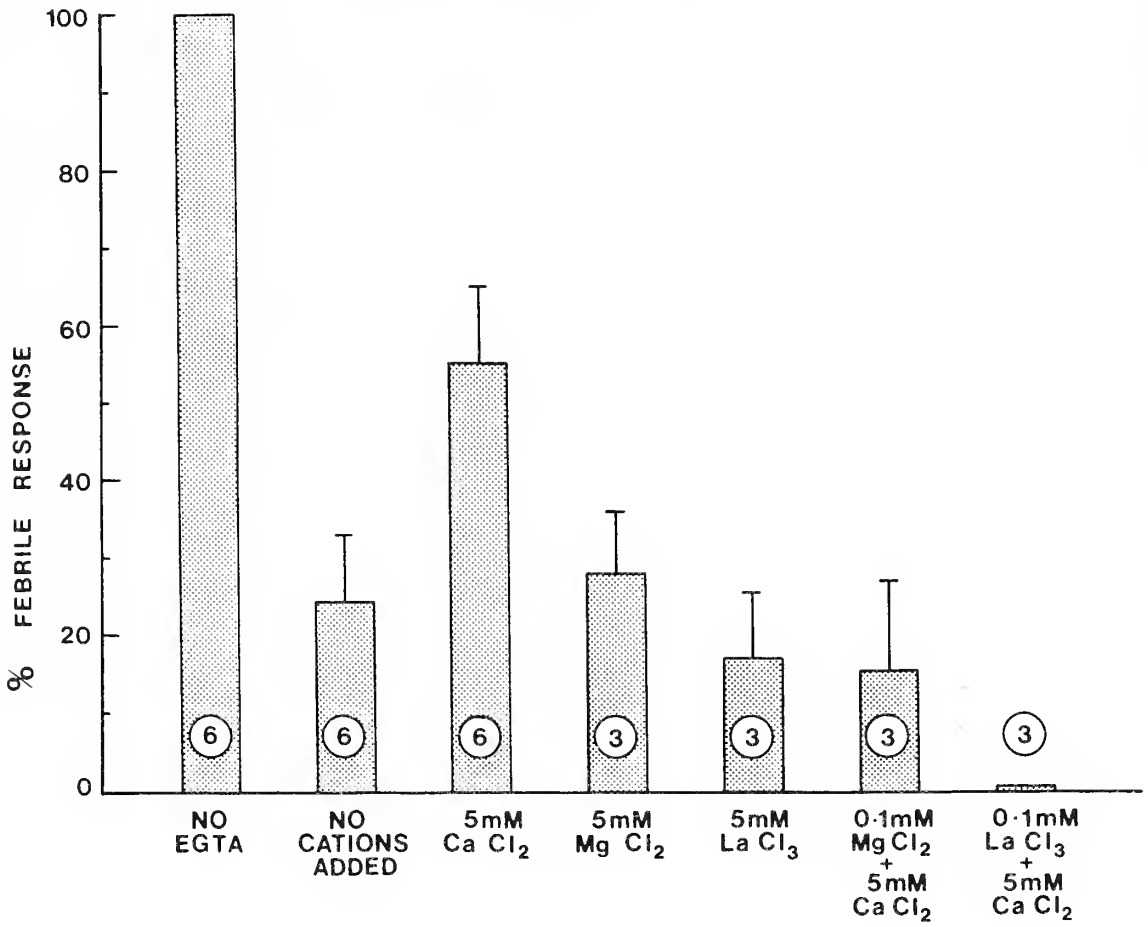

% FEBRILE RESPONSE
100
80
60
40
20
0
6
6
6
3
3
3
3
NO EGTA
NO CATIONS ADDED
5mM Ca Cl2
5mM Mg Cl2
5mM La Cl3
0·1mM Mg Cl2 + 5mM Ca Cl2
0·1mM La Cl3 + 5mM Ca Cl2

TABLE 6

Effects of A23187 on Pyrogen Production

Mean fevers produced by supernatants from 2 X 10^6 monocytes incubated with 0.20 uM A23187 and/or LPS in the presence of 25 uM $CaCl_2$ and/or $MgCl_2$ in HBSS. There were four recipients for each supernatant.

Potential Activator(s)	Cations Present	Fever (S.E.) (°C)
None	Ca^{2+}, Mg^{2+}	0.16 (0.01)
A23187	Ca^{2+}	0.15 (0.02)
A23187	Mg^{2+}	0.08 (0.04)
A23187	Ca^{2+}, Mg^{2+}	0.14 (0.06)
A23187 + LPS	Ca^{2+}	0.31 (0.05)°
LPS	----	0.68 (0.13)°

° $P<0.05$ by paired Student's t test

stable. Twenty hour supernatants did not show any EP activity, although an aliquot of the same cells incubated with LPS produced EP (mean fever 1.08°C; 2 recipients).

$^{45}Ca^{2+}$ Tracer Study of Ionophore Action

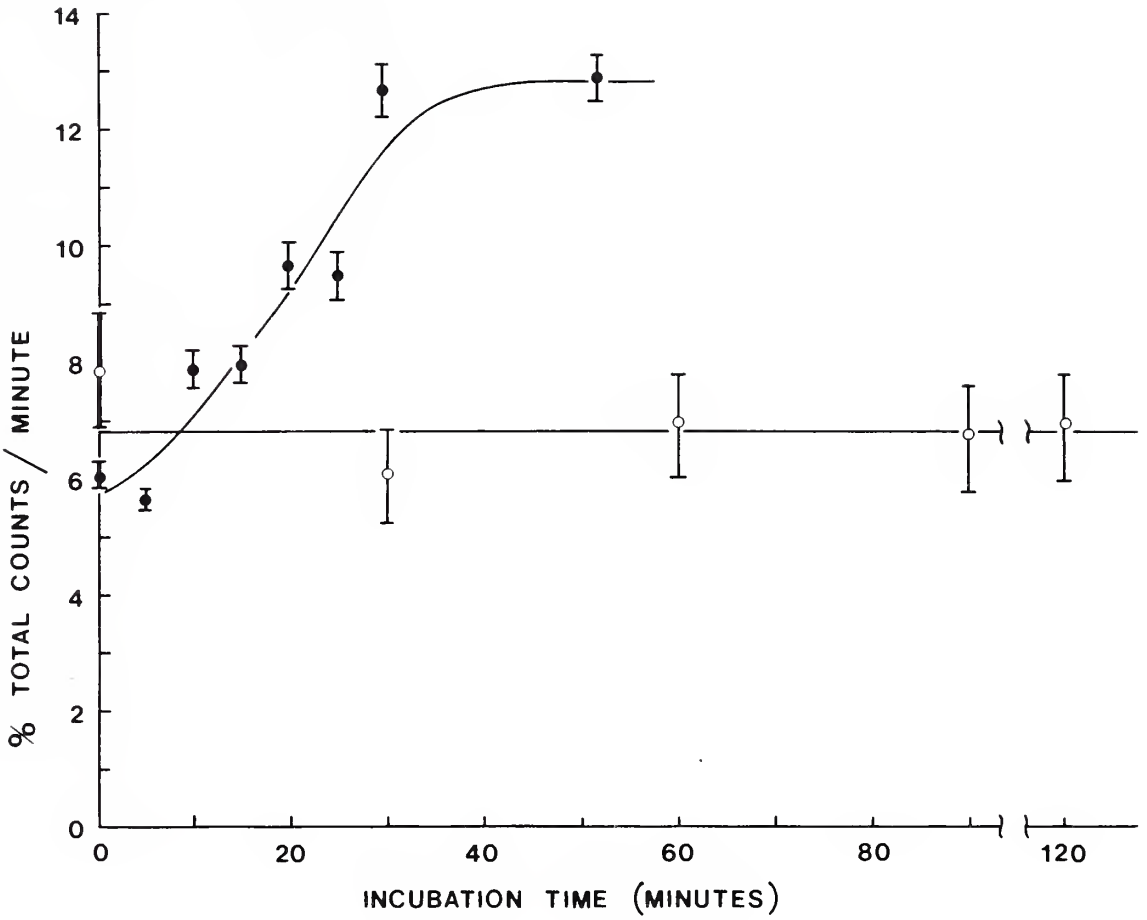
To confirm that added A23187 did interact with cells during incubation, rabbit mononuclear cells isolated on Percoll gradients were incubated with $^{45}Ca^{2+}$ in the presence and absence of 0.36 uM A23187. Cells treated with ionophore showed an increase in cell-associated counts during the first thirty minutes of incubation, while the counts in untreated cells did not increase during a two hour incubation period (Figure 6).

$^{45}Ca^{2+}$ Tracer Studies of LPS Stimulation

Rabbit mononuclear cells isolated on Ficoll-Hypaque gradients were incubated with $^{45}CaCl_2$ for two hours to allow equilibration before the addition of 100 pg/ml LPS and/or 500 units PMB. No flux of Ca^{2+} could be demonstrated by changes in cell-associated $^{45}Ca^{2+}$ upon the addition of LPS (data not shown). Separate aliquots of cells were incubated with or without LPS, and the twenty hour supernatants were assayed for EP. The aliquots incubated with LPS produced EP (mean fever 0.5°C; 2 recipients), while the aliquots without LPS were non-pyrogenic.

Figure 6: Ionophore-Induced Calcium Uptake in Monocytes

Counts per minute of filtrates of 50 ul aliquots of cells incubated with (.) or without (o) 0.36 uM A23187, expressed as a percentage of the total counts in an unfiltered 50 ul aliquot. Total counts (standard errors) were 46043 (1284) for the cells incubated with ionophore and 57516 (7137) for the cells incubated alone. Standard errors are shown.


14
12
10
8
6
4
2
0
% TOTAL COUNTS / MINUTE
0
20
40
60
80
120
INCUBATION TIME (MINUTES)

Effects of cAMP on EP Production

Buffy coat cells incubated for twenty hours with 0.01 mg/ml EPI and/or 0.01 mg/ml PROP produced no detectable EP (Table 7). Direct intravenous injection of these drugs at this dose produced no changes in baseline body temperature of test rabbits. To investigate the effects of EPI and PROP as modulators of an independently-stimulated response, cells were incubated with 100 pg/ml LPS with or without the addition of EPI and/or PROP (0.01 mg/ml). These drugs did not affect the response of monocytes to LPS (Table 8). Similarly, we found that 14 uM PGE_1 did not affect LPS stimulation of EP production (Table 8).

10 ug/ml SE stimulated release of EP sufficient to cause a mean fever of 0.78 ± 0.18°C, but the addition of 200 units/ml PMB before incubation abolished the effect. Similarly, supernatants from cells incubated with 3 mM db-cAMP produced fevers of 0.47 ± 0.11°C, but this was prevented by including 200 units/ml PMB in the incubation medium (Table 9). Because of the presumed presence of LPS contaminating these agents, direct testing of their ability to augment LPS-stimulated release of EP was not practical. However, they clearly did not prevent the release of EP.

TABLE 7

Effects of Epinephrine and Propranolol on EP Production

Mean fevers produced by supernatants from 2 X 10^6 monocytes incubated with 0.01 mg/ml EPI and/or 0.01 mg/ml PROP alone or in the presence of 100 pg/ml LPS. Except where noted, there were four recipients for each supernatant.

Potential Activator(s)	Fever (S.E.) (°C)
EPI	0.03 (0.08)[+]
PROP	0[°]
EPI + PROP	0[-]
LPS	0.86 (0.10)
LPS + EPI	0.84 (0.09)[=]
LPS + PROP	0.73 (0.15)[=]
LPS + EPI + PROP	0.76 (0.10)[=]

[+] There were five recipients for this supernatant.
[°] There were three recipients for this supernatant.
[-] There were two recipients for this supernatant.
[=] Not significantly different from fever when cells were incubated with LPS alone, by Student's t test for paired samples

TABLE 8

Effects of Prostaglandin E_1 on EP Production

Mean fevers produced by supernatants from 2 X 10^6 monocytes incubated with 14 uM PGE_1, alone or in the presence of LPS. Except where noted, there were three recipients for each supernatant.

Potential Activator(s)	Fever (S.E.) (°C)
None	0[+]
PGE_1	0.10 (0.06)
LPS	0.73 (0.12)°
LPS + PGE_1	0.78 (0.07)°

[+] There were two recipients for this supernatant.
° Not significantly different by Student's t test for paired samples

TABLE 9

Pyrogenicity of SE and db-cAMP due to LPS Contamination

Mean fevers produced by supernatants from 2 X 10^6 monocytes incubated with 10 ug/ml SE or 3 mM db-cAMP, alone or in the presence of 200 units/ml PMB.

Potential Activator(s)	Fevers (S.E.) (°C)
None	0.05 (0.05)[+]
SE	0.78 (0.18)[°'-]
SE + PMB	0.03 (0.03)[-'=]
db-cAMP	0.47 (0.11)[°'(]
db-cAMP + PMB	0.03 (0.03)[°'(]

[+] There were two recipients for this supernatant.
[°] There were six recipients for this supernatant.
[-] Significantly different by Student's t test for independent samples (P<0.025)
[=] There were three recipients for this supernatant.
[(] Significantly different by Student's t test for independent samples (P<0.025)

DISCUSSION

The Role of Calcium in EP Production

Using a standard dose of LPS as a pyrogenic stimulus we found that rabbit blood monocytes can produce EP in media with free calcium concentrations calculated to be in the 10^{-8} to 10^{-3} M range. As in studies reported by Wood and colleagues (48-50), we found that EP production was inhibited at greater than physiological concentrations of free calcium. Studies of macrophage lysosomal enzyme release stimulated by incubation with zymosan have also shown that extracellular Ca^{2+} is not required, and that high levels of extracellular Ca^{2+} (5mM) inhibited enzyme release (54). That we saw more inhibition in HBSS than in MEM can be attributed to calcium-binding components in the latter medium, notably succinic acid and the amino acids present, which reduce the concentration of free calcium. MEM also contains more phosphate and sulfate anions, which complex with calcium, as well. In both media, however, we found maximal EP production with a calculated free $[Ca^{2+}]$ of 0.4 - 0.5 mM. This is lower than the average value of 1.15 mM free Ca^{2+} Moore measured in the serum of normal volunteers (70). Although all buffy coat preparations were washed twice in Ca^{2+}-free solutions (probably containing uM levels

of Ca^{2+} as a trace contaminant), significant amounts of Ca^{2+} may have remained bound to cell membranes. This would increase the actual amount of Ca^{2+} present and, perhaps, raise the local concentration of free Ca^{2+}, in addition. The fall in pH seen during the course of incubation, to as low as 6.9, would increase free Ca^{2+} further by decreasing the stability of calcium complexes. In our HBSS incubations, the difference in fever stimulated by EP generated with 0.5 and 1.0 mM free calcium was not statistically significant ($P>0.05$ by Student's t test for paired samples), and no comparable incubation with 1 mM free Ca^{2+} was performed in MEM. Therefore, we cannot exclude the possibility that EP production is maximal at approximately physiological concentrations of free Ca^{2+}.

While monocytes could be stimulated to produce submaximal amounts of EP with extremely low levels of free extracellular Ca^{2+}, preincubation of cells with a Ca^{2+} chelator inhibited subsequent stimulation of EP production. The degree of inhibition was related to the time of preincubation. It was partially reversed by later addition of Ca^{2+}, but not other cations, to the system. In fact, incubation with small amounts of Mg^{2+} or La^{3+} prevented the reversal of inhibition by later addition of Ca^{2+}. The prolonged incubation of cells depleted of Ca^{2+} during these experiments may have had other effects which prevented subsequent stimulation, although in the experiments

summarized in Table 5 there was no evidence that delayed addition of LPS to cells reduced the amount of EP produced. These results suggest that the mere presence of Ca^{2+} in the extracellular space where it would be available to fulfill any required role in the binding of LPS to the cell membrane was not sufficient to cause EP production, and that re-entry of Ca^{2+} into the cells was necessary for EP production to occur.

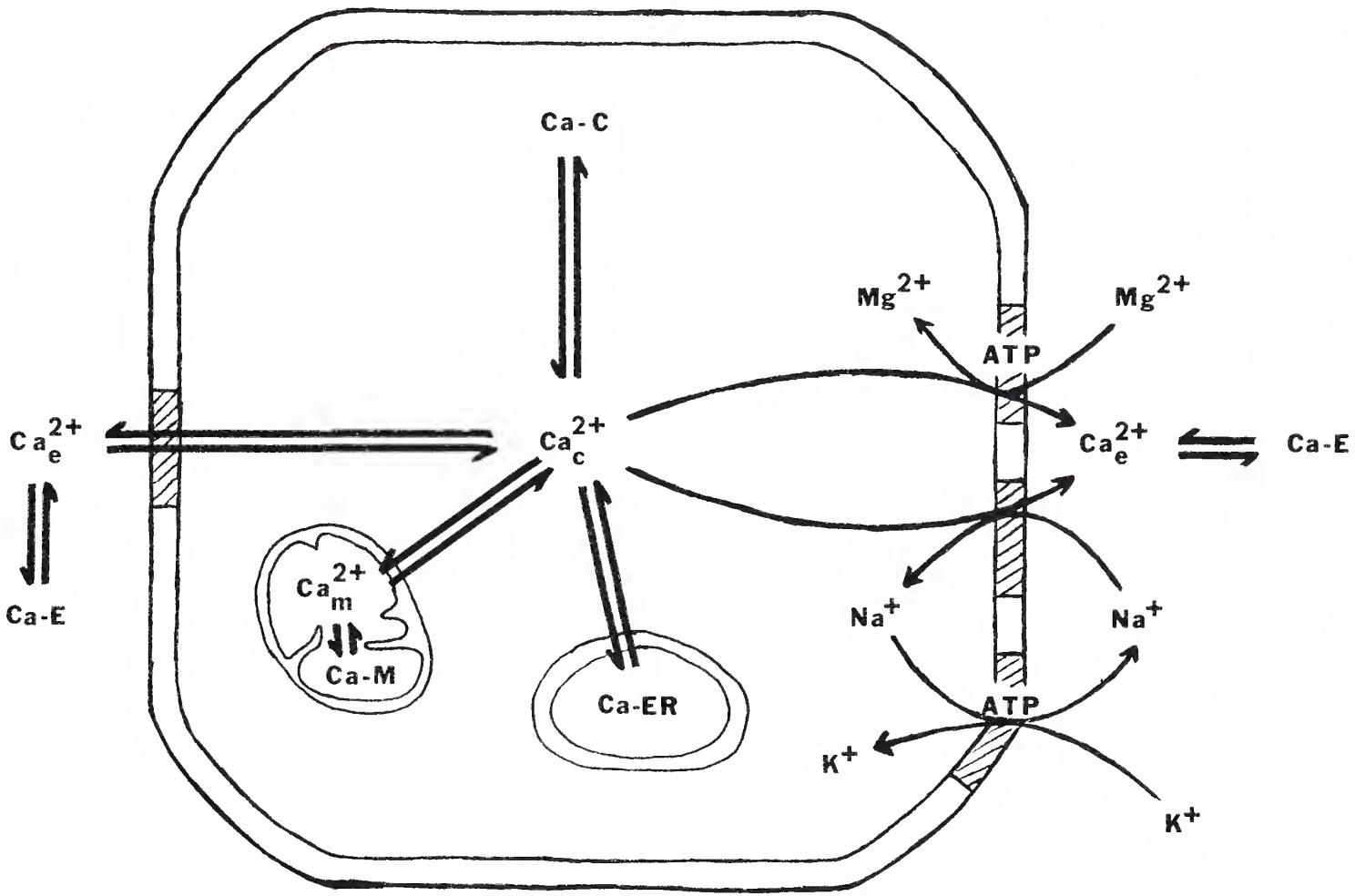
While the role of extracellular Ca^{2+} in biological systems can be difficult to determine (71), these observations suggest that there is a pool of intracellular Ca^{2+} important to the process of pyrogenic stimulation and/or EP production which is somehow protected from rapid equilibration with extracellular Ca^{2+}. It may be analagous to the intracellular pool thought to play a role in pancreatic acinar cell secretion of enzymes (72). This pool may be bound to the plasma membrane, or may be sequestered in another intracellular site, such as the mitochondria or endoplasmic reticulum. Figure 7 is a simplified schematic diagram of calcium compartments in the macrophage. Macrophages have a membrane-bound Mg^{2+} and ATP dependent Ca^{2+} pump which transports free Ca^{2+} from the cytoplasm to the extracellular surface (73), and over time the action of this pump could deplete the intracellular stores of Ca^{2+} if reentry is prevented by formation of EGTA-Ca complexes extracellularly. Borle has shown that mitochondria lose

Ca^{2+} slowly with a half-time of 152 minutes (74), while Mela and Chance have shown that mitochondrial Ca^{2+} uptake has a half-time of 8 seconds (75). These observations are consistent with our results showing that a lengthy preincubation with EGTA is required to deplete the necessary Ca^{2+} pool, but that the pool is replenished rapidly as evident by restoration of the response when Ca^{2+} is added together with the pyrogenic stimulus.

In some systems where calcium is thought to be important in stimulus-secretion coupling, Ca^{2+} ionophores have been used to elicit a response in the place of a normal stimulus. Their action is thought to be due to increasing intracellular Ca^{2+} levels. For example, ionophore A23187 has been shown to stimulate the release of histamine from mast cells (76) and the release of catecholamines from adrenal medulla (77). A23187 has also been reported to stimulate the release of platelet activating factor from rabbit, rat, and human alveolar macrophages (78). We found that A23187, in doses that allowed influx of a radioactive Ca^{2+} tracer, was not a sufficient stimulus for EP production by rabbit monocytes. Similarly, Foreman, et al., reported that A23187 did not stimulate lysosomal enzyme secretion by macrophages (55), and MacMillan and his associates found that A23187-induced release of the lysosomal acid hydrolase B-N-acetyl-glucosaminidase from macrophages was accompanied by the release of lactate dehydrogenase, a cytosolic enzyme,

Figure 7: Calcium Metabolism in the MNPC

Schematic diagram of the calcium compartments within a MNPC (modified from 53). $Ca^{2+}e$ = free extracellular Ca^{2+}, Ca-E = bound extracellular Ca^{2+}, $Ca^{2+}c$ = free cytosolic Ca^{2+}, Ca-C = bound cytosolic Ca^{2+}, $Ca^{2+}m$ = free mitochondrial Ca^{2+}, Ca-M = bound mitochondrial Ca^{2+}, and Ca-ER = bound Ca^{2+} in the endoplasmic reticulum.


Ca-C
Ca_e^{2+}
Ca-E
Ca_c^{2+}
Ca_m^{2+}
Ca-M
Ca-ER
Mg^{2+}
Mg^{2+}
ATP
Ca_e^{2+}
Ca-E
Na^+
Na^+
ATP
K^+
K^+

signifying the loss of cell integrity (54). Our observation that EP production induced by LPS was reduced in the presence of A23187 may have been due to a similar toxic effect of the ionophore on monocytes. Alternatively, the presence of ionophore and a low Ca^{2+} extracellular medium may have caused depletion of intracellular stores of Ca^{2+} as cells were stimulated by LPS and released their intracellular pools. Hand and co-workers reported that incubation of alveolar macrophages with A23187 resulted in a rise in intracellular acid phosphatase and an increased rate of intracellular bactericidal activity, but only after 40 to 72 hours of incubation, and only in the presence of mM Mg^{2+} (56). We found that Mg^{2+}, like Ca^{2+}, was ineffective in stimulating EP production with A23187. Since detectable EP is released in the first two to three hours after stimulation of the monocyte (79), it is unlikely that prolonging incubations would yield a different result.

While we were unable to demonstrate either influx or efflux of $^{45}Ca^{2+}$ from LPS-stimulated monocytes, this does not exclude the possibility that transient rises of free cytoplasmic Ca^{2+} occur during stimulation on a time scale which is shorter than the one we observed. Changes in plasma membrane permeability allowing influx of Ca^{2+} that were matched by increased activity of the membrane-bound Ca^{2+} pump, or release of intracellular stores with rapid re-uptake would not necessarily cause changes in cell-

associated Ca^{2+} observable in our experiments. This would require a system in which intracellular Ca^{2+} was very tightly controlled. The lack of increase in cell-associated Ca^{2+} during stimulation is consistent with the observation of Wood that cells secreting EP had lower total Ca^{2+} levels than cells in which secretion had been suppressed (50).

The Role of Cyclic Nucleotides in EP Production

Adenosine 3', 5' cyclic monophosphate (cAMP) has been reported to inhibit macrophage migration (57), to decrease secretion of plasminogen activator by mouse exudate cells (58) and a macrophage-like tumor cell line (J774.2) (59), and to decrease the secretion of lysosomal enzymes by mouse exudate cells (54). cAMP has also been reported to stimulate secretion of T-cell activating factors from J774.2 cells, suggesting that it might be the second messenger involved in LPS-stimulated release of such factors from MNPC's (80), and in later experiments both cAMP and LPS were shown to induce phosphorylation of non-histone proteins by these cells (81). However, no attempts were made to exclude the possibility that the analogues of cAMP used may have been contaminated with LPS, and that all of the effects seen were LPS-induced. For example, LPS reduces plasminogen activator secretion by mouse peritoneal exudate macrophages (82).

We found that db-cAMP, which is more resistant to phosphodiesterase degradation and crosses cell membranes more readily than cAMP, did not stimulate EP production in the presence of polymyxin B (PMB), an antibiotic which blocks the pyrogenicity of LPS in vitro (64). Further, epinephrine and prostaglandin E_1, in doses greater than those needed to stimulate adenylate cyclase maximally in macrophage homogenates (60) or in intact cells (54, 83), failed to stimulate EP production or to alter EP production in response to a standard dose of LPS. Staphylococcal enterotoxin B, another agent thought to activate adenylate cyclase, did not generate EP in the presence of PMB, confirming earlier observations that it did not stimulate EP production in vitro (84).

Duff performed a parallel set of experiments in our laboratory to investigate the role of guanosine 3', 5' cyclic monophosphate (cGMP) in EP production. His results, summarized in Table 10, show that incubation of cells with phorbol myristic acid (PMA), an agent which stimulates endogenous cGMP production, in a concentration of 100 ng/ml for 24 or 48 hours did not cause EP production (85). This concentration of PMA was active, however, in stimulating the metabolic burst in neutrophils and monocytes. Others have found that PMA does not activate macrophages unless LPS is present in the system (86).

TABLE 10

Effects of cGMP on EP Production

Mean fevers produced by supernatants from 2 x 10^6 monocytes incubated with 1 mM b-cGMP, 1 mM IBMX, or 100 ng/ml PMA with or without 500 units/ml PMB (from 85).

Potential Activator(s)	Fevers (S.E) (°C)
PMA (24 hour incubation)	0.13 (0.03)
PMA (48 hour incubation)	0.13 (0.01)
b-cGMP	1.17 (0.12)
b-cGMP + PMB	0.18 (0.06)
IBMX	0.40 (0.08)
IBMX + PMB	0.07 (0.02)
IBMX + PMB + b-cGMP	0.08 (0.04)

Further, Duff found that 1 mM 8-bromo-guanosine 3', 5' cyclic monophosphate (b-cGMP) stimulated production of EP by cells, but this was suppressed by addition of 200 units/ml PMB during incubation. Similar results were obtained with 1 mM IBMX, a phosphodiesterase inhibitor, with and without the addition of b-cGMP (Table 10). Since the b-cGMP and IBMX used were contaminated with LPS, it was not possible to investigate their effects on EP production independently-stimulated by LPS. However, there is an earlier brief report that IBMX does not affect LPS-stimulated EP production (63).

The Second Messenger Hypothesis

With the discovery that many diverse hormones stimulated responses in target cells by the common pathway of increasing intracellular cAMP levels, Sutherland and his colleagues proposed a set of four criteria to establish the presence of such a second messenger in a given system (87). These criteria can be restated in a more general fashion to encompass other potential second messengers, such as Ca^{2+} or cGMP. First, a membrane-bound entity which responds to the first messenger (cell stimulator) by generation of the second messenger should be demonstrated. Second, the level of the second messenger within the cell should change appropriately with stimulation by the first messenger. Third, the effect of the first messenger (cell response)

should be enhanced by agents which inhibit clearance of the second messenger, if the target cell is not already maximally stimulated. Fourth, exogenous addition of the proposed second messenger should reproduce the action of the first messenger.

In the experiments reported here, we have shown that Ca^{2+}, cAMP, and cGMP cannot fulfill these criteria, and, therefore, they should not be considered second messengers for LPS-stimulated EP production by MNPC's. While there is a role for Ca^{2+} in this process, as evidenced by the reversible inhibition of EP production by prolonged chelation with EGTA, addition of extracellular Ca^{2+} or ionophore A23187 to increase the intracellular level of Ca^{2+} was not sufficient to stimulate EP production. Further, MNPC's stimulated with LPS failed to demonstrate calcium uptake, suggesting that an influx of extracellular Ca^{2+} is not important to the process. Addition of exogenous db-cAMP or agents shown to stimulate cAMP generation in MNPC's were also not sufficient to initiate EP production. Nor did they enhance or inhibit EP production from cells submaximally stimulated with LPS. Similarly, Duff's experiments show that exogenous b-cGMP and PMA, an agent which stimulates endogenous generation of c-GMP, do not stimulate EP production in the absence of LPS.

PROSPECTS FOR FUTURE WORK

Except for the poorly-defined role of Ca^{2+} in this system, the mechanism of derepression of the EP genome in MNPC's remains unclear. Our work suggests that an intracellular pool of Ca^{2+} is important to the process, but no release of intracellular stores could be demonstrated by measuring Ca^{2+} efflux with a radioactive tracer. Recent evidence indicates that Dantrolene Sodium, an agent used to treat malignant hyperthermia, may act by preventing Ca^{2+} release from the sarcoplasmic reticulum in muscle cells (88), and that it may similarly prevent release of intracellular stores from other tissues, such as adrenal medulla (89) and pancreatic islet cells (90). If Dantrolene can be shown to inhibit LPS stimulation of EP production, the intracellular release of Ca^{2+} as a step in the process would be more plausible.

It is possible that another second messenger will be revealed in future work. Intracellular Na^{+}, as suggested by Wood and his associates (48, 49), may prove to be important. There is some evidence that Na^{+} may play such a role in pancreatic acinar cell secretion (reviewed in 91). While it is possible that the effect of LPS is a direct one, not requiring the existence of a second messenger to interact with the EP genome, as suggested by the results of some

(81), the broad diversity of agents which can stimulate EP production makes this less likely.

Since this work was begun, others have shown the existence of calcium-dependent protein kinases in a variety of tissues (92). While they do not require cyclic nucleotides for activity, phospholipids and some of their breakdown products have a marked effect. While 10^{-3} M - 10^{-4} M Ca^{2+} is needed to activate the kinases in the absence of phospholipid, 10^{-6} M Ca^{2+} is sufficient in the presence of phophatidylinositol (PI), phosphatidyl serine (PS), and diacylglycerols (92, 93). Thus, under proper circumstances, these Ca^{2+}-dependent kinases can be activated with normal cytosolic concentrations of ionized Ca^{2+}. There has long been speculation that PI metabolism is an important step in cell activation, since activators of many types of cells increase rates of PI turnover in the cell membrane (94). Karnovsky and his colleagues have shown that LPS induces an increase in incorporation of inorganic phophate into monocyte membrane lipids, particularly into PI and PS (95). They have shown, further, that this process requires Ca^{2+}.

Thus, one can put these observations into a hypothesis of monocyte activation which begins with binding of LPS or another agent to the cell surface, thereby inducing a breakdown of PI in the membrane. The PI breakdown products subsequently released into the cytosol might activate a protein kinase which requires small amounts of calcium, such

as already exist in the cytosol. Thus, consistent with our results, no influx of calcium would be required, and no significant release of intracellular stores, with consequent net efflux of calcium from the cell, would be necessary either. Such an activated protein kinase could then phosphorylate a histone or other controller of DNA expression to derepress the EP genome. The inhibitory effect of corticosteroids on EP production may be exerted through effects on PI metabolism, a hypothesis which could be tested by trying to duplicate Karnovsky's results in the presence of steroids.

Given the widespread ability of most vertebrates to produce EP and the potent role it plays as a controller of immune function, further work to define the mechanism of EP production should be undertaken.

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Given the widespread ability of most vertebrates to produce EP and the potent role it plays as a controller of immune function, further work to define the mechanism of EP production should be undertaken.

GUIDE TO ABBREVIATIONS

b-cGMP	8-bromo-guanosine 3', 5' cyclic monophosphate
Ca^{2+}	calcium
cAMP	adenosine 3', 5' cyclic monophosphate
cGMP	guanosine 3', 5' cyclic monophosphate
db-cAMP	N^6, O^2 - dibutyryl adenosine 3', 5' cyclic monophosphate
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethyleneglycol-bis-(B-amino-ethyl ether) N, N' tetra-acetic acid
EP	endogenous pyrogen
EPI	epinephrine
HBSS	Hank's balanced salt solution
IBMX	isobutyl-methyl-xanthine
Il-1	Interleukin-1
K^+	potassium
La^{3+}	lanthanum
LAF	lymphocyte activating factor
LP	leukocytic pyrogen
LPS	endotoxin, lipopolysaccharide
LT	leukotriene
MEM	Eagle's minimum essential medium
Mg^{2+}	magnesium
MNPC	mononuclear phagocytic cell
Na^+	sodium
PE	peritoneal exudate
PG	prostaglandin
PGE_1	prostaglandin E_1
PI	phosphatidylinositol
PMA	phorbol myristic acid
PMB	polymyxin B
PMN	polymorphonuclear cell
PROP	propranolol
PS	phosphatidyl serine
SE	Staphylococcal enterotoxin B
SFH	stock Ficoll-Hypaque
SIP	stock isotonic Percoll

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