JCI The Journal of Clinical Investigation

Increased levels of cyclic adenosine-3',5'-monophosphate in human polymorphonuclear leukocytes after surface stimulation.

J E Smolen, … , H M Korchak, G Weissmann

J Clin Invest. 1980;65(5):1077-1085. https://doi.org/10.1172/JCI109760.

Research Article

Levels of cyclic AMP (cAMP) (but not cyclic GMP) in suspensions of human polymorphonuclear leukocytes (PMN) increased promptly after exposure of the cells to stimuli such as the chemotactic peptide N-formyl methionyl leucyl phenylalanine, the immune complex bovine serum albumin/anti-bovine serum albumin and calcium ionophore A23187. cAMP increased rapidly, reaching a maximum of twice the basal level 10--45 s after stimulation; after 2--5 min the amount of cAMP had subsided to basal levels. Elevations in cAMP levels were concurrent with, or followed, membrane hyperpolarization (measured by uptake of the lipophilic cation triphenylmethyl phosphonium) and always preceded lysosomal enzyme release and superoxide anion (O2) production. Elevated cAMP levels could be uncoupled from these later events by removal of extracellular divalent cations, replacement of extracellular Na+ with K+ or choline+, and by use of low concentrations of stimulus; each of these conditions virtually abolished lysosomal enzyme release and O2 generation, while leaving the stimulated elevation of cAMP levels unimpaired. Calcium ionophore A23187 did not provoke membrane hyperpolarization, thus uncoupling changes in membrane potential from changes in cAMP levels. These data suggested that cAMP is not a critical component in the earliest steps of stimulus-secretion coupling. Surface stimulation of cells pretreated with prostaglandins E1 or I2 yielded very high levels of cAMP; these high levels may be an important part of the mechanism […]

Increased Levels of Cyclic Adenosine-3′,5′-Monophosphate in Human Polymorphonuclear Leukocytes after Surface Stimulation

JAMES E. SMOLEN, HELEN M. KORCHAK, and GERALD WEISSMANN, *Department of Medicine, Division of Rheumatology, New York University School of Medicine, New York 10016*

ABSTRACT Levels of cyclic AMP (cAMP) (but not cyclic GMP) in suspensions of human polymorphonuclear leukocytes (PMN) increased promptly after exposure of the cells to stimuli such as the chemotactic peptide *N*-formyl methionyl leucyl phenylalanine, the immune complex bovine serum albumin/anti-bovine serum albumin and calcium ionophore A23187. cAMP increased rapidly, reaching a maximum of twice the basal level 10–45 s after stimulation; after 2–5 min the amount of cAMP had subsided to basal levels. Elevations in cAMP levels were concurrent with, or followed, membrane hyperpolarization (measured by uptake of the lipophilic cation triphenylmethyl phosphonium) and always preceded lysosomal enzyme release and superoxide anion (O_2^-) production. Elevated cAMP levels could be uncoupled from these later events by removal of extracellular divalent cations, replacement of extracellular Na^+ with K^+ or choline$^+$, and by use of low concentrations of stimulus; each of these conditions virtually abolished lysosomal enzyme release and O_2^- generation, while leaving the stimulated elevation of cAMP levels unimpaired. Calcium ionophore A23187 did not provoke membrane hyperpolarization, thus uncoupling changes in membrane potential from changes in cAMP levels. These data suggested that cAMP is not a critical component in the earliest steps of stimulus-secretion coupling. Surface stimulation of cells pretreated with prostaglandins E_1 or I_2 yielded very high levels of cAMP; these high levels may be an important part of the mechanism by which stable prostaglandins inhibit lysosomal enzyme release and O_2^- generation.

Dr. Smolen and Dr. Korchak are Arthritis Foundation Fellows.

Received for publication 23 July 1979 and in revised form 26 December 1979.

INTRODUCTION

Surface stimulation of human polymorphonuclear leukocytes (PMN)[1] provokes a variety of responses, of which lysosomal enzyme release and superoxide anion radical (O_2^-) generation are particularly pertinent to their biological function. To elucidate the biochemical and physiological pathways responsible for the transmission of stimulatory signals from receptors on the plasma membrane and the translation of these signals into cellular responses, we have employed methods which allow a detailed examination of several processes during the first minute of PMN stimulation. The first measurable responses of cells exposed to particulate and soluble stimuli were changes in membrane potential (1): hyperpolarization was observed within 10 s. A distinct stimulus-dependent lag period of 20–60 s elapsed before the initiation of O_2^- generation (1, 2). We have also reported that the initial kinetics of lysosomal enzyme release could be timed and showed that degranulation took place after a lag period of 20–60 s (3). These lag periods were also stimulus-dependent and were similar in duration to those seen for O_2^- generation.

A number of investigators have reported that human PMN have unchanged levels of cyclic AMP (cAMP) after exposure to phagocytic stimuli for several minutes (4–8); elevated levels of cyclic GMP have been reported by one group (9–11). In contrast to these findings, Herlin et al. (12) showed the exposure of PMN

[1] *Abbreviations used in this paper*: BSA/anti-BSA, an immune complex of bovine serum albumin and anti-bovine serum albumin; cAMP, cyclic AMP; cGMP, cyclic GMP; FMLP, *N*-formyl methionyl leucyl phenylalanine; PiCM, phosphate-buffered saline containing Ca^{++} and Mg^{++}; PMN, polymorphonuclear leukocytes.

to latex particles provoked a rapid twofold increase in cAMP levels. This increment was prompt (maximal within 15 s) and brief. The fact that basal cAMP levels were restored within 1–2 min explained why previous investigators, who had assayed the cell suspensions after 2–5 min, were unable to detect this response. Herlin et al. suggested that the brief increment in cAMP might have a regulatory role in glycogen metabolism (12, 13).

Since the reported increment in cAMP levels appeared to coincide with membrane hyperpolarization and to precede lysosomal enzyme release and O_2^- generation, we decided to investigate the possibility that cAMP was a mediator of these other events. In summary, we found that prompt, brief elevations in cAMP levels were concurrent with or followed membrane hyperpolarization, depending upon the stimulus employed. These cAMP increments always preceded the onset of lysosomal enzyme release and O_2^- generation and could be uncoupled from these later events by several methods; therefore, it seems unlikely that cAMP is an important mediator of stimulus-secretion coupling. Finally, pretreatment of PMN with prostaglandin(PG) E_1 or PGI_2 produced modest elevations of cAMP content which were greatly augmented by subsequent exposure to a surface stimulus.

METHODS

Materials. Cytochalasin B was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wis. Concanavalin A and theophylline were obtained from Sigma Chemical Co., St. Louis, Mo. and *N*-formyl methionyl leucyl phenylalanine (FMLP) was purchased from Peninsula Laboratories, Inc., San Carlos, Calif. Calcium ionophore A23187 was a gift from Eli Lilly & Co., Indianapolis, Ind. PGE_1 and PGI_2 were generous gifts from Dr. John Pike, the Upjohn Company, Kalamazoo, Mich. All other materials were reagent grade.

Preparation of cell suspensions. Heparinized (10 U/ml) venous blood was obtained from healthy adult donors. Purified preparations of PMN were isolated from this blood by means of Hypaque/Ficoll gradients (14) followed by standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes (15). This allowed studies of cell suspensions containing 98±2% PMN with few contaminating erythrocytes or platelets. The cells were suspended in a buffered salt solution consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1 mM $MgCl_2$, and 0.6 mM $CaCl_2$, pH 7.4 (PiCM).

Human mononuclear cells were also isolated using Hypaque/Ficoll gradients (14). Purified platelet suspensions were prepared by a minor modification of the method of Hamberg et al. (16).

Determination of cAMP levels. cAMP levels were determined by a modification of the method of Herlin et al. (12). In general, purified PMN were suspended in PiCM (2×10^7 cells/ml) and preincubated at 37°C for 5 min. An aliquot of this suspension (200 μl) was taken as a zero-time control sample and was placed in a test tube for extraction of cyclic nucleotides. This and all other extraction tubes contained 1 ml of phosphate-buffered saline with 0.5 mM theophylline preheated and maintained at 100°C in a vigorously boiling water bath. A stimulus was added to the remaining cells whereupon aliquots were sequentially removed and placed in preheated extraction tubes at the indicated times. The tubes were kept in the boiling water bath for 5 min. Upon removal, extract volumes were increased to 6 ml with 0.05 M sodium acetate buffer, pH 6.2. The samples were cooled in an ice-bath, sonicated for 5 s (~20 W; sonicator by Kontes Co., Vineland, N. J.) and centrifuged at 3,000 *g* for 15 min. The supernates, decanted from the pellets of denatured protein, were assayed for cAMP content by means of an acetylated radioimmunoassay (New England Nuclear, Boston, Mass.). cGMP was similarly assayed using more concentrated extracts derived from a larger number of PMN.

This procedure allowed samples to be taken reliably as soon as 5 s after stimulation and at time intervals as short as 5 s thereafter. The extraction procedure ensured that the cells were promptly denatured and that all biochemical reactions were immediately terminated. The extracts contained at least 95% of the cellular cyclic nucleotides, as determined by recovery of tritiated standards. Artifactual increases in apparent cAMP levels, which can be obtained by boiling adenylate cyclase reaction mixtures (17), were not found with this system, as determined by boiling cell extracts for time periods ranging from 15 s to 10 min.

Kinetics of lysosomal enzyme release. Details concerning the measurement of the initial kinetics of lysosomal enzyme release will be published elsewhere. In brief, we used a modified flow dialysis system, which permitted semicontinuous monitoring of enzyme secretion. The apparatus consisted of a flow dialysis cell, the upper and lower chambers separated by a Millipore filter with a 1-μm pore size (Millipore Corp., Bedford, Mass.). The lower chamber, fed by a reservoir, was continuously eluted, with the eluent directed to a fraction collector. The upper chamber of the dialysis cell contained a suspension of cytochalasin B-treated PMN; the hydrostatic pressure of the eluting system was adjusted such that small amounts of extracellular medium continuously traversed the filter into the lower chamber. Thus, the extracellular medium was continuously sampled as eluent was directed to the fraction collector. The 6-s stepping frequency of the collector provided a nominal time resolution of the same magnitude.

The PMN and the entire apparatus were preincubated at 37°C, after which a bolus containing a concentrated stimulus and [^{14}C]inulin was added. The inulin served as the extracellular space marker; it immediately began to cross the filter and thus served to mark the moment of stimulation and to calibrate the entire apparatus. The appearance of [^{14}C]inulin in collected fractions preceded the appearance of lysosomal enzymes by 15 s or more. Linear extrapolation of the rising portions of the curves to base line permitted an easy, reliable means of determining the lag period of lysosomal enzyme release relative to stimulation ([^{14}C]inulin elution). The linear extrapolation technique effectively averaged together six to eight data points, providing a practical resolution of 1–2 s (using the same lot of cells). The lag periods were not artifacts due to selective retention of lysosomal enzymes within the apparatus, since no lag period was seen for PMN that were introduced into the upper chamber after stimulation.

All [^{14}C]inulin recovery curves (used to mark the moment of stimulus addition and thus to calibrate the entire apparatus) are omitted for the sake of clarity; the zero-time point for all figures depicting lysosomal enzyme release corresponds to that time at which [^{14}C]inulin was first eluted.

Miscellaneous procedures. The membrane potential of PMN was determined by uptake of the lipophilic cation triphenylmethyl phosphonium bromide (1) and continuous measurement of superoxide anion production was performed as previously described (1).

RESULTS

Changes in cyclic nucleotides after PMN stimulation. Although some investigators have reported that cAMP levels of PMN do not change several minutes after surface stimulation (4–8), more recent work has indicated that significant changes do take place at short time intervals (12). Consequently, we determined whether such transient changes correlated with other early cellular responses to surface stimuli. These responses included membrane hyperpolarization, lysosomal enzyme release, and superoxide anion (O_2^-) generation.

Purified resting PMN had cAMP levels of 6.7±1.8 pmol/10^7 cells (n = 44) when measured by an acetylated radioimmunoassay. cGMP levels were found to be 0.32 ±0.12 pmol/10^7 cells (n = 16). Boiling of cell suspensions was used to ensure prompt termination of biochemical processes; this boiling did not produce any artifactual increases in cAMP levels (17). The extraction procedure was efficient, since recoveries of trace quantities of tritiated cyclic nucleotides averaged 100 ±11% (n = 11) for cAMP and 98±8% (n = 7) for cGMP.

When suspensions of PMN were exposed to the chemotactic peptide FMLP (0.1 μM), a very rapid increase in cAMP levels was observed (Fig. 1). Significant increments in cAMP were observed at time intervals as short as 5 s. The increase in cAMP peaked at up to twice the basal level in 10–15 s; the average maximum

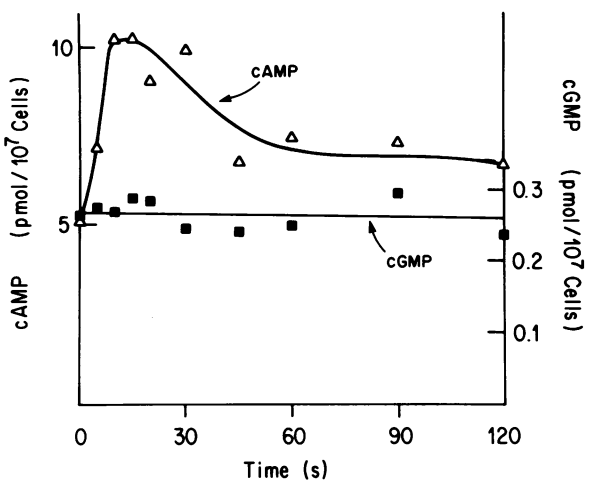

FIGURE 1 Effect of FMLP on cyclic nucleotides in suspensions of PMN. Aliquots of a suspension of purified PMN were taken at the indicated times after exposure to FMLP (100 nM). These samples were immediately extracted by heating at 100°C and then assayed for cAMP contents as detailed in Methods. The zero-time sample was extracted before addition of the stimulus; an appropriate amount of FMLP was added to it during the extraction process. cGMP was determined in an identical fashion. The results shown are from a single typical experiment. The average peak increment in cAMP content was 78±38% (SD) above basal levels (n = 17).

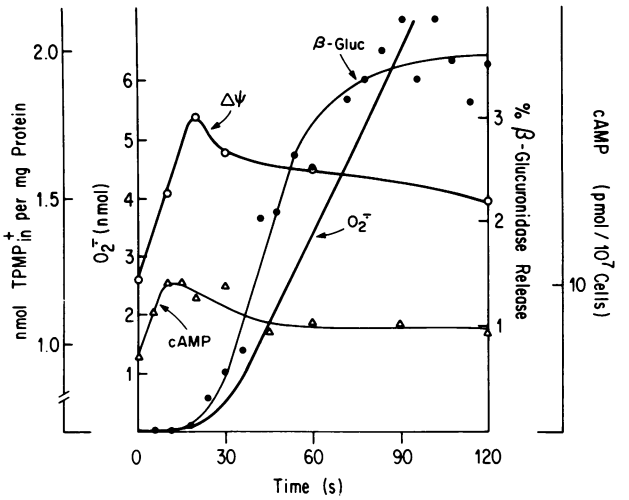

FIGURE 2 Effect of FMLP on cAMP, transmembrane potential, lysosomal enzyme release, and O_2^- generation in PMN. cAMP was assayed as described in the legend to Fig. 1 and Methods. Membrane potential ($\Delta\psi$) was measured by the intracellular content of [^{3}H]TPMP (1). Superoxide anion (O_2^-) was measured by the continuous assay of ferricytochrome c reduction (1, 2). The semicontinuous assay of β-glucuronidase secretion (β-gluc) was performed using the modified flow dialysis technique outlined in Methods (3). All four responses were measured at the indicated times after exposure of the PMN to FMLP (100 nM). The results shown are from single typical experiments.

increase, measured at 10 or 15 s, was 78±38% (SD) (n = 17) above the basal levels. cAMP content declined rapidly thereafter and approached base line at 1–2 min, a process which was complete by 5 min (not shown). The cAMP levels of unstimulated cells did not change during the course of the experiment. Cytochalasin B (5 μg/ml) did not affect the time-course or the peak level observed (102±23% (n = 4) above the basal level). No changes in cGMP levels were observed during this time period with FMLP or with any other stimulus.

Relationship between cyclic nucleotide changes and other PMN responses. The data from Fig. 1 were plotted along with other early responses of PMN to place them all within a common temporal framework (Fig. 2). Stimulation with FMLP provoked a rapid increase in membrane potential ($\Delta\psi$), as measured by enhanced cellular uptake of the lipophilic tritiated triphenylmethyl phosphonium cation. Initiation of hyperpolarization, which was virtually immediate for all stimuli, was concurrent with the rapid increase in cAMP levels. However, both of these prompt responses clearly preceded the onset of O_2^- generation, which had a lag period of ~20 s when monitored continuously (1–3). Release of β-glucuronidase, an enzyme found predominantly in azurophil granules, was assayed by means of a semicontinuous flow dialysis system described previously (3). Like O_2^- generation, release of β-glucuronidase also had a lag period of ~20 s. Thus, the increases in cAMP levels

clearly preceded lysosomal enzyme release and O_2^- generation, but were concurrent with membrane hyperpolarization.

When an immune complex consisting of bovine serum albumin and anti-bovine serum albumin (BSA/anti-BSA) was used as the stimulus, membrane hyperpolarization again appeared immediately (Fig. 3). Both lysosomal enzyme release and O_2^- generation were initiated after distinct lag periods of ~40 s. Unlike the case with FMLP, the increment in cAMP level induced by an optimum secretory concentration of BSA/anti-BSA (150 μg/ml) had a lag period. The length of this lag period was variable but it was always distinct. For the case shown in Fig. 3, the lag period for the increase in cAMP relative to membrane hyperpolarization (LcAMP) was 35 s. Thus, since hyperpolarization preceded elevation of cAMP content, changes in cAMP levels are unlikely to be the cause of changes in membrane potential for this stimulus.

The calcium ionophore A23187 (10 μM, the optimum secretory concentration) did not provoke rapid changes in membrane potential (Fig. 4), perhaps because induced calcium fluxes bypassed the need for the usual cell surface receptor-ligand interactions which indirectly lead to hyperpolarization. Nonetheless, lysosomal enzyme release and O_2^- generation were both elicited after relatively long lag periods. Increments in cAMP

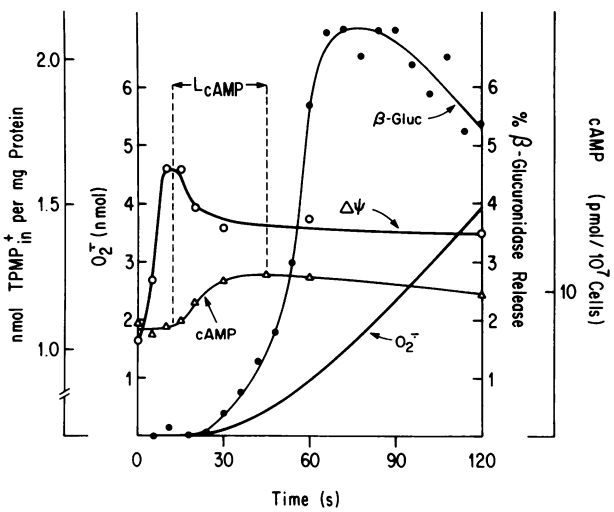

FIGURE 3 Effect of BSA/anti-BSA on cAMP, transmembrane potential, lysosomal enzyme release, and O_2^- generation in human PMN. cAMP, membrane potential, O_2^- generation, and β-glucuronidase (β-gluc) release were measured as described in the legends to Figs. 1 and 2 and in Methods. All four responses were measured at the indicated times following exposure of PMN to BSA/anti-BSA immune complex (150 μg/ml) (18). The results shown are from a single typical experiment. The average peak increment in cAMP induced by BSA/anti-BSA was 47±4% (SD) above basal levels (n = 3). LcAMP, lag period for increase in cAMP relative to membrane hyperpolarization.

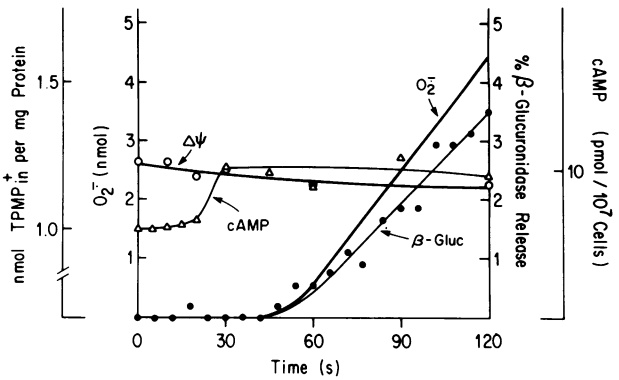

FIGURE 4 Effect of A23187 on cAMP, transmembrane potential, lysosomal enzyme release, and O_2^- generation in human PMN. cAMP, membrane potential, O_2^- generation, and β-glucuronidase (β-gluc) secretion were measured as described in the legends to Figs. 1 and 2 and Methods. All four responses were measured at the indicated times following exposure of PMN to calcium ionophore A23187 (10 μM). The results shown are from a single typical experiment. The average peak increment in cAMP induced by A23187 was 67±18% (SD) above basal levels (n = 3).

were also observed, again with relatively long lag periods.

Effects of mononuclear cells and platelets. Elevations of cAMP levels were not due to the presence of contaminating mononuclear cells. Isolated mononuclear cells, which contain substantial amounts of cAMP and accumulate more cyclic nucleotide in response to some stimuli (4), were exposed to FMLP, BSA/anti-BSA, and A23187. A quantity of these mononuclear cells equivalent to twice the normal level of contamination was stimulated and extracted along with the usual number of unstimulated PMN. No systematic increases in cAMP were observed (Fig. 5) when mononuclear cells were exposed to these stimuli. PMN, which were obtained from the same blood sample from which the mononuclear cells were isolated, responded fully to FMLP (upper line). Similar experiments indicated that platelets at concentrations up to twofold the normal level of contamination could not account for the observed increases in cAMP (data not shown).

All other stimuli tested were capable of provoking increments of cAMP (but not cGMP) together with lysosomal enzyme release and O_2^- generation. Zymosan-activated serum (10%) induced a large immediate increase in cAMP levels, which was virtually identical to that seen for FMLP (data not shown). The lectin concanavalin A produced a weak response with a lag period comparable to that of A23187. Opsonized zymosan particles produced the smallest increments in cAMP content (only 20±14% above basal levels, n = 3); lag periods were also observed with this stimulus.

Are elevations of cAMP required for neutrophil activation? These results allowed us to conclude that

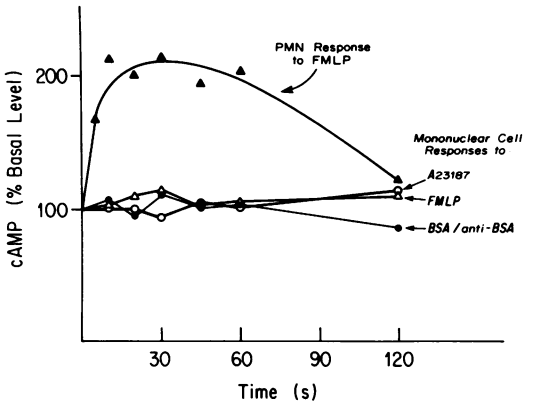

FIGURE 5 Effect of stimuli on cAMP levels of contaminating mononuclear cells. A purified PMN suspension containing 3% mononuclear cells as normal contaminants was exposed to FMLP and cAMP was generated as indicated by "PMN response to FMLP." A number of purified mononuclear cells corresponding to 6% contamination was similarly exposed to FMLP (100 nM), BSA/anti-BSA (150 μg/ml), and A23187 (10 μM). These mononuclear cells were extracted along with the usual number of unstimulated PMN (to assure similar extraction conditions) and assayed for cAMP. Results are expressed as percentages of basal levels for each experiment; this was done for the sake of clarity since basal levels varied slightly due to the addition of mononuclear cells. The results shown are from a single typical experiment.

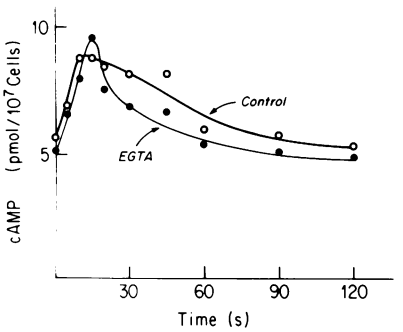

FIGURE 6 Effect of EGTA on cAMP response to FMLP. A sample of PMN was prepared and divided into two lots. The first lot was washed in phosphate-buffered saline (without divalent cations); EGTA was then added to a concentration of 1 mM. The other lot was washed and otherwise prepared normally in PiCM (control). The EGTA-treated and control cells were exposed to FMLP (100 nM) and cAMP was determined as described in the legend to Fig. 1 and Methods. The results shown are from a single typical experiment. The average peak increment in cAMP induced by FMLP in the presence of EGTA was 59±28% (SD) above the basal level (n = 3).

the observed increases in cAMP levels were concurrent with, or followed, membrane hyperpolarization and were thus unlikely to be the cause of this earlier event. While the possibility remained that the early membrane hyperpolarization could be the cause of cAMP changes, the results of experiments in which A23187 was the stimulus (Fig. 4) indicated that changes in membrane potential were not necessary for any of the later responses.

The following experiments showed that the stimulus-dependent increments in cAMP levels could be similarly uncoupled from the two subsequent responses. The presence of EGTA effectively blocks lysosomal enzyme release and O_2^- production. Yet the removal of divalent cations did not affect the hyperpolarization response (1), nor did it affect the stimulated increase in cAMP levels (Fig. 6); the time course and peak amplitudes of the responses were similar in the presence or absence of EGTA. Replacement of Na^+ in the medium with either K^+ or choline$^+$ inhibited lysosomal enzyme release, O_2^- production, and membrane hyperpolarization,[2] but did not substantially affect the increment in cAMP (Fig. 7). Finally, by use of appropriate concentrations of FMLP, it was possible to obtain a large increase in cAMP levels without attendant lysosomal enzyme release or O_2^- generation (Fig. 8). Approximately 100 nM FMLP was required to provoke optimal enzyme release or O_2^- production; both of these later responses were almost undetectable when 1 nM FMLP was employed. However, a large increment of cAMP (observed 10 s after stimulation) was induced by 1–100 nM FMLP; at 1 nM, the increment of cAMP was normal, while virtually no lysosomal enzyme release or O_2^- generation was found. A substantial increase in cAMP level was obtained using as little as 0.1 nM FMLP.

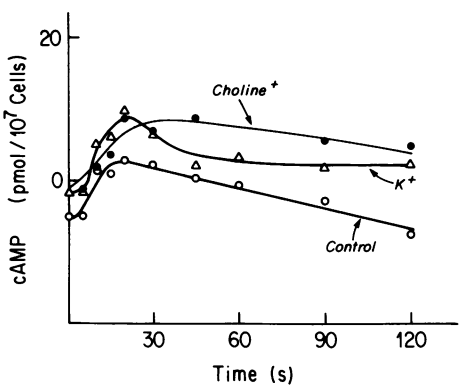

FIGURE 7 Effect of Na^+-free media on cAMP response to FMLP. PMN designated K^+ were prepared and washed in 155 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM Hepes-HCl, pH 7.4. Cells designated "choline" were washed in an identical medium except that choline chloride replaced KCl. Both lots of cells were exposed to FMLP (100 nM)) and cAMP was determined as outlined in the legend to Fig. 1 and Methods. The results shown are from a single typical experiment. The average peak increment in cAMP induced by FMLP was 52±23% (SD) in the presence of choline (n = 3) and 48±16% in the presence of K^+ (n = 3).

[2] Korchak, H. M., and G. Weissmann. Stimulus-response coupling in human neutrophil. Membrane potential changes and the role of extracellular Na^+. Submitted for publication.

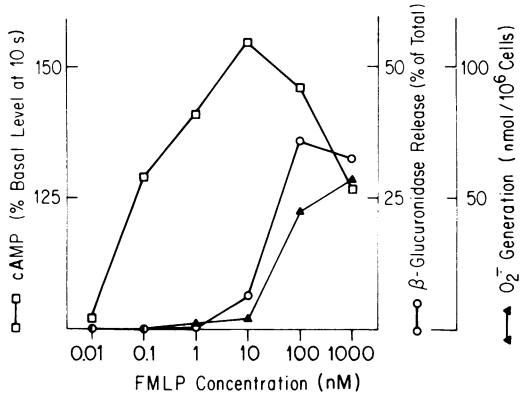

FIGURE 8 Effect of FMLP concentration on increments in cAMP, lysosomal enzyme release, and superoxide anion generation. PMN were exposed to the indicated concentrations of FMLP. The amount of cAMP generated at 10 s was measured and is expressed as a percentage of the basal (zero-time) level. β-Glucuronidase release and O_2^- generation were measured 5 min after stimulation using standard techniques (19). The results shown are from a single typical experiment. The average peak increments in cAMP (above basal levels) induced by FMLP were 78±38% (SD) (n = 17) at 100 nM, 72±40% (n = 3) at 10 nM, and 63±37% (n = 4) at 1 nM. Uncertainties in O_2^- generation and lysosomal enzyme release are typically ±5%.

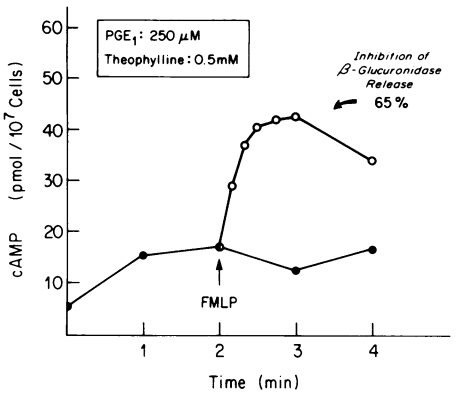

FIGURE 9 Effect of PGE_1 and FMLP on cAMP levels and lysosomal enzyme release in PMN. Purified PMN were preincubated in PiCM containing 0.5 mM theophylline for 5 min at 37°C. At time zero PGE_1 (250 μM) was added and samples were taken every minute for four min (lower curve). After two min, a sample of these cells was removed and stimulated with FMLP (100 nM) as shown by the upper curve. Lysosomal enzyme release induced by FMLP (in the presence of theophylline), determined as described (19), was inhibited by pretreatment with PGE_1 (as indicated). The results shown are from a single typical experiment. The average peak increments induced by FMLP above the basal level established by PGE_1 + theophylline is 187±45% (SD) (n = 7).

Thus, a cAMP increment at 10 s is not a sufficient prerequisite for subsequent lysosomal enzyme release and O_2^- generation.

Exposure of PMN to PGE_1 or PGI_2 occasionally produced both modest increases in cellular cAMP levels and inhibition of lysosomal enzyme release. This day-to-day and subject-to-subject variability was rectified by routine preincubation of the cells with theophylline for 5 min at 37°C. Theophylline itself (0.1–2 mM) had little or no effect upon the customary increment in cAMP in response to FMLP; average peak levels were 109±71% (SD) above basal levels (n = 3). Fig. 9 shows that when PMN pretreated with 0.5 mM theophylline were exposed to PGE_1, cAMP levels increased two- to threefold over the span of 4 min. If PGE_1-treated cells were subsequently stimulated with FMLP, a large increment in cAMP was observed. This response was prompt and peaked at a level of 8–10-fold the normal basal quantity. The PGE_1-treated PMN also released 65% less β-glucuronidase and lysozyme in response to FMLP (in the presence of theophylline) than did those cells which were not exposed to the PG. As shown in Fig. 10, PGI_2 induced a modest increase in cAMP levels in theophylline-treated cells (higher concentrations of theophylline were necessary to obtain inhibition of enzyme secretion by this prostaglandin); once again, stimulation with FMLP produced a large additional increment in cAMP and inhibition of lysosomal enzyme release was observed. Decreasing the amount of PGE_1 from 250 to 100 μM reduced the amount of inhibition of lysosomal enzyme release without any similar reduction of either base-line or peak FMLP-induced cAMP levels (data not shown); this indicates that inhibition of degranulation is not strictly determined by the level of cAMP induced by the PG or by the peak level provoked by subsequent stimulation.

DISCUSSION

Increases in the cAMP levels of human PMN provoked by surface stimulation have not been regularly observed previously. Table I shows a list of selected references, the first five of which reported no changes in cAMP. This is easily attributable to the fact that these investigators measured cyclic nucleotide levels 2 min or more after stimulation, by which time the increments in cAMP had already subsided (12; this work). The increment observed by Manganiello et al. (4) was attributed to contaminating mononuclear cells; the small number of these cells present in purified PMN preparations were not responsible for the increments reported in this study (Fig. 5). We did not observe the increases in cGMP which Ignarro and his co-workers have reported (9–11, 20).

We found that prompt increments in cAMP levels were provoked by each stimulus employed. These increments are unlikely to be associated with the microtubule assembly which generally accompanies stimulation, since microtubule disassembly has been suggested as mechanism for elevating cAMP levels (21); however,

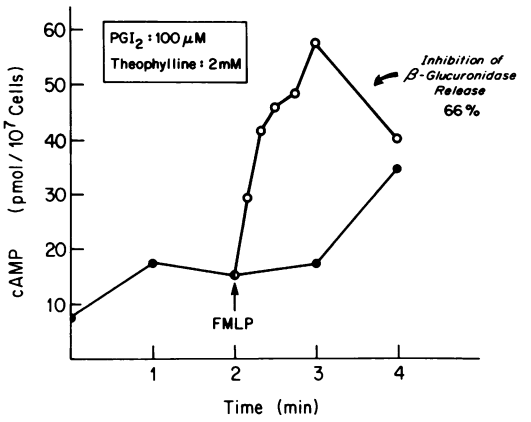

FIGURE 10 Effect of PGI_2 and FMLP on cAMP levels and lysosomal enzyme release in PMN. Purified PMN were preincubated with 2 mM theophylline, then exposed to 100 μM PGI_2 (dissolved in PiCM 30 s before addition) and FMLP (100 nM). See legend to Fig. 9 for further details. The results shown are from a single typical experiment. The average peak increments induced by FMLP above the basal level established by PGI_2 + theophylline is 195±91 (n = 3).

it is possible that transient disassembly is responsible for the brief increment in cAMP.

The increments in cAMP were concurrent with or followed membrane hyperpolarization and preceded lysosomal enzyme release and O_2^- generation. This temporal sequence suggested that cAMP might be a secondary messenger responsible for transmission of the stimulatory signal to the mechanisms responsible for the later events. However, several experiments suggested that this may not be the case: lysosomal enzyme release and O_2^- generation could be virtually abolished whereas the prompt increments in cAMP were unchanged (*a*) in the absence of the divalent cations (Fig. 6), (*b*) in the absence of extracellular Na^+ (Fig. 7), and (*c*) when low concentrations of FMLP (1 nM) were used (Fig. 8). These data showed that an increment in cAMP is not a sufficient condition for secretion and O_2^- generation. That elevation of cAMP content is not necessary for these events is suggested (but not proven) by the fact that serum-treated zymosan produces little or no change in cyclic nucleotide levels. The finding that the absence of Ca^{++} and Na^+ have little or no effect upon the stimulated increment in cAMP is also noteworthy, in view of the important roles these cations play in stimulation of PMN. Degranulation of rabbit PMN is impaired by the absence of both Na^+ (22) and Ca^{++} (23), and enhanced influxes of both cations take place after stimulation (24).

Increments in cAMP and the hyperpolarization response were clearly uncoupled when A23187 was used as the stimulus (Fig. 4). This suggests that some process other than membrane hyperpolarization, such as an increase in intracellular Ca^{++} (achieved either by stimulated Ca^{++} influx or by mobilization of intracellular Ca^{++} stores), is responsible for elevated cAMP contents.

It is well known that exogenous cAMP or agents that elevate intracellular levels of cAMP inhibit lysosomal enzyme release (25). Indeed, it seems paradoxical that stimulated cells have elevated cAMP levels at just the moment when lysosomal enzyme release and O_2^- generation is initiated. We would suggest that a mere doubling of cAMP levels is innocuous insofar as these later events are concerned. A "threshold" somewhere above this doubled level of cAMP would have to be reached before secretion is inhibited. The increment of cAMP could be either an accidental or vestigial response to surface stimulation or could have some other metabolic significance (13). Consistent with this explanation, it should be noted that the elevation in cAMP seen in PG-treated cells (Figs. 9 and 10), though often considered the mechanism by which these agents inhibit secretion, is not much greater than that obtained with stimuli such as FMLP alone. It is far more likely that the large increments of cAMP obtained when PG-treated cells are subsequently exposed to stimulation (Figs. 9

TABLE I
Effect of Surface Stimulation on cAMP Levels of Human PMN: Reported Values

Reference	Stimulus	Basal	After stimulation	Period of stimulation
		pmol/10⁷ cells		
Ignarro and George (10)	Zymosan	0.7–1	0.7–1	2 min
Manganiello et al. (4)	Latex	3–5*	12–27*	5 min
Stolc (5)	Latex	10	13	15 min
Ignarro et al. (8)	Zymosan	~1.0	~1.3	15 min
Zurier et al. (22)	Zymosan	5	7	20 min
Herlin et al. (12)	Latex	7	14	15 s
This work	FMLP, BSA/anti-BSA, A23187	7	14	15 s

* Values reported as pmol/mg cell protein.

and 10), a phenomenon also reported by Zurier et al. (25), are responsible (either directly or indirectly) for inhibition of granule discharge and O_2^- generation. Thus, cells pretreated with PG are "primed," such that stimuli evoke large amounts of cAMP and ironically contribute to inhibition of secretion.

One possible explanation for this phenomenon is that the decrease in membrane microviscosity which accompanies surface stimulation (26) permits a closer coupling of PG receptors and adenyl cyclase, in a manner similar to that suggested by Hirata et al. (27); stimulation of PG-treated PMN would thus lead to greatly enhanced production of cAMP. While this is an explanation consistent with our data, it should be remembered that we do not know what effect a doubling of cAMP levels per se has on secretion. We do not know if the intracellular/extracellular distribution of cAMP is identical with PGE_1 and FMLP stimulated PMN. Furthermore, the fact that lower levels of prostaglandins produce correspondingly less inhibition of lysosomal enzyme release, while giving rise to identical increments in cAMP levels, indicates that these agents have some inhibitory effects other than the mere elevation of cAMP. Investigations into the mechanisms and significance of this phenomenon are continuing.

ACKNOWLEDMENTS

This work was aided by grants (AM-11949, GM-23211, HL-19072, and HL-19721) from the National Institutes of Health, The National Foundation-March of Dimes, The National Science Foundation (76-05621), and the Arthritis Foundation.

REFERENCES

1. Korchak, H. M., and G. Weissmann, 1978. Changes in membrane potential of human granulocytes antecede the metabolic responses to surface stimulation. *Proc. Natl. Acad. Sci. U. S. A.* **75:** 3818–3822.
2. Cohen, H. J., and M. E. Chovaniec. 1978. Superoxide generation by digitonin-stimulated guinea pig granulocytes. A basis for a continuous assay for monitoring superoxide production and for the study of the activation of the generating systems. *J. Clin. Invest.* **61:** 1081–1087.
3. Smolen, J. E., H. M. Korchak, and G. Weissmann. 1978. The onset of lysosomal enzyme release and superoxide production in cytochalasin B-treated human granulocytes. *J. Cell. Biol.* **79:** 208a.
4. Manganiello, V., W. H. Evans, T. P. Stossel, R. J. Mason, and M. Vaughan. 1971. The effect of polystyrene beads on cyclic 3′,5′-adenosine monophosphate concentration in leukocytes. *J. Clin. Invest.* **50:** 2741–2744.
5. Stolc, V. 1971. Regulation of iodine metabolism in human leukocytes by adenosine-3′,5′-monophosphate. *Biochem. Biophys. Acta.* **264:** 285–288.
6. Schell-Frederick, E., and J. Van Sande. 1974. Evidence that cyclic AMP is not the chemical mediator of the metabolic changes accompanying phagocytosis. *J. Reticuloendothel. Soc.* **15:** 139–148.
7. Stolc, V. 1974. Restrained adenyl cyclase in human neutrophils: stimulation of cyclic adenosine-3′,5′-monophosphate formation and adenyl cyclase activity by phagocytosis and prostaglandins. *Blood.* **43:** 743–748.
8. Ignarro, L. J., R. J. Paddock, and W. J. George. 1974. Hormonal control of neutrophil lysosomal enzyme release: effect of epinephrine on adenosine-3′,5′-monophosphate. *Science (Wash. D. C.).* **183:** 855–857.
9. Ignarro, L. J., T. F. Lint, and W. J. George. 1974. Hormonal control of lysosomal enzyme release from human neutrophils. Effects of autonomic agents on enzyme release phagocytosis, and cyclic nucleotide levels. *J. Exp. Med.* **139:** 1395–1414.
10. Ignarro, L. J., and W. J. George. 1974. Hormonal control of lysosomal enzyme release from human neutrophils: elevation of cyclic nucleotide levels by autonomic neurohormones. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 2027–2031.
11. Ignarro, L. J., and W. J. George. 1974. Mediation of immunologic discharge of lysosomal enzymes from human neutrophils by guanosine-3′,5′-monophosphate. Requirement of calcium, and inhibition by adenosine-3′,5′-monophosphate. *J. Exp. Med.* **140:** 225–238.
12. Herlin, T., C. S. Petersen, and V. Esmann. 1978. The role of calcium and cyclic adenosine 3′,5′-monophosphate in the regulation of glycogen metabolism in phagocytizing human polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* **542:** 63–76.
13. Petersen, C. S., T. Herlin, and V. Esmann. 1978. Effects of catecholamines and glucagon on glycogen metabolism in human polymorphonuclear leukocytes. *Biochim. Biophys. Acta.* **542:** 77–87.
14. Boyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by combining centrifugation and centrifugation at Ig. *Scand. J. Clin. Lab. Invest.* **21:** 77.
15. Zurier, R. B., S. Hoffstein, and G. Weissmann. 1973. mechanism of lysosomal enzyme release from human leukocytes. I. Effect of cyclic nucleotides and colchicine. *J. Cell Biol.* **58:** 27–41.
16. Hambert, M., J. Svensson, T. Wakabayashi, and B. Samuelsson. 1974. Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 345–349.
17. Larner, E. H., and C. Rutherford. 1978. An artifact produced by boiling adenyl cyclase reaction mixtures prior to the cyclic AMP-radioimmunoassay. *Anal. Biochem.* **91:** 684–690.
18. Ward, P. A., and N. J. Zvaifler. 1973. Quantitative phagocytosis by neutrophils. I. A new method with immune complexes. *J. Immunol.* **III:** 1771–1776.
19. Goldstein, I. M., S. Lind, S. Hoffstein, and G. Weissmann. 1977. Influence of local anesthetics upon human polymorphonuclear leukocyte function *in vitro*. Reduction of lysosomal enzyme release and superoxide anion production. *J. Exp. Med.* **146:** 483–494.
20. Smith, R. J., and L. J. Ignarro. 1975. Bioregulation of lysosomal enzyme secretion from human neutrophils: roles of guanosine 3′,5′-monophosphate and calcium in stimulus-secretion coupling. *Proc. Natl. Acad. Sci. U. S. A.* **72:** 108–112.
21. Malawista, S. E., J. M. Oliver, and S. A. Rudolph. 1978. Microtubules and cyclic AMP in human leukocytes: On the order of things. *J. Cell Biol.* **77:** 881–886.
22. Showell, H. J., P. H. Naccache, R. I. Sha'afi, and E. L. Becker. 1977. The effects of extracellular K^+, Na^+, and Ca^{++} on lysosomal enzyme secretion from polymorphonuclear leukocytes. *J. Immunol.* **119:** 804–811.
23. Becker, E. L., and H. J. Showell. 1974. The ability of chemotactic factors to induce lysosomal enzyme release.

II. The mechanism of release. *J. Immunol.* **112:** 2055–2062.
24. Naccache, P. H., H. J. Showell, E. L. Becker, and R. I. Sha'afi. 1977. Changes in ionic movements across rabbit polymorphonuclear leukocyte membranes during lysosomal enzyme release. Possible ionic basis for lysosomal enzyme release. *J. Cell. Biol.* **75:** 635–649.
25. Zurier, R. B., G. Weissmann, S. Hoffstein, S. Kammerman, and H.-H. Tai. 1974. Mechanism of lysosomal enzyme release from human leukocytes. II. Effects of cAMP and cGMP, autonomic agonists, and agents which affect microtubule function. *J. Clin. Invest.* **53:** 297–309.
26. Berlin, R. D., and J. P. Fera. 1977. Changes in membrane microviscosity associated with phagocytosis: effects of colchicine. *Proc. Natl. Acad. Sci. U. S. A.* **74:** 1072–1076.
27. Hirata, F., W. J. Strittmatter, and J. Axelrod. 1979. β-Adrenergic receptor agonists increase phospholipid methylation, membrane fluidity, and β-adrenergic receptor-adenylate cyclase coupling. *Proc. Natl. Acad. Sci. U. S. A.* **76:** 368–372.