CYCLIC ADENOSINE MONOPHOSPHATE IN PHAGOCYTIZING GRANULOCYTES AND ALVEOLAR MACROPHAGES

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The ingestion of particles by phagocytizing cells is accompanied by marked changes in such metabolic parameters as oxygen consumption, glycolysis, glycogenolysis, H_2O_2 production, and activity of the hexose-monophosphate shunt, with the extent of these events depending on the particular cell type studied (for references see 1, 4, 6, 7). In general, these metabolic changes can be observed within a few minutes or less after the addition of particles and are considered to reflect an increased energy demand during phagocytosis. It is not clear, however, in which way the contact of particles with the outer membrane or the initial phase of phagocytosis is signaled to the interior of the cell, where these metabolic changes take place.

It is now well established that the interaction of many hormones with specific receptor sites at the outer membrane is signaled to the interior by changes in the intracellular concentration of adenosine 3′,5′-monophosphate (Ado-3′,5′-P, cyclic AMP) via stimulation of the membrane-located adenylate cyclase (13). This system is not restricted to hormone effects, e.g., lymphocytic transformation by stimulation of phytohemagglutinins (13). It was therefore tempting to speculate that cyclic AMP may have a messenger function in the process of phagocytosis. Indeed, results presented by Park et al. (11) suggest that during phagocytosis of polystyrene-latex particles by crude leukocyte preparations, the intracellular concentration of cyclic AMP is increased. Similar results were reported by Manganiello et al. (9). It was not clear, however, whether the observed increase in cyclic AMP concentration was in fact related to phagocytosis. On the other hand, Stossel and colleagues (14) were unable to detect significant changes of cyclic AMP concentration in phagocytizing guinea pig peritoneal leukocytes.

In view of these conflicting observations, it was of interest to reinvestigate the possible participation of cyclic AMP in the phagocytosis of polystyrene-latex particles by two different cell types, pig peripheral leukocytes and rabbit alveolar macrophages.

MATERIALS AND METHODS

Alveolar macrophages were obtained from female New Zealand rabbits sensitized with bacille Calmette-Guérin (BCG) according to Myrvik et al. (10). Leukocyte suspensions were prepared from pig blood by the method of Fallon et al. (5) and subjected to density gradient centrifugation in Ficoll to obtain granulocyte-rich preparations (15). Differential counts of this suspension gave 70–92% granulocytes, 6–30% lymphocytes (crude pig leukocyte preparations contain up to 70% lymphocytes), and 1–3% monocytes. Contamination was less than one platelet per granulocyte and one erythrocyte per 100 granulocytes. The viability was checked with a dye exclusion test (0.3% trypan blue). Preparations containing more than 3% trypan blue-positive cells were discarded. The cell isolation procedures were performed at 0°–4°C and cells were suspended in modified Krebs-Ringer phosphate buffer (pH 7.4) with 8.3 mM glucose (KRP) to contain 100 mg wet cells/ml.

Incubation Conditions

The incubation mixtures contained 500 μl cell suspension (5×10^7–1×10^8 granulocytes or about 5×10^6 macrophages). When granulocytes were incubated, 100 μl autologous serum were added. KRP was added to give a final volume of 900 μl. The tubes were placed in a metabolic shaker (100 oscillations/min) at 37°C. At the time indicated 100 μl of a suspension of polystyrene-latex particles (0.481 μm diameter, 7.8×10^{10} particles in KRP or KRP [controls]) were added. For the estimation of latex ingestion, the reaction was stopped by transferring the incubation mixture to 4 ml ice cold KRP without glucose and subsequently centrifuged at 0°C. For the estimation of cyclic AMP, the incubation was stopped

by adding 100 μl ice cold 5 M perchloric acid and placing the tube in a methanol-dry ice bath. 20 μl [^{3}H]cyclic AMP were added for the estimation of the recovery from purification.

Latex uptake was measured by the dioxane procedure of Roberts and Quastel (12). The oxygen consumption was monitored in a standard Warburg respirometer.

Isolation of Cyclic AMP

After sonication for 15 s, 100 mM $ZnCl_2$ and K_2CO_3, to give a final pH of 7.0 (3), were added to precipitate-interfering nucleotides. After centrifugation the clear supernatant was loaded onto a AG 50 WX 4 column (0.5 × 12 cm). The column was eluted with water and the 6th–9th ml were collected and lyophilized. The recovery was between 57 and 73%.

Cyclic AMP was assayed by measuring the stimulation of a protein kinase with γ-[^{32}P]ATP as phosphate donor and histone as acceptor (modified from Kuo and Greengard [8]). The incubation mixture (total volume 50 μl) contained sodium glycerophosphate 250 μM pH 6.0, 10 mM Mg-acetate, 2 mM theophylline, 0.3 mM EGTA, 40 μg histone, and 10 μl protein kinase (50 μg protein), and 10 μl of either cyclic AMP standards or the unknown extract. After incubation for 15 min at 30°C, the reaction was stopped by the addition of bovine serum albumin (640 μg in 100 μl) and 1 ml 20% TCA and cooling the mixture to 0°C. After centrifugation the pellet was washed with 1 ml 5% TCA, hydrolyzed by the addition of 400 μl 1 N NaOH and 100 μl 1 mM KH_2PO_4 at 100°C, and the total hydrolysate was transferred into counting vials containing 15 ml water. Utilizing the Cerenkov effect, the radioactivity was measured in a Packard 3003 liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Internal standards were completely recovered in the final enzymatic assay. Protein kinase was prepared from beef heart according to the method of Kuo and Greengard (8).

Substances Used

Histone type II a (Sigma Chemical Co., St. Louis, Mo.); prostaglandin E_1 (kindly donated by Upjohn Co., Kalamazoo, Mich.); [^{3}H]cyclic AMP, sp act 24.1 Ci/mmol (New England Nuclear, Boston, Mass.); γ-[^{32}P]ATP, sp act 12–20 Ci/mmol (Amersham Buchler KG, Braunschweig, Germany); other substances were obtained from standard suppliers.

RESULTS AND DISCUSSION

Both cell types studied rapidly phagocytized polystyrene-latex particles. Uptake of particles was linear from 0 to 10 min and declined thereafter to reach a plateau after 15–30 min. Maximum particle uptake was 35 and 38% of the amount added (2 mg) in granulocytes and alveolar macrophages, respectively. Phagocytosis was accompanied by the expected increase in oxygen consumption from 3.5 to 8.5 μl O_2 per h per mg cell protein in granulocytes and from 14 to 15.8 μl O_2 per h per mg cell protein in alveolar macrophages.

Cyclic AMP concentration in granulocytes rose sharply at the start of the incubation without addition of particles, reached peak values at 10 min which were almost ten times that of O°C controls, and returned to a stable level after 30 min of incu-

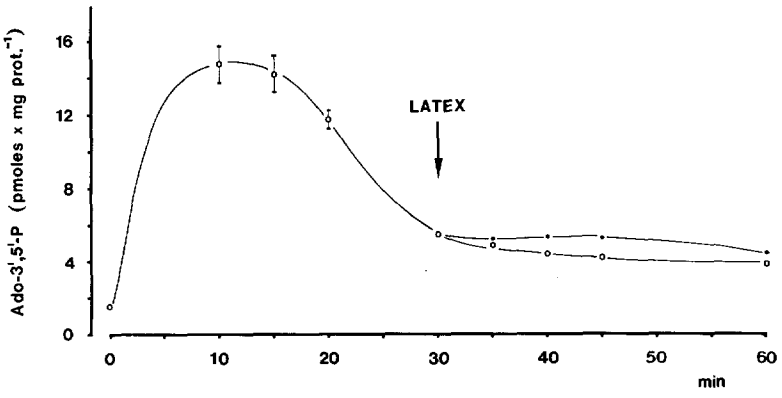

FIGURE 1 Concentration of cyclic AMP in polymorphonuclear leukocytes during phagocytosis. Cells were suspended in KRP pH 7.4 containing 10% serum and 5 U/ml heparin. After 30 min of preincubation 2 mg/ml latex particles were added (closed circles). Open circles represent controls. Each point is the mean ± standard error of six experiments.

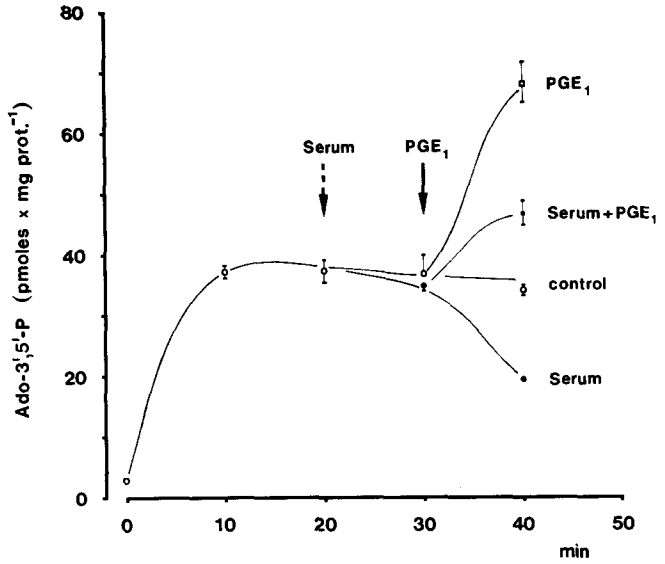

FIGURE 2 Influence of serum and prostaglandin E_1 on cyclic AMP levels in polymorphonuclear leukocytes. Cells were incubated in KRP pH 7.4 at 37°C. Autologous serum (10% of final volume) was added at 20 min. To both serum and serum-free incubates prostaglandin E_1 was added at 30 min (final concentration 10^{-5} M). n = four determinations from two cell preparations.

bation (Fig. 1). Assuming that there was a better chance to detect changes in cyclic AMP elicited by phagocytosis during this stable phase rather than after the beginning of the incubation, the addition of particles and the subsequent measurements were started after 30 min of preincubation. Under these conditions, the addition of latex particles resulted in only a small increase in cyclic AMP concentration above controls. In view of the results obtained with macrophages (see below), shorter time intervals were also studied in a separate series of experiments. No increase of the cyclic AMP concentration was detectable at either 30 s, 1 min, or 2 min after the addition of particles. These results may indicate that in granulocytes phagocytosis is not accompanied by significant changes in cyclic AMP concentration.

In support of this negative conclusion is the observation that these cells can respond to an appropriate stimulus such as prostaglandin E_1 (2) with the expected increase in cyclic AMP concentration (Fig. 2).

The cause of the tremendous increase in cyclic AMP concentration after the warming up to 37°C is not known. One possible explanation, namely that the serum added to standard incubations contains substances which stimulate the adenylate cyclase system, can be excluded by the finding that this effect also occurs in the absence of serum (Fig. 2).

These results are in apparent contradiction to those reported in the literature (9, 11). Park et al. found a marked increase above controls of cyclic AMP concentration in a crude human leukocyte preparation to which particles had been added (11). Phagocytosis was not ascertained in these experiments. Similarly Manganiello et al. (9) observed an increase in cyclic AMP in phagocytizing human leukocytes 5 min after latex particle addition. This increase was highest in preparations rich in mononuclear cells, including nonphagocytizing lymphocytes. The authors therefore speculated that the observed increase in cyclic AMP may not be related to phagocytosis at all. In support of this view is a previous investigation from the same laboratory in which no significant change in cyclic AMP concentration was found in phagocytizing rabbit peritoneal leukocytes, a preparation containing predominantly (more than 90%) polymorphonuclear leukocytes (14). Our own results are in accordance with this view. In preliminary experiments with crude pig leukocyte preparations (leaving the Ficoll step out), we occasionally observed an increase in cyclic AMP

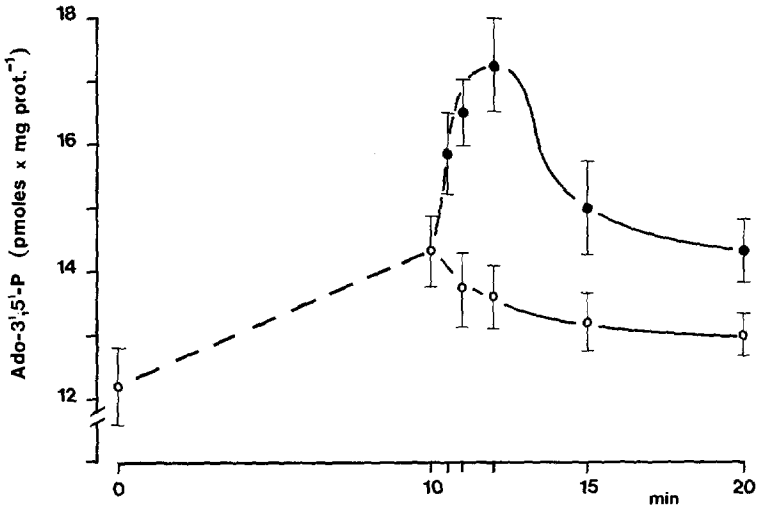

FIGURE 3 Concentration of cyclic AMP in alveolar macrophages during phagocytosis. Cells were suspended in KRP pH 7.4 at 37°C. After 10 min of preincubation polystyrene-latex beads (2 mg/ml) were added. n = six experiments.

concentration upon particle addition, too, whereas the results shown in detail have been obtained with preparations enriched in polymorphonuclear leukocytes.

In preceding experiments with alveolar macrophages in which phagocytosis was not measured, it was found that the concentration of cyclic AMP increased only by about 10–20% upon transfer from 0° to 37°C. Therefore the preincubation period was reduced to 10 min (Fig. 3).

In contrast to granulocytes, in alveolar macrophages phagocytosis is accompanied by a significant increase in cyclic AMP concentration (Fig. 3). This difference may reflect either the known difference in energy metabolism during phagocytosis or differences in the biochemical mechanisms of phagocytosis in these two cell types (7).

It seems justified to conclude that changes in cyclic AMP concentration are not a prerequisite for or a consequence of phagocytosis in all types of phagocytizing cells. This, however, does not exclude the possibility that in alveolar macrophages the rise in cyclic AMP concentration is the necessary link between contact of particles with the membrane and initiation of phagocytosis and/or metabolic changes. This view is supported by the short latency period between particle addition and onset of cyclic AMP rise.

SUMMARY

The uptake of polystyrene-latex beads, oxygen consumption, and concentration of cyclic AMP were measured during phagocytosis in pig peripheral blood leukocyte preparations rich in polymorphonuclear cells and in rabbit alveolar macrophages. Both cell types took up particles very rapidly and exhibited the expected increase in the oxygen consumption. The cyclic AMP content of the polymorphonuclear fraction did not immediately change after incubation with polystyrene-latex beads. Alveolar macrophages exhibited a small but significant increase in the level of cyclic AMP as early as 30 s after addition of particles.

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