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BIOCHEMICAL STUDIES ON THE EFFECT OF PAPAVERINE ON POLYMORPHONUCLEAR LEUCOCYTES

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Abstract—Papaverine, an inhibitor of phosphodiesterase, inhibits phagocytosis in polymorphonuclear leucocytes and the stimulation of oxidative metabolism associated with phagocytosis. The inhibitory effect of papaverine is reversible. Papaverine also inhibits the metabolic stimulation of leucocytes elicited by membrane perturbing agents. The results are related to the role of plasma membranes in the process of polymorphonuclear leucocyte activation.

PHAGOCYTOSIS is a multistep process including sequential events such as recognition of particles, attachment of particles to the phagocyte surface, formation of the phagocytic vacuole and, finally, its fusion with primary lysosomes. It is well established that phagocytosis by polymorphonuclear (PMN) leucocytes is accompanied by increased respiratory and hexose monophosphate shunt (HMS) activities. It is less clear with which step(s) of the phagocytic process those metabolic changes are associated.

On the basis of indirect evidence it has been postulated that stimulation of the respiratory and HMS activities are triggered by contact between particles and phagocyte surface.[1–8] The use of drugs that interfere with specific pathways in the cell has proved to be very helpful in separating the events of the phagocytic process.

Colchicine and vinblastine, for example, which are known to disrupt microtubules inhibit the formation of the digestive vacuole without inhibiting the ingestion process itself,[9,10] while cytochalasin B, which interferes with the contractile system of microfilaments,[11–13] inhibits both uptake of particles and the phagocytosis-associated metabolic stimulation.[14,15] These techniques, therefore, can also provide information about the step(s) of the phagocytic process involved in the "metabolic burst". We report here the effect of papaverine, an inhibitor of phosphodiesterase,[16,18] on phagocytosis and the phagocytosis-associated metabolic burst in guinea-pig PMN leucocytes. The effect of papaverine on the metabolic stimulation of PMN by phospholipase C[5] and Concanavalin A[19] will also be described.

MATERIALS AND METHODS

Leucocytes were obtained from sterile guinea-pig peritoneal exudates as previously described.[1,2] Leucocytes were suspended in calcium-free Krebs–Ringer-phosphate

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(KRP) or in Tris–HCl buffered saline which was prepared by substitution of phosphate buffer in KRP for 16·6 mM Tris–HCl pH 7·4.

For metabolic studies leucocytes were stimulated with the following agents: heat-killed bacteria (*B. mycoides*; ratio bacteria to cells 100:1), phospholipase C purchased from Sigma Chemical Co. (0·5–0·7 units/4 $\times$ 10^7 cells in 2·0 ml), Concanavalin A (300 μg/20 $\times$ 10^6 cells in 2·0 ml) and paraffin oil emulsion (see later).

Procedures for determination of oxygen uptake with a Clark oxygen electrode, of $^{14}CO_2$ production from labelled substrates, and of lactate production, have been described already.[1,2,5] In these experiments the radioactivity of $^{14}CO_2$ trapped by KOH was measured in a Beckman LS-100 liquid scintillation spectrometer using Bray's medium as a scintillation cocktail.[20]

The initial rate of phagocytosis was measured using a paraffin oil emulsion (POE) in which the dye Oil Red O had been dissolved as described by Stossel *et al.*[21] At various intervals of incubation of PMN leucocytes with POE phagocytosis was stopped with 1 mM *N*-ethylmaleimide (NEM) dissolved in isotonic saline. After washing away the extracellular POE the cell pellet was extracted with *p*-dioxane, centrifuged and the clear supernatant read at 524 nm. The molar extinction coefficient of Oil Red O in *p*-dioxane, with 1 cm light path, is 2·29 $\times$ 10^4 at 524 nm.

Glucose-6-phosphate dehydrogenase (G-6-PD) activity was assayed according to the method described by Löhr and Waller.[22] The activity of NADPH oxidase was assayed as previously described.[1,2,6]

Papaverine was used either as the free base or as the hydrochloride derivative. Papaverine base was dissolved in 98 per cent ethyl alcohol and was stable when added to the KRP medium. Papaverine hydrochloride tended to come out of solution, at high concentrations, in KRP. This was overcome by using Tris–HCl buffered saline as basic assay medium. For electron microscopy suspensions of leucocytes were fixed by adding to them an equal volume of a 3·0 per cent solution of purified glutaraldehyde in 0·2 M cacodilate buffer pH 7·2 containing 2 per cent sucrose. After 1 hr the suspension was centrifuged and the pellet resuspended in a 1 per cent osmium tetroxide solution in 0·1 M cacodilate buffer 7·2. After dehydration in graded ethanols the cell pellet was embedded in DER 332.[23] Ultrathin sections, prepared with an ultrotome III (LKB), were doubly stained with an aqueous solution of 2 per cent uranyl acetate for 30 min and with lead citrate,[24] and examined in a Philips EM 300 electron microscope.

RESULTS

Effect of papaverine on respiration and glucose oxidation. Table 1 shows the effect of papaverine base, dissolved in ethyl alcohol, and papaverine hydrochloride on the respiration and $^{14}CO_2$ production from [1-^{14}C]glucose in phagocytizing and non phagocytizing leucocytes.

At both 10^{-4} and 5 $\times$ 10^{-4} M papaverine hydrochloride inhibits respiration and $^{14}CO_2$ production in non-phagocytizing leucocytes, the magnitude of the effect being proportional to drug concentration. The inhibitory effect of papaverine base appears less pronounced. This could be due to an inhibitory effect of alcohol on respiration and $^{14}CO_2$ production which indeed are both markedly depressed in control alcohol-treated cells. In view of that possibility papaverine hydrochloride was used in further experiments unless otherwise stated.

TABLE 1. EFFECT OF PAPAVERINE ON $^{14}CO_2$ PRODUCTION FROM [1-^{14}C]-GLUCOSE AND RESPIRATION OF POLYMORPHONUCLEAR LEUCOCYTES

Addition	Resting cells		Phagocytizing cells	
	$^{14}CO_2$ production (cpm/10^8 cells/10 min)	Oxygen uptake (natoms O/10^8 cells/min)	$^{14}CO_2$ production (cpm/10^8 cells/10 min)	Oxygen uptake (natoms O/10^8 cells/min)
None	31,600 ± 7300 (5)	70·7 ± 3·7 (3)	94,500 ± 10,470 (4)	387·2 ± 10·7 (5)
Papaverine × HCl 10^{-4} M	15,650 ± 3350 (3)	48·0 (1)	52,500 (1)	225·1 (1)
Papaverine × HCl 5 × 10^{-4} M	2331 ± 1030 (5)	13·3 ± 0·7 (3)	7555 ± 3523 (4)	39·2 ± 1·7 (5)
50 μl EtOH	6650 ± 1280 (4)	35·4 ± 0·7 (3)	101,640 ± 25,250 (4)	302·4 ± 6·0 (5)
Papaverine 10^{-4} M	4393 ± 360 (3)	35·0 (1)	48,972 ± 18,400 (3)	139·4 ± 6·0 (3)
Papaverine 5 × 10^{-4} M	3907 ± 793 (4)	24·0 ± 0·8 (3)	28,887 ± 15,200 (3)	13·0 ± 0·4 (5)

The assay medium for both $^{14}CO_2$ production and oxygen uptake was 2·0 ml and contained 2·5 mM glucose and 2–4 × 10^7 cells. The specific activity of glucose was 3·15 × 10^5. In experiments where papaverine dissolved in alcohol was used, the incubation medium was regular KRP and each assay received 50 μl of 98% ethyl alcohol. In experiments with papaverine hydrochloride, Tris–HCl buffered saline was used as medium (see Materials and Methods). Heat-killed bacteria (*B. mycoides*) opsonized with guinea-pig serum were used as phagocytizable particles. The ratio bacterial leucocytes was 100:1. Results are given as mean ± S.E.M. Number of experiments in parentheses.

Phagocytizing cells show the usual increase in respiration and $^{14}CO_2$ production. The increase is markedly inhibited by either papaverine or papaverine hydrochloride. However at variance with resting cells, values of respiration and $^{14}CO_2$ production of phagocytizing cells in the presence of ethyl alcohol are close to those of phagocytizing cells in absence of alcohol. This may suggest that alcohol interferes with mechanisms of the respiratory and HMS activities in resting cells but not with the metabolic burst due to phagocytosis.

Figure 1 shows the effect of varying papaverine concentration on the inhibition of respiration and $^{14}CO_2$ production from [1-^{14}C]glucose in resting and phagocytizing leucocytes. The inhibitory effect on respiration parallels that on $^{14}CO_2$ production. At a concentration of 10^{-4} M papaverine is about 50 per cent inhibitory and at a concentration five times as high both respiration and $^{14}CO_2$ production are almost suppressed.

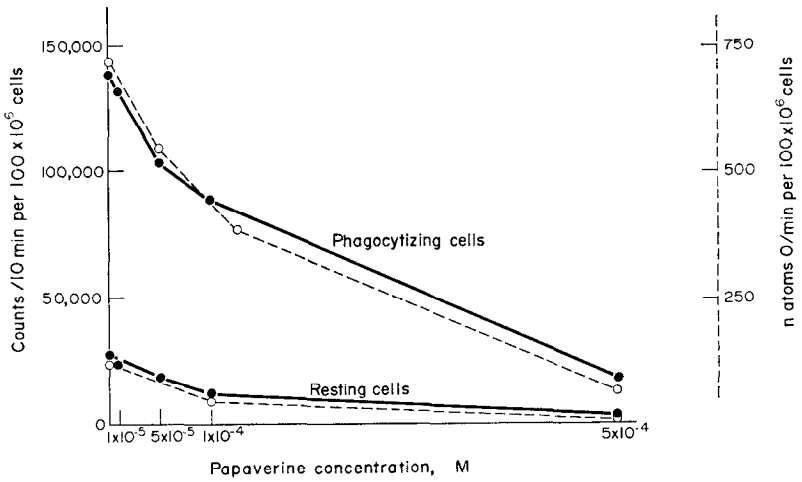

FIG. 1. Effect of varying concentration of papaverine × HCl on $^{14}CO_2$ production from 1-[^{14}C]-glucose and oxygen consumption in resting and phagocytizing PMN leucocytes. Experimental conditions as in Table 1.

Figure 2 is a recording, with a Clark oxygen electrode, of the respiration of cells at rest and after addition of bacteria. It is shown that papaverine inhibits the stimulated respiration within a few seconds when added either after (B) or before (C) bacteria. Data shown so far were obtained with glucose 2·5 mM present in the assays. Glucose however was by no means essential since its omission did not alter the stimulation of oxidative metabolism by bacteria or the effect of papaverine.

Effect of papaverine on phagocytosis. A new method, which employs a paraffin oil emulsion as phagocytizable particles, was used to quantitate the amount of ingested material. Table 2 shows the effect of paraffin oil emulsion on the respiration and HMS activities of leucocytes. Both activities are stimulated in presence of paraffin oil particles and the stimulation is inhibited by papaverine. This indicates that the emulsion used for our experiments on phagocytosis behaved similarly to bacteria or any other phagocytizable particle with respect to the "metabolic burst" that accompanies phagocytosis.

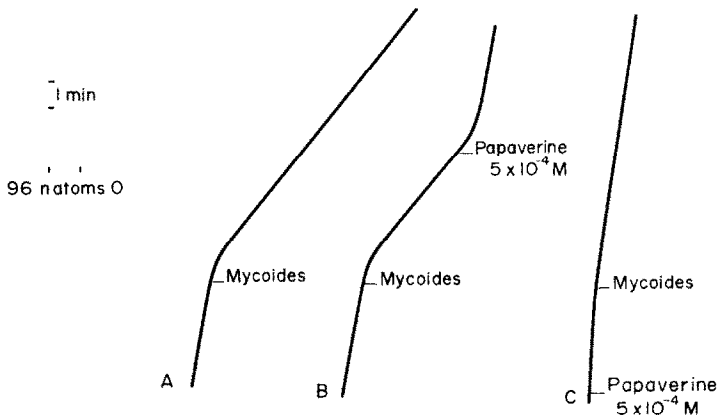

FIG. 2. Early effect of papaverine on the oxygen consumption of phagocytizing PMN leucocytes. Recording of oxygen uptake with polarographic technique using a Clark oxygen electrode. Number of PMN leucocytes in the assay 2×10^7. Other experimental details as in Table 1.

TABLE 2. INHIBITION BY PAPAVERINE OF $^{14}CO_2$ PRODUCTION FROM [1-^{14}C]-GLUCOSE AND RESPIRATION STIMULATED BY PARAFFIN OIL EMULSION (POE) IN POLYMORPHONUCLEAR LEUCOCYTES

	Control		+ Papaverine 5×10^{-4} M	
	− POE	+ POE	− POE	+ POE
$^{14}CO_2$ production (cpm/10^8 cells/10 min)	25,000	70,000	2190	2210
Respiration (natoms O/10^8 cells/min)	70	208	13	8·0

Experimental procedure as in Table 1 except that paraffin oil emulsion was used as phagocytizable particles in a ratio of 0·1 ml of emulsion to 1 ml of incubation mixture.

Figure 3 shows the effect of papaverine on phagocytosis of paraffin particles. The rate of uptake is linear over 6–7 min. In five different experiments measurements of the amount of material ingested gave values ranging from $0{\cdot}25 \times 10^{-7}$ to $0{\cdot}5 \times 10^{-7}$ moles Oil Red O/5 min/10^8 cells. In the presence of 5×10^{-4} M papaverine the uptake of paraffin particles is completely suppressed. Again the presence or absence of glucose in the incubation medium did not make any difference.

The effect of papaverine on phagocytosis was also checked by electron microscopy. Figure 4 is an electron micrograph of PMN leucocytes phagocytizing heat killed *B. mycoides* in absence of papaverine (a), and in presence of 5×10^{-4} M papaverine (b). Each of the untreated leucocytes shown in the figure contains several bacteria enclosed in a well defined phagocytic vacuole, whereas no bacteria are visible inside the papaverine-treated leucocytes. Also treated cells do not have bacteria adhering to their surface as occurs in untreated cells. Untreated leucocytes have few granules and their cytoplasm is markedly damaged. This is due to the well known phenomenon of degranulation that accompanies phagocytosis.

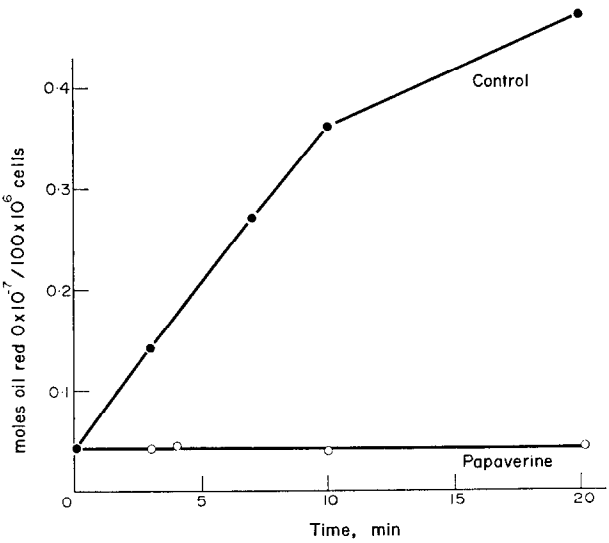

FIG. 3. Effect of 5×10^{-4} M papaverine × HCl on phagocytosis of paraffin oil particles by PMN leucocytes. Cells ($25–50 \times 10^6$) were preincubated in 1 ml Tris-HCl buffered saline for 5 min with or without papaverine. Then 0·1 ml emulsion, containing dissolved Oil Red O, was added. Phagocytosis was stopped at various intervals by adding 12 ml of 1 mM *N*-ethyl-maleimide (NEM) dissolved in isotonic saline. Cells were washed 3–4 times with isotonic saline. Pellets were extracted for 1 hr with 2–3 ml dioxane and then centrifuged. The concentration of the dye in the supernatant was read at 524 nm.

Effect of papaverine on glycolysis. In view of the inhibition of particle uptake by papaverine it was interesting to investigate the effect of papaverine on glycolysis. Glycolysis is regarded as the pathway that provides energy for phagocytosis.[25,26] This conclusion has been reached on the basis of the results of experiments with inhibitors of glycolysis.

Table 3 shows that lactate production in untreated cells is higher in the presence of glucose than without it. Despite this, the rate of phagocytosis of POE particles is not affected by glucose, as discussed previously, suggesting that the problem of relationships between glycolysis and phagocytosis has to be revised.

TABLE 3. EFFECT OF PAPAVERINE ON GLYCOLYSIS OF POLYMORPHONUCLEAR LEUCOCYTES

Addition	μmoles Lactate/10^8 cells	
	Control	5×10^{-4} Papaverine
None	0·330	0·633
Glucose 2·5 mM	0·935	0·866

Cells ($20–40 \times 10^6$) suspended in Tris–HCl buffered saline were incubated with or without papaverine at 37° for 20 min. The incubation was stopped by adding perchloric acid (0·3 M final concentration). Lactate was determined in the clear supernatant. Assay for lactate: 0·44 M glycine buffer pH 9·0, 0·34 M hydrazine, 1·0 mM NAD. Volume 3 ml. Incubation 1 hr at 25°. Reduced NAD was measured at 340 nm.

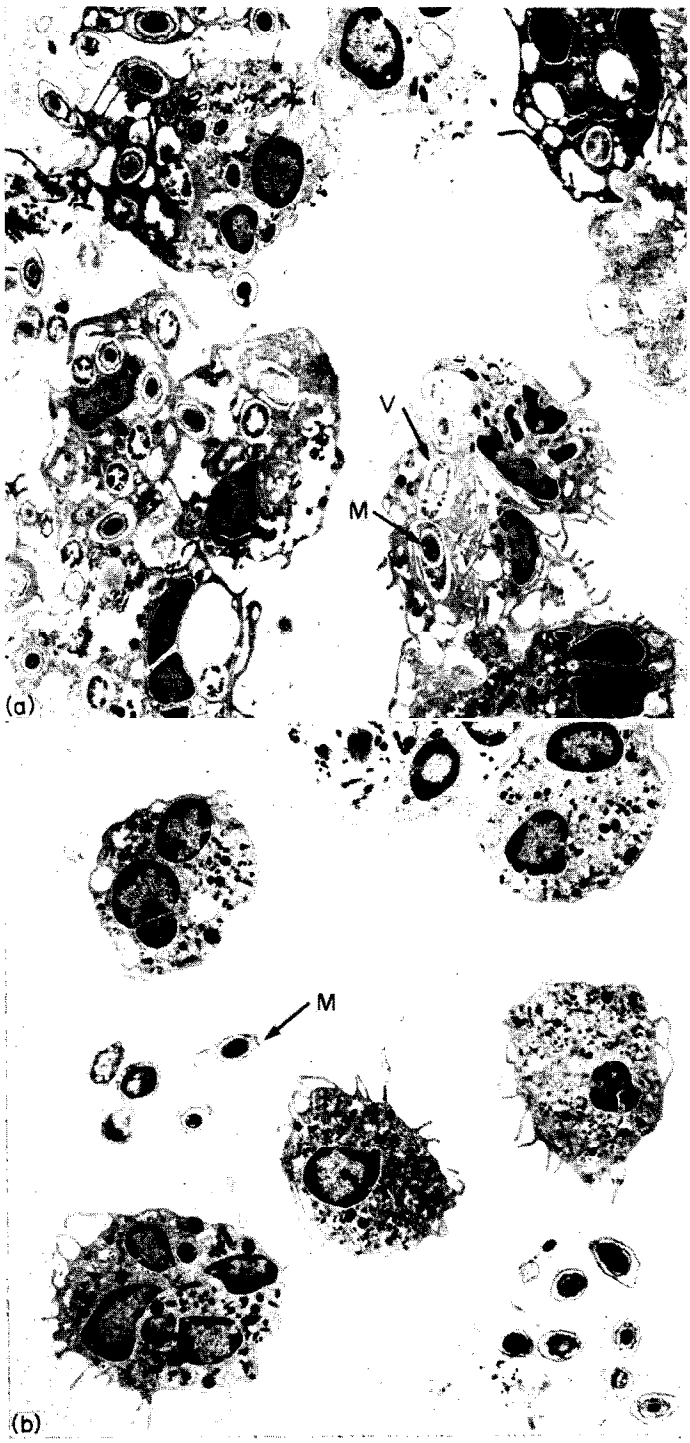

FIG. 4. Electron micrograph of PMN leucocytes phagocytizing *B. mycoides* (M) without (a) and with 5×10^{-4} M papaverine × HCl (b). Leucocytes were incubated with *B. mycoides* (ratio cells to bacteria 1:50) for 5 min at 37°. Each of the untreated PMN contains several bacteria enclosed in a phagocytic vacuole (V). Bacteria are not seen within treated cells.

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Papaverine, at the same concentration that inhibits phagocytosis and the phagocytosis-associated respiratory burst, that is 5×10^{-4} M, stimulates lactate production in a medium without glucose but slightly inhibits it when glucose is present. This inhibition is due perhaps to decreased glucose entry into the cell in the presence of papaverine. Accordingly incorporation of radioactivity from labeled exogenous glucose into lactate was depressed by papaverine in a medium containing 2·5 mM glucose with an increase of the ratio of lactate produced from endogenous sources to lactate produced from exogenous glucose. Again phagocytosis and the respiratory burst was inhibited by papaverine to the same extent regardless of the presence or absence of glucose in the medium.

Reversibility. Table 4 shows that respiration and $^{14}CO_2$ production of cells treated with papaverine and then washed, subsequently behave similar to those of control cells.

TABLE 4. REVERSIBILITY OF THE INHIBITORY EFFECT OF PAPAVERINE ON $^{14}CO_2$ PRODUCTION FROM [1-^{14}C]-GLUCOSE AND RESPIRATION OF POLYMORPHONUCLEAR LEUCOCYTES

	$^{14}CO_2$ production (% of control)		Oxygen uptake (% of control)	
	Resting cells	Phagocytizing cells	Resting cells	Phagocytizing cells
Control (no preincubation)	100	363	100	350
+ Papaverine 5×10^{-4} M	5	14	18	20
Preincubation + washing	88	326	101	340
Preincubation with papaverine 5×10^{-4} M + washing	75	310	95	300

Preincubation of cells with or without papaverine was carried out at 37° for 5 min. Cells were diluted out with cold Tris buffered saline, centrifuged, resuspended in Tris buffered saline and used for assays. Parallel determinations of $^{14}CO_2$ production and oxygen uptake were carried out on preincubated cells and non preincubated cells.

TABLE 5. EFFECT OF PAPAVERINE 5×10^{-4} M ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G-6-PD) AND NADPH OXIDASE ACTIVITIES OF POLYMORPHONUCLEAR LEUCOCYTES

	Percent of control
G-6-PD	$-10{\cdot}5 \pm 2{\cdot}5$ (8)
NADPH oxidase	$-9{\cdot}5 \pm 0{\cdot}5$ (7)

Assay medium for G-6-PD: 0·05 M glycylglycine buffer pH 7·6, 0·1 M KCl, 0·05 M $MgCl_2$, 1·3 mM glucose-6-phosphate, 0·3 mM NADP. Volume 3 ml. Reduction of NADP was followed at 340 nm in a Hitachi–Perkin Elmer double beam spectrophotometer at 37°. Assay for NADPH oxidase: 65 mM phosphate buffer pH 5·5, 0·17 M sucrose, 1 mM NADPH, 2 mM KCN and 0·5 mM $MnCl_2$. Temperature 37°. Oxygen uptake was monitored with a Clark oxygen electrode. Results are given as mean ± S.E.M. Number of experiments in parentheses.

Effect of papaverine on glucose-6-phosphate dehydrogenase (G-6-PD) and NADPH oxidase. These two enzymes are responsible for HMS activity and for oxygen consumption respectively. Table 5 shows that papaverine does not inhibit appreciably either G-6-PD or NADPH oxidase.

Effect of papaverine on the metabolic stimulation of leucocytes elicited by agents other than particles. Several agents, which perturb the assembly or the chemical composition of leucocyte plasma membrane, can mimic the metabolic stimulation associated with phagocytosis. Examples of such agents are phospholipase C and Concanavalin A. Table 6 shows that papaverine also inhibits respiration of leucocytes treated with either phospholipase C or Concanavalin A.

TABLE 6. EFFECT OF PAPAVERINE ON THE PMN LEUCOCYTE RESPIRATION STIMULATED BY PHOSPHOLIPASE C and CONCANAVALIN A

Addition	Oxygen uptake (natoms O/min/10^8 cells)
None	65
Phospholipase C*	280
Phospholipase C + 5×10^{-4} M papaverine × HCl	60
Concanavalin A†	140
Concanavalin A + 5×10^{-4} M papaverine × HCl	55

* 0·7 units/2 × 10^7 cells/ml.
† 300 μg/2 × 10^7 cells/ml.

DISCUSSION

Incubation of PMN leucocytes with the miolytic drug papaverine, 6,7-dimethoxy-1-veratrylisoquinoline, produced a reversible dose-dependent inhibition of phagocytosis, of increased oxygen uptake and of increased glucose oxidation through the HMS which normally accompany phagocytosis.

Phagocytosis is believed to be an energy-requiring process and this energy would be provided via glycolysis in the form of adenosine triphosphate.[25,26]

Our results show that papaverine markedly stimulates glycolysis in the absence of added glucose. This stimulatory effect is probably due to increased glycogenolysis with increased availability of substrate for glycolysis. In fact papaverine, a potent inhibitor of cyclic nucleotide phosphodiesterase,[16,18] would cause an increase in cyclic AMP (cAMP) concentration which, in turn, would stimulate phosphorylase.[27] The glycogenolytic activity of papaverine has been shown already to parallel the phosphodiesterase inhibitory effect of the drug in rat diaphragm.[17]

When glucose was present in the incubation medium the glycogenolytic effect of papaverine was not evident as it was masked by a decrease of lactate production from exogenous glucose. Whatever the mechanism of the dual response of lactate production to papaverine, it is relevant that phagocytosis and the metabolic response of PMN leucocytes to phagocytosis is consistently inhibited by papaverine whether glycolysis is stimulated or slightly inhibited. This suggests that the inhibitory effect of papaverine on phagocytosis must have a mechanism other than a curtailment of energy supply in the form of adenosine triphosphate. Several possibilities should be

considered. Papaverine is a potent inhibitor[16-17] of cyclic nucleotide phosphodiesterase and, therefore, it increases cAMP concentration in the cell. It has been shown that other agents which raise the level of cAMP in the cell, that is theophylline and prostaglandin, and cAMP itself, at a concentration above 10^{-4} M, significantly retard uptake of certain particles by phagocytic cells.[28] Also cAMP and agents that raise the level of cAMP in the cell, either inhibitors of phosphodiesterase or activators of adenyl cyclase, inhibit migration of macrophages.[29]

Data not shown in this paper suggest that papaverine may inhibit PMN motility. About 50 per cent of cells treated with papaverine were devoid of pseudopods which were quite prominent in all the untreated cells. Moreover direct observations with the phase microscope showed that motion of PMN was appreciably reduced by addition of papaverine.

However a possible inhibition of cell motility is not likely to be the only reason of inhibition of particle uptake since contact between particles and phagocytes was insured by adequate mixing in suspension.

Therefore an impairment of the other two steps of particle uptake, that is attachment to the phagocyte surface and engulfment of attached particles, must be implicated. The almost complete absence of particles associated with papaverine-treated cells suggests that the attachment step is greatly affected by the drug.

Inhibition of phagocytosis by papaverine is accompanied by marked reduction of HMS and respiratory activities.

The current view is that stimulation of those two pathways is triggered by conformational changes of plasma membrane which occur during attachment of particles to the phagocyte surface. Experimental evidence has been provided that supports such a hypothesis.[8,30]

The possibility of an inhibition of HMS and respiratory activities due to a general toxic effect of papaverine on the enzymes of these two pathways has been ruled out. In fact neither NADPH oxidase, which is responsible for oxygen uptake, or the HMS oxidereductases (G-6-PD and 6-phosphogluconate dehydrogenase) are appreciably affected by papaverine. Moreover the inhibitory effect of papaverine was easily reversed by washing the cells.

Therefore the likelihood is that the effect of papaverine is secondary to an interference of the drug with leucocyte plasma membrane where it would impair those rearrangements which occur in phagocytosis and generate the signal for the metabolic burst to take place.

A stabilizing effect of papaverine on biological membranes has been described already. Liver lysosomes exposed *in vitro* to papaverine release their hydrolytic enzymes into the incubation medium slower and to a lesser extent than untreated lysosomes.[31]

This effect has been attributed to an increase in cAMP concentration. However, the effect of cAMP alone was found to be smaller than that of papaverine. Therefore either the concentration of cAMP in the preparation exposed to papaverine was higher than that of added cAMP or a direct effect of the drug on lysosomal membrane should be considered, in addition to inhibition of phosphodiesterase, to explain the stabilization of lysosomal membrane.

However, the effects of papaverine described in this paper are evident within few seconds after addition of the drug. This would support a direct action of the drug on

the cell membrane rather than a mechanism requiring generation of a chemical mediator.

Furthermore we have found that papaverine also inhibits PMN stimulation induced by phospholipase C[5] and concanavalin A.[19] Leucocyte stimulation by these two agents is triggered by well documented changes of the leucocyte membrane. Phospholipase C cuts off the polar head groups of membrane phospholipids and concanavalin A reversibly binds to glycoprotein receptor sites on the membrane causing clustering of the protein molecules normally dispersed in the lipid environment of the membrane. Papaverine was effective when added either before phospholipase C or concanavalin A or during the stimulation induced by the two agents. This indicates that papaverine did not inhibit the enzymatic activity of phospholipase C since its inhibitory action was still evident after phospholipid hydrolysis had occurred to a certain extent. These data give further support to the idea that papaverine interferes with the changes of the leucocyte membrane which are considered to be the trigger mechanism of leucocyte stimulation.

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