JCI The Journal of Clinical Investigation

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J Clin Invest. 1971;50(5):1091-1099. https://doi.org/10.1172/JCI106580.

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The Effects of the Morphine Analogue Levorphanol on Leukocytes

METABOLIC EFFECTS AT REST AND DURING PHAGOCYTOSIS

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ABSTRACT Studies on bacteria have suggested that morphine-like drugs have effects on the cell membrane. To determine the effect of this class of drugs on a mammalian cell, we selected the rabbit peritoneal exudate granulocyte, which undergoes striking membrane changes during phagocytosis. We examined the effect in vitro of the morphine analogue, levorphanol on phagocytosis and metabolism by granulocytes incubated with and without polystyrene particles or live *Escherichia coli*. Levorphanol (1 or 2 mmoles/liter) decreased: (*a*) acylation of lysolecithin or lysophosphatidylethanolamine in the medium (which is stimulated about two-fold during phagocytosis) both at rest (40%) and during phagocytosis (60%); (*b*) uptake of latex particles and *Escherichia coli*, as judged by electron microscopy; (*c*) killing of live *Escherichia coli* (10-fold); (*d*) $^{14}CO_2$ production from glucose-1-^{14}C during phagocytosis by at least 80%; (*e*) K^+ content of granulocytes (35%); (*f*) oxidation of linoleate-1-^{14}C by 50%, and its incorporation into triglyceride by more than 80%. However, levorphanol stimulated 2 to 3-fold the incorporation of linoleate-1-^{14}C or palmitate-1-^{14}C into several phospholipids. Glucose uptake, lactate production, and adenosine triphosphate (ATP) content are not affected by the drug. Thus, levorphanol does not appear to exert its effects through generalized metabolic suppression.

Removal of levorphanol by twice resuspending the granulocytes completely reverses all inhibition.

In line with observations on bacteria, it appears that the complex effects of levorphanol on granulocytes may be due at least in part to an effect on the cell membrane.

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Received for publication 12 June 1970 and in revised form 9 November 1970.

INTRODUCTION

During phagocytosis numerous biochemical and morphological changes take place that indicate that engulfment and killing of microorganisms require a set of active metabolic responses (1, 2). Recently we reported from this laboratory that these responses include net synthesis of new membrane lipids (3, 4). It was shown that a quantitatively important source of membrane phospholipid of granulocytes is provided by lysocompounds in the extracellular environment. These compounds, specifically lysolecithin (LPC)[1] and lysophosphatidylethanolamine (LPE), are acylated to the major membrane phospholipids, phosphatidylcholine (PC), and phosphatidylethanolamine (PE) by granulocytes at rest. This reaction is stimulated up to three-fold during engulfment of particles.

A series of observations has shown that levorphanol, an analogue of morphine, exerts a profound inhibitory effect on growth and on the biosynthesis of ribosomal RNA in *Escherichia coli*, (5–7). More recently, an additional effect of the drug on the bacterial membrane has been suggested by the findings that levorphanol interferes with the accumulation of various substances by *E. coli* (8, 9) and that levorphanol markedly alters the labeling pattern of the phospholipids of this bacterial species from radioactive precursors such as palmitate-1-^{14}C, glycerol-^{14}C, and $^{32}P_i$ (unpublished observations). Similar results have been reported for *Staphylococcus aureus* by Gale (10, 11). Changes in phospholipid metabolism of brain tissue under the influence of narcotic drugs have also been reported (12, 13).

[1] *Abbreviations used in this paper:* LPC, lysolecithin; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMN, polymorphonuclear; TEA, triethanolamine.

To explore further the possibility that levorphanol exerts an effect on mammalian cell membranes, we chose polymorphonuclear leukocytes. These cells can engulf particles in vitro and thus can be examined while engaged in a process during which the cellular membranes undergo extensive changes. The results indicate that levorphanol indeed suppresses the acylation of lysocompounds, exerts a number of other metabolic effects, and inhibits ingestion of particles and bacterial killing by granulocytes.

METHODS

Cells

Polymorphonuclear (PMN) Leukocytes

PMN leukocytes were collected from sterile peritoneal exudates produced in rabbits as described before (14). The granulocytes, which comprised more than 90% of the total cell population, were resuspended in Hanks'[2] solution plus 4% bovine albumin[3] to give a final concentration of from 4 to 8×10^7 cells per 0.5 ml of complete incubation mixture. A cell number of 2×10^7 corresponds to approximately 1 mg of protein.

Homogenates were prepared in 0.25 M sucrose in a glass homogenizing tube, using a motor driven teflon pestle. Homogenization of granulocytes was carried out for at least three periods of 30 sec.

Bacteria. A leucine auxotroph of *E. coli* B was grown in minimal medium buffered with triethanolamine (TEA) at a pH of about 7.9 as reported by Simon and Van Praag (6). Growth was followed by optical density measurements at 550 mμ in a Lumetron colorimeter and by viable cell counts on nutrient agar plates. An OD of 1.0 is equivalent to 3×10^8 bacteria. Stationary phase cultures were harvested by centrifugation, washed, and resuspended in Hanks' solution at a cell density of approximately 5×10^9/ml, and prepared for addition to granulocyte suspensions as described below.

Biosynthesis and preparation of radioactive substrates. PC and PE were biosynthetically labeled by incubating rat liver slices with orthophosphate-^{32}P[4] as described in detail before (15–17). The LPC-^{32}P and LPE-^{32}P were obtained by breakdown of the corresponding ^{32}P-labeled diacylphosphatides with *Crotalus adamanteus* phospholipase A.[5] Radiochemical purity of the ^{32}P lysocompounds as determined by thin-layer chromatography on Silica Gel G[6] (see below) was at least 98% for LPC and at least 95% for LPE.

Incubation procedure. Radioisotopically labeled LPC or LPE of known specific activity, dissolved in chloroform: methanol (2:1) or palmitic acid-1-^{14}C[7] (55.5 mCi/mmole) or linoleic acid-1-^{14}C[6] (204 mCi/mmole) in benzene were taken to dryness under nitrogen and complexed to bovine albumin in phosphate buffer (0.05 mole/liter, pH 7.4) or in Hanks' solution. When $^{14}CO_2$ collections were carried out, the fatty acid was washed before use according to the method of Dole and Meinertz (18) to remove any contaminating water-soluble radioactivity. In other experiments glucose-1-^{14}C[6] (260 μCi/mg) or glucose-6-^{14}C[6] (30 μCi/mg) in ethanol were dissolved in Hanks' solution after evaporation of the ethanol under nitrogen.

Incubation mixtures in a total volume of 0.5 ml, contained 0.1 ml of the labeled albumin solution and granulocytes (usually 5×10^7 cells) suspended in Hanks' solution. Other additions, including the narcotic levorphanol,[8] were as indicated in the text or in the legends.

Phagocytosis in vitro was initiated by adding 0.1 ml of polystyrene latex particles[9] (1.1 μ diameter, 3.6×10^9 particles) suspended in physiological saline, or about 5×10^8 *E. coli*, in 0.1 ml of Hanks' solution, giving a 10:1 ratio of bacteria to granulocytes. In experiments involving killing of live bacteria, *E. coli* were treated with rabbit serum at a concentration of 10% by volume in Hanks' solution and incubated at 37°C for 20 min. The suspending medium was removed by centrifugation, and the bacteria were resuspended in Hanks' solution alone. Unless stated otherwise, drugs and bacteria were added at the same time. At appropriate intervals, the leukocyte suspension that contained bacteria was thoroughly mixed, and a portion was transferred into cold TEA buffer. Suitable dilutions were made, and viable counts were determined by conventional techniques.

Collection of $^{14}CO_2$. Suspensions of leukocytes were placed in Erlenmeyer flasks containing polyethylene cups suspended from rubber stoppers.[10] At 0 min., a portion of glucose-1-^{14}C, glucose-6-^{14}C. or linoleic acid-1-^{14}C was added. As soon as the radioactivity was added, the flasks were stoppered and incubated at 37°C. At the indicated time intervals, the reaction was stopped by injecting 0.2 ml of 10 N H_2SO_4 through the rubber stopper into the main chamber. Hyamine was added to the collection cups and incubation at 37°C was continued for 1 hr. At this time, the cups that contained hyamine were removed and placed in counting vials and shaken well with 12 ml toluene-BBOT[11] mixture. Counting in a Packard liquid scintillation spectrometer was carried out immediately after addition of scintillation mixture and cooling of the vials. Under these circumstances quenching was minimal. Radioactivity of portions of glucose-^{14}C was determined also in the presence of hyamine and thus provided an internal standard.

K^+ content. To determine the K^+ content of granulocytes incubated with or without various agents, the cells were separated by centrifugation. The packed cells were then digested with concentrated nitric acid and dissolved in 0.02 M LiCl. K^+ content was determined by conventional flame photometry using LiCl as an internal standard.

Glycolysis. Glucose levels in the medium were measured in duplicate using the "AutoAnalyzer."[12] Lactate determinations were carried out on medium and cells using Boehringer (Mannheim, Germany) test kits. The cells contained less than 3% of the lactate found in the medium.

[2] Hanks' balanced salt solution (without phenol red), Microbiological Associates, Inc., Bethesda, Md.

[3] Armour Pharmaceutical Co., Kankakee, Ill.

[4] International Chemical & Nuclear Corporation, Burbank, Calif.

[5] Lights and Company, Colnbrook, Bucks., England.

[6] E. Merck A. G. Darmstadt, Germany.

[7] New England Nuclear Corp., Boston, Mass.

[8] Levorphanol tartrate, a generous gift of Hoffmann-La Roche, Inc., Nutley, N. J.

[9] Dow Chemical Co., Midland, Mich.

[10] Kontes Glass Co., Vineland, N. J.

[11] 2,5-bis-2-(5-tert-butylbenzoxazolyl) thiophene, Packard Instrument Co., Downers Grove, Ill.

[12] We are greatly indebted to Dr. Norman Altszuler of the Department of Pharmacology at New York University School of Medicine, New York, in whose laboratory the glucose determinations were carried out.

Radiochemical assays. Lipids were extracted and conversion of the labeled lysocompounds to diacyl derivatives was determined by thin-layer chromatography as described in detail previously (3, 15–17). Incorporation of ^{14}C-labeled fatty acids into various cellular lipids was determined in a similar manner. Triglycerides were separated from fatty acids and phospholipids in a solvent system (I) consisting of petroleum ether: ethyl ether: glacial acetic acid (160:80:2, v/v). Phospholipids were further separated in a solvent system (II) consisting of chloroform: methanol: glacial acetic acid: water (100:56:20:10, v/v). To prevent contamination of phospholipids with ^{14}C-labeled free fatty acid, portions of lipid extract were spotted 6.5 cm from the top edge of the plate and developed first with solvent system I. Fatty acids and triglycerides were made visible by exposure to iodine vapor and were scraped from the plate into counting vials, leaving the phospholipids at the point of application. The plate was placed in a desiccator for ½ hr, and then run in the reverse direction in solvent system II, using enough solvent to permit the new lower edge of the thin layer to be wetted. Radioactivity was determined by liquid scintillation counting. Quenching due to silica gel was minimal and closely similar for each fraction since care was taken to scrape off approximately equal amounts of powder. The radioactivity in each fraction was expressed as per cent of the total radioactivity recovered in all fractions and converted to nmoles on the basis of the known amounts of radioactive substrate added. In most experiments the whole incubation mixture was extracted. Where indicated, cells and medium were separately extracted.

TABLE I

Effect of Levorphanol on the Acylation of Lysolecithin-^{32}P by Granulocytes

	−Polystyrene		+Polystyrene	
Control	100	(15)	241 ±28	(15)
2 mM levorphanol	57 ±4.5	(15)	91 ±7.1	(15)
2 mM levorphanol preincubation*	63 ±8.3	(4)	84 ±6.2	(4)
Levorphanol removed	110 ±16	(7)	208 ±33	(7)

Granulocytes were incubated for 30 min in the presence or absence of polystyrene latex particles. LPC-^{32}P complexed to albumin was added in a concentration ranging from 15 to 100 μmoles/liter in different experiments. In experiments in which recovery from the effect of levorphanol was examined, the cell suspension was divided into two portions incubated with or without levorphanol for 30 min, after which the cells were centrifuged at 200 *g*, resuspended twice in fresh medium, and reincubated for 30 min, with or without levorphanol as shown. Assay of PC-^{32}P formation was carried out as described in the section on Methods. Results are expressed as per cent of control values without polystyrene and are given as mean ±SE of the indicated number of experiments (n).

* Levorphanol added 30 min before addition of LPC-^{32}P and particles or saline.

RESULTS

Effect of levorphanol on acylation of lysocompounds. Levorphanol tartrate[13] inhibits acylation of albumin-bound ^{32}P-labeled LPC to PC by rabbit granulocytes (Table I). In the absence of levorphanol, addition of polystyrene particles produces the stimulation ($P < 0.001$) of acylation of LPC (Table I) observed previously (3). In the presence of levorphanol at a concentration of 2 mmoles/liter, resting leukocytes convert less LPC to PC ($P < 0.001$). Whereas addition of latex particles increased acylation by 50%, the values after stimulation remained below the resting values observed in the absence of levorphanol. Preincubation of leukocytes with levorphanol for 30 min before addition of ^{32}P-labeled LPC and particles or saline gave similar results, indicating that the drug exerted its effect rapidly. The inhibition of acylation of LPC was completely reversed when levorphanol was removed by resuspending the cells twice in fresh medium.

Acylation of LPE to PE was also inhibited by levorphanol, both at rest (by about 40%) and during ingestion of polystylene particles (by about 60%). The enantiomorph of levorphanol, dextrorphan had identical inhibitory effects.

The results shown in Table II suggest that the apparent inhibition of synthesis of PC and PE from their respective monoacyl derivatives cannot be attributed to increased breakdown of the products. In this experiment, leukocyte PC and PE were labeled during initial incubation with linoleate-1-^{14}C. Previous studies have revealed that more than 80% of radioactivity from linoleate-1-^{14}C incorporated into phospholipids, is recovered in PC and PE (19, 20). After removal of the radioactive precursor and reincubation for 2 hr in fresh medium with or without levorphanol, neither the total radioactivity of each cell sample, nor the distribution of radioactivity among various lipid fractions were indicative of breakdown of PC and PE.

A comparison was made of the inhibition by levorphanol of the acylase reaction in homogenates of leukocytes and by intact cells (Table III). If levorphanol directly affects the enzyme, a greater sensitivity to the drug might be expected in the broken cell preparation because of more ready interaction between enzyme and drug. As seen previously (3), acylase activity in homogenized cells is greater than that of intact cells and further differs in that addition of ATP and CoA stimulates the reaction. In contrast to the usual inhibition of acylation in intact cells, levorphanol (2 mmoles/liter) manifests little or no inhibitory effect on homogenized leukocytes, both with and without added ATP and CoA. It appears unlikely therefore that levorphanol has a direct effect on the acylase.

Experiments with human peripheral blood leukocytes showed an identical and also reversible inhibitory effect by levorphanol on acylation.

[13] No effect was seen when 2 mM Na tartrate was added.

TABLE II

Effect of Levorphanol on the Stability of Lecithin and Phosphatidylethanolamine Prelabeled with Linoleic Acid-1-^{14}C

Fraction	SPH* + LPC		PC		PE		FFA		TG	
	Control	Levorphanol	Control	Levorphanol	Control	Levorphanol	Control	Levorphanol	Control	Levorphanol
0 min	3.4		27.7		3.6		14.7		35.5	
30 min	4.7	4.5	32.6	30.5	4.8	4.1	9.7	12.6	31.8	33.0
60 min	3.5	4.9	32.8	31.5	4.9	4.4	11.4	12.5	31.4	32.3
120 min	4.9	4.8	34.0	34.3	5.4	6.5	6.2	5.8	36.7	35.3

Granulocyte lipids were labeled with linoleate-1-^{14}C (1μCi) during incubation of a suspension of 5×10^8 cells in ascitic fluid for 1 hr at 37°C. At the end of this time cells and medium were separated by centrifugation, and the cells were resuspended in ascitic fluid without labeled fatty acid and reincubated for 30 min to allow remaining free fatty acid-^{14}C to enter ester positions. Finally the cells were spun again, resuspended in 3.5 ml of Hanks' solution with serum albumin (4g/100 ml), and divided into seven equal portions that were incubated with or without levorphanol (2 mmoles/liter) for the indicated periods of time. The suspensions were extracted with chloroform: methanol (1:1, v/v), and the various lipid fractions were separated by thin-layer chromatography as described in Methods. The radioactivity of each fraction is given as per cent of total radioactivity recovered from the plate. Radioactivity in minor fractions is not recorded.

* SPH = sphingomyelin.

Effect of levorphanol on killing of E. coli by granulocytes. The killing of *E. coli* by granulocytes was used as a means of determining the effect of levorphanol on the over-all process of phagocytosis. Suspensions of granulocytes were incubated with live *E. coli* in a ratio of 1 granulocyte to 10 bacteria. Table IV shows that levorphanol reduces bacterial killing approximately 10-fold. Fig. 1 shows a similar experiment, except that the number of bacteria per leukocyte was increased to 50 to allow a greater percentage of surviving microorganisms in the presence of leukocytes not treated with levorphanol. As in the case of inhibition of acylation of LPC, removal of levorphanol results in disappearance of the inhibitory effect on killing. The possibility was explored that bactericidal activity of the cell suspension in the presence of levorphanol might be due to leakage of bactericidal substances into the medium rather than to ingestion and subsequent killing. Incubation for 30 min of *E. coli* in cell free media from leukocytes previously incubated with or without levorphanol did not decrease the bacterial viable counts.

Electron microscopic studies show a marked reduction in uptake of polystyrene particles and a moderate but distinct decrease in engulfment of *E. coli*.[14] The extent of protection by levorphanol against bacterial killing can thus be accounted for by the observed decrease in uptake.

Effect of levorphanol on energy metabolism. Engulfment requires intact glycolysis (1), and bacterial killing is markedly enhanced by H_2O_2 formation linked to oper-

[14] Zucker-Franklin, D., P. Elsbach, and E. J. Simon. Manuscript in preparation.

TABLE III

Comparison of the Effect of Levorphanol on Acylation of Lysolecithin-^{32}P by Intact and by Homogenized Granulocytes

	Whole cells		Homogenate, no additions		Homogenate plus ATP, CoA	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	nmoles of PC formed per 2 × 10^7 cells					
Control	3.3;	3.5	5.1;	3.9	9.6;	9.2
2.0 mM levorphanol	2.0;	1.5	4.4;	4.1	10.1;	10.3

Intact granulocytes and homogenates of granulocytes prepared in 0.25 M sucrose (see section on Methods) were incubated for 30 min with 0.12 mmoles/liter lysolecithin-^{32}P complexed to albumin. ATP, CoA, and $MgCl_2$ were added in concentrations of 10, 0.2, and 10 mmoles/liter, respectively.

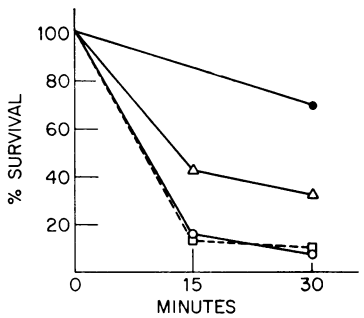

FIGURE 1 Effect of levorphanol on killing of *E. coli* by granulocytes. Granulocytes were incubated at 37°C for 30 min with and without levorphanol (2 mmoles/liter), after which cells and medium were separated by centrifugation at 200 *g* for 10 min. The granulocytes were then resuspended in fresh medium. Cells previously incubated with levorphanol were divided into two equal portions. To one of these levorphanol was again added. At this point (0 min in the figure) bacteria treated with serum (see Methods) were added, where indicated, in a ratio of 50 bacteria to 1 granulocyte. Each incubation mixture contained 8×10^7 granulocytes in a total volume of 0.5 ml of Hanks'-albumin solution. Portions of the suspensions were removed for determination of viable counts at the indicated times. ● = *E. coli* alone, with or without levorphanol; ○ = *E. coli* and granulocytes; △ = *E. coli* and granulocytes and levorphanol; □ = *E. coli* and granulocytes from which levorphanol was removed.

ation of the hexosemonophosphate shunt (21–24). Levorphanol might interfere with either or both of these pathways.

Table V contains the results of one of two closely similar experiments in which glucose utilization and lactate production by granulocytes in the absence and presence of levorphanol were determined. Neither with nor without polystyrene particles in the incubation mixture was there a detectable effect of levorphanol on the two variables examined. These findings are in agreement with those of Greene and Magasanik in *E. coli* (9). The absence of a distinct increase in glucose utilization and lactate production during phagocytosis is not in conflict with the putative role of glycolysis as a source of energy for phagocytosis, since it has been emphasized that the burst of increased glycolytic activity lasts only a few minutes after addition of the particles (1, 25). In line with the fact that glycolysis, the main source of energy for the granulocyte, is unimpaired when levorphanol is added, is the finding that the ATP content of leukocytes treated with the drug was the same as that of untreated cells in two experiments.[15]

In contrast to these negative observations, a marked inhibitory effect was noted on the production of $^{14}CO_2$ from glucose-1-^{14}C (Fig. 2). The familiar and dramatic

[15] We are indebted to Dr. Federico Welsch of Dartmouth Medical School, for the ATP determinations.

TABLE IV

Effect of Levorphanol on Killing of E. coli B Leucine⁻ by Granulocytes

Time of incubation...	15 min	30 min	40 min	60 min
Survival* +levorphanol/ −levorphanol	9	11	12	12

Granulocytes were incubated at 37°C for the indicated periods of time with and without levorphanol (2 mmoles/liter). Bacteria treated with serum as described in the section on Methods were added in a ratio of 10:1. At appropriate intervals, portions were removed for determination of viable counts. Each value represents an average of at least two experiments. The range of protection observed was from 5- to 17-fold. Survival in the control ranged from 5 to 0.3% of the total bacteria added. The viable count of serum treated bacteria incubated in the absence of leukocytes was the same in the presence and absence of levorphanol.

* Ratio of surviving bacteria in the presence and absence of levorphanol.

increase in CO_2 production through direct oxidation of glucose that accompanies phagocytosis was eliminated by levorphanol at a concentration of 1 or 2 mmoles/liter. Removal of levorphanol by washing the leukocytes restored the stimulation of $^{14}CO_2$ production by ingestion of *E. coli* to the same values reached by untreated phagocytizing leukocytes. Consistent with these results, reduction of the dye nitrobluetetrazolium (26) was also reversibly inhibited by levorphanol (Fig. 3).

TABLE V

Glucose Uptake and Lactate Production by Granulocytes in the Presence and Absence of Latex Particles and/or Levorphanol

Levorphanol	Latex particles	Glucose 15 min	Glucose 30 min	Lactate 15 min	Lactate 30 min
		nmoles		*nmoles*	
−	−	780	1550	1120	2070
−	+	610	1390	1500	2330
+	−	780	1450	1150	1960
+	+	780	1500	1170	2250

Granulocytes (1×10^8 cells per tube) suspended in Hanks' solution were incubated in a total volume of 1.0 ml that also contained 40 mg of bovine serum albumin, and either 0.1 ml of polystyrene particles or 0.1 ml of saline. Levorphanol was added in a concentration or 2 mmoles/liter. The whole experiment was carried out in duplicate. At the end of the indicated periods of time cells and medium were separated by centrifugation. Glucose uptake and lactate production at 15 and 30 min were calculated from the difference in medium content with respect to zero time. The cell pellets contained negligible amounts of lactate.

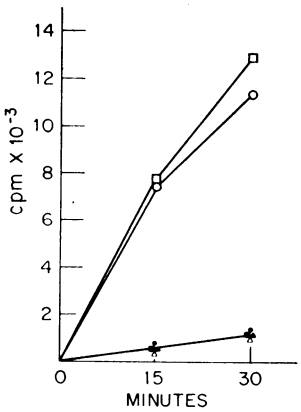

FIGURE 2 Effect of levorphanol and its removal, on $^{14}CO_2$ production from glucose-1-^{14}C by granulocytes incubated with and without *E. coli*. Granulocytes were incubated as described in the legend of Fig. 1. After preincubation for 30 min with and without levorphanol (2 mmoles/liter), and resuspension in fresh medium (with and without levorphanol) heat-killed *E. coli* in a ratio of 10 bacteria to 1 granulocyte, and 0.1 μCi of glucose-1-^{14}C were added. Each incubation mixture further contained 5×10^7 granulocytes in Hanks'-albumin solution and 5.5 mM glucose in a total volume of 0.5 ml. Collection and counting of $^{14}CO_2$ was carried out as described in Methods. Heat-killed *E. coli* produced negligible quantities of $^{14}CO_2$. ● = granulocytes alone; ▲ = granulocytes alone and levorphanol; ○ = granulocytes and *E. coli;* △ = granulocytes and *E. coli* and levorphanol; ■ = granulocytes from which levorphanol was removed; □ = granulocytes from which levorphanol was removed and *E. coli.*

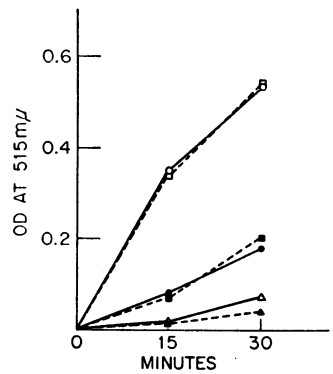

FIGURE 3 Effect of levorphanol, and its removal, on reduction of nitrobluetetrazolium by granulocytes incubated with and without polystyrene particles. This experiment was carried out as described in the legends of FIG. 1, except that at time 0, 0.2 ml of a 0.2% solution of nitrobluetetrazolium dye was added to each incubation mixture and either 0.2 ml of polystyrene particles in saline or saline alone. KCN was present in all samples at a concentration of 1 mmole/liter. Each incubation mixture contained 5×10^7 granulocytes in Hanks'-albumin solution in a total volume of 1.0 ml. Determination of reduction of the dye was carried out as described by Baehner and Nathan (26). ● = granulocytes alone; ▲ = granulocytes alone and levorphanol; ○ = granulocytes and polystyrene particles; △ = granulocytes and polystyrene particles and levorphanol; ■ = granulocytes from which levorphanol was removed; □ = granulocytes from which levorphanol was removed and polystyrene particles.

Production of $^{14}CO_2$ from glucose labeled in the 6-position was so small that no meaningful assessment of any inhibition of mitochondrial glucose combustion by levorphanol was possible. However, an effect on mitochondrial substrate oxidation was suggested by a two-fold inhibition of $^{14}CO_2$ production from linoleate-1-^{14}C by 2 mM levorphanol (Table VI).

Effect of levorphanol on incorporation of fatty acids into granulocyte lipids. Esterification of exogenous fatty acid-1-^{14}C into various cellular lipids under the influence of levorphanol appears affected in a rather complex way. Table VII demonstrates that incorporation of palmitate-1-^{14}C into total phospholipids is stimulated by levorphanol. This increased labeling is mainly attributable to enhanced incorporation of palmitate into PE and into phosphatidylinositol and/or phosphatidylserine. There is no consistent effect on labeling of PC. Table VII shows that, by contrast, labeling of triglyceride is markedly inhibited by levorphanol. To determine whether levorphanol also has a stimulatory effect on phospholipid synthesis at higher fatty acid concentrations, the experiments were repeated with linoleate at a concentration of 0.125 mmole/liter. This unsaturated fatty acid is more readily complexed to albumin in higher concentrations than the saturated palmitate. Vir-

TABLE VI

Effect of Levorphanol on $^{14}CO_2$ Production from Linoleic Acid-1-^{14}C by Granulocytes

	15 min				30 min				60 min			
	Exp. 1	%	Exp. 2	%	Exp. 1	%	Exp. 2	%	Exp. 1	%	Exp. 2	%
Control	0.073		0.019		0.160		0.049		0.37		0.11	
2 mM Levorphanol	0.031	42	0.008	42	0.093	58	0.025	51	0.21	57	0.058	54

Granulocytes were incubated at 37°C for the indicated periods of time with and without levorphanol. Linoleic acid-1-^{14}C was added as an albumin complex, in a concentration of 50 mmoles/liter. Values are expressed as nmoles of $^{14}CO_2$ produced per 2×10^7 cells. %, per cent of control.

TABLE VII

Effect of Levorphanol on the Incorporation of Palmitic acid-1-^{14}C into the Lipids of Granulocytes

	Total PL		PC		PE		PI and PS		TG	
Minutes...	30	60	30	60	30	60	30	60	30	60
Control	0.45	0.74	0.25	0.40	0.044	0.063	0.047	0.055	0.37	0.80
2 mM levorphanol	0.67	0.95	0.17	0.28	0.15	0.23	0.27	0.29	0.026	0.17

Granulocytes were incubated with and without levorphanol for the indicated periods of time. Palmitic acid-1-^{14}C was added at a concentration of 18 nmoles in a total volume of 1.0 ml. Cells and medium were separated before extraction. Total PL were separated from triglycerides by thin-layer chromatography on neutral Silica Gel H, in petroleum ether: ethyl-ether: glacial acetic acid (70:30:1, v/v). Phospholipids were separated in chloroform-methanol-glacial acetic acid-water (100:56:20:10, v/v). In this latter solvent system unidentified compounds that migrated with the solvent front carried the same radioactivity in the absence and presence of levorphanol and have not been included in the Table. Phosphatidylinositol and phosphatidylserine were incompletely separated and cut as one spot. Results are expressed as nmoles of lipid formed per 2×10^7 cells.

tually identical results were obtained at the higher fatty acid concentrations.

The possibility was considered that the increased labeling of phospholipids was due to the formation of complexes between fatty acid-1-^{14}C and levorphanol, with R_f values closely similar to those of the phospholipids.

This appears to be excluded by the following experiment: levorphanol was added after the period of incubation with labeled fatty acid was completed, either before or after the reaction was stopped with chloroform: methanol (1:1). The labeling of lipids extracted in this manner was the same as that of control leukocyte extracts to which levorphanol had not been added.

Effect of levorphanol on the K^+ content of granulocytes. Previous observations in our laboratory have indicated that active transport of K^+ is dependent on glycolysis (14). Since no effect of levorphanol on either glycolysis or ATP stores of leukocytes has been detected, it was of interest to examine K^+ uptake in the presence of levorphanol. Leakage of K^+ in the face of normal lactate production would lend further support to the concept that levorphanol exerts an effect on the membrane itself. In Table VIII the effects are compared of levorphanol and of NaF (the latter in a concentration that abolishes glucose uptake and lactate production) on the K^+ content of granulocytes. It is evident that levorphanol (2 mmoles/liter) causes a significant loss of cellular K^+. Although in these experiments lactate determinations were not carried out, it is noteworthy that the typical drop in pH of the cell suspension during incubation, was abolished by NaF, but not by levorphanol.

DISCUSSION

Levorphanol reversibly inhibited several leukocytic functions. Some of these inhibitory effects were evident at rest, while others were apparent during phagocytosis. At rest levorphanol caused a marked reduction of acylation of lysophosphatides, a decrease in cellular K^+ content, a decreased oxidation of fatty acid-1-^{14}C present in the medium as albumin complexes, and a decreased reduction of nitrobluetetrazolium. In addition, fatty acid incorporation into total and several individual phospholipids including PE and phosphatidylinositol and/or phosphatidylserine was increased, whereas fatty acid incorporation into triglycerides was decreased.

When the granulocytes were engaged in phagocytosis levorphanol impaired bacterial killing, and inhibited particle uptake as determined by electron microscopy.[14] Levorphanol also markedly reduced or eliminated the striking stimulation, normally seen during phagocytosis, of the acylation of lysophosphatides, of the direct oxidation of glucose-1-^{14}C, and of the reduction of nitrobluetetrazolium.

Inhibition of acylation of lysocompounds by levorphanol could result from a reduction of energy metabolism in the cell. However, levorphanol had no effect on glycolysis, the major energy-producing pathway in gran-

TABLE VIII

Effect of Levorphanol on Potassium Content of Granulocytes

	μEq K^+ per 2×10^7 cells ±SE	% of Control	*P*
Control	0.66 ±0.032 (3)	100	
2 mM levorphanol	0.43 ±0.043 (4)	65	<0.01
20 mM NaF	0.28 ±0.073 (3)	42.5	<0.01

Granulocytes (from 5 to 8×10^7 cells in different experiments) were incubated in Hanks' solution for 60 min with or without the indicated agents. At the end of incubation, cells and medium were separated by centrifugation, and the K^+ content of the cell pellet was determined as described in the section on Methods. The medium contained 7 μEq K^+ per ml. Results are expressed as mean ±SE of the indicated number of experiments (n).

ulocytes. Thus, glucose uptake and lactate production were unaffected by a concentration of levorphanol that inhibits acylation. Moreover, levorphanol does not behave like known glycolytic inhibitors since such agents have been shown to reduce the incorporation of ^{14}C fatty acids into leukocyte phospholipids (27), whereas levorphanol actually increased incorporation of labeled fatty acids into several phospholipids.

The fact that inhibition of acylation is probably not due to accelerated breakdown of phospholipids (Table II), to a direct effect on the acylase (Table III), or to inhibition of glycolysis, leads us to suggest that levorphanol may exert a direct effect on the cell membrane, as has been suggested for bacteria (8, 9). Furthermore, since glycolysis provides the energy for K^+ transport by the granulocyte, inhibition of K^+ transport under conditions of apparently intact glycolysis is compatible with this hypothesis.

The effects of levorphanol on phagocytosis are of considerable interest. Electron microscopic evidence shows a markedly diminished uptake of latex particles and a moderate decrease in engulfment of bacteria. This, in the face of apparently intact glycolysis, presumably the source of energy for engulfment (1), once again supports the concept of drug induced alterations in the membrane. A decreased response in the presence of particles, of the direct oxidative pathway for glucose and of the stimulation of the reduction of nitrobluetetrazolium would be readily explained by inhibition of uptake of particles. It is not obvious however why these metabolic responses were completely eliminated since ingestion and degranulation, while less than in the absence of the drug, were not entirely abolished. These aspects require further study including examination of the effect of levorphanol on killing of other bacterial species (both producers and nonproducers of H_2O_2 [24]).

We have previously postulated that acylation of lysocompounds is involved in net membrane synthesis required during the engulfment phase of phagocytosis (3). If this postulate is correct, any agent that interferes with ingestion should also inhibit the stimulation of the acylase activity of the leukocytes. In this respect it is important that several inhibitors of both glycolysis and engulfment are also inhibitors of the acylase reaction.[16] Conversely, inhibition of the acylase should be associated with reduced engulfment. Thus, the morphological evidence of decreased uptake of particles under the influence of levorphanol also supports these concepts. It should be pointed out that while phospholipid synthesis by acylation of medium lysocompounds was inhibited both at rest and during phagocytosis, in the presence of polystyrene particles a 50% stimulation relative to the resting value was usually observed. Further, medium fatty acid incorporation into total and several individual phospholipids was actually increased when levorphanol was added. The remaining contribution of lecithin formation from medium lysolecithin, plus the enhanced synthesis of phospholipids, presumably via the phosphatidic acid pathway, may provide sufficient membrane material to account for the fact that some engulfment continued. It is not clear why uptake of latex particles was inhibited more strikingly than that of bacteria.

The present work on mammalian leukocytes, as well as the studies on bacteria, indicate clearly that morphine-like drugs produce, in addition to their effects on metabolic pathways, alterations in the properties of cell membranes. The relevance of these observations to the clinical effects of narcotic analgesics is uncertain, since the concentrations of the drug used in these studies were far higher than those encountered in body fluids after pharmacological dosage and since the effects were also shown by the nonnarcotic enantiomorph, dextrorphan.

[16] Unpublished results.

ACKNOWLEDGMENTS

This work was supported by U. S. Public Health Service Grants AM 05472 and MH 10227.

Dr. Wurster submitted this paper in partial fulfillment of the requirements for the Ph.D. degree.

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