Cyclic 3′,5′-Adenosine Monophosphate in the Human Leukocyte: Synthesis, Degradation, and Effects on Neutrophil Candidacidal Activity

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Abstract Prostaglandins E_1 and E_2 (PGE_1 and PGE_2) stimulate adenyl cyclase activity in broken cell preparations of normal human leukocytes, whereas prostaglandin $F_{1\alpha}$ produces no effect. PGE_1 and PGE_2 also cause increased accumulation of cyclic 3′,5′-adenosine monophosphate–^{3}H (^{3}H-labeled AMP) in intact leukocytes which have been preincubated with adenine-^{3}H in vitro. Theophylline inhibits leukocyte phosphodiesterase activity and potentiates the stimulatory effect of the prostaglandins on intracellular accumulation of cyclic 3′,5′-AMP–^{3}H.

The ability of human granulocytes in vitro to kill *Candida albicans* was consistently inhibited by PGE_1 and theophylline. This effect was reproduced by dibutyryl cyclic 3′,5′-AMP, a lipid-soluble analogue of the endogenous nucleotide. The inhibition of candidacidal activity could not be accounted for by drug effects on phagocytosis, oxygen consumption, or hexose monophosphate shunt activity. These results are consistent with the hypothesis that increased intracellular concentrations of cyclic 3′,5′-AMP impair the granulocyte's ability to kill *C. albicans*, but the precise mechanism of inhibition has not yet been defined.

INTRODUCTION

Several observations are compatible with the possibility that cyclic 3′,5′-adenosine monophosphate (AMP) may mediate hormonal effects as a "second messenger" in the leukocyte. (*a*) Glucagon activates human leukocyte phosphorylase in vitro, and may do so by the same mechanism as in the liver (1). (*b*) Methylxanthines potentiate epinephrine's inhibitory effect on the release of granulocyte histamine triggered by antigen-reagin complexes in vitro (2). Such potentiation can usually be demonstrated in tissues in which an effect of epinephrine is mediated by cyclic 3′,5′-AMP. (*c*) Epinephrine and prostaglandin E_1 (PGE_1) stimulate accumulation of cyclic 3′,5′-AMP in human leukocytes (3). (*d*) Cyclic AMP and theophylline inhibit release of lysosomal β-glucuronidase by phagocytic leukocytes (4).

We have found that adenyl cyclase and phosphodiesterase are present in the human leukocyte, and that intact leukocytes can synthesize radioactive cyclic 3′,5′-AMP derived from radioactive adenine. Accumulation of intracellular cyclic 3′,5′-AMP is increased by compounds that activate adenyl cyclase; this effect is markedly potentiated by methylxanthines, which inhibit phosphodiesterase. These same compounds also inhibit the activity of human granulocytes to kill *Candida albicans*, an effect which is reproduced by dibutyryl cyclic 3′,5′-AMP, a lipid-soluble analogue of the endogenous nucleotide. This inhibitory action is probably independent of changes in rate of phagocytosis or in postphagocytic events such as increased oxygen consumption and hexose monophosphate shunt activity in granulocytes; however, the actual mechanism remains unknown.

METHODS

All experiments were performed on cells isolated from heparinized venous blood of hematologically normal subjects.

Isolation of leukocytes for enzyme assays. Blood was sedimented with 3% dextran in 0.9% saline (1 volume dextran solution per 2 volumes of blood) for 45–60 min, and the leukocyte-rich supernatant was centrifuged at 150 *g* for 10 min at 4°C. (The remainder of the isolation procedure was performed at this temperature.) The cell button was

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resuspended in 20 ml of 0.32 M sucrose and again centrifuged at 150 g for 10 min, after which the button contained 15–20 leukocytes to one platelet (see Results). Contaminating erythrocytes were then removed by hypotonic lysis (5), and the final leukocyte button was resuspended in 0.32 M sucrose ($2–3 \times 10^7$ leukocytes per ml). The cell suspension was subjected to sonication for 30 sec in a Biosonik sonicator (Brownwill Scientific, Rochester, N. Y.) at a setting of 40, which was sufficient to break up 95% of the leukocytes. This sonicate served as the source of enzyme in both the adenyl cyclase and phosphodiesterase assays.

Isolation of leukocytes for studies other than adenyl cyclase and phosphodiesterase. After sedimentation with dextran as described above, the leukocyte-rich supernatant was washed twice by centrifugation (150 g for 10 min at 20°C) in Hanks' balanced salt solution (BSS) containing 20% fetal calf serum and 2 U/ml of sodium heparin (Riker Laboratories, Northridge, Calif.). For studies of respiration and glucose oxidation, contaminating red cells were removed by hypotonic lysis (5). For measurement of candidacidal activity and of conversion of adenine-^{3}H to cyclic 3',5'-AMP-^{3}H, the cells were resuspended in Hanks' BSS containing 25% normal AB human serum. For the other studies McCoy's or Spinner-modified minimal essential medium was substituted for Hanks' BSS, and cells were present at 1–2 $\times 10^7$/ml.

Isolation of platelets. In two experiments the supernatant fluid after centrifugation (150 g) of leukocyte-rich plasma was used as a source of platelets for the adenyl cyclase assay. This supernatant fluid was centrifuged at 1200 g for 10 min at 4°C, and the platelet button was washed with sucrose and exposed to hypotonic lysis of red cells, followed by sonication, exactly as described above.

Isolation of lymphocytes. After sedimentation of heparinized blood with dextran, lymphocytes were separated from other leukocytes on a glass-wool column (6). The resulting lymphocyte preparations, 97–100% pure, were resuspended in ice-cold 0.32 M sucrose and subjected to repeated centrifugation, hypotonic lysis of erythrocytes, and sonication under the same conditions described for leukocytes and platelets.

Assay of adenyl cyclase. Adenyl cyclase was measured as described by Krishna, Weiss, and Brodie (7). The incubation mixture contained, in a final volume of 0.6 ml: tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.3 (4×10^{-2} mole/liter); theophylline (1×10^{-2} mole/liter); $MgCl_2$ (3.3×10^{-3} mole/liter); 8-adenosine triphosphate-^{14}C (2×10^{-3} mole/liter, 1–5 μCi/μmole, obtained from Schwarz Bio Research Inc., Orangeburg, N. Y.); and enzyme (the equivalent of 0.5–5 mg leukocyte protein). Drugs such as NaF or prostaglandins (obtained from the The Upjohn Pharmaceutical Co.) were added immediately before the reaction was started by adding substrate (ATP). Tubes were incubated at 37°C for various times (0–15 min), and the reaction was terminated by immersion of tubes in boiling water for 3 min. "Carrier" cyclic 3',5'-AMP, 0.5 mg, was added to each tube just before boiling. After centrifugation of the boiled reaction mixture the supernatant fluid was chromatographed on a Dowex 50-H$^+$ column as described by Krishna et al. (7); 70–80% of nonradioactive "carrier" cyclic 3',5'-AMP appeared in the third 2 ml fraction, as measured by optical density at 260 mμ. After other adenine nucleotides were removed from this fraction by coprecipitation with $Ba(OH)_2$ and $ZnSO_4$, 1 ml of supernatant was mixed with 15 ml of phosphor mixture and the radioactivity was determined in a liquid scintillation spectrometer. The amount of cyclic 3',5'-AMP-^{14}C formed was corrected for recovery, determined by optical density (at 260 mμ) of the carrier nucleotide present in the $Ba(OH)_2$-$ZnSO_4$ supernatant. Each experimental point was determined in duplicate, the values differing by not more than 5%.

Measurement of degradation of cyclic 3',5'-AMP. This assay measured the disappearance of cyclic 3',5'-AMP-^{3}H after exposure to sonicated leukocytes. The substrate (cyclic 3',5'-AMP-^{3}H) was separated from products such as adenosine-^{3}H and 5'-AMP-^{3}H by Dowex 50-H$^+$ chromatography and precipitation with $Ba(OH)_2$ and $ZnSO_4$, as described by Krishna et al. (7) and outlined above. The incubation mixture included, in addition to enzyme (sonicated leukocytes), tris(hydroxymethyl)aminomethane-HCl buffer, pH 7.4 (4×10^{-2} mole/liter); $MgCl_2$ (2×10^{-3} mole/liter); and cyclic 3',5'-AMP-^{3}H (4×10^{-4} mole/liters, 0.05–0.2 μCi/μmole, obtained from New England Nuclear) in a total volume of 1 ml. The reactions was initiated by addition of cyclic 3',5'-AMP-^{3}H, followed by incubation at 37°C for various times, and terminated by immersion of the tubes in boiling water for 3 min. Just before boiling, 0.1 ml of a carrier solution of cyclic 3',5'-AMP-^{14}C (5 mg/ml, 0.01 μCi/μmole, obtained from New England Nuclear) was added. After Dowex 50-H$^+$ chromatography and $Ba(OH)_2$-$ZnSO_4$ precipitation of the third 2 ml fraction of eluate, 1 ml of supernatant was added to 15 ml of a phosphor mixture and the radioactivity of both ^{3}H and ^{14}C was determined by standard techniques in a liquid scintillation spectrometer. The ratio of ^{3}H to ^{14}C was proportional to the amount of cyclic 3',5'-AMP remaining in the incubation mixture after enzymatic degradation.

Incorporation of adenine-^{3}H into cyclic 3',5'-AMP by intact leukocytes. A method previously applied to adipocytes (8, 9) and brain slices (10) was adapted to human leukocytes as follows. To a 20 ml suspension of leukocytes (1×10^7/ml) in Hanks' BSS containing 25% human AB serum, 10 μCi of adenine-^{3}H (6 Ci/mmole) was added, and the mixture was incubated for 40 min (unless otherwise noted) at 37°C. At the end of this preincubation, 2-ml aliquots of the cell suspension were added to 25-ml Erlenmeyer flasks containing appropriate amounts of the drugs to be studied, and the incubation was continued at 37°C for another 10 min. The reaction was terminated by centrifugation at 0°C and 800 g for 1 min. An aliquot of the supernatant fluid was removed for measurement of radioactivity, the cell button was resuspended in 0.7 ml of water containing 0.5 mg nonradioactive cyclic 3',5'-AMP, and the tube was immersed in boiling water for 3 min. After centrifugation the supernatant fluid was subjected to the same Dowex 50-H$^+$ chromatography and $Ba(OH)_2$-$ZnSO_4$ precipitation described above. Recovery of cyclic 3',5'-AMP was measured by optical density, as in the adenyl cyclase assay. Radioactivity of 1 ml of the $Ba(OH)_2$-$ZnSO_4$ supernatant was determined in a liquid scintillation spectrometer. Results were expressed as per cent of adenine-^{3}H radioactivity recovered in cyclic 3',5'-AMP per 10^8 leukocytes per 10 min.

Confirmation that measured radioactivity was cyclic 3',5'-AMP. ^{14}C and ^{3}H radioactivity in the third column fraction after $Ba(OH)_2$-$ZnSO_4$ precipitation was tested for authenticity by comparison with authentic cyclic 3',5'-AMP in four ways.

(*a*) Samples were placed on a Dowex 1-Cl$^-$ column and eluted with 0.01 N HCl as described by Krishna et al. (7). Recovery from the column of either the ^{3}H or the ^{14}C radioactivity and the authentic cyclic 3',5'-AMP was quantitative (95–100%).

(*b*) Samples were subjected to chromatography on Whatman No. 1 paper in two different solvent systems: (*i*) isobutyric acid-NH_3 (sp gr 0.88)-EDTA (0.1 mole/liter)-H_2O

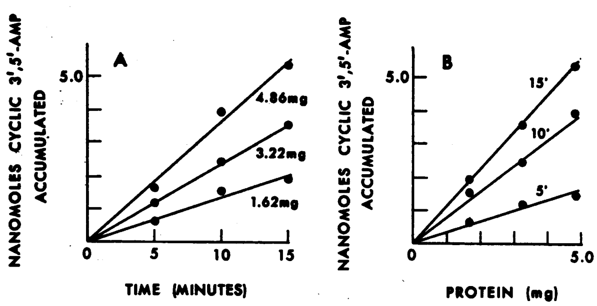

FIGURE 1 Production of cyclic 3',5'-AMP by sonicated leukocytes. Each tube contained 10^{-2} M NaF. (A) Cyclic 3',5'-AMP vs. time, for three different protein concentrations. (B) Cyclic 3',5'-AMP vs. protein concentration, at three different time intervals. Each value represents the mean of duplicate determinations which differed by not more than 5%.

(100:42:1.6:55.8); (*ii*) ethanol-NH_4 acetate (1 mole/liter, pH 7.4) (75:30).

(*c*) Radioactivity was added to 100 mg authentic nonradioactive cyclic 3',5'-AMP and dissolved by heating in 4 ml water; the solution was cooled and poured through Whatman No. 1 filter paper. The crystals on the paper were washed repeatedly with ice-cold absolute ethanol and dried. A small amount (about 5 mg) of the remaining crystalline material was weighed and redissolved in 0.1 N NaOH and counted in a liquid scintillation counter. The remaining material was recrystallized two more times, and its specific activity was determined in the same way.

(*d*) The "unknown" ^{14}C or ^{3}H radioactivity was mixed with authentic radioactive cyclic 3',5'-AMP (unknown ^{14}C with authentic ^{3}H, and unknown ^{3}H with authentic ^{14}C). The mixtures were subjected to partial degradation by purified beef heart phosphodiesterase (supplied by Dr. Gopal Krishna), which converts cyclic 3',5'-AMP to 5'-AMP. Incubations were continued at 37°C for 2 hr under conditions exactly similar to those described above for measurement of degradation of cyclic 3',5'-AMP. After boiling, the incubation mixtures were subjected to Dowex 50-H^+ chromatography followed by $Ba(OH)_2$-$ZnSO_4$ precipitation.

Measurement of phagocytosis. *Candida albicans* were cultivated as previously described (11) and washed with saline before use. ^{32}P-labeled microorganisms were obtained by incubating log phase cultures for 18 hr in medium containing 100 μCi/ml of carrier-free ^{32}P. Unlabeled heat-killed *Candida albicans* were added to neutrophils (1×10^7/ml) suspended in McCoy's medium containing 20% normal human AB serum at a ratio of 1.8 *Candida* to 1 neutrophil. After incubation for 15 min at 37°C, permanent methanol-fixed Giemsa preparations were made from the mixture using a cytocentrifuge (Shandon Instrument Co., London). Ingestion of *Candida* was measured (in a blinded fashion) by counting the number of ingested organisms in 150 neutrophils. Uptake of ^{32}P-labeled *Candida* by glass-adherent leukocyte monolayers at 37°C was assessed by adding radioactive yeast for periods up to 15 min and then thoroughly washing the monolayers with warm BSS and quantitating the leukocyte-associated radioactivity by counting in a liquid scintillation spectrometer.

Metabolic studies. Leukocyte preparations were freed of contaminating erythrocytes by hypotonic lysis (5) and of platelets by differential centrifugation as described above. Production of $^{14}CO_2$ from glucose-1-^{14}C (obtained from New England Nuclear) was determined as previously described (12), except that *C. albicans* (1.8 yeasts per neutrophil) was the test particle. Oxygen consumption was measured with a Clark electrode and a Gilson Model KM recorder (Gilson Medical Electronics, Inc., Middleton, Wis.) (12).

Candidacidal activity. Leukocytes were tested against 3- to 5-day-old broth cultures of yeast-phase *C. albicans,* strain 820, as described previously (11). Between 97 and 99.5% of the organisms were viable by the test of methylene blue exclusion (11). Plastic test tubes containing 2.5×10^6 neutrophils in 0.75 ml of Hanks' BSS with 33% normal AB serum and any drug or drug combination to be tested were first incubated in a 37°C water bath for 30 min. After this preincubation, 2.5×10^6 *Candida* cells (in 0.25 ml of BSS) were added and the tubes were rotated at 30 rpm and 37°C. After 15 min of rotation, permanent slides were prepared from a drop of the mixture with a cytocentrifuge (Shandon, London), and after 60 min the incubate was treated with sodium deoxycholate and 2×10^{-4} M methylene blue to permit determination of the percentage of killed *C. albicans* (11). In a few experiments, the drug effects were confirmed by simultaneous evaluation by standard colony counting techniques, thus ruling out a direct effect of the drugs used on staining characteristics of the organisms. In each experiment the "60 min" candidacidal activity of the subject's leukocytes in the absence of drugs served as the control against which the drug effects were evaluated.

RESULTS

Cyclic 3',5'-AMP metabolism

Adenyl cyclase. Preliminary experiments estalished that Mg^{++} ion was required for optimal adenyl cyclase activity. A maximum effect was reached when Mg^{++} concentration was 1.5–2.0 times that of ATP, as shown previously by Birnbaumer, Pohl, and Rodbell (13) in adipocyte ghosts. Substrate (ATP) concentrations of 1–2 $\times 10^{-3}$ mole/liter, produced maximal activity, and a 2×10^{-3} M concentration was routinely used. NaF (at 1×10^{-2} mole/liter) maximally stimulated adenyl cyclase activity. Production of cyclic 3',5'-AMP was linear with

TABLE I
Recrystallization of Radioactivity in $Ba(OH)_2$-$ZnSO_4$ Supernatant

	Specific activity	
	A	B
	cpm/mg	
Before recrystallization	63.2	72.0
First recrystallization	61.0	73.8
Second recrystallization	61.6	75.9
Third recrystallization	67.8	74.2

Radioactivity was mixed with authentic cyclic 3',5'-AMP, dissolved, and recrystallized as described in the text. (A) ^{14}C in $Ba(OH)_2$-$ZnSO_4$ supernatant from adenyl cyclase assay. (B) ^{3}H in Ba-Zn supernatant from experiment measuring incorporation of adenine-^{3}H into cyclic 3',5'-AMP.

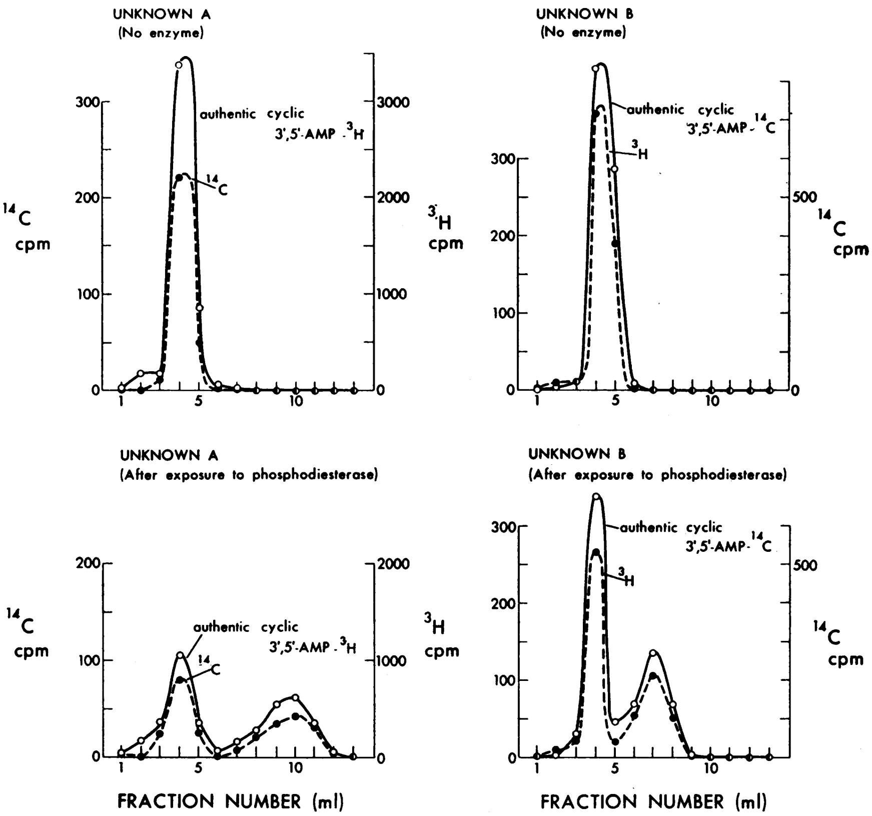

FIGURE 2 Effect of purified phosphodiesterase on chromatography of radioactivity in supernatant after $Ba(OH)_2$-$ZnSO_4$ precipitation. Unknown A (left) = ^{14}C radioactivity obtained from adenyl cyclase assay, combined with authentic cyclic 3′,5′-AMP-^{3}H. Unknown B (right) = ^{3}H radioactivity obtained from metabolism of adenine-^{3}H by intact leukocytes, combined with authentic cyclic 3′,5′-AMP-^{14}C. Samples were incubated with water (upper panels) or with purified beef heart phosphodiesterase (lower panels), then placed on Dowex 50-H^+ columns and eluted with water. See text for details.

both protein concentration (0–4.8 mg protein per tube) and time (0–15 min) (Fig. 1).

The ^{14}C radioactivity obtained from incubation of sonicated leukocytes with ATP-^{14}C was identified as cyclic 3′,5′-AMP-^{14}C in four ways.

(*a*) It was bound by a Dowex 1-Cl^- column at pH 7.8 and eluted by 0.01 N HCl in the same fractions as authentic cyclic 3′,5′-AMP.

(*b*) Paper chromatography in isobutyric acid-NH_3-EDTA showed that the "unknown" ^{14}C migrated with the same R_f (0.62) as authentic cyclic 3′,5′-AMP. Both the ^{14}C and the authentic nucleotide migrated with an R_f of 0.46 in the ethanol-NH_4-acetate solvent system. In both systems the ^{14}C and authentic cyclic 3′,5′-AMP were easily separated from adenosine, ATP, ADP, and 5′-AMP.

(*c*) The specific activity of a mixture of "unknown" ^{14}C and authentic nonradioactive cyclic 3′,5′-AMP remained constant through three successive recrystallizations (Table I, column A).

(*d*) Further evidence that this ^{14}C radioactivity was authentic cyclic 3′,5′-AMP was obtained by incubating a mixture of the "unknown" ^{14}C and authentic ^{3}H nucleotide with a purified preparation of phosphodiesterase. In the absence of enzyme the ^{14}C was eluted from Dowex 50-H^+ in the same fractions as authentic cyclic 3′,5′-AMP-^{3}H (Fig. 2 A, top). The phosphodiesterase, however, converted about 50% of both the ^{14}C and the ^{3}H to a product which appears in a later peak (Fig. 2 A, bottom). The second peak coincides with that of authentic 5′-AMP. Treatment of the column fractions with $Ba(OH)_2$ and $ZnSO_4$ caused both the ^{14}C and ^{3}H in the second peak to precipitate quantitatively (like 5′-AMP [5]), while radioactivity in the first peak remained in the supernatant. The phosphodiesterase reaction was not carried to completion because the supply of purified en-

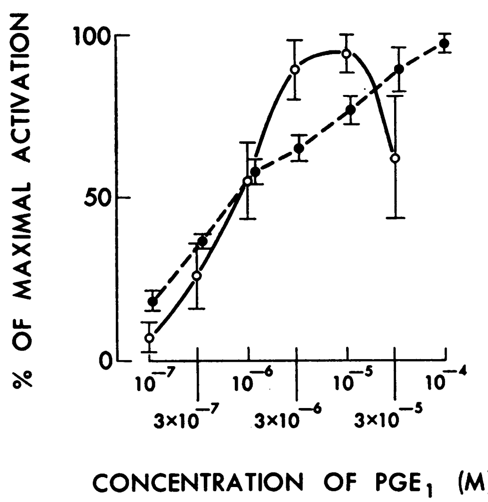

FIGURE 3 Effect of varying concentrations of PGE_1 on adenyl cyclase activity (open circles) in sonicated leukocytes and on accumulation of cyclic 3',5'-AMP-^3H (solid circles) by intact leukocytes after preincubation with adenine-^3H. 10^{-2} M theophylline was present in all reaction tubes. Values are normalized as per cent of the maximal activation caused by any concentration of PGE_1 in an individual experiment. Each point on the curve of adenyl cyclase activity represents the mean ±SE of values from four subjects studied on different days. Each point on the curve of cyclic 3',5'-AMP-^3H accumulation represents the mean ±SE of four experiments performed on cells from a single subject. Significance of differences from control (i.e. zero activation) are as follows: adenyl cyclase activity (paired t test): 10^{-6} mole/liter ($P < 0.025$), 3×10^{-6} mole/liter ($P < 0.005$), 10^{-5} mole/liter ($P < 0.001$), 3×10^{-5} mole/liter ($P < 0.05$); cyclic 3',5'-AMP-^3H accumulation (standard t test): 3×10^{-7} mole/liter and higher concentrations ($P < 0.01$).

zyme was limited. A parallel experiment with the same enzyme indicated comparable degradation of authentic nonradioactive cyclic 3',5'-AMP, thus ruling out the distant possibility that the "unknowns" and both authentic radioactive nucleotides all contained equal amounts of a chromatographically indistinguishable major contaminant.

In order to assess the contribution of platelet adenyl cyclase to the total enzyme activity measured in our usual preparations, the enzyme was measured in both platelets and leukocytes of two patients. The ratio of leukocytes to platelets was measured by phase microscopy of the "leukocyte" suspension just before sonication. The results (Table II) confirmed earlier reports (14) that adenyl cyclase is present in blood platelets, but platelets could not have contributed more than 1% of the total enzyme activity measured in our leukocyte preparations.

Similarly, the relative contribution of lymphocytes to the adenyl cyclase measured in our mixed population of leukocytes was determined by measuring adenyl cyclase in purified preparations of lymphocytes from two patients. On a per-cell basis lymphocytes contained less than 20% of the adenyl cyclase activity present in the preparation of mixed leukocytes (Table II), although enzyme activity per milligram cell protein (not shown) was unchanged. Since lymphocytes accounted for 26–30% of the total cells present in such preparations, they contributed less than 5% of the total adenyl cyclase activity in our mixed leukocyte suspensions.

The stimulatory effects of three prostaglandins on leukocyte adenyl cyclase activity was examined. A dose-response curve for prostaglandin E_1, the most powerful stimulator of the three, is shown in Fig. 3. Activation of cyclase was maximal at 10^{-5} M PGE_1, and half-maximal at about 10^{-6} mole/liter. The effects on leukocyte adenyl cyclase of two other prostaglandins, PGE_2 and $PGE_{1\alpha}$, are shown in Table III. Leukocytes of four subjects varied in basal as well as drug-stimulated enzyme activity, when values were expressed on the basis of leukocyte protein. When values were normalized, with enzyme activity in the presence of NaF being taken as 100% for each individual subject, the relative effects of the prostaglandins and NaF were quite consistent from subject to subject. NaF was the most powerful stimulator. At 10^{-5} mole/liter, PGE_1 was a more active stimulator than PGE_2, and $PGF_{1\alpha}$ was inactive.

Degradation of cyclic 3',5'-AMP by sonicated leukocytes. Disappearance of cyclic 3',5'-AMP was linear with time (0–40 min) and increasing amounts of sonicate protein (0–7 mg). Like adenyl cyclase, leukocyte degradative activity varied but averaged 800 ±280 (SD) pmoles/mg protein per min in four subjects. Theophylline (1×10^{-2} mole/liter) produced 44 ±10% (SD) inhibition of nucleotide degradation in leukocytes from these four subjects ($P < 0.01$ by paired t test). In one

TABLE II

Relative Contributions of Platelets and Lymphocytes to Leukocyte Cyclase Activity (in Presence of NaF)

Experiment.......	1	2	3	4
Adenyl cyclase activity, *pmoles/10⁷ cells per min*				
Leukocyte preparation	54	28	86	48
Platelets	0.38	0.39	—	—
Purified lymphocytes	—	—	11	9.1
Leukocyte: platelet ratio in leukocyte preparation	14:1	20:1	—	—
Lymphocytes in leukocyte preparation, %	—	—	30	26
Relative contribution to measured adenyl cyclase activity by				
Platelets	0.027/54	0.020/28	—	—
%	0.05	0.07	—	—
Lymphocytes, %	—	—	3.8	4.9

TABLE III
Effect of Prostaglandins and NaF on Leukocyte Adenyl Cyclase Activity

Drug	Experiment No. 1	2	3	4	Mean ±SE	Per cent of maximal activity (NaF) mean ±SE
	pmoles/mg protein per min					
None	7.40	2.02	1.66	3.70	3.70 ±1.32	11.9 ±1.04
$PGF_{1\alpha}$, 10^{-5} mole/liter	8.00	2.36	2.10	4.01	4.12 ±1.36	13.4 ±1.23
PGE_2, 10^{-5} mole/liter	11.0	3.69	3.64	4.84	5.79 ±1.76	19.7 ±2.07*
PGE_1, 10^{-5} mole/liter	13.1	4.87	5.23	6.86	7.52 ±1.91	26.7 ±3.0‡
NaF, 10^{-2} mole/liter	49.5	18.2	15.5	35.0	29.6 ±7.93*	100§

Difference from no drug (paired *t* test):
* $P < 0.05$.
‡ $P < 0.025$.
§ $P < 0.001$.

additional experiment, caffeine (1×10^{-2} mole/liter) produced similar inhibition of degradation (68%).

Incorporation of adenine-³H into cyclic 3',5'-AMP–³H. Intact leukocytes removed 80% of adenine-^3H from the extracellular medium in 40 min (Fig. 4 A). Fig. 4 B illustrates the effect of increasing time of preincubation with adenine-^3H on the accumulation of intracellular radioactive cyclic 3',5'-AMP during a subsequent exposure of the cells to theophylline (1×10^{-2} mole/liter) and PGE_1 (1×10^{-5} mole/liter). Accumulation of radioactive cyclic 3',5'-AMP was maximal after 40 min preincubation.

The radioactivity present in the $Ba(OH)_2$-$ZnSO_4$ supernatant of the third column fraction obtained from such experiments was identified as cyclic 3',5'-AMP–^3H by the same methods used to identify the ^{14}C obtained in the adenyl cyclase assay. (*a*) Dowex 1–Cl^- chromatography showed supernatant ^3H to be bound by the exchange resin and eluted by 0.01 N HCl in the same fashion as authentic nucleotide. (*b*) The ^3H compound migrated with authentic nucleotide in two paper chromatographic systems ($R_f = 0.62$ in isobutyric acid–NH_3–EDTA, and 0.46 in ethanol-NH_4-acetate), and was separated from ATP, ADP, 5'-AMP, and adenosine. (*c*) The "unknown" ^3H was successively recrystallized to constant specific activity (Table I, column B). (*d*) The purified phosphodiesterase converted both the ^3H and authentic ^{14}C-labeled nucleotide to 5'-AMP (Fig. 2 B).

After the standard 40 min preincubation, PGE_1 in the presence of theophylline (1×10^{-2} mole/liter) produced a dose-dependent increase in intracellular cyclic 3',5'-AMP–^3H (Fig. 3). As with adenyl cyclase, half-maximal effect was produced by about 10^{-6} M PGE_1. If theophylline inhibited degradation of cyclic 3',5'-AMP in intact cells as it does in broken cell preparations, it might be expected that ^3H-labeled nucleotide would accumulate in the presence of theophylline alone, but it did not (Table IV). Lack of accumulation could be due to a relatively low basal rate of synthesis of the nucleotide, incomplete inhibition of degradation, or leakage of cyclic 3',5'-AMP from the cell (9). PGE_1 (1×10^{-5} mole/liter) produces a marked increase in accumulation of cyclic 3',5'-AMP–^3H; the increase is potentiated by theophylline (Table IV), an effect which is consistent with stimulation of nucleotide synthesis combined with inhibition of nucleotide degradation.

The three prostaglandins tested showed the same order of potency in stimulating intracellular accumulation of the cyclic nucleotide as on adenyl cyclase. At 10^{-5} mole/liter, PGE_1 was a better stimulator than PGE_2, and $PGF_{1\alpha}$ had relatively little effect (Table IV). As was the case with adenyl cyclase, the accumulation of nucleotide-^3H by intact cells varied in different subjects,

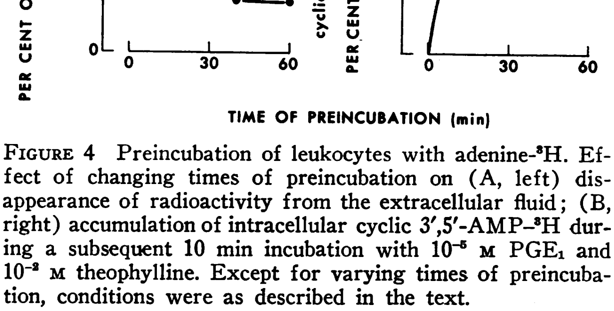

FIGURE 4 Preincubation of leukocytes with adenine-^3H. Effect of changing times of preincubation on (A, left) disappearance of radioactivity from the extracellular fluid; (B, right) accumulation of intracellular cyclic 3',5'-AMP-^3H during a subsequent 10 min incubation with 10^{-5} M PGE_1 and 10^{-2} M theophylline. Except for varying times of preincubation, conditions were as described in the text.

TABLE IV

Effect of Prostaglandins and Theophylline on Accumulation of Cyclic 3',5'-AMP–^3H in Intact Leukocytes

Drug	Experiment No. 1	2	3	4	Mean ±SEM	Per cent of maximum in each experiment mean ±SEM
	% adenine-^3H in cyclic 3',5'-AMP per 10^8 cells per 10 min					
None	0.12	0.37	0.31	0.11	0.23 ±0.07	5.03 ±0.86
Theophylline, 10^{-2} mole/liter	0.21	0.35	0.34	0.15	0.26 ±0.05	6.18 ±0.63
$PGF_{1\alpha}$, 10^{-5} mole/liter	0.13	0.32	0.25	0.14	0.21 ±0.05	5.03 ±0.93
$PGF_{1\alpha}$, 10^{-5} mole/liter + theophylline, 10^{-2} mole/liter	0.33	0.50	0.70	0.23	0.44 ±0.11	9.95 ±0.53*
PGE_2, 10^{-5} mole/liter	0.47	0.80	0.76	0.23	0.57 ±0.14‡	12.6 ±1.14§
PGE_2, 10^{-5} mole/liter + theophylline, 10^{-2} mole/liter	2.14	2.76	4.31	1.12	2.58 ±1.34‖	56.7 ±1.26*
PGE_1, 10^{-5} mole/liter	0.55	1.19	1.49	0.32	0.89 ±0.27	18.6 ±1.82§
PGE_1, 10^{-5} mole/liter theophylline, 10^{-2} mole/liter	3.60	5.16	7.47	2.00	4.55 ±1.17‖	100

* Difference from theophylline alone $P < 0.05$, standard t test.
‡ Difference from no drug $P < 0.05$, paired t test.
§ Difference from no drug $P < 0.05$, standard t test.
‖ Difference from theophylline alone $P < 0.05$, paired t test.

both in the presence and absence of drugs, when values were expressed in absolute terms (per cent of adenine-^3H converted to cyclic 3',5'-AMP–^3H per 10^8 cells per 10 min incubation). The relative effects of the drugs became quite consistent when results were normalized, with the effect of PGE_1 plus theophylline being taken as 100% (Table IV).

In three experiments (not shown) purified lymphocytes also incorporated radioactive adenine into cyclic 3',5'-AMP and responded to PGE_1 and theophylline in the same way. On a per-cell basis, this incorporation was slightly less than that of the mixed leukocyte preparations. This suggests that in the mixed leukocyte preparations the contribution by lymphocytes to the measured accumulation of cyclic nucleotide was of the same order of magnitude as their percentage in the differential count of leukocytes in the individual subjects (25–30%).

TABLE V

Effect of Drugs on Phagocytosis of C. albicans by Neutrophils

Drug	Phagocytosis* Microscopic	^{32}P uptake
Dibutyryl cyclic 3',5'-AMP, 3 × 10^{-3} mole/liter	77.1 ±13.8(7)‡	74.3 ±6.2(4)‡
PGE_1, 1 × 10^{-5} mole/liter	70.8 ±18.2(7)‡	59.0(2)
Theophylline, 3 × 10^{-3} mole/liter	74.6 ±21.7(8)§	70.5(2)

* Expressed as per cent of control. Mean ±SD. The number in parentheses indicates the number of subjects whose granulocytes were studied.
‡ Difference from control significant at $P < 0.01$, by paired t test.
§ Difference from control significant at $P < 0.05$, by paired t test.

Granulocyte function

Phagocytosis. Preincubation of neutrophils for 30 min with dibutyryl cyclic 3',5'-AMP (3 × 10^{-3} mole/liter), PGE_1 (1 × 10^{-5} mole/liter), and theophylline (3 × 10^{-3} mole/liter) produced a slight but consistent inhibitory effect on ingestion of *C. albicans* ($P < 0.01$), whether measured at a single time point (15 min) (Table V) or over 15 min, using radioactively labeled yeasts (Fig. 5). Reduction of phagocytosis was achieved without observable impairment of leukocyte viability (trypan blue exclusion).

Metabolic studies. Neutrophils (1.5 × 10^7/ml) were preincubated with or without drug for 15 min before the addition of *Candida albicans* in an enclosed chamber containing an oxygen electrode. Dibutyryl cyclic 3',5'-AMP, PGE_1, and theophylline produced no change in the slow rate of basal oxygen consumption or in the magnitude of the respiratory burst associated with phagocytosis (Table VI).

The production of $^{14}CO_2$ from glucose-1-^{14}C by leukocytes was measured during the 30 min after addition of *Candida*. Leukocytes were preincubated with saline or drug for 30 min before the addition of particles. PGE_1 had a modest inhibitory effect on glucose-1-^{14}C oxidation by phagocytic leukocytes, whereas theophylline and dibutyryl cyclic 3',5'-AMP had no effect (Table VI).

Electron microscopy after phagocytosis. Thin sections were prepared from pellets of phagocytic leukocytes incubated in the presence or absence of dibutyryl cyclic 3',5'-AMP (3×10^{-3} mole/liter), theophylline (3×10^{-3} mole/liter), or PGE_1 (1×10^{-5} mole/liter) and examined by electron microscopy. When sections were coded and examined as unknowns, we could detect no difference between control and drug-treated samples in regard to extent of degranulation or the discharge of granule contents into the phagocytic vacuoles.

Granulocyte candidacidal activity. Theophylline and dibutyryl cyclic 3',5'-AMP greatly diminished the ability of normal leukocytes to kill ingested *Candida albicans.* PGE_1 was less potent but produced statistically significant inhibition (Table VII). Since stock solutions of PGE_1 (3×10^{-3} mole/liter) were made with absolute ethanol, incubation tubes containing 1×10^{-5} M PGE_1 also contained 0.33% ethanol (v/v); however, this concentration of ethanol did not affect candidacidal activity (Table VII).

Candida cells preincubated with the various drugs and then washed with Hanks' BSS before their addition to leukocytes were killed to the same extent as organisms incubated in Hanks' BSS alone, indicating that the drugs acted to impair leukocyte function rather than to increase the resistance of the ingested fungi to intraleukocytic events. The inhibition of candidacidal activity could not be attributed to alterations in phagocytosis, because ingestion occurred to completion by 15 min after addition of *Candida* to cells in a ratio of 1:1, even in the presence of drugs.

The possibility that theophylline might potentiate the effect of PGE_1 was examined by using a combination of the two drugs at concentrations which individually produced small and not statistically significant changes in leukocyte candidacidal activity. In eight experiments the average inhibitory effect of the two drugs combined was greater than the sum of their individual inhibitory effects, but this difference was not statistically significant (Table VII).

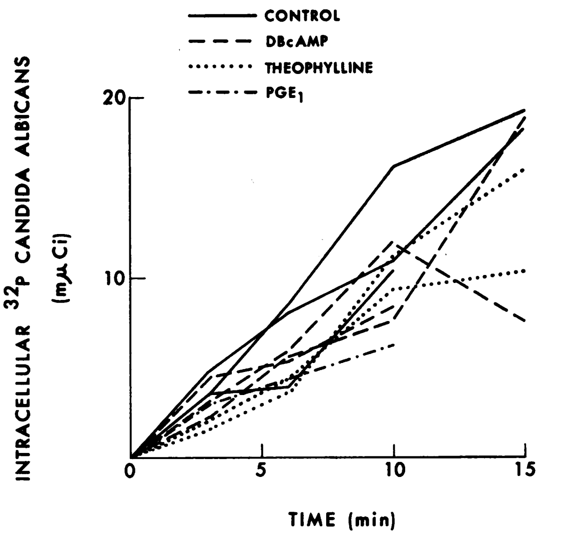

FIGURE 5 Uptake of ^{32}P-labeled *Candida albicans* by glass-adherent leukocyte monolayers as a function of time. See text for details. Each line represents the mean of duplicate determinations using the cells of single subject on one day. Cells were preincubated with saline or drug for 30 min before the addition of *Candida albicans* (1.8 yeasts per granulocyte). DBcAMP = dibutyryl cyclic 3',5'-AMP, 3×10^{-3} mole/liter; theophylline, 3×10^{-3} mole/liter; PGE_1 = prostaglandin E_1, 1×10^{-5} mole/liter.

DISCUSSION

These experiments were designed to answer two questions. (*a*) Does the human leukocyte contain the enzymatic machinery for synthesis and degradation of cyclic 3',5'-AMP? We found that it does. (*b*) What role does

TABLE VI

Drug Effects on Leukocyte Metabolism after Phagocytosis of C. albicans

Drug	O_2 consumption*	$^{14}CO_2$ production*
Dibutyryl cyclic 3',5'-AMP,		
3×10^{-3} mole/liter	108 ±27(7)	72.4 ±24(5)
PGE_1, 1×10^{-5} mole/liter	110 ±17(6)	62.3 ±24(5)‡
Theophylline,		
3×10^{-3} mole/liter	114 ±30(6)	117 ±26(5)

* Expressed as per cent of control ±SD. Number in parentheses indicates number of subjects whose leukocytes were studied.
‡ Difference from control significant at $P < 0.025$. None of the other differences are statistically significant (paired *t* test).

TABLE VII

Inhibitory Effect of Drugs on Leukocyte Candidacidal Activity

Drug	Candidacidal activity	Significance*
	% of control‡	$P <$
Dibutyryl cyclic 3',5'-AMP,		
1×10^{-3} mole/liter	29.7 ±2.1(6)	0.001
Theophylline		
1×10^{-3} mole/liter	32.8 ±11.8(5)	0.01
1×10^{-4} mole/liter	93.9 ±6.9(8)	NS
PGE_1 (in 0.33% ethanol)		
1×10^{-5} mole/liter	75.0 ±7.1(9)	0.01
5×10^{-6} mole/liter	84.1 ±7.3(8)	NS
PGE_1, 5×10^{-6} mole/liter + theophylline, 1×10^{-4} mole/liter		
Calculated "additive" effect	78.0 ±9.2(8)	§
Actual effect in combination	67.3 ±8.8(8)	§
Ethanol, 0.33%	101.5 ±6.8(5)	NS

* Paired *t* test.
‡ Mean ±SE (n).
§ Not statistically different by paired *t* test ($0.1 < P < 0.05$).

Table VIII
Relative Stimulatory Effects of Drugs on Synthesis of Cyclic 3′,5′-AMP

Compound	Leukocyte adenyl cyclase	Cyclic 3′,5′-AMP–^{3}H accumulation in leukocytes (in presence of theophylline, 1×10^{-2} mole/liter)	Platelet adenyl cyclase (from Wolfe and Shulman [12])
	Stimulation relative to PGE₁		
PGE_1*	100	100	100
PGE_2	49	54	59
$PGF_{1\alpha}$	13	5	4
NaF, 10^{-2} mole/liter	680	—	55

* Concentration of prostaglandins: in leukocyte experiments, 1×10^{-5} mole/liter; in platelets, 3×10^{-6} mole/liter.

the cyclic nucleotide have in regulating granulocyte function? We cannot definitely answer this second question. Our data strongly suggest, however, that cyclic 3′,5′-AMP can mediate the pharmacologic effects of several compounds on granulocytes.

The presence of adenyl cyclase in the human leukocyte has been denied by Wolfe and Shulman (14) and recently demonstrated in a brief note by Scott (3). The present experiments agree with Scott. In our studies the activity per milligram of tissue protein of the leukocyte enzyme in the presence of NaF is comparable to that of rat cerebrum (7), and somewhat less than that described in human platelets (14). Wolfe and Shulman had leukocyte mixtures that were considerably contaminated by platelets (platelet: leukocyte ratio of 20:1 vs 1:14 in our experiments), and they may not have used a high enough concentration of leukocyte protein to allow detection of the adenyl cyclase in leukocytes.

The relative activities of the prostaglandins in stimulating leukocyte adenyl cyclase is the same as that described in platelets (Table VIII), although NaF produced a much greater relative stimulation in leukocytes than in platelets (14). PGE_1, the most potent prostaglandin tested, produced small but detectable stimulation of enzyme activity at about 1×10^{-7} mole/liter (Fig. 3), several orders of magnitude higher than the concentrations of prostaglandins described in human plasma. Accordingly, PGE_1 may prove useful as a pharmacologic tool for investigation of the metabolism and function of cyclic 3′,5′-AMP, but a "physiologic" effect of the compound on adenyl cyclase in leukocytes cannot be proposed on the basis of these experiments. The same qualification holds true for platelets.

Sonicates of human leukocytes are capable of degrading cyclic 3′,5′-AMP. Since the simple assay procedure we employed measured disappearance of the nucleotide rather than the appearance of product, it is not possible to be sure that degradation of the nucleotide was entirely due to a specific phosphodiesterase. However, the degradative activity was partially inhibited by theophylline and caffeine, an effect which has been described for phosphodiesterases from other tissues (15).

Present knowledge of the steps by which granulocytes kill *C. albicans* is incomplete (16), but suggests several steps at which these drugs might act. Ingestion of the organism to be killed is obviously important, but the slight inhibition of phagocytosis caused by these drugs (Table V, Fig. 5) does not seem (in the case of theophylline and dibutyryl cyclic 3′,5′-AMP, at least) sufficient to account for the more pronounced inhibition of candidacidal activity. Effects of these drugs on postphagocytic phenomena, such as increased oxygen consumption and increased activity of the hexosemonophosphate shunt (Table VI), were inconsistent or small relative to the inhibition of candidacidal activity (Table VII). Optimal candidacidal activity is presumed to require production of H_2O_2 and the action of granulocyte myeloperoxidase (16). Although we found no inhibition of myeloperoxidase activity by these drugs in vitro, an effect on H_2O_2 generation or the in vivo exposure of intracellular *Candida* to the action of myeloperoxidase has not been ruled out.

It is possible, as recently suggested by May, Levine, and Weissman (4), that cyclic 3′,5′-AMP may interfere with "degranulation," or release of granule enzymes into the phagocytic vacuole (or out of the cell). Quantitative measurement of this process is difficult. Experiments (not shown) measuring intracellular and extracellular enzymes (lysozyme and myeloperoxidase) after phagocytosis showed no clearcut effect of theophylline, PGE_1, or the dibutyryl analogue. The electron microscopy studies were similarly inconclusive.

Our experiments suggest that whatever the specific mechanism may be, the inhibitory effects of PGE_1 and theophylline may be mediated by a rise in intracellular concentrations of cyclic 3′,5′-AMP. Dibutyryl cyclic 3′,5′-AMP may act by mimicking the action of endogenous nucleotide. Critical examination of the present evidence suggests questions which must be resolved before the mediating role of cyclic 3′,5′-AMP can be proved.

(*a*) Theophylline (1×10^{-3} mole/liter) is a better inhibitor of candidacidal activity than is PGE_1, but the converse is true when accumulation of cyclic 3′,5′-AMP–^{3}H derived from adenine-^{3}H is examined. It is difficult to know how much weight to place on this discrepancy, which might be resolved by measurement of intracellular cyclic 3′,5′-AMP concentrations.

(*b*) Although the inhibitory effect of theophylline on leukocyte candidacidal activity was additive to that of PGE_1 (Table VII), a synergistic effect of the two drugs was not demonstrated (as it should be if the drugs act only by inhibition of phosphodiesterase and stimulation of adenyl cyclase, respectively). The failure to demonstrate synergism could be due to incorrect choice of concentration of either drug, as pointed out by Sutherland, Robison, and Butcher (15). Practical considerations would make more detailed examination of many doses of the drugs individually and in combination extremely difficult.

(*c*) PGE_1's effects on adenyl cyclase and candidacidal activity may be independent of one another. Additional compounds which stimulate adenyl cyclase must be studied. Our laboratory is presently investigating the effect of catecholamines on adenyl cyclase and leukocyte function, since it has been suggested that isoproterenol and epinephrine inhibit histamine release from leukocytes by stimulating adenyl cyclase (2). When a specific inhibitor of adenyl cyclase activation (e.g. a beta-adrenergic blocking agent vs. isoproterenol) becomes available, more critical experiments can be designed.

(*d*) Although the dibutyryl derivative of cyclic 3′,5′-AMP inhibits granulocyte function at the relatively high concentrations required to mimic the effects of endogenous nucleotide in other tissues (15), it could produce its effect by mechanisms not related to cyclic 3′,5′-AMP. Nonspecific or inconsistent effects of dibutyryl cyclic 3′,5′-AMP have been described in heart (16).

If cyclic 3′,5′-AMP does prove to mediate any or all of the pharmacologic effects of theophylline and the prostaglandins on leukocyte function, the question of the possible physiologic role of the endogenous nucleotide in leukocytes may still remain open. Exploration of this question will involve further investigation of other possible influences on adenyl cyclase or phosphodiesterase activity (e.g. immune complexes, catecholamines, and phagocytosis) and functions of the leukocyte in addition to candidacidal activity (e.g. motility, release of possible mediators of inflammation).

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