INHIBITOR OF ADENOSINE3',5'-MONOPHOSPHATE BINDING AND PROTEIN KINASE ACTIVITY IN LEUCOCYTE LYSOSOMES

PI-KWANG TSUNG and GERALD WEISSMANN

Department of Medicine, New York University of Medicine, New York, N. Y. 10016

Received February 22, 1973

SUMMARY: In contrast to other tissues (e.g. brain, heart), no cAMP dependent protein kinase activity and little cAMP-binding activity could be detected in crude homogenates of purified human PMN leucocytes. This was due to the presence of an inhibitor of cAMP binding and protein kinase activity in PMN leucocytes. Since the inhibitor was entirely segregated in PMN lysosomes (rich in β-glucuronidase and acid phosphatase), lysosome-free supernatants yielded cAMP-dependent protein kinase (>5-fold stimulation with 5 μM cAMP) and considerable cAMP binding activity. The inhibitor was not dialyzable, and unlike the usual protein kinase modulators, was heat-labile. Preparations of beef-heart protein kinase, treated with the PMN inhibitor, lost cAMP-binding and protein kinase activities simultaneously. The presence of this lysosomal inhibitor may invalidate studies of cAMP binding and protein kinase activities in crude homogenates prepared from lysosome-rich tissues.

INTRODUCTION

Exogenous cyclic adenosine 3',5'-monophosphate (cAMP), or agents which elevate endogenous cAMP in mixed populations of human peripheral blood leucocytes inhibit the release of various mediators of inflammation and tissue injury. These include lysosomal hydrolases (1), histamine (2), lymphocyte cytotoxins,(3) and the slow-reacting substance of anaphylaxis, SRS-A (4). We have previously suggested that these effects of cAMP are mediated by cAMP-dependent protein kinases which regulate microtubule function and thereby inhibit secretion of mediators (1, 5). Indeed, we have isolated, and partially purified,a protein kinase (6) from human polymorphonuclear leucocytes (PMN's). It was impossible, however, to express with any degree of confidence the increments in specific activity of the cAMP-dependent protein kinase, since little or no enzyme activity could be detected in crude homogenates of the cells. Nor did these homogenates display cAMP-binding activity, although preparations obtained by means of ammonium sulfate precipitation and various chromatographic separations

displayed both protein kinase and cAMP-binding activity (6). These observations proved to be due to an inhibitor of protein kinase and cAMP-binding present in PMN lysosomes, and the experiments described below will detail the evidence for this conclusion.

MATERIALS AND METHODS

PMN leucocytes and subcellular fractions. PMN leucocytes were obtained from freshly drawn, heparinized, human peripheral blood by the method of Böyum (7), using Hypaque-Ficoll density gradients. PMN fractions (5×10^8 cells/ml) contained 98-99% PMN's, with 1-2% eosinophiles; lymphocyte fractions (6×10^6 cells/ml) contained 98-99% lymphocytes, with 1-2% basophiles and monocytes; MLB (monocyte-lymphocyte-basophile) fractions (2.7×10^7 cells/ml) contained 79-85% lymphocytes, 10-15% monocytes, and 1-6% basophiles; platelet-plasma fractions contained 3×10^5 platelets/ml; and erythrocyte fractions contained 4.3×10^7 cells/ml. Subcellular fractionation of purified human PMN's was carried out essentially as previously described (8) except that lysosomal granules were sedimented at 27,000 x g for 15', and a small granule fraction was sedimented at 105,000 x g for 2 hours. Homogenization was carried out in five volumes of 0.25M sucrose containing 5 mM potassium phosphate buffer (pH 7.0) containing 6 mM mercaptoethanol and 10% glycerol (PMG) buffer. To avoid contamination of final supernatants by lysosomal materials, only those preparations were employed in which over 90% of total β-glucuronidase was sedimentable: this accounted for the presence of unruptured cells in the 800 x g "nuclei and debris" fractions. Lysosomal enzymes and proteins were assayed as previously (6, 8) described.

Protein kinase, cAMP binding, etc. Protein kinase activity with calf thymus histone (Worthington) as substrate was assayed in a final volume of 0.2 ml as previously described (6). Cyclic AMP-binding was determined by the method of Gilman (9) in the absence of protein kinase inhibitor, except that 50 mM phosphate buffer, pH 6.0, and 30 pmoles of ^{3}H-cAMP were used. Frozen and thawed cell fractions and beef heart were homogenized as above in PMG buffer for 20 strokes in a glass/Teflon homogenizer. The 800 x g supernatant was used for cAMP-dependent protein kinase and cAMP binding assays, except when the ammonium sulfate precipitate step was added for the preparation of beef heart protein kinase (10). In the latter case, the homogenate was centrifuged at 27,000 x g for 30 min. in the cold. Solid ammonium sulfate was added slowly with stirring to the supernatant fraction to 50% saturation. After 30 min. of stirring, the precipitate was collected by centrifugation at 16,000 x g for 20 min. and dissolved in 6% of the crude extract volume of PMG buffer. After dialysis (14 hrs., 20 volumes) the preparation was tested for protein kinase and cAMP binding activity in the absence or presence of various PMN preparations. Cyclic AMP was purchased from Boeringer-Mannheim, γ-^{32}P-ATP from New England Nuclear; ^{3}H-cAMP from Schwartz-Mann was purified by means of Dowex AG 50W-X8 before use; Sephadex G-75 was from Pharmacia.

RESULTS AND DISCUSSION

Human PMN's were subjected to subcellular fractionation and the fractions subjected to assay for cAMP-dependent protein kinase and cAMP-

TABLE I

DISTRIBUTION OF PROTEIN KINASE ACTIVITY*, CYCLIC AMP BINDING**, ACTIVITY, AND LYSOSOMAL ENZYME ACTIVITY IN SUBCELLULAR FRACTIONS OF HUMAN LEUCOCYTES

Fraction	Total Protein (mg)	^{32}P incorporated*** -histone -cAMP	-histone +cAMP	+histone -cAMP	+histone +cAMP	cAMP Binding Activity+	β-glucuronidase‡	Acid Phosphatase‡
Total homogenate	30	12.4	15.5	57	57	0.180	80	140
Nuclear & debris fraction	10.8	18.2	20.1	79	96	0.158	164	211
27,000 x g pellet	7.2	20.6	25.9	127	95	0.159	397	375
105,000 x g pellet	2.0	40.0	47.0	200	180	0.492	168	221
105,000 x g supernatant	8.0	17.8	23.3	135	315	3.109	8	80
Ammonium sulfate ppt.	3.9	--	--	106	348	--	--	--
G-75 eluate	0.2	--	--	141	776	--	--	--

* 20 μg of each fraction incubated 5' at 37°C with or without cAMP at 5 X 10^{-6}M for protein kinase assay in the presence of 100 μg calf thymus histone (mean of four experiments). ** Cyclic AMP binding activity was determined in the absence of protein kinase inhibitor, 50 mM phosphate buffer pH 6.0 and 30 pmoles of ^{3}H-cAMP at 0°, 1 hr. *** pmoles/mg protein/5 min. + pmoles/mg protein. ‡ μg phenolphthalein/mg protein/hr. ‡ μmoles Pi/mg protein/hr.

TABLE II

EFFECT OF SUBCELLULAR FRACTIONS OF HUMAN POLYMORPHONUCLEAR (PMN) LEUCOCYTES ON CYCLIC AMP BINDING BY BEEF HEART SUPERNATANTS

Fraction	Protein (μg)	cAMP binding* -beef heart sup.	+ beef heart sup.	Percent inhibition
Beef heart sup.	125	-	41,232	-
PMN nuclei and debris	30	860	32,282	23
PMN 27,000 x g pellet	30	820	23,061	46
PMN 105,000 x g pellet	30	1,997	36,275	14
PMN 105,000 x g supernatant	40	6,141	44,570	0

* Cyclic AMP binding as cpm of ^{3}H-cAMP (30 pmoles) bound at 0°, 60', in 50 mM phosphate buffer (pH 6.0) using indicated amounts of 800 x g supernatants from frozen thawed peripheral blood cell fractions (Hypaque/Ficoll gradient).

binding activities as well as for lysosomal hydrolases (Table 1). True cAMP-dependent protein kinase activity could only be demonstrated in the 105,000 x g supernatant, this fraction also showed maximum cAMP binding. In contrast, the lysosomal fraction (27,000 x g pellet) richest in β-glucuronidase and acid β-glycerol phosphatase,demonstrated as little binding or cAMP-dependent protein kinase activity as did the whole homogenate. When the cAMP-dependent protein kinase was further purified (6) using

TABLE III

EFFECT OF VARIOUS HUMAN BLOOD CELLS ON cAMP BINDING BY BEEF HEART SUPERNATANTS

Cell Type	Protein (μg)	cAMP binding* -beef heart sup.	cAMP binding* +beef heart sup.	Percent inhibition
Beef heart sup.	125	-	31,939	-
Polymorphonuclear leucocytes	48	1,885	17,826	47
Lymphocytes	45	5,440	39,427	0
MLB	40	1,750	35,683	0
Platelet-rich plasma	48	0	36,209	0
Erythrocytes	43	127	32,916	0

* Cyclic AMP binding as cpm of ^{3}H-cAMP (30 pmoles) bound at 0°, 60', in 50 mM phosphate buffer (pH 6.0) using indicated amounts of 800 x g supernatants from frozen-thawed peripheral blood cells (Hypaque/Ficoll gradient).

the 105,000 x g supernatant as starting material, ammonium sulfate precipitation and Sephadex G-75 chromatography yielded preparations 5.5-fold as active in the presence of cAMP as in its absence (Table I). In contrast, when the starting material was the 27,000 x g supernatant, cAMP produced only a 2-fold increment. Since the crude homogenate showed no cAMP-dependent protein kinase activity, and sedimentable fractions showed only cAMP-independent kinase activity, it was likely that the cells contained either (a) an inhibitor of the kinase and/or cAMP binding or (b) a dissociative factor, e.g. cationic protein, which rendered protein kinase independent of added cAMP (11). The latter possibility was unlikely, since the specific activity of the cAMP-independent kinase in sedimentable fractions never approached that of cAMP-dependent kinase in the supernatants, and since cAMP binding activity was also limited to the latter. Consequently (Table II), subcellular fractions of human PMN's were added to preparations of beef heart, and it became clear that cAMP-binding of beef heart supernatants was inhibited only by the lysosomal fractions of PMN's. Moreover (Table III), only PMN's contained this inhibitory factor: other formed elements of the blood did not contain detectable inhibitory activity.

In order to determine whether inhibition of cAMP binding and inhibition of protein kinase activity were coincidental, both parameters

TABLE IV

EFFECT OF PMN LYSOSOMAL LYSATE ON BEEF HEART PROTEIN KINASE AND cAMP-BINDING ACTIVITY*

Amount of PMN lysate added (μg)	0	8	16	32	48	64
Percent protein kinase activity	100**	55	37	30	25	22
Percent cAMP-binding activity	100***	50	35	29	18	15

* Frozen/thawed lysate of PMN lysosomes in varying amounts added to 90 μg of ammonium sulfate precipitate of beef heart protein kinase. Calf thymus histone (100 μg) as acceptor, incubated for 5 min. at 37°C in the presence of 5 μM cAMP. Binding assay carried out at 0°C for 1 hr. with 30 pmoles of ^{3}H-cAMP in a final volume of 200 ml. ** = 30 pmoles ^{32}P incorporated. *** =27160cpm ^{3}H-cAMP bound.

TABLE V

EFFECT OF HEAT AND DIALYSIS ON INHIBITION OF cAMP BINDING BY BEEF HEART SUPERNATANTS INDUCED BY PMN's AND THEIR LYSOSOMES

Fraction*	Treatment	cAMP binding -beef heart sup.	cAMP binding +beef heart sup.	Percent inhibition
Beef heart sup.	None		15,339	--
Whole PMN's	None	2,433	8,919	50
Whole PMN's	Dialyzed	1,902	9,103	47
Whole PMN's	Heated	610	15,113	5
PMN lysosomes	None	1,775	8,173	53
PMN lysosomes	Dialyzed	815	7,186	56
PMN lysosomes	Heated	748	15,274	5

* Freeze-thawed preparations of whole PMN's or their lysosomes (30 μg protein), 27,000 x g pellet after removal of nuclei and debris (see Table 1), mixed with beef heart 800 x g supernatant (90 μg protein) before assay for binding of cyclic ^{3}H-cAMP (30 pmoles). Fractions heated at 80°C for three minutes and/or dialyzed 18 hours against buffer (see text) for 60', 0° in 50 mM phosphate buffer (pH 6.0).

were tested on a partially purified (ammonium sulfate step) preparation of beef heart protein kinase. Table IV indicates that increasing amounts of the lysosomal lysate inhibited both kinase and binding activity to the same extent.

The presence in several tissues of a heat-stable inhibitor of protein kinase activity upon histone is widely appreciated (12, 13), and recently this material has been further characterized as a "modulator" of protein kinases by virtue of its differential effects upon cGMP and cAMP-dependent enzymes employing various substrates (14). Consequently

we determined the heat-stability (80°C for 3 min.) and dialyzability of inhibitor, and found (Table V) that the factor was heat-labile and non-dialyzable.

These experiments thus serve to distinguish the PMN lysosomal inhibitor from the protein kinase "modulator" described by Donnelly et al (14): in contrast to their findings, the PMN preparation is heat labile and does affect cAMP-binding. Since binding is affected at 0°, and inhibitory activity correlates with kinase inhibition, it is not necessarily due to the presence of phosphatases or histonases (8) active at near-neutral pH present in PMN lysosomes. Further experiments are required to determine whether the lysosomal inhibitor affects the substrate, the subunits of protein kinase, or the cyclic nucleotide itself. The data do suggest, however, that when crude homogenates are prepared from tissues containing PMN's, or perhaps other lysosome-rich cells, formal cell fractionation is required to eliminate lysosomes before assays of cAMP-dependent protein kinases are attempted or cAMP-binding proteins are isolated. Furthermore, they suggest that when lysosomes release their contents into intra- or extracellular spaces (15) substances usually sequestered within these organelles can potentially interfere with cAMP-dependent processes.

Acknowledgments: Aided by grants from the National Institutes of Health (AM-11949), The New York Heart Association, The National Tuberculosis and Respiratory Disease Association, and The Whitehall Foundation.

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