Vol. 88, No. 2, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1979 Pages 462-469

VARIABLE DEPENDENCE ON PROTEIN KINASE STIMULATORY MODULATOR FOR CYCLIC GMP STIMULATION OF HISTONE PHOSPHORYLATION BY RAT LIVER CYCLIC GMP-DEPENDENT PROTEIN KINASE

Charles W. Mackenzie III and Thomas E. Donnelly, Jr.*
Department of Pharmacology
University of Nebraska Medical Center
Omaha, Nebraska 68105

Received March 19, 1979

SUMMARY: Various histone fractions from several sources differ markedly in their degree of dependence on protein kinase stimulatory modulator for maximum phosphorylation by rat liver cyclic GMP-dependent protein kinase in the presence of cyclic GMP. DEAE-cellulose and QAE-Sephadex chromatography of arginine-rich and mixed histones resulted in the histones displaying increased dependence on the modulator. This increased dependence was apparently due to the removal of contaminating modulator as heat-stable modulator activity could be eluted from the DEAE-cellulose column. Lysine-rich histone was not markedly dependent on the modulator before or after QAE-Sephadex chromatography.

INTRODUCTION: Donnelly et al. (1) reported that homogeneous protein kinase modulator from lobster tail muscle significantly stimulated the phosphorylation of arginine-rich histone by lobster cyclic GMP-dependent protein kinase (cGPK) in the presence of optimal levels of cyclic GMP. This protein also inhibited the phosphorylation of arginine-rich histone by cyclic AMP-dependent protein kinase (cAPK) from lobster tail muscle and bovine heart. Kuo and coworkers (2,3) have more recently demonstrated that separate protein kinase modulators are present in mammalian tissues which stimulate the phosphorylation of histones by mammalian cGPK and inhibit the phosphorylation by cAPK. It has been suggested that protein kinase stimulatory modulator (PKSM) is absolutely required for cyclic GMP to stimulate histone phosphorylation by cGPK (2-4). However other workers have questioned the importance of PKSM as they have reported that cyclic GMP significantly stimulates histone phosphorylation by cGPK

*To whom correspondence should be addressed.

ABBREVIATIONS: cGPK, cyclic GMP-dependent protein kinase; cAPK, cyclic AMP-dependent protein kinase; PKSM, protein kinase stimulatory modulator; PKM, crude intestinal protein kinase modulator.

0006-291X/79/100462-08$01.00/0

in the absence of added PKSM (5-10). Kuo has suggested that this could be due to their enzyme preparations being contaminated with PKSM (4,11), or due to their assay systems containing unphysiologically high concentrations of Mg^{2+} under which conditions cyclic GMP has some stimulatory activity but PKSM has little effect (12). However stimulation of histone phosphorylation by cyclic GMP has been noted with homogeneous cGPK (8-10) and physiological levels of Mg^{2+} (9,10) in the absence of exogenous PKSM. In this report we demonstrate that histone fractions vary in their degree of dependence on PKSM for cyclic GMP to stimulate their phosphorylation by rat liver cGPK and that further variability is observed due to some histone preparations being contaminated with differing amounts of PKSM activity and other histone fractions.

MATERIALS AND METHODS: Cyclic AMP-Agarose (attached through the N^6-amino group), DEAE-cellulose, QAE-Sephadex A-50 and cyclic GMP were obtained from Sigma. [γ-^{32}P] ATP was purchased from New England Nuclear. Histones were obtained from the sources indicated in the Tables and Figures.

Cyclic GMP-dependent protein kinase was purified from rat liver (Mackenzie, C.W., III and Donnelly, T.E., Jr., unpublished data) approximately 100,000-fold by affinity chromatography by a method adapted from Gill et al. (7) and Flockerzi et al. (10). Using the method of Kuo et al. (4), the standard assay for kinase activity contained 50 mM potassium phosphate, pH 7.0, 2.5 mM theophylline, 5 mM magnesium chloride, 40 µg histone (type as indicated), and 5 µM [γ-^{32}P] ATP (0.15 µCi), with appropriate amounts of protein kinase, protein kinase modulator or cyclic GMP (0.4 µM) as indicated, in a volume of 0.2 ml. The samples were incubated at 30°C for 5 min, the reaction stopped with 20% trichloroacetic acid and the samples processed as described previously (4) with 20% trichloroacetic acid.

Arginine-rich histone, free of PKSM, was purified from frozen calf thymus glands by the method of Johns et al. (13). Following the first precipitation with ethanol, the precipitate was resuspended in 1.25N HCl-ethanol (20:80), centrifuged for 15 min at 10,000 x g and reprecipitated with ethanol. The precipitate was washed with ether and dried in a vacuum desiccator as described by Johns et al. (13). Crude protein kinase modulator (PKM) was obtained from rat intestine by the method of Donnelly et al. (1) as described previously for various mammalian tissues. Kuo et al. (4) have reported that rat intestine has predominantly PKSM with little protein kinase inhibitory modulator present.

To demonstrate PKSM contamination of arginine-rich histones, 3 ml of ICN arginine-rich histone (2 mg/ml in 10 mM potassium phosphate, pH 7.0) were passed over a 1 ml DEAE-cellulose column at room temperature. Equal amounts of protein, based on the absorbance at 280 nm, were used for the assay shown in Table II. This treatment was less effective in reducing the cyclic GMP stimulation when using ICN mixed histones. In a large scale study, 500 mg of ICN mixed histones (5 mg/ml in 10 mM potassium phosphate buffer, pH 7.0) were passed over a 1.5 ml DEAE-cellulose column 5 times in the cold room (4°C). Histone PKSM activity was prepared by first washing the column with 10 mM

potassium phosphate buffer, pH 7.0 plus 20% glycerol. Elution of adsorbed histone PKSM was with 4 ml of 1.5M NaCl in 10 mM potassium phosphate, pH 7.0, plus 20% glycerol. The sample was dialyzed against 10 mM potassium phosphate pH 7.0, plus 20% glycerol before assay for PKSM activity.

QAE-Sephadex A-50 chromatography was also used to remove contaminating PKSM activity (Table II). Two ml of the histones (5 mg/ml in 10 mM potassium phosphate buffer, pH 7.0, plus 20% glycerol) were passed over a 1 ml column of QAE-Sephadex A-50 ten times at room temperature.

RESULTS: In the presence of cyclic GMP, the addition of crude intestinal protein kinase modulator (PKM) caused variable increases in the phosphorylation of several histone preparations by highly purified cyclic GMP-dependent protein kinase (Table I). In the absence of cyclic GMP, the addition of exogenous PKM increased the phosphorylation of some histones while decreasing that of others.

The concentration dependence of exogenous PKM on the phosphorylation of two preparations of arginine-rich histone is shown in Fig. 1. Arginine-rich histone purified in our laboratory by the method of Johns et al. (13) was completely dependent on the addition of exogenous PKM for cyclic GMP to stimulate its phosphorylation. In the absence of cyclic GMP, its phosphorylation was also increased by exogenous PKM but less than that in the presence of cyclic GMP. High levels of PKM caused a decrease in enzyme activity both in the absence and presence of cyclic GMP. In the absence of exogenous PKM, the phosphorylation of ICN arginine-rich histone showed a significant stimulation by cyclic GMP. While the activity in the presence of optimal PKM (6.25 µl) was only increased by 20% in the presence of cyclic GMP, basal activity was reduced by 50% resulting in an increase in cyclic GMP stimulation to 4.6-fold. Increasing the PKM to 12.5 µl/assay increased the cyclic GMP stimulation even further to over 12-fold. These results suggested that commercially available histones might be variably contaminated with PKSM activity.

DEAE-cellulose and QAE-Sephadex chromatography (Methods) were used to directly demonstrate contamination of ICN arginine-rich and Sigma mixed histones by PKSM activity. Following chromatography these histones displayed increased dependence upon exogenous PKM for cyclic GMP to stimulate their phosphorylation (Table II). In contrast, lysine-rich histone was unaffected by QAE-Sephadex

Table I

Effects of crude intestinal protein kinase modulator (9 μg) on the phosphorylation of various histone preparations (40 μg) by cyclic GMP-dependent protein kinase (0.1 μg) in the absence and presence of cyclic GMP.

Histone	-Modulator			+Modulator		
	-cGMP	+cGMP	+cG/-cG	-cGMP	+cGMP	+cG/-cG
Arginine-Rich (f_3)						
Worthington HA Lot 21B	2.66	4.18	1.6	0.45	6.04	13.4
Lot 2LA	1.48	1.58	1.1	2.32	5.59	2.4
ICN	2.95	4.16	1.4	0.92	4.65	5.1
Sigma Type VIII-S	0.18	0.27	1.5	2.69	3.47	1.3
Johns et al. (13)	1.18	0.80	0.7	1.71	6.18	3.6
Lysine-Rich (f_1)						
Worthington HL	0.71	6.19	8.7	0.34	4.96	14.6
Sigma Type V	2.64	10.83	4.1	1.21	14.49	12.0
Slightly Lysine-Rich(f_{2a})						
Sigma Type VI	1.06	1.68	1.6	1.40	2.97	2.1
Slightly Lysine-Rich(f_{2b})						
Worthington HF2B	5.30	6.27	1.2	1.27	8.98	7.1
Sigma Type VII	2.41	3.65	1.5	1.08	9.87	9.1
Mixed						
ICN Lot 2570	1.34	1.93	1.4	0.84	7.51	8.9
Lot 5147	1.54	2.48	1.6	0.82	7.66	9.3
Sigma Type II	0.70	0.62	0.9	1.62	4.98	3.1

The results are expressed as pmoles of ^{32}P incorporated per 5 min.

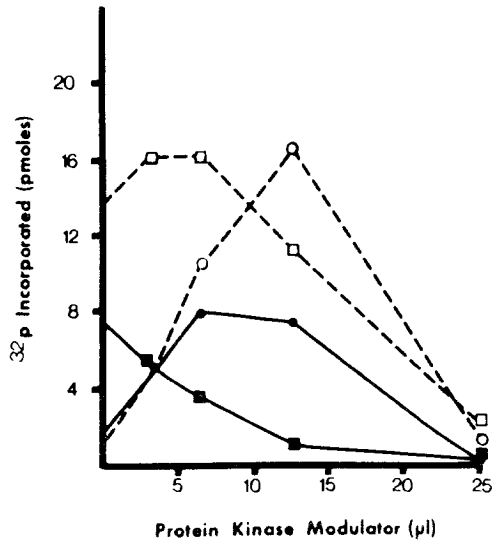

Figure 1. Effects of increasing amounts of crude intestinal protein kinase modulator (0.7 μg/ul) on the phosphorylation of 40 μg ICN arginine-rich histone (□, ■) and arginine-rich histone (○, ●) purified according to Johns et al. (13) by cyclic GMP-dependent protein kinase (0.2 μg) in the absence (closed symbols) and presence (open symbols) of cyclic GMP.

Table II

Effects of DEAE-cellulose and QAE-Sephadex chromatography on the dependence of histones (40 μg) on exogenous crude intestinal protein kinase modulator (9 μg) for phosphorylation by cyclic GMP-dependent protein kinase (0.1 μg) in the absence and presence of cyclic GMP.

	- Modulator			+ Modulator		
Treatment	-cGMP	+cGMP	+cG/-cG	-cGMP	+cGMP	+cG/-cG
(Experiment 1)						
DEAE-cellulose						
ICN Arginine-Rich						
Untreated	2.46	3.26	1.3	0.33	3.32	10.1
Treated	2.19	2.44	1.1	0.23	3.52	15.3
ICN Mixed						
Untreated	1.54	2.48	1.6	0.82	7.66	9.3
Treated	1.29	1.64	1.3	0.42	3.22	7.7
(Experiment 2)						
QAE-Sephadex						
ICN Arginine-Rich						
Untreated	1.44	2.35	1.6	0.60	4.97	8.3
Treated	0.86	1.05	1.2	0.95	3.97	4.2
ICN Mixed						
Untreated	1.50	3.55	2.4	0.59	7.70	13.1
Treated	0.68	0.92	1.4	0.36	5.04	14.0
Worthington Lysine-Rich						
Untreated	0.42	4.61	11.0	0.62	5.64	9.1
Treated	0.55	4.90	8.9	0.43	6.05	14.1

The results are expressed as pmoles ^{32}P incorporated per 5 min.

chromatography. In a large-scale study with ICN mixed histone, a heat-stable (10 min at 95°C) PKSM activity was eluted from the DEAE-cellulose column (Methods). This PKSM activity caused a pattern of increased phosphorylation (Fig. 2) similar to that observed with crude intestinal PKM both in the absence and presence of cyclic GMP using the arginine-rich histone purified in our laboratory as substrate.

DISCUSSION: This study indicates that the ability of cyclic GMP to markedly increase the phosphorylation of arginine-rich, slightly lysine-rich and mixed histones by cGPK from rat liver is largely dependent on the presence of PKSM. Preparations of these histones from various commercial sources differed in their degree of stimulation by cyclic GMP, apparently due to their being contaminated with varying amounts of PKSM. Arginine-rich histone purified by the method of Johns et al. (13) is almost completely dependent on the presence of PKSM. In contrast, a preparation of lysine-rich histone obtained from Worthington was nearly independent of PKSM.

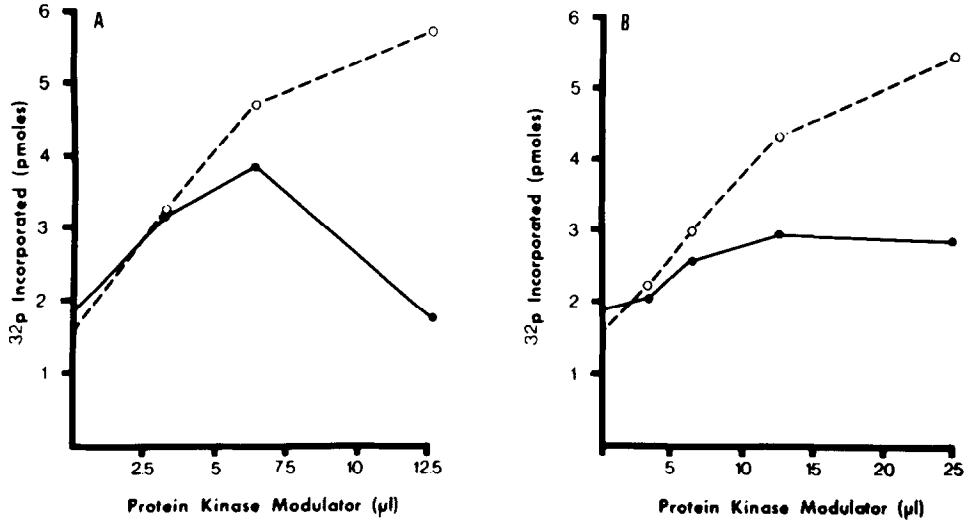

Figure 2. Effects of increasing amounts of A) crude intestinal protein kinase modulator (0.7 ug/ul) and B) protein kinase modulator (0.01 µg/ul) obtained from mixed histones on the phosphorylation of arginine-rich histone (40 µg) purified according to Johns et al. (13) by cyclic GMP-dependent protein kinase (0.1 µg) in the absence (●) and presence (○) of cyclic GMP.

PKSM activity was isolated from calf thymus mixed histone by DEAE-cellulose chromatography, however complete dependence on added PKSM was not observed, apparently due to the fact that lysine-rich histone represented a significant amount of the preparation. Panyim and Chalkley (14) estimated that lysine-rich histone represented 21% of the histones present in calf thymus, however the amount present in the preparation obtained from ICN and Sigma is not known. It is also to be expected that lysine-rich histone may represent a minor contaminant in most commercially available histone preparations as no claim of homogeneity is made by most suppliers.

Yamamoto et al. (15), using histones purified in their laboratory, noted that in the presence of cyclic GMP, the modulator increased the phosphorylation of all histone types using cGPK from silkworm pupae. They did not examine the dependence of stimulation by cyclic GMP on the presence of modulator, however they noted that the phosphorylation of arginine-rich histone in the presence of cyclic GMP was almost completely dependent on the presence of modulator.

Chihara-Nakashima et al. (16) have found that modulator is apparently

almost completely required for the phosphorylation of rat liver ribosomal proteins by cGPK isolated from silkworm pupae. Other proteins have been found to be substrates for cGPK from mammalian sources however no requirement for modulator has been indicated. For example, hormone-sensitive lipase, phosphorylase b kinase and cholesterol esterase, which are generally assumed to be physiological substrates for cAPK, have been shown to be phosphorylated and activated by cGPK from bovine lung (17). Similarly the activities of pyruvate kinase and glycogen synthase are inhibited following phosphorylation by cAPK or cGPK from bovine lung (18). In both studies (17,18) it was suggested that the proteins were probably physiological substrates for cAPK and not cGPK as much greater amounts of cGPK activity were required to phosphorylate and alter the properties of the substrates. These workers did not examine the effect of exogenous PKSM on the phosphorylation and alteration of substrate activity. The possibility also exists that the substrate preparations were contaminated with endogenous PKSM allowing them to be substrates for cGPK. Therefore further investigation is necessary to determine whether PKSM is required for the phosphorylation of many or a limited number of substrates of cGPK.

ACKNOWLEDGEMENTS: The authors are indebted to Stanley Swanson and Stephen Scott for their excellent technical assistance in this study. The study was supported by grant GM 24287 from the National Institutes of Health to T.E.D.,Jr.

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