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STIMULATORY MODULATOR-REQUIRING CYCLIC NUCLEOTIDE-INDEPENDENT PROTEIN KINASE: PARTIAL PURIFICATION FROM FETAL CALF HEARTS AND COMPARISON WITH VARIOUS PROTEIN KINASES.

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SUMMARY: A stimulatory modulator-requiring cyclic nucleotide-independent protein kinase was purified over 400-fold from the extract of fetal calf hearts by the steps of DEAE-cellulose and Sephadex G-100 chromatographies. The enzyme was activated by stimulatory modulator of cGMP-dependent protein kinase. Inhibitory modulator (protein inhibitor) of cAMP-dependent protein kinase, calcium, phosphatidyl serine and cyclic nucleotides were without effect. The enzyme (3.2 S) was much smaller than the holoenzymes of cGMP- and cAMP-dependent protein kinases. This new species of enzyme thus appears to be similar to the putative catalytic subunit of cGMP-dependent protein kinase previously reported.

During the course of investigating G-PK[1], we observed (unpublished) that certain fractions of tissue extracts contained a cyclic nucleotide-independent protein kinase activity which was augmented by stimulatory modulator, a protein stimulator of the holoenzyme (1,2) and the putative catalytic subunit (3) of G-PK. The present paper describes a partial purification of this species of protein kinase. Data are also presented showing that the enzyme in question was different from other classes of protein kinases reported to date, and that it appeared to be similar to the putative catalytic subunit of G-PK based upon all criteria examined.

EXPERIMENTAL PROCEDURE

Materials: ^{32}Pi (orthophosphoric acid, carrier free) was purchased from New England Nuclear; cAMP, cGMP, mixed histone (type II), lysine-rich histone (type III-S), phosphatidyl serine and marker proteins were from Sigma; fetal calf hearts were from Pel-Freez Biologicals.

Purification of SM-PK: The calf fetal heart (450 g) was cut into small cubes and homogenized in one-volume of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.03% Triton X-100. The homogenate was kept at 4° for 1 hr with intermittent mixing. Crude extract was obtained by centrifuging the homogenate for 30

[1]The following abbreviations are used: G-PK, cGMP-dependent protein kinase; SM-PK, stimulatory modulator-requiring, cyclic nucleotide-independent protein kinase; A-PK, cAMP-dependent protein kinase; Ca-PK, calcium-dependent protein kinase.

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min at 13,700 x g, followed by centrifuging the supernatant for 30 min at 34,800 x g. The extract was then directly charged onto a DEAE-cellulose column (3.7 x 27 cm), which had been previously washed with 1 M potassium phosphate buffer (pH 7.0) and subsequently equilibrated with 50 mM potassium phosphate buffer (pH 7.0) (equilibration buffer). A linear gradient of the phosphate buffer (pH 7.0), from 100 to 500 mM (total volume: 800 ml), was employed for the chromatography after the column was washed with 3 bed volumes of the equilibration buffer. Active fractions (Fig. 1) were pooled and concentrated to 5 ml in an Amicon Stirred Cell (Model 402) using YM 10 membrane. The concentrate was then charged onto a Sephadex G-100 column (4.5 x 43 cm), which had been washed and equilibrated with the equilibration buffer. The enzyme was eluted using the same buffer (Fig. 2).

Other methods: [γ-^{32}P]ATP was prepared from $^{32}P_i$ according to the method of Post and Sen (4). Stimulatory modulator of G-PK from the dog heart was purified through the step of Sephadex G-100 (2). G-PK from the fetal guinea pig lung was purified through the step of Sephadex G-200 (5). A-PK from the fetal calf heart was from the step of DEAE-cellulose (Fig. 1). Phospholipid-requiring Ca-PK from the bovine heart was purified by the steps of ammonium sulfate, DEAE-cellulose and Sephadex G-200 (6). Ultracentrifugation of protein kinases was carried out in a 5 to 20% sucrose gradient at 47,000 rpm and 4°, using a Beckman SW 60Ti rotor. The sedimentation coefficient was determined by the method of Martin and Ames (7), using marker proteins as indicated (Fig. 3). Protein was determined by the method of Lowry et al. (8).

Assay for protein kinases: The standard assay system (5) for A-PK, G-PK and SM-PK contained, in a final volume of 0.2 ml, potassium phosphate (pH 7.0), 8 µmol; theophylline, 0.5 µmol; mixed histone, 40 µg; $MgCl_2$, 2 µmol; [γ-^{32}P]ATP, 1 nmol, containing about 1 x 10^6 cpm; with or without appropriate amounts of cAMP, cGMP, inhibitory modulator or stimulatory modulator, as indicated. The assay system for phospholipid-requiring Ca-PK (9) contained, in a volume of 0.2 ml, Tris/Cl (pH 7.5), 5 µmol; $MgCl_2$, 4 µmol; lysine-rich histone, 40 µg; $CaCl_2$, 0.1 µmol; phosphatidyl serine, 1 µg; [γ-^{32}P]ATP, 1 nmol, containing about 1 x 10^6 cpm. The reaction was carried out for 10 min at 30°. One unit of the protein kinase activity is defined as that amount of enzyme that transferred 1 pmol of ^{32}P from [γ-^{32}P]ATP to recovered histone under the assay conditions.

RESULTS AND DISCUSSION

The activity of SM-PK in the heart extract was largely separated from the overwhelming A-PK activity by means of DEAE-cellulose chromatography (Fig. 1). A small peak of G-PK activity overlapping with the large A-PK peak was also separated from the SM-PK activity. The enzyme in question was further purified by Sephadex G-100 gel filtration, which effectively removed the contaminating A-PK (Fig. 2). The active fractions devoid of A-PK activity were pooled and used as the source of SM-PK in studies reported herein. The enzyme was purified 440-fold by these steps, with an overall activity recovery of 18% (Table 1).

The activity of SM-PK, as that of G-PK (1-3,5), was augmented by stimulatory modulator, whereas it was not affected by inhibitory modulator or phosphatidyl

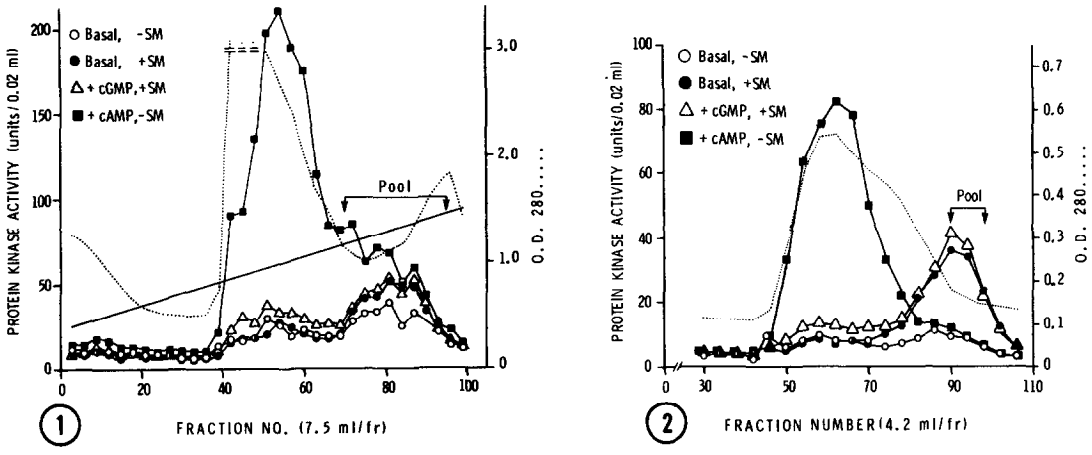

Fig. 1. Chromatography of the crude extract of fetal calf hearts on DEAE-cellulose column (3.7 x 27 cm). The linear gradient used was 100 to 500 mM potassium phosphate buffer (pH 7.0). The flow rate was 0.75 ml/min, and the fraction size was 7.5 ml. An aliquot (0.02 ml) from the fractions was used for assaying the protein kinase activity in the presence or absence of cGMP (0.5 μM), cAMP (0.5 μM) and stimulatory modulator (SM, 14 μg) prepared from dog hearts, as described in Experimental Procedure. Dashed line indicates absorbance (280 nm).

Fig. 2. Gel filtration of the enzyme preparation (pooled DEAE fractions 70-95) from the fetal calf heart on Sephadex G-100 column (4.5 x 43 cm). The flow rate was 0.42 ml/min. and the fraction size was 4.2 ml. An aliquot (0.02 ml) from the fractions was used for assaying the enzyme activity under the conditions described in Fig. 1 and Experimental Procedures. Dashed line, absorbance (280 nm).

TABLE I

Summary of purification of SM-PK from fetal calf hearts

For experimental details, see the text. The enzyme activity was assayed in the presence or absence of stimulatory modulator (14 μg) and in the absence of cyclic nucleotides. The activity stimulated by the modulator are presented. The starting material was 450 g of the frozen heart.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (-fold)	Recovery (%)
Crude extract	6,400	160,000	25	1	100
DEAE-Cellulose eluate	164	107,000	652	26	67
Sephadex G-100 eluate	3	28,000	11,000	440	18

TABLE II.

Comparison of effects of various factors on different protein kinases.

Protein kinases were assayed as described under Experimental Procedure, in the presence or absence of stimulatory modulator (SM, 14 µg), inhibitory modulator (IM, 5 µg), phosphatidyl serine (PS, 1 µg), cGMP (0.5 µM), cAMP (0.5 µM) and Ca^{2+} (0.5 mM), as indicated. The enzymes used were SM-PK (1.5 µg), G-PK (10 µg), A-PK (19 µg) and phospholipid-requiring Ca-PK (26 µg).

Enzyme	Addition	Protein kinase activity (units) None	SM	IM	PS
SM-PK	None	10.3	37.2	12.8	13.3
G-PK	cGMP	23.2	58.9	25.3	24.6
A-PK	cAMP	20.2	15.7	6.3	16.3
Ca-PK	Ca^{2+}	4.6	5.0	4.7	49.7

serine (Table 2). A-PK and Ca-PK, on the other hand, were inhibited by inhibitory modulator (10) and stimulated by phosphatidyl serine (9,11), respectively, as previously reported. The difference between SM-PK and other classes of protein kinases was further demonstrated (Table 3). Thus, its activity was not influenced by cGMP, cAMP and Ca^{2+}, specific activators for G-PK (5), A-PK (12) and Ca-PK (9,11), respectively, as reported elsewhere. The data clearly indicated that SM-PK appeared to be a new species of protein kinase which was not activated by cAMP or cGMP as in the case of the cyclic nucleotide-independent class of protein kinase, and at the same time was stimulated by stimulatory modulator as in the case of G-PK holoenzyme (1-3,5). We have shown (3) that the putative catalytic subunit of G-PK is independent of cGMP and is stimulated by the modulator, properties shared by the present SM-PK. It is possible that they may be also similar

TABLE III

Comparison of effects of various activators on different protein kinases.

Assay conditions were the same as in Table 2 and Experimental Procedure.

Enzyme	Addition	Protein kinase activity (units) None	cGMP	cAMP	Ca^{2+}
SM-PK	SM	31.0	30.5	31.6	33.3
G-PK	SM	13.1	61.4	26.7	12.2
A-PK	None	5.4	6.9	20.5	3.7
Ca-PK	PS	12.3	12.8	20.3	47.9

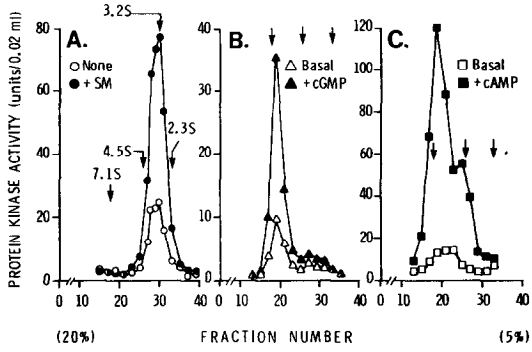

Fig. 3. Linear sucrose density gradient ultracentrifugation of protein kinases. Sucrose (5 to 20%) was dissolved in 50 mM potassium phosphate buffer, pH 7.0. A, SM-PK (0.2 ml, 16 µg) purified from fetal calf hearts; fractions assayed in the presence or absence of stimulatory modulator (SM, 14 µg). B, G-PK (0.2 ml, 220 µg) purified from fetal guinea pig lungs; fractions assayed with stimulatory modulator (14 µg) in the presence or absence of 0.5 µM cyclic GMP. C, A-PK (0.2 ml, 800 µg) purified from fetal calf hearts; fractions assayed in the presence or absence of 0.5 µM cyclic AMP. The marker proteins used were: human gamma globulins Cohn fraction II (7.1 S); bovine serum albumin (4.5 S); trypsin inhibitor from soybean (2.3 S).

in molecular size. This was found to be indeed the case. The enzyme had a sedimentation coefficient of 3.2 S (Fig. 3), similar to that of the putative catalytic subunit of G-PK reported earlier (3). The molecular size of the holo-enzymes of A-PK and G-PK was compared with that of SM-PK in the same experiment (Fig. 3); they were both 6.9 S, as reported elsewhere (3). In addition, the present enzyme was devoid of cAMP- or cGMP-binding activity (data not shown), as the catalytic subunits of G-PK (3) and A-PK (13). Although the catalytic subunit of A-PK has a similar sedimentation coefficient (14) as that of the present enzyme, the phosphotransferase activity of the former is inhibited by inhibitory modulator and is not influenced by stimulatory modulator (1,2). In spite of similarities in the sedimentation coefficient and lack of effects of inhibitory modulator and cyclic nucleotides, the present SM-PK appears to be also different from protein kinase M, a proteolytic product of protein kinase C (11) or phospholipid-requiring Ca-PK (11). This was based largely upon the observations that protein kinase C requires reducing agents for its activity (15) whereas SM-PK does not (unpublished).

Unlike activation of A-PK by cAMP (13), it has been shown that, at least in vitro, cGMP activates G-PK without causing dissociation of the G-PK "holo-

enzyme" (16,17). Nevertheless, formation of the putative catalytic and/or regulatory subunit of G-PK has been shown to occur under certain incubation conditions, such as in the presence of both cGMP and histone (3), protamine or histone (18), both cGMP and mercaptoethanol (19) and trypsin (20). It is possible, therefore, that G-PK may undergo activation and dissociation in vivo in the presence of certain cellular components, acting in concert with cGMP, to yield the catalytic subunit. Identification in the present studies of SM-PK having properties similar to those of the putative catalytic subunit of G-PK is intriguing. The establishment of whether SM-PK is the catalytic subunit or catalytic fragment of G-PK is clearly in order. If this is the case, it would not only shed light on the in vivo regulation of G-PK by cGMP, but also suggest biological significance of the reaction. The latter notion seems to be hinted at by recent observations that the activity levels of SM-PK in Morris hepatomas were much higher than in the control normal liver (21). It has been reported that hepatomas contain elevated cGMP (22), which may lead to a higher degree of activation and perhaps dissociation of G-PK, resulting in a greater formation of the putative catalytic subunit, or presumably SM-PK.

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