A NEW SPECIFIC ENDOGENOUS INHIBITOR OF A CYCLIC NUCLEOTIDE INDEPENDENT (G TYPE) PROTEIN KINASE

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1. Introduction

ATP:protein phosphotransferases (EC 2.7.1.37) (protein kinases, PK) have emerged as important regulatory factors of many cellular processes [1]. In addition to cAMP- and cGMP-dependent enzymes, cyclic nucleotide-independent PK have been characterized in several mammalian tissues [1] and it has been suggested that they may play important roles in glycogen [2] and protein [3] metabolism. As suggested [4], in vivo modulation of PK activities may involve endogenous protein factors, acting either as inhibitor or activator of specific PK activities. Two types of small molecular weight inhibitors have been characterized in mammalian tissues: the heat stable inhibitory protein (HSIP) [5] which selectively inhibits the catalytic subunit of cAMP-dependent PK; and a type II heat-stable factor active on both cAMP-dependent and -independent PK [6].

This paper reports the isolation and characterization from bovine adrenal cortex cytosol of yet another heat-stable macromolecular PK inhibitor. However, in contrast to the factors mentioned, it was found to specifically inhibit a particular type of cyclic nucleotide-independent PK activity (PK G, which uses GTP as well as ATP as phosphoryl donor) isolated from the same tissue. After purification to homogeneity, the PK G inhibitor (PK G I) was obtained as a polydisperse aggregate which dissociated into a 16 500 M_r moiety upon sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

In addition to its interest as a tool for the biochemical study of protein kinases, this specific endogenous factor may be of great biological significance for the modulation of cAMP-independent PK G activity and hence for the regulation of intracellular phosphorylation–dephosphorylation processes.

2. Materials and methods

[γ-^{32}P]ATP (20 Ci/mmol) and [γ-^{32}P]GTP (25 Ci/mmol) were purchased from the Radio-Chemical Center (Amersham) and Na ^{125}I from the CEA (Saclay). Phosphocellulose (P-11) and DEAE cellulose (DE-52) were obtained from Whatman, Sepharose (6 B) from Pharmacia and Biogel (P-6) from Biorad Labs. Hydroxylapatite was prepared according to [7]. Histone (IIa) and bovine serum albumin (Cohn fraction V) were purchased from Sigma, pronase (CB) from Calbiochem and other marker proteins from Boehringer. Casein (Merck) was prepared according to [8]. Pure ox brain calcium-dependent regulator (CDR) preparation was a generous gift from Professor J. Demaille.

2.1. *Bovine adrenal cortex cytosol*

This was prepared as in [9] in TDG buffer (10 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol and 2% glycerol). The catalytic subunit of the cAMP-dependent protein kinase was isolated from cytosol adopting the procedure in [10].

2.2. *Casein kinases of the A (PK A) and G (PK G) types*

These were isolated from adrenal cortex cytosol after adsorption onto a phosphocellulose column as

in [11]. The bulk of cAMP-dependent histone kinase was removed by washing with TDG buffer containing 0.2 M NaCl; PK A and PK G were then eluted in a stepwise manner with TDG buffer containing 0.6 and 1 M NaCl, respectively.

2.3. *Dodecyl sulfate (0.1%) slab gel electrophoresis*

This was performed in a discontinuous Tris–glycine buffer according to [12] using either a 7.5–15% gradient (molecular size screening) or a 15% (study of small M_r moieties) polyacrylamide gels.

2.4. *Protein ^{125}I labeling*

This was carried out using 5 μg protein and the Na ^{125}I (0.5 mCi)–chloramine T procedure in [13]. Free iodine was removed by filtering the reaction mixture through a Biogel P-6 column (0.9 × 25 cm) in TDG buffer containing 0.1 M NaCl.

2.5. *Protein kinase assay*

This was performed under the incubation conditions in [14] with the TCA precipitation procedure in [15]. For inhibition studies, the 80 μl standard reaction mixture contained either 50 μl enzyme preparation or 50 μl mixture of enzyme and inhibitor in the indicated proportion and 30 μl TDG buffer containing either [γ-^{32}P]ATP or [^{32}P]GTP (spec. act. 1000 cpm/pmol). $MgCl_2$ and 60 μg casein were also present in this 30 μl aliquot unless otherwise stated. The final concentrations were 0.01 mM ATP (or GTP) and 50 mM $MgCl_2$. When the catalytic subunit of cAMP-dependent protein kinase was used, the standard reaction mixture was the same except that it contained 1 mM $MgCl_2$ and 60 μg histone. The reaction was initiated by addition of the enzyme preparation and incubated at 30°C under linear conditions with regard to time and enzyme concentration.

2.6. *Protein determinations*

These were performed by the method in [16] using bovine serum albumin as the standard, except for the later purification steps where the procedure in [17] was adopted.

2.7. *Inhibitory activity*

This was estimated by calculation of the 50% inhibitory dose (ID_{50}, mg protein) by the method in [18]. Total inhibitory activity of a given preparation was then expressed as the ratio of its total protein content to its ID_{50}. Thus, this ratio represents the volume to which the preparation must be diluted to yield a 50% inhibition of PK activity under standard assay conditions. This method of expressing the results was chosen since it takes into account non-linearity of inhibitory dose–response curves, as seen with most of the usual types of inhibition.

3. Results and discussion

3.1. *Purification of the PK G inhibitory factor (PK G I) from bovine adrenal cortex cytosol*

Previous experiments have shown that PK G I is not retained on phosphocellulose resin at low ionic strength, in contrast to the A- and G-type casein kinases. Consequently the described preparation procedure was devised to yield the inhibitor as a by-product of the PK A and PK G isolation (using the same cytosol batch).

3.1.1. Phosphocellulose chromatography

Crude adrenal cortex cytosol (2600 ml) was 70% saturated with ammonium sulfate; after centrifugation (20 000 × *g*, 30 min), the precipitate was dissolved in TDG–0.1 M NaCl buffer (1300 ml) and dialysed against the same medium. The dialysate was transfered onto a 5 × 20 cm phosphocellulose column pre-equilibrated with the same buffer and the collection started immediately. The bulk of inhibitory activity was recovered in the flow-through volume and the following 400 ml wash with TDG–0.2 M NaCl buffer, whereas the A and G casein kinases remained on the column and could be obtained independently [11].

3.1.2. Heat treatment, pH 4.7 precipitation and DEAE-cellulose chromatography

The inhibitory fractions were heat treated for 5 min in a boiling water bath and the resulting precipitate discarded after centrifugation (20 000 × *g*, 10 min). The clear supernatant was brought to pH 4.7 by dropwise addition of 0.1 M acetic acid with constant stirring and pH control. The resulting precipitate was collected (20 000 × *g*, 20 min), dissolved in 12 ml distilled water and dialysed against 10 mM HCl–imidazole

buffer (pH 6.5). The preparation was then transfered to a 2 X 2 cm DEAE-cellulose column equilibrated in the same buffer. After washing with 30 ml HCl–imidazole buffer containing 0.2 M NaCl, a linear 0.2–0.5 M NaCl gradient was applied and the PK G I activity eluted (0.28–0.45 M NaCl, 42 ml).

3.1.3. Hydroxylapatite chromatography and gel filtration

After dialysis against 10 mM phosphate buffer (pH 6.8) the preparation was added to a 2 X 3.7 cm hydroxylapatite column prepared in the same medium. After a 50 ml wash with 10 mM phosphate buffer, a linear 10–200 mM phosphate gradient was applied. The active fractions were eluted between 20–120 mM (56 ml) phosphate, pooled, dialysed against 10 mM phosphate buffer and adsorbed onto a 0.9 X 2 cm hydroxylapatite column where they were concentrated. After a 5 ml washing (10 mM phosphate), PK G I was recovered in a small volume (4.3 ml) of 150 mM phosphate buffer. The preparation was then transfered to a 1.6 X 92 cm Sepharose (6B) column and eluted with 10 mM phosphate buffer (pH 6.8) containing 0.2 M NaCl. The pooled active fractions (54 ml) were dialysed against 10 mM phosphate buffer (pH 6.8) and concentrated using the above hydroxylapatite procedure with a 0.6 X 1.1 cm column. The final PK G I preparation, eluted with a small volume of 150 mM phosphate buffer (750 μl containing ~200 μg protein), was equilibrated by dialysis against 10 mM phosphate buffer (pH 6.8).

The quantitative data corresponding to the purification scheme are given in table 1. Since it was not possible to evaluate the specific inhibitory activity in crude cytosol, the purification factors were calculated on the basis of the activity found in the heat-treated sample. Taking into account the overall yield, it was calculated that the PK G I represented <1% of the cytosolic heat-stable proteins. The loss occuring at the pH 4.7 precipitation step was not due to partial precipitation since no PK G I activity could be detected in the corresponding supernatant. The pH 4.7 treatment had been introduced since:

(i) Separate experiments with crude PK G I preparation had shown that 4.7 was the apparent pH_i of PK G I activity;
(ii) pH 4.7 treatment was very efficient in precipitating PK G I whereas the bulk of the HSIP in [5] remained in the supernatant.

Although hydroxylapatite chromatography apparently did not improve the purification factor (table 1), two protein contaminents (as judged by A_{280}) were eliminated at this stage. The final gel filtration through Sepharose 6B (fig.1) shows that this step eliminated a protein contaminent whereas no protein (A_{280}) could be detected in the diluted fraction containing the PK G I activity. The final inhibitor preparation appeared homogeneous upon

Table 1
Purification scheme of adrenal cortex PK G inhibitor

Step	ID_{50} (μg/ml) (A)	Protein (mg) (B)	Total act. (B/A X 1000) (ml)	Purif. factor	Overall yield (%)
Heat treatment	33.3	1545	46 846	1	100
pH 4.7 precipitate	7.29	74.50	10 205	4.6	21.8
DEAE cellulose	2.00	3.87	1935	16.7	4.1
Hydroxyl-apatite	1.92	2.71	1412	16.7	3.0
Sepharose 6B	0.254	0.19	740	126.2	1.6

Conditions of assays, determination of inhibitory activity (ID_{50}) and expression of total inhibitory activity as in section 2

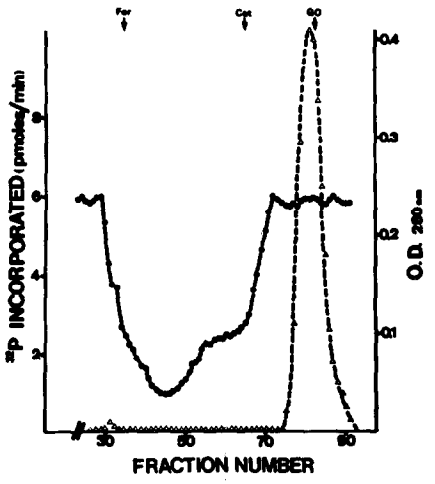

Fig.1. Gel filtration of PK G I through Sepharose 6B (final purification step). Gel filtration was performed as in section 3; the PK G I activity (●——●) was assayed (see section 2) using 30 μl collected fractions after a 6-fold dilution. A_{280} was monitored (△- - -△). The column was calibrated using ferritin (Fer: mol. wt 460 000), Catalase (Cat: mol. wt 232 000) and glucose oxidase (GO: mol. wt 140 000).

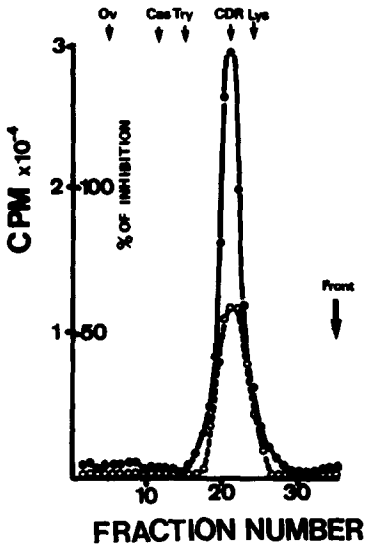

Fig.2. SDS (0.1%)–polyacrylamide (15%) gel electrophoresis of PK G I. ^{125}I-Labeled PK G I (200 000 cpm), PK G I (6 μg) and marker proteins were analysed in parallel lanes of the gel slab. At the end of the electrophoresis run, the lane containing PK G I was cut into 2 mm long slices: the slices were extracted overnight by 50 μl TDG buffer which was then extensively dialysed against TDG buffer and assayed for PK G I activity (o- - -o). The remaining gel slab was stained by Coomassie blue (0.25%), and the lane containing ^{125}I-labeled PK G I was sliced as before, each slice counted for radioactivity (●——●). Calibration was done using ovalbumin (OV: M_r 45 000); casein (Cas: M_r 30 000); trypsin (Try: M_r 24 000); CDR (M_r 16 500) and lysozyme (Lys: M_r 14 300).

polyacrylamide gel electrophoresis under denaturing conditions (see below).

3.2. *Characterization of the PK G inhibitor*

3.2.1. Molecular properties

The isolated PK G I was obtained as a large size macromolecular moiety, exhibiting a polydispersity ranging from 2.5–4 X 10^5 app. mol. wt upon gel filtration (fig.1). Increasing ionic strength (0.5 M NaCl) did not modify this characteristic. Treatment with pronase (5 mg/ml, 15 h at 37°C) abolished the inhibitory activity of the preparation. No active smaller size product occured during proteolysis, as judged by Sepharose gel filtration. DNase and RNase had no effect on the inhibitor; thus, a protein core appears to be necessary for PK G inhibitory activity. This was further confirmed by the ultraviolet absorption spectra of the PK G I, exhibiting an A_{280} max.

Slab gel electrophoresis of the PK G I under denaturating conditions (0.1% SDS) was carried out in both 7.5–15% gradient and 15% polyacrylamide gels. Since the inhibitor was very poorly stained by Coomassie brillant blue (0.25%), ^{125}I labeling of the PK G I preparation was performed. As shown in fig.2, a single labeled moiety was detected under these conditions, co-migrating with the PK G inhibitory activity as detected on a parallel lane of the same gel. Using marker proteins, an app. mol. wt of M_r 16 500 was obtained for the PK G I (fig.2). In addition to showing homogeneity of the isolated PK G I, these results also suggest that the purified polydisperse preparation (fig.1) may represent an aggregate which is dissociated in the presence of SDS. The lack of sensitivity to Coomassie blue may not be surprising since it has been reported for other regulatory polypeptides such as the HSIP [5,19] and CDR [20].

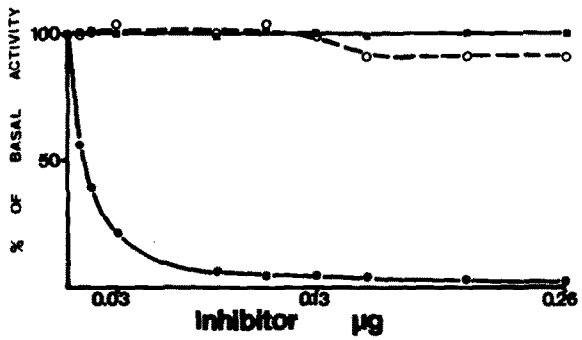

Fig.3. Specificity of PK G inhibitor. Aliquots (20 µl) of adrenal cortex cAMP-independent PK A (▪——▪) and PK G (●——●) and cAMP-dependent catalytic subunit (○- - -○) preparations were assayed for protein kinase activity after addition of PK G I (increasing concentrations in 30 µl). Each value is the mean of 3 replicates with regard to control values established with 8 replicates.

3.2.2. Protein kinase inhibitory properties

The inhibitory activity of increasing concentrations of PK G I was evaluated using three adrenal cortex protein kinase preparations, namely cAMP-independent A and G casein kinases and the catalytic subunit of the cAMP-dependent histone kinase, isolated as above. As shown in fig.3, the inhibitory effect was specific to the casein kinase of the G-type and resulted in a rapid total inhibition of this activity. Under similar conditions, a HSIP preparation [2,5] inhibited the catalytic subunit of the cAMP-dependent PK, whereas PK A and PK G were not affected. It was observed that the inhibitory effect was not dependent upon ATP (or GTP) concentrations (10^{-4}–10^{-6} M) but could be reversed when the substrate (casein) concentration was increased. However, further study is needed to define the PK G inhibition mechanism at the molecular level and possibly demonstrate a substrate–inhibitor competition. It should be mentioned that PK G I itself was not phosphorylated by PK G when the reaction was carried out in the absence of casein.

These data demonstrate the presence of a new protein kinase inhibitory factor in the bovine adrenal cortex cytosol; to our knowledge this is the first inhibitory factor specific for a particular cAMP-independent protein kinase activity. PK G I is clearly different from the known inhibitors of PK activities, namely the HSIP [5,19] and the non-specific type II inhibitor in [6] although some properties (e.g., heat stability, small molecular size) are common to these inhibitory factors. Another heat-stable regulatory protein, namely the calcium-dependent regulator [20] appeared similar to our PK G I when analysed under SDS–polyacrylamide electrophoresis (fig.2). However, CDR (with and without calcium) had no effect upon our PK G activity.

Although the presence of PK G and its specific inhibitor in the same cellular compartment and a possible enzyme–inhibitor complex remains to be demonstrated under in vivo conditions, the PK G–PK G I system would represent a new possible regulatory pathway of cellular protein kinase activities. Since preliminary results (unpublished) have shown the presence of PK G I in many bovine tissues in addition to the adrenal cortex, this regulatory system may be of general interest.

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