Vol. 100, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1981 Pages 613-620

IDENTIFICATION OF A SPECIFIC ENDOGENOUS INHIBITOR OF A CASEIN KINASE (G TYPE) IN BOVINE ADRENAL CORTEX AS A GLYCOSAMINOGLYCAN MIXTURE.

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Received March 18, 1981

SUMMARY

Bovine adrenal cortex contains an endogenous factor which selectively inhibits a particular class of cyclic nucleotide independent protein kinase activity (G type casein kinase). This previously purified inhibitor (D. JOB et al., FEBS Lett., 98, 303-308) was found to contain typical glycosaminoglycan components. Electrophoretic analysis and selective degradation procedures showed that the inhibitor preparation contained two major glycosaminoglycan moieties identified as heparin-like and chondroitin sulfate-like structures. The casein kinase G inhibitory properties appeared to require intact glycosaminoglycan chains. These findings may suggest a potential role of glycosaminoglycans in the modulation of intracellular protein phosphorylation processes by a particular type of messenger independent protein kinase.

INTRODUCTION

ATP-protein phosphotransferases (protein kinases) have emerged as important regulators of cellular protein activities by covalent modification (1,2). Several types of protein kinases have been characterized and may be classified according to their dependence upon specific effectors such as cyclic nucleotides (cAMP or cGMP), calcium and calmodulin or ds RNA (2). Activities for which no specific effector has yet been recognized may be termed messenger independent protein kinases (2). We have previously isolated from bovine adrenal cortex cytosol two types of protein kinases belonging to this category and characterized them as casein kinases of the A (using only ATP as phosphate donor) and G (using GTP as well as ATP) types (3). In addition to positive effectors, cellular protein kinase activities may be modulated by endogenous inhibitory factors (4). Two heat stable, small molecular weight inhibitors have been isolated: the specific inhibitor of cAMP dependent protein kinase (5) and a type II inhibitor active on both cAMP dependent and cAMP independent protein kinases (6). Recently, two cAMP independent protein kinase inhibitory factors have been extracted from rat liver nuclei, and one of them identified as a family of oligonucleotides(7). We have previously purified from bovine adrenal cortex cytosol a new heat stable factor which selectively inhibits the G type casein kinase and showed no action upon casein kinase A or the cAMP dependent system (8). This specific inhibitor was

0006-291X/81/100613-08$01.00/0

thus termed casein kinase G inhibitor (CKG I) and was purified as a small molecular weight moiety with acidic properties (8). An enzyme-inhibitor interaction was demonstrated in vitro and shown to be reversed in the presence of polyamines which were thus suggested as potential modulators of the G type casein kinase activity in the intact cell (9).

This paper reports the identification of the previously purified adrenal cortex casein kinase G inhibitor as an heterogeneous glycosaminoglycan structure, based on chemical analysis and selective degradation procedures of glycosaminoglycan chains. These findings may suggest a possible role of intracellular glycosaminoglycans as potential modulators of casein kinase G activity in the intact cell.

EXPERIMENTAL PROCEDURES

[γ^{32}P]ATP (20 Ci/mmol) and [γ^{32}P]GTP (25 Ci/mmol) were purchased from the Radiochemical Center (Amersham); Na ^{125}I was from the CEA (Saclay). Casein (Merck) was treated according to ASHBY and WALSH (10) before use. Alcian blue, chondroitin sulfate A, B and C, hyaluronic acid (III s), chondroitinase ABC (Proteus vulgaris), chondroitinase AC (Arthrobacter aurescens), hyaluronidase (bovine type VI S) were from Sigma. Heparin was from Hoffman La Roche Laboratories. Pronase (Streptomyces griseus) and proteins used as molecular weight markers were purchased from Boehringer. Glucosamine (HCl) was from Fluka. Cellulose acetate strips were from Chemetron and 3-methyl-2-benzothiazolinone hydrazone (HCl) from Eastman. Crude heparinase (Flavobacterium heparinum) was a gift from Dr. A. LINKER.

Enzyme preparations. Bovine adrenal cortex casein kinase of the A and G types were purified as previously described (3,8). Protein kinase assays and protein kinase inhibitor activity measurements were performed as previously described (3,8,9).

Casein kinase inhibitor was purified from bovine adrenal cortex cytosol following the previously described procedure (8) except that lyophilization was used instead of the final hydroxyapatite concentration step.

Degradative treatment of the inhibitor. Pronase digestion was carried out with an aliquot of the inhibitor preparation (65 µl containing 4 µg protein) for 6 h at 37°C in the presence of 5 µg pronase, in 20 mM, pH 8.0 tris-HCl buffer containing 2 mM $CaCl_2$. The reaction was stopped by a 5 min heating at 100°C. Other enzymatic treatments were performed with 10 µl aliquot of inhibitor (containing 26 µg glycosaminoglycan) after mixing with enzyme solutions as follows: chondroitinase ABC: 0.22 units/ml in pH 8.0 tris acetate buffer (11); chondroitinase AC: 0.22 units/ml in pH 7.3 tris acetate buffer (11): hyaluronidase: 7500 units/ml in pH 5.4 acetate buffer (12): crude heparinase: 1 mg/ml in pH 7.0 sodium acetate buffer (13). Incubations were carried out at 37°C for 90 min except in the case of hyaluronidase (17 h) and heparinase (17 h at 30°C) and stopped by a 1 min treatment at 100°C. Reaction of the inhibitor with nitrous acid (30 min at 25°C) was performed with aliquot (10 µl) of the inhibitor preparation and nitrous acid as in (14).

Analytical methods.
. Total glycosaminoglycans (GAG) were measured according to GOLD (15); hexosamine content following SMITH and GILKERSON (16), uronic acid content according to BITTER and MUIR (17) and sulfate content with the rhodizonate method of TERHO and HARTIALA (18). Proteins were measured according to LOWRY et al. (19).

. Electrophoretic analysis of GAG on cellulose acetate was performed according to WESSLER (20) with 3 hour runs. GAG were stained by 5 min treatment of the strips with a 0.1% alcian blue solution in 0.3% acetic acid and destaining carried out with 10% acetic acid washings. Quantitative scanning was performed using a VERNON densitometer. Dodecyl sulfate (0.1%) slab gel electrophoresis was performed in discontinuous tris-glycine buffer according to LAEMMLI (21) using 15% polyacrylamide gel. - Protein ^{125}I labeling was carried out following the Na ^{125}I-chloramine T procedure (22), free iodine being removed by filtration of the reaction mixture through a Biogel P-6 column.

RESULTS

1. Presence of glycosaminoglycan structures in the casein kinase G inhibitor.

Table I gives the values obtained when total GAG and typical components of GAG chains (hexosamines, uronic acids, sulfate groups) were measured in the purified bovine adrenal cortex casein kinase G inhibitor preparation. The preparation contains also a measurable amount of protein which suggests a proteoglycan structure for casein kinase G inhibitor, which has been previously found homogeneous according to several criteria usually used for proteins (7). However, the protein content of the inhibitor preparation is less than 5% of the mass of the preparation as expressed as GAG. As seen in table I, the molar ratio of characteristic GAG components found in casein kinase G inhibitor preparation are rather similar to those obtained with model chondroitin sulfate of the A type.

The casein kinase G inhibitor was then analysed by cellulose acetate electrophoresis as illustrated in figure 1. After alcian blue staining, the native preparation (fig. 1, lane 1) exhibited two colored spots; the minor one had the electrophoretic mobility of heparin used as model GAG structure on a parallel lane, whereas the major spot migrated like a chondroitin sulfate of the A type. Pronase treatment of the inhibitor prior to the analysis did not modify this pattern (fig. 1, lane 2). It may be noticed that the casein kinase G inhibitor could not be detected on the electrophoretic strips after protein staining under these conditions but was revealed after autoradiography when a [^{125}I]-labeled inhibitor preparation was analysed under identical conditions (fig. 1, track 3). The casein kinase G inhibitor activity could be measured after fractionation and extraction of a parallel strip lane. As shown in figure 1, the inhibitory activity co-migrates with the components detected after GAG staining. It may thus be concluded that the inhibitor preparation contains glycosaminoglycan chains. The GAG structures involved appear heterogeneous but the casein kinase G inhibitory activity has always been found to comigrate with the GAG components upon cellulose acetate

TABLE I

Chemical characterization of glycosaminoglycan chains in the casein kinase G inhibitor preparation as compared to commercially available model GAG (heparin; CSA: chondroitin sulfate A; CSC: chondroitin sulfate C). Determination of total GAG and GAG components was performed as described under methods.

	Total GAG	Protein	Uronic acids	Hexosamines	Sulfates	Molar Ratios		
						Uronic acids: Hexosamines	Sulfates: Hexosamines	Sulfates: Uronic acids
Inhibitor preparation								
- per ml preparation	2.6 mg	0.06 mg	4.5 µmol	4.3 µmol	3.6 µmol	1.03	0.85	0.82
- per mg GAG	-	0.023 mg	1.7 µmol	1.6 µmol	1.4 µmol			
Heparin (/mg GAG)	-	-	1.1 µmol	1.1 µmol	2.3 µmol	1.00	2.09	2.09
CSA (/mg GAG)	-	-	1.9 µmol	1.8 µmol	1.5 µmol	1.05	0.83	0.79
CSC (/mg GAG)	-	-	1.2 µmol	1.3 µmol	1.3 µmol	0.92	1.00	1.08

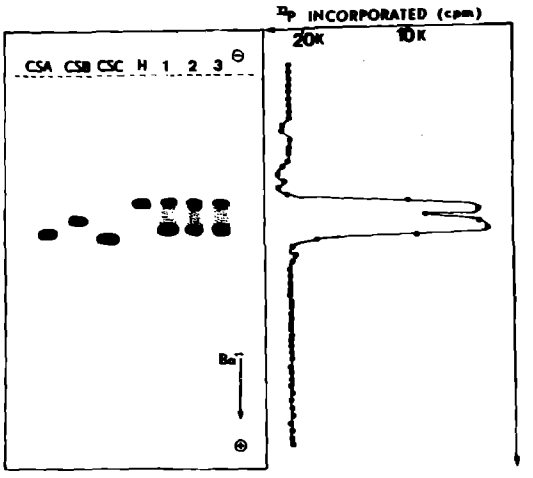

Fig. 1 - Electrophoretic study of the glycosaminoglycan content of the adrenal cortex casein kinase G inhibitor.
The left part of the figure illustrates the electrophoretic analysis on cellulose acetate of model GAG (CSA: chondroitin sulfate A; CSB: chondroitin sulfate B; CSC: chondroitin sulfate C; H: heparin) and the inhibitor preparation (6 µg GAG and 0.17 µg protein) before (1) and after (2) treatment with pronase. Analysis and staining with alcian blue were as described under methods. Autoradiogram of the ^{125}I labeled inhibitor preparation was performed on a parallel lane (3). The right part of the figure gives the distribution of casein kinase G inhibitory activity extracted from a parallel lane of the cellulose strip as described under methods. Casein kinase G activity in the presence of the extract from the strip is represented. The arrow indicates the direction of the electrophoretic migration in the presence of barium (Ba^{++}) as described under methods.

electrophoresis. Similar analysis on polyacrylamide gel was not satisfactory due to the spreading of both the alcian blue staining and the casein kinase G inhibitory activity along the gel.

2. Further characterization of the GAG structures present in the inhibitor.

Further characterization of the GAG component found in the inhibitor was carried out using selective degradation procedures, as illustrated in figure 2. Chondroitinase ABC as well as chondroitinase AC and hyaluronidase treatments resulted in the disappearance of the higher mobility inhibitor component after alcian blue staining and of the corresponding biological activity. Crude heparinase led to the total disappearance of the material detected after staining of the gel or by inhibitory activity measurement. Nitrous acid treatment selectively destroyed the lower mobility component, whereas a combination of chondroitinase ABC digestion followed by nitrous acid treatment totally destroyed all detectable inhibitory material. These data indicated that the inhibitor contains a chondroitin-like and an heparin-like structures, in agreement with the aforementioned suggestion based on electrophoretic pro-

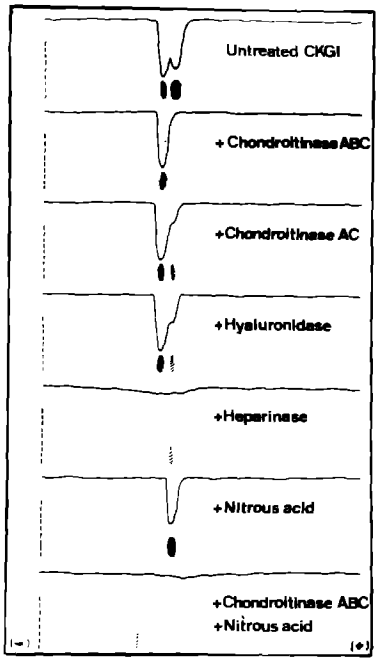

Fig. 2 - Electrophoretic analysis of the casein kinase G inhibitor preparation after various degradation procedures.
The preparation (6 µg GAG and 0.17 µg protein) was analysed by electrophoresis on cellulose acetate strip as in figure 1. Parallel lanes were either stained with alcian blue giving the pattern drawn as black spots or extracted for determination of casein kinase G inhibitory activity along the cellulose strip (full line trace).

perties (fig. 1). They also showed that the inhibitory activity is related to the presence of intact GAG structures in the inhibitor preparation.

DISCUSSION

The specific G type casein-kinase inhibitor previously purified from adrenal cortex cytosol has been shown in this study to be made of two major components characterized as heparin-like and chondroitin sulfate-like structures respectively. An heterogeneous glycosaminoglycan structure is thus proposed for the endogenous casein kinase G inhibitor previously found apparently homogeneous according to several protein criteria (8). Selective inhibition of casein kinase G appeared to require the presence of GAG chains in the proteoglycan structure. These data are consistent with the previous observation that the inhibitory activity was resistant to trypsin digestion and only partly destroyed by pronase after drastic incubation conditions (8). The present findings are in agreement with the fact that naturally occurring GAG such as heparin and chondroitin sulfate have been found to selectively inhibit casein kinase G activity whereas A type casein kinase

and cAMP dependent protein kinase were not affected (24). Inhibition of a phosvitin kinase from rooster liver by heparin has been previously reported (25) and recently selective inhibition of a reticulocyte casein kinase II by the same GAG has also been observed (26). In all these cases, the GAG-sensitive protein kinases were messenger independent and the casein kinase II is a G type enzyme (27). The polyanionic GAG nature of the inhibitor is also in agreement with the fact that its interaction with casein kinase G could be reversed in the presence of polycations such as naturally occuring polyamines (9). This property was the basis of the proposal that casein kinase G activity in the intact cell may be modulated through an interaction of polyamines with the enzyme-endogenous inhibitor complex (9).

The fact that the inhibitor could be identified as a glycosaminoglycan mixture focuses the interest on a possible interaction of intracellular GAG with the G type casein kinase phosphorylation system. Interaction of GAG and especially heparin with several enzymatic systems has been recognized (28) and various pharmacological effects of these compounds are known in vivo beside the widely used action on the blood coagulation cascade (29). Although a scanty knowledge of GAG intracellular distribution in mammalian tissues is available (30), these structures have been found not only attached to plasma membrane but associated with intracellular compartments (30-33). Although both G type casein kinase and its endogenous inhibitor have been isolated from adrenal cortex cytosol (3,8), the precise cellular location of both moiety remains to be established. However, it may be mentioned that a partially purified inhibitor with identical properties toward casein kinase G has been isolated from bovine adrenocortical cells in primary culture (unpublished). Further studies are thus needed to define a possible functional relationship between the messenger independent G type casein kinase protein phosphorylation system and intracellular GAG since both qualitative and quantitative modifications of the latter have been suggested to correlate with normal and/or pathological cellular proliferation (29,30). On the other hand, the purified endogenous inhibitor and naturally occurring GAG, particularly heparin, appear as useful biochemical tools for the characterization of protein kinases in mammalian tissues, since their inhibitory action appears selectively directed toward G type, messenger independent protein (casein)-kinases.

Acknowledgments - This work was possible thanks to the support of the INSERM (U-244, ATP 77-84 and 79-114), the CNRS and the Fondation pour la Recherche Médicale Française. We are indebted to Dr. A. LINKER for a generous gift of heparinase.

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