SELECTIVE INHIBITION OF A CYCLIC NUCLEOTIDE-INDEPENDENT PROTEIN KINASE (G-TYPE CASEIN KINASE) BY NATURALLY OCCURRING GLYCOSAMINOGLYCANS

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1. Introduction

Several types of nucleoside triphosphate–protein phosphotransferases (protein kinases, PK) activities have been recognized in various mammalian tissues [1]. These activities may be classified according to their dependence upon specific effectors [2] such as cyclic nucleotides (cAMP or cGMP), calcium and calmodulin or ds RNA [2]. On the other hand, a number of PK activities have been characterized for which no specific effector has yet been demonstrated [1,2]; these activities may thus be termed messenger-independent protein kinases [2]. We had isolated in bovine adrenal cortex cytosol 4 casein kinases belonging to this category [3]. These enzymatic moieties could be classified into an A-type casein kinase (using only ATP as phosphate donor, CKA) and a G-type (CKG, using GTP as well as ATP). The CKG activity was then shown to be in an inhibited form in crude tissue extract, due to the presence of a specific endogenous inhibitory factor (CKG I) isolated in homogeneous form [4]. In addition, it was found that polycations such as naturally occuring polyamines can stimulate purified CKG activity [3] and also release the CKG I inhibitory effect on the enzyme [5]. Polyamines (e.g., spermine) were thus suggested as possible mediators of the intracellular CKG–CKGI system [5].

In the course of these studies, it was observed that glycosamino glycan structures such as heparin can inhibit CKG activity. In view of the possible interference of the CKG system in vivo with the known cellular glycosaminoglycans (GAG) [6] we decided to examine the interaction of available naturally-occurring GAG structures on 3 types of protein kinase activities using purified enzyme preparations. This paper reports the results obtained with 3 different protein kinases namely the catalytic subunit of cAMP-dependent PK and the bovine adrenal cortex casein kinases of the A- and G-types. Several naturally occuring GAG appear to selectively inhibit CKG activity and may thus be proposed as useful biochemical tools for the characterization of cellular protein kinase activities. On the other hand, these observations point to a possible role of proteoglycans as potential modulators of a specific protein kinase activity in intact cells.

2. Materials and methods

[γ^{32}P]ATP (20 Ci/mmol) was purchased from the Radiochemical Centre (Amersham). Casein (Merck) was treated according to [7] before use. Spermine (tetrahydrochloride), chondroitin sulfate A, B and C, hyaluronic acid (sodium salts) and histone (II A) were from Sigma. Heparin (sodium salt) was from Hoffmann LaRoche. AG 50 W X 8 and AB 1 X 2 ionexchange resins were from Bio-Rad.

2.1. *Enzymatic preparations*

Casein kinase of the A (CKA)- and G (CKG)-types were isolated from bovine adrenal cortex cytosol after a stepwise elution from a phosphocellulose column in [4] with 0.6 and 1 M buffered NaCl, respectively. These preparations were stored at −20°C in TDG buffer (10 mM Tris–HCl (pH 7.5) containing 1 mM dithiothreitol, 2% glycerol and 1 mg bovine serum albumin/ml). Pure catalytic subunit (C) of bovine heart cAMP-dependent protein kinase was a generous gift from Professor J. Demaille (Montpellier).

2.2. *Protein kinase assay*

This was performed under the incubation conditions in [8] with the trichloroacetic acid precipitation procedure of [9], as in [3–5], either with casein (with a 50 mM $MgCl_2$ final conc.) or histone (with a 5 mM $MgCl_2$ final conc.) as the substrate. Various amounts of model glycosaminoglycans were added to the assay, as indicated in the text and figure legends. Casein and histone kinase activity unit was defined as the amount of enzyme which catalysed the incorporation of 1 pmol ^{32}P/min in the protein substrate under the assay conditions. Inhibitory activities were quantitated by calculation of the 50% inhibitory dose (I_{50}) following the method in [10].

2.3. *Acid–base titration of glycosaminoglycans*

Commercial GAG (sodium salts) and spermine (tetrahydrochloride) were desalted before titration by passage through an AG 50 W X 8 (hydrogen form), or an AG 1 X 2 (hydroxide form) ion-exchange resin, respectively. GAG concentrations were determined as in [11] and spermine recovery was measured using [^{3}H]spermine (New England Nuclear) added as tracer before ion-exchange treatment. Average recoveries were 60–80% for protonated GAG and 100% for tetrahydroxylated spermine. Titration of protonated GAG (100 mg/10 ml) was performed either by 50 mM NaOH or 15 mM spermine. Hyaluronic acid (0.5 mg/ml) was titrated by 10 mM NaOH and 3 mM spermine solutions.

Radioactive counting and all ancillary methods were as in [3–5].

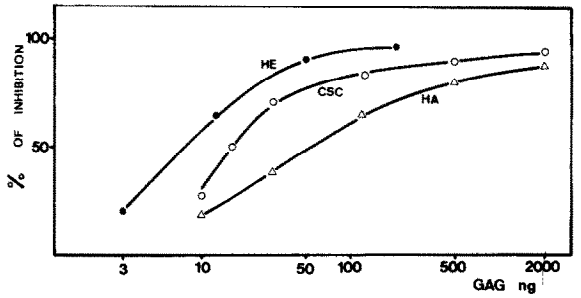

Fig.1. Inhibition of G-type casein kinase activity by glycosaminoglycan structures. The incubation conditions were as in section 2, in a final 80 μl vol. containing casein (60 μg), [$\gamma^{32}P$]ATP (10^{-4} M) and various amount of GAG in TDG buffer (50 mM $MgCl_2$). The reaction was initiated by addition of 10 units of purified adrenal cortex CKG. Each point is the mean of 3 replicates: HE, heparin; CSC, chondroitin sulfate C; HA, hyaluronic acid.

3. Results

3.1. *Effect of various glycosaminoglycan structures on G-type casein kinase activity (CKG)*

Fig.1 illustrates the effect of increasing concentrations of heparin, a chondroitin sulfate and hyaluronic acid on the activity of CKG isolated from bovine adrenal cortex cytosol. Heparin appeared to be a powerful inhibitor of CKG activity, being potent in the ng-range in the assay and yielding an almost complete inhibition of the enzyme for the highest concentrations used. Hyaluronic acid was markedly less effective whereas chondroitin sulfates (A and B, not shown in fig.1) exhibited an intermediate potency. The concentration of GAG needed to yield a 50% inhibition of CKG under our assay conditions (I_{50}) were: chondroitin sulfate A 325 ng/ml; chondroitin sulfate B 350 ng/ml; chondroitin sulfate C 275 ng/ml; heparin 94 ng/ml; hyaluronic acid 687 ng/ml.

3.2. *Selectivity of CKG inhibition by various GAG*

Table 1 gives the results obtained when 3 different protein kinases (C, CKA, CKG) activities were

Table 1
Effect of several glycosaminoglycans and dextran sulfate on 3 different protein kinases: C (catalytic subunit of the cAMP-dependent PK); CKA and CKG (casein kinases of the A- and G-types)

Compound added	Protein kinase act. (pmol [^{32}P]incorporated/min)		
	C	CKA	CKG
None (control)	62.1	1.1	10.0 (0)
Heparin 3.1 ng	61.9	1.2	7.7 (23)
12.5 ng	62.0	1.1	3.3 (65)
Chondroitin sulfate C			
31 ng	62.5	1.0	2.7 (73)
125 ng	62.3	0.9	1.6 (84)
Hyaluronic acid			
31 ng	61.8	0.9	4.4 (56)
125 ng	62.1	1.1	2.6 (74)
Dextran sulfate			
31 ng	61.9	1.1	2.7 (73)
125 ng	62.2	1.3	1.1 (89)

Incubations were done as in section 2 either with histone (C) or casein (CKA and CKG) as the substrate. In parentheses: % inhibition with regard to the control

assayed in the absence or in the presence of various GAG at 2 different concentrations. The inhibitory effect appeared selectively directed toward CKG activity whereas C and CKA were not affected, even at GAG concentrations which yielded 80–90% inhibition of CKG. In addition to naturally occuring GAG structures, dextran sulfate was introduced in this study to check whether the polyanionic sulfate character was a common requirement for the inhibitory activity. This sulfated polysaccharide was indeed a potent inhibitor of CKG, although ~10-times less active than heparin. The suggestion that a polyanionic structure may be required in the inhibition process was further suggested by the lack of effect of characteristic monomeric GAG components such as glucuronic acid and glucosamine (not shown).

3.3. *Reversal of the GAG effect on CKG*

When an increasing amount of substrate (casein) was used in the assay of CKG in the presence of inhibitory doses of GAG, a progressive release of the inhibitory effect was observed, as illustrated in table 2. These data suggested that glycosaminoglycan structures act through a competitive inhibitory mechanism toward casein at the enzyme active site.

Since the inhibitory activity appeared related to a negatively charged polymeric structure, the effect of naturally occuring polycations (namely polyamines) on the GAG inhibitory activity was examined. As illustrated in fig.2, addition of increasing amounts of spermine to a glycosaminoglycan-inhibited CKG resulted in a rapid release of inhibition in the case of heparin. However, spermine was not able to release the CKG inhibition due to the presence of chondroitin sulfate A, whereas the effect of C and B chondroitin sulfates was only partly reversed by the polycation. It may thus be suggested that although common to

Table 2
Effect of substrate (casein) concentration on the inhibition of CKG activity (%) by different glycosaminoglycans

GAG added (ng/ml) [Casein]	CSA (312.5)	CSB (375)	CSC (312.5)	HE (75)	HA (1875)
0.75 mg/ml	62	60	66	45	70
7.5 mg/ml	5	4	8	4	7

Assay conditions and abbreviations as in table 1. Each value is the mean of 3 replicates

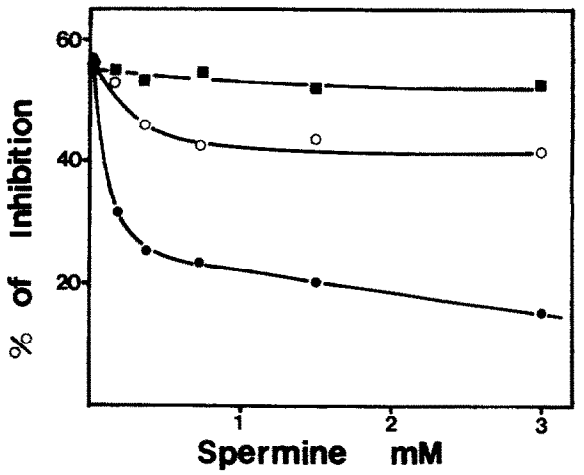

Fig.2. Effect of a polyamine on the inhibition of CKG by various glycosaminoglycans. Casein kinase activity was measured as in section 2 in the presence of 15 ng heparin (●——●), 200 ng chondroitin sulfate A (■——■) or 200 ng chondroitin sulfate C (○——○) in a standard reaction mixture (75 μl) to which was added increasing amounts of spermine in 5 μl of 25 mM Tris–HCl (pH 7.4) buffer. Each point is the mean of 3 replicates.

all inhibitory GAG structures tested, the polysulfate character is not the unique molecular determinant for CKG inhibition. This point was further examined by acid–base titration of the glycosaminoglycans used. As shown in table 3, a good correlation was found for the acidic property of individual GAG titrated either by sodium hydroxide or spermine. However, although the most potent CKG inhibitor

Table 3
Acidic character and CKG inhibitory potency of glycosaminoglycan structures

GAG structure	HE	CSA	CSC	HA
NaOH titr. (mmol/g GAG)	8.15	4.46	5.2	5.06
Spermine titr. (mmol/g GAG)	2.16	1.20	1.30	1.10
NaOH / spermine	3.77	3.72	4.03	4.62
I_{50} for CKG (ng/ml)	94	325	275	687

(i.e., heparin) exhibited the highest acidic titer, there was no linear relationship between the acidic character as measured by these titration experiments and the CKG inhibitory potency (I_{50}) in the case of chondroitin sulfates and hyaluronic acid. It may thus be suggested that, although the polysulfate structure appears sufficient to selectively inhibit CKG activity, the overall inhibitory properties of naturally occuring GAG structures most likely involve additional and more specific molecular parameters.

4. Discussion

Naturally occuring glycosaminoglycan structures (e.g., heparin, chondroitin sulfates, hyaluronic acid) have been found to exhibit a selective inhibitory property directed toward G-type casein kinase activity, whereas another casein kinase (CKA) and the cAMP-dependent protein kinase activities are not affected. This specific CKG inhibition can be reversed by an excess of protein substrate, suggesting that GAG act through a competitive mechanism. Polycations such as naturally occuring polyamines (e.g., spermine) are able to release the GAG effect on CKG activity, although this does not appear a general property for all GAG examined in this study. These observations suggest that the active site of CKG is specifically sensitive to polyanionic structures such as polysulfated polysaccharides. This is especially true of heparin, which specifically inhibits CKG activity at low concentration and whose action can be fully reversed upon polyamine addition. These properties toward CKG are reminiscent of what had been found with a specific endogenous inhibitor of CKG (CKG I) purified from adrenal cortex cytosol [3–5]. Current work is in progress toward the structural identification of CKG I, which appears also as a negatively charged moiety [3–5], unpublished). With regard to the biological significance of the findings reported herein, it remains to be established whether glycosaminoglycans and particularly heparin could be potent modulators of CKG activity in the intact cell. Many pharmacological properties of GAG have been reported in vivo [12] and interaction of these polyanions with several cellular enzymatic systems have been described [13]. Although a scanty knowledge of GAG intracellular distribution is available [13], these structures appear not only attached to plasma membranes [13,14] but have been found associated with intracellular components [13–15]. On the other hand, due to their marked and specific inhibitory action on CKG activity GAG may be proposed as a useful biochemical tool in the characterization of various types of protein kinase activities in mammalian tissues.

Further studies are needed along these lines to define a possible relationship between CKG activity (and thus intracellular protein phosphorylations) and glycosaminoglycans. This would also help to define the possible role of CKG in the cellular functions since both qualitative and quantitative variations of GAG cellular content have been suggested to be related to normal and pathological cellular proliferating activity [12,13].

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