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INHIBITION OF MAMMARY GLAND CYCLIC AMP-DEPENDENT PROTEIN KINASE BY ARGININE-RICH HISTONES

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SUMMARY

The cyclic AMP-dependent protein kinase activity from lactating bovine mammary gland efficiently phosphorylates lysine-rich histones but not arginine-rich histones. It is shown that arginine-rich histones in fact inhibit phosphorylation of lysine-rich histones. Polyarginine and a range of low molecular weight cationic molecules are also inhibitors. Inhibition of histone H2b phosphorylation by histones H4 and H3 is competitive with respect to H2b. This inhibition behaviour may be tissue-specific since the protein kinase activity in crude extracts from lactating bovine mammary gland, although heterogeneous, may be completely inhibited (> 95%) by arginine-rich histones and polyarginine.

INTRODUCTION

Protein kinase activities able to phosphorylate basic substrates such as histones and, in many cases, stimulated by cyclic AMP have been detected in a wide range of animal tissues.[1] The best characterised of these, the enzyme from rabbit muscle, has been shown to recognise primarily a short sequence of amino acids containing a serine residue located 2-5 residues on the carboxyl terminal side of one or more basic amino acids.[2] Comparison of the sites phosphorylated in a variety of substrates by this and several other protein kinases shows that those enzymes which exhibit a preference for basic substrates have a common property of recognising phosphorylation sites close to lysine and arginine residues.[2,3] This appears to be the basis for the ability of both lysine- and arginine-rich histones to serve with varying efficiencies as phosphate acceptors.

In this paper we show that the histone-preferring protein kinase of lactating bovine mammary gland, while able to efficiently phosphory-

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late lysine-rich histones at multiple sites, is strongly inhibited by arginine-rich histones. Polyarginine, protamine and a variety of small cationic molecules also are inhibitors. It is suggested that the activity of this enzyme or group of enzymes *in vivo* may be controlled or restricted by one or more arginine-rich proteins.

MATERIALS AND METHODS

Extracts containing protein kinase activity were prepared from lactating bovine mammary gland as described previously.[4] The addition of 10 mM magnesium acetate was found to stabilize the activity during storage at -20°C.

Protein kinase activity was assayed in reaction mixtures of final volume 0.1 ml containing 50 mM Tris-HCl buffer (pH 8.0), 20 mM sodium fluoride, 2 mM theophylline, 0.3 mM ethylene glycol bis-(β-aminoethyl ether)-N, N-tetraacetic acid, 5 mM magnesium acetate, 0.25 mM [γ-^{32}P]-ATP (10-150 cpm/pmole) and 0.25 mM cyclic AMP. Phosphate acceptor proteins and inhibitors were present at concentrations as indicated. The reaction was initiated by addition of enzyme and incubation was for 5 minutes at 30°C. Reaction was terminated by the addition of 0.4 ml 0.1 M sodium pyrophosphate followed by 0.5 ml of 50% trichloroacetic acid. The suspension was mixed and centrifuged for 1 minute at 12,000 g. The pellets were washed three times in 25% trichloroacetic acid then dissolved in 1 ml 0.2 M NaOH and radioactivity was determined by Cerenkov counting in a scintillation counter.

Histones were isolated from calf thymus glands by the method of Oliver *et al*, [5]. Histones H1, H2a, H2b, and H4 were found to yield a single band after electrophoresis by the method of Panyim and Chalkley [6]. Histone H3 also contained two faster migrating bands which represented about 5% of the total protein. β-caseins A_2 and B were prepared by the method of Manson and Annan [7]. The identity of the β-casein B sample was checked in the rabbit muscle protein kinase assay.[2] Reduced carboxymethylated maleylated lysozyme was prepared as described by Bylund and Krebs [8]. Polyarginine (Type IIB) polylysine (Type II) protamine (Grade I), and low molecular weight amines were obtained from Sigma. Tryptic digestion and fingerprint analysis were carried out using standard procedures [9]. Radioactive peptides were located by autoradiography.

RESULTS

1. Inhibition of lysine-rich histone phosphorylation by arginine-rich histones

Mammary gland protein kinase is able to efficiently phosphorylate the lysine-rich histones H1 and H2b but is completely inactive towards the arginine-rich histones H4 and H3 [4]. Further investigation has shown that H4 and H3 are able to strongly inhibit phosphorylation of the lysine-rich histones. Table 1 shows that H4 is somewhat more effective in this regard than H3, while polyarginine is a very potent inhibitor. In order

TABLE 1. INHIBITION OF MAMMARY GLAND PROTEIN KINASE BY ARGININE-RICH HISTONES

Phosphate acceptor.	Acceptor concentration (mg/ml)	Inhibitor	Inhibitor concentration (mg/ml)	Inhibition (%)
H1	5	H3	5	69.7
H1	5	H3	10	97.7
H1	5	H4	5	97.5
H1	5	H4	10	98.4
H2b	5	H3	5	41.4
H2b	5	H3	10	96.3
H2b	5	H4	5	94.7
H2b	5	H4	10	96.3
H2b	4	polylysine	4.0	20.9
H2b	4	polylysine	0.8	0
H2b	4	polyarginine	1.0	98.2
H2b	4	polyarginine	0.1	40.0

to study this effect further an attempt was made to locate the site of phosphorylation in H2b histone. Fingerprint analysis of tryptic digests of H2b histone phosphorylated by two subfractions of the crude activity (obtained by Ultrogel AcA34 gel chromatography) revealed a distinctive pattern of radioactive peptides for each fraction. A total of 9-10 radioactive peptides were present. The heterogeneous pattern of labelling might be due to a relative lack of specificity of the enzymes or it may indicate further heterogeneity within the two enzyme subfractions or both. Attempts to further fractionate the crude activity have been hampered by its lability. In any case it is clear that all of the kinase activity present in the crude extract is inhibited by H4 and H3 histones. Histone H2a was less effective as a phosphate acceptor (see Table 2) than histones H1 and H2b and did not inhibit phosphate incorporation into these histones.

Separate experiments showed that the inhibition by arginine-rich histones was not due to activation of an ATPase or of a phosphoprotein phosphatase. Sedimentation analysis indicated that under the conditions of the assay no interaction occurs between H1 and H4 histones, in agreement with previous reports [10] that H1 histone does not interact with other histones

TABLE 2. INHIBITION OF MAMMARY GLAND PROTEIN KINASE BY SMALL MOLECULES

Acceptor*		Inhibitor	Inhibitor concn.(mM)	Inhibition (%)
H2b histone		Spermine	10	33.5
H2b histone		Spermidine	10	28.4
H2b histone		Cadaverine	20	6.5
H2b histone		Guanidine	20	29.1
H2b histone		Tyramine	20	33.8
H2b histone		Tryptamine	20	42.1
H2b histone		Histamine	20	62.4
H2b histone	(60)	Histamine	4	37.4
H1 histone	(100)	Histamine	4	21.9
H2a histone	(41)	Histamine	4	21.9
RCMM-lysozyme	(43)	Histamine	4	2.6
Protamine	(97)	Histamine	4	5.2
β-Casein-B	(24)	Histamine	4	-13.1#

* All acceptors present in assay at 4 mg/ml.

\# Stimulation.

RCMM- = reduced carboxymethylated maleylated -

Numbers in brackets indicate relative incorporation into phosphate acceptors in the absence of inhibitor.

under similar conditions. Inhibition, therefore, cannot be due to the formation of histone aggregates. Kinetic analysis of the inhibition of H2b phosphorylation by histones H4 and H3 showed that the inhibition was competitive with respect to H2b. The apparent Ki values were 0.06 mM for H4 and 0.14 mM for H3; the apparent Km for histone H2b was determined to be 0.2 mM (Fig.1).

In view of the strong inhibition observed for polyarginine, the effect of protamine on the phosphorylation of lysine-rich histone was investigated. When the level of histone H2b was held constant (4 mg/ml) and increasing levels of protamine added, total incorporation remained approximately constant. Separation of protamine and H2b, by Sephadex G-50 filtration following incubation showed that, when both acceptors were present, protamine

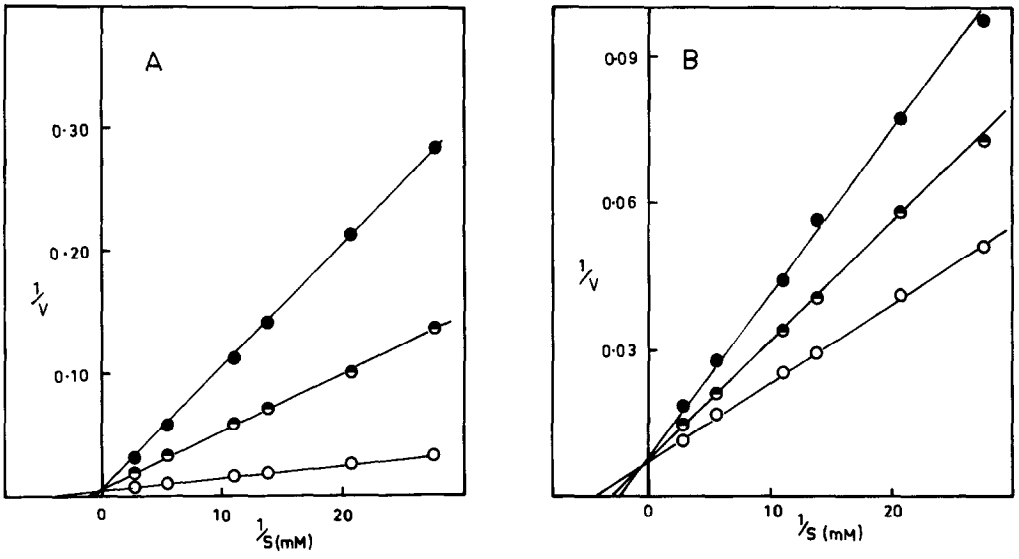

Fig. 1. Double reciprocal plots for initial velocity of phosphorylation of histone H2b by histone-preferring protein kinase from lactating bovine mammary gland versus concentrations of H2b at constant levels of H4 and H3 histones.

A, variable H2b concentration, no H4.
variable H2b concentrations plus 0.18 mM H4 histone.
variable H2b concentrations plus 0.44 mM H4 histone.

B, variable H2b concentration no H3 histone added.
variable H2b concentration plus 0.05 H3 histone.
variable H2b concentration plus 0.10 mM H3 histone.

produced a progressive inhibition of H2b phosphorylation. When both acceptors were present at equal concentrations 86% of the incorporation was into protamine. Thus protamine is an effective inhibitor of lysine-rich histone phosphorylation. It is not known whether the same enzyme activity phosphorylates both H2b histone and protamine.

Kemp et al. [2] showed that β-casein-B, which contains the substitution Arg in place of Ser at position 122, is specifically phosphorylated by rabbit muscle protein kinase at Ser-124 approximately 75 times more rapidly than are the other genetic variants of β-casein. When β-caseins A_2 and B were tested as acceptors for the mammary gland enzyme no difference in incorporation was observed either at pH 6.5 or at pH 8.0, indicating that Arg-122 in β-casein-B does not serve as a recognition site for the mammary gland enzyme.

2. Inhibition by small cationic molecules.

The effect of a range of small basic molecules on the mammary gland kinase was investigated and the results (Table 2) indicate that a variety of such molecules are able to inhibit phosphorylation when lysine-rich histones are used as acceptors. While this work was in progress Murray et al [11] reported a similar effect of polyamines on cyclic AMP-dependent protein kinases from a variety of tissues including mouse epidermis and rabbit muscle.

DISCUSSION

Most protein kinases appear to be able to phosphorylate both lysine-rich and arginine-rich histones although it is not unusual for a marked preference for one or other histone to be exhibited. The bovine mammary gland kinase not only fails to phosphorylate arginine-rich histones but is subject to strong inhibition by both H4 and H3 histones. Kemp et al., [12] have shown, using synthetic peptide substrates that arginine residues are important in determining substrate recognition by rabbit muscle protein kinase. Substitution of the serine in a peptide substrate by alanine produced a competitive inhibitor of phosphorylation of the original peptide and of RCMM-lysozyme. It seems likely that sequences in H4 and H3 histones are sufficiently similar to those surrounding phosphorylation sites in H1 and H2b to cause competitive binding to the active site of the mammary gland kinase. Failure of the enzyme to phosphorylate such sequences in H4 and H3 could be due to the presence of arginine instead of lysine residues or to the absence of serine or threonine residues within such sequences. Examples of such homologous sequences are:

H2b : 85
Lys Arg Ser Thr Ile

H3 : 115
Lys Arg Val Thr Ile

H3 : 8
Arg Lys Ser Thr Gly

Several other similar partially homologous sequences are apparent when the amino acid sequences of the lysine-rich and arginine-rich histones are compared

The enzyme activity studied here resembles other cyclic AMP-dependent protein kinases in its apparent preference for arginine residues within sequences which bind at the active site, and it seems probable that similar sequences might characterise its in vivo substrates(s). Nevertheless the substrate specificity of this enzyme appears to be distinct from that of, for example, the rabbit muscle enzyme since it will not specifically phosphorylate β-casein B. It is also distinguished from many other protein kinases by its complete inability to phosphorylate arginine-rich histones.

Several proteins able to modulate or inhibit protein kinases have been reported, including two from rat mammary gland which inhibit the mammary cytosol cyclic AMP-dependent protein kinase from that species.[13]. One of these has been shown to inhibit non-competitively. The rat mammary gland kinase is able to phosphorylate both lysine- and arginine-rich histones [13, 14]. Inhibitors which do act competitively have been reported to be present in a number of tissues [15]. In bovine mammary gland such inhibitors, containing arginine-rich sequences, might play a role in restricting what appears to be a relatively non-specific activity directed towards lysine-rich sequences.

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