Volume 243, number 2, 385–388 FEB 06757 January 1989

Two physiological substrate-specific casein kinases are present in the bovine mammary gland

Charles L. Brooks

Department of Veterinary Pathobiology, The Ohio State University, Columbus, OH 43210, USA

Received 26 November 1988

Two species of casein kinase from lactating bovine mammary gland have been identified; a Ca^{2+}- and CM-independent casein kinase and a Ca^{2+}- and CM-dependent casein kinase. The Ca^{2+}- and CM-independent casein kinase phosphorylates previously dephosphorylated α_{s1}-, β- or κ-casein while the Ca^{2+}- and CM-dependent casein kinase prefers previously dephosphorylated β- or κ-casein as substrates. Two activities are indicated by their substrate specificity, sensitivity to Ca^{2+} and CM, pH maxima, and differential solubilization by anionic detergents. The presence of a regulated casein kinase in the lactating mammary gland suggests that casein phosphorylation may be a regulator of micelle formation or secretion.

Casein; Protein kinase; Calmodulin; (Bovine)

1. INTRODUCTION

The kinases responsible for the physiological phosphorylation of bovine caseins have not been thoroughly characterized. A casein kinase that prefers previously dephosphorylated substrates has been described and solubilized [1–4]. A second casein kinase in lactating bovine mammary gland is regulated by Ca^{2+} and CM and prefers previously dephosphorylated $\varkappa$-casein as substrate [5]. A similar activity in the lactating rat mammary gland has been reported [6].

The purpose of this work is to differentiate between these two activities in terms of substrate specificity, enzymatic characteristics and solubility in various anionic detergents.

2. MATERIALS AND METHODS

2.1. *Materials*

Caseins (α_{s1}, β and $\varkappa$) were isolated from defatted milk of individual cows by DEAE-cellulose (Whatman DE-52) chromatography (pH 6.5) [7] and repurified on DEAE-cellulose at a lower pH (5.0). The caseins appeared homogeneous by alkaline-urea acrylamide gel electrophoresis [3]. The amino acid composition and that determined from sequence data of the α_{s1}-, β- and $\varkappa$-caseins correlated with values of 0.864, 0.971 and 0.961, respectively. Caseins were dephosphorylated and assayed for phosphate content as described [5]. CM was purified from bovine brain by fluphenazine affinity chromatography [6,10].

Correspondence address: C.L. Brooks, Department of Veterinary Pathobiology, The Ohio State University, 1925 Coffey Road, Columbus, OH 43210, USA

2.2. *Methods*

Microsomal membranes were prepared by differential centrifugation [5] from mammary tissue from Jersey cows in the second to fourth weeks of lactation. Protein concentrations were determined by the method of Bradford [11].

Membranes (100 μg) were used as the source of casein kinase activities and assayed as in [5] using a 100 μl mixture containing 50 mM Pipes, 10 mM $MgCl_2$, 100 μg casein substrate, 80 μM [^{32}P]ATP (New England Nuclear, Boston, MA) and when appropriate approx. 1 mM Ca^{2+} and 0.9 μM CM. Reaction mixtures were incubated for 15 s at 30°C, and concluded by addition of SDS, 2-mercaptoethanol and by heating. The products were separated by SDS-containing 12% polyacrylamide gel electrophoresis and the phosphorylation of the caseins quantified by either autoradiography or dissolution of the excised protein containing gel followed by determination of the incorporated [^{32}P]phosphate.

Solubilization of the mammary gland membranes was performed with 1% detergent in buffer containing 300 mM sucrose, 10 mM Tris (pH 7.0), 1 mM EGTA and 10 μg/ml aprotinin at room temperature for 1 h with constant agitation. The solubilized protein was collected after centrifugation at 4°C for 1 h at 148500 × g.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

3. RESULTS

3.1. *Phosphorylation of native and dephosphorylated α_{s1}-, β- and κ-caseins by mammary microsomes*

A greater than 99% dephosphorylation of isolated α_{s1}-, β- and κ-caseins was accomplished by incubation with bovine intestinal alkaline phosphatase followed by trichloroacetic acid precipitation. The acid precipitation eliminated all detectable phosphatase activity. The Ca^{2+}- and CM-dependent casein kinase activity was increased by 7- and 8-fold, respectively, when dephosphorylated rather than native β- and κ-caseins were used as substrates (table 1). Dephosphorylation of α_{s1}-casein did not allow an increase in the Ca^{2+}- and CM-dependent casein kinase. A large biological variation was observed between preparations from cows in early lactation. A Ca^{2+}- and CM-independent casein kinase utilizing dephosphorylated α_{s1}-, β- and κ-casein substrates was present and appeared to be similar to those observed [1–4].

3.2. *Characteristics of the Ca^{2+}- and CM-dependent casein kinase*

The Ca^{2+}- and CM-dependent casein kinase activity was Mg^{2+}-dependent and required the simultaneous presence of Ca^{2+} and CM. Trifluoperazine reduced the Ca^{2+}- and CM-dependent activity to background but did not influence the Ca^{2+}- and CM-independent activities.

3.3. *Influence of H^+ concentration on casein kinase activities*

The activity of the Ca^{2+}- and CM-independent casein kinase was observed over a wide pH range with maximal activity at 6.6 (fig.1). The Ca^{2+}- and CM-dependent casein kinases gave maximal activity at pH 6.8 while the activity rapidly fell under more acidic conditions. The ratios of Ca^{2+}- and CM-independent activities at pH 6.8 and 6.0 were 1.1, 1.5 and 1.0 for α_{s1}-, β- and κ-caseins respectively; while the ratios of Ca^{2+}- and CM-dependent activities were 8.9 and 10.4 for β- and κ-caseins.

Table 1

Ca^{2+}- and CM-dependent casein kinase activities utilizing native and dephosphorylated substrates

Substrate	Activity[a] (pmol/min per mg) (mean ± SD)
Native α_{s1}-casein	43 ± 37
Dephosphorylated α_{s1}-casein	43 ± 24
Native β-casein	9 ± 9
Dephosphorylated β-casein	65 ± 32
Native κ-casein	6 ± 7
Dephosphorylated κ-casein	48 ± 28

[a] n = preparations from 3 cows

3.4. *Solubilization of casein kinase activities*

The solubilization of Ca^{2+}- and CM-dependent and -independent casein kinases from bovine mammary membranes were markedly different and dependent on the detergent (table 2). The Ca^{2+}- and CM-independent casein kinase using α_{s1}-caseins as substrate was enriched per unit protein by 153, 261 and 310% during the solubilization process by extraction with digitonin, octylglucopyranoside or Triton X-100, respectively. Deoxycholate was less effective. The membranes had substrate activity ratios of 0.75 (α_{s1}/β) while the ratios for all the detergent-solubilized preparations were between 0.72 and 0.77, suggesting that the activities phosphorylating α_{s1} and β in a Ca^{2+}- and CM-independent manner were one and the same.

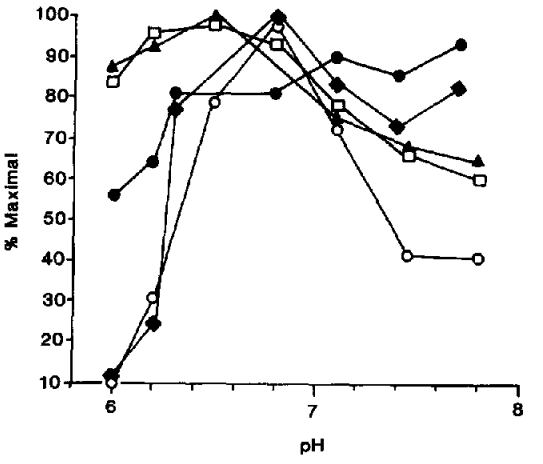

Fig.1. Effect of pH on Ca^{2+}- and CM-independent and -dependent casein kinase activities for various dephosphorylated substrates. Data from studies of three animals have been normalized and averaged. (○) Ca^{2+}- and CM-dependent κ-casein kinase, (♦) Ca^{2+}- and CM-dependent β-casein kinase, (▲) Ca^{2+}- and CM-independent κ-casein kinase, (●) Ca^{2+}- and CM-independent β-casein kinase and (■) Ca^{2+}- and CM-independent α_{s1}-casein kinase.

Table 2

Effects of detergent solubilization on Ca^{2+}- and CM-independent and -dependent casein kinase activities utilizing dephosphorylated substrates

Preparation	Ca^{2+} and CM dependence	Substrate	
		α_{s1}-Casein	β-Casein
Membranes	–	52.6	39.4
	+	ND	31.1
Deoxycholate	–	28.4	22.0
	+	ND	0.0
Triton X-100	–	163.4	121.7
	+	ND	21.3
Digitonin	–	133.4	95.7
	+	ND	32.2
Octylglucopyranoside	–	137.7	104.6
	+	ND	21.7

Results expressed as pmol/min per mg protein; ND, not determined

The solubilization of Ca^{2+}- and CM-dependent β-casein kinase activity in contrast was less efficient with any of the detergents tested. Only digitonin provided a marginal extractive enrichment of activity (104%); octylglucopyranoside (69%) and Triton X-100 (68%) did not selectively solubilize the Ca^{2+}- and CM-dependent β-casein kinase activity. No Ca^{2+}- and CM-dependent casein kinase activity was extracted with deoxycholate. The ratios of Ca^{2+}- and CM-independent and -dependent β-casein kinase activities are 0.79 for the membrane preparation and 0.34, 0.21 and 0.18 for digitonin-, octylglucopyranoside- and Triton X-100-solubilized preparations, respectively.

4. DISCUSSION

Two distinct casein kinases are present in the lactating bovine mammary gland as judged by substrate specificity, Ca^{2+} and CM dependence, pH sensitivity and properties of detergent solubilization. Previously, a divalent cation-dependent casein kinase has been described [1–4] and is most likely the activity we describe as a Ca^{2+}- and CM-independent casein kinase. I previously reported a second casein kinase that utilizes κ-casein as substrate and is Ca^{2+}- and CM-independent [5]. This report compares the characteristics of the Ca^{2+}- and CM-independent and -dependent casein kinases.

The removal of phosphate from casein phosphoproteins should allow a substantial increase in phosphorylation during kinase reactions if the enzymes phosphorylate the same residues as in vivo. Both dephosphorylated β- and κ-caseins served as substrates for the Ca^{2+}- and CM-dependent casein kinase having activities 7–8-fold higher with dephosphorylated substrates. α_{s1}-casein is not a physiological substrate for the Ca^{2+}- and CM-dependent casein kinase activity as α_{s1} dephosphorylation did not allow an increase in activity. The similar magnitudes of Ca^{2+}- and CM-independent and -dependent casein kinase activities suggest a physiological role for each.

Unique phosphorylation sites for the Ca^{2+}- and CM-dependent and -independent κ-casein kinase activities are difficult to rationalize as sequence analysis reveals a single phosphoserine at residue 149 [9]. Two kinases competing for the same site are unlikely but phosphate analysis suggests a stoichiometry of 2 mol phosphate per mol κ-casein [12] suggesting a second phosphorylation site on κ-casein.

Both Ca^{2+}- and CM-dependent and -independent casein kinase activities require divalent cations [1–5] while the -dependent casein kinase also requires the combination of Ca^{2+} and CM for full activity. Others have characterized bovine mammary casein kinases as being activated by either Mg^{2+} or Ca^{2+} [1,2,10]. Perhaps this difference can be explained by the lack of thorough chelation of Ca^{2+} during the preparation of membranes and the continued association of CM with the casein kinase. We use a buffer containing 1 mM EDTA to remove divalent cations and prepare membranes free of CM.

Recent work of Bingham et al. [13] demonstrated that all sites of phosphorylation previously described by sequence analysis can be phosphorylated by a Ca^{+}- and CM-independent casein kinase preparation. The preparation of the membranes was performed in 1 mM EDTA, suggesting that the Ca^{2+}- and CM-dependent enzyme may not participate in phosphorylation of the familiar sites. The meaning of these data needs to be interpreted by phosphorylation and mapping experiments under conditions where the Ca^{2+}- and CM-

dependent β-casein kinase activity can also be determined.

Data involving enzymatic activity at various H^+ concentrations fall into two patterns that suggest the presence of two casein kinases. The Ca^{2+}- and CM-dependent casein kinase activity (β- and $\varkappa$-casein substrates) exhibits a sharp decline in activity at pH values below 6.6. In contrast, the Ca^{2+}- and CM-independent casein kinase activity (α_{s1}-, β- and $\varkappa$-casein substrates) retain their activities at these lower pH values.

The differential solubilization of Ca^{2+}- and CM-dependent and -independent casein kinase activities suggests that they reside on unique proteins. Consistent Ca^{2+}- and CM-independent casein kinase activity ratios (0.72–0.77) using α_{s1}- and β-casein substrates in various detergent-solubilized preparations indicate that one enzyme can utilize either substrate. The data from the pH studies support this interpretation.

Two physiologically relevant casein kinase activities are present in the lactating bovine mammary gland. The primary function of the enzymes appears to be to phosphorylate caseins in preparation for micelle formation. The presence of a Ca^{2+}- and CM-regulated casein kinase in the bovine mammary gland specific for physiologic sites on β- and $\varkappa$-casein suggests that processes such as micelle formation or intracellular protein routing may be regulated features of the bovine mammary gland.

Acknowledgement: This work was supported by a grant (HD21130) from the NIH.

REFERENCES

[1] Chew, L.F. and MacKinlay, A.G. (1974) Biochim. Biophys. Acta 359, 73–82.
[2] MacKinlay, A.G., West, D.W. and Manson, W. (1977) Eur. J. Biochem. 76, 233–243.
[3] Bingham, E.W. and Groves, M.L. (1979) J. Biol. Chem. 254, 4510–4515.
[4] Szymanski, E.S. and Farrell, H.M., jr (1982) Biochim. Biophys. Acta 702, 163–172.
[5] Brooks, C.L. (1987) J. Dairy Sci. 70, 2226–2232.
[6] Brooks, C.L. (1984) Biochem. J. 224, 195–200.
[7] Thompson, M.P. (1966) J. Dairy Sci. 49, 792–795.
[8] Bidlingmeyer, B.A., Cohen, S.A. and Tarvin, T.L. (1984) J. Chromatogr. 336, 93–104.
[9] Eigel, W.N., Butler, J.E., Ernstrom, C.A., Farrell, H.M., jr, Harwalar, V.R., Jenness, R. and Whitney, R.M. (1984) J. Dairy Sci. 67, 1999.
[10] Charbonneau, H. and Cormier, M.J. (1979) Biochem. Biophys. Res. Commun. 90, 1039–1047.
[11] Bradford, M. (1976) Anal. Biochem. 72, 248–254.
[12] Vreeman, H.J., Both, P., Brinkhuis, J.A. and Van der Spek, C. (1977) Biochim. Biophys. Acta 491, 93–103.
[13] Bingham, E.W., Parris, N. and Farrell, H.M., jr (1988) J. Dairy Sci. 71, 324–336.