Ca²⁺-Stimulated Ribonuclease

A NEW MARKER ENZYME OF DIFFERENTIATED RAT MAMMARY TISSUES

By DAI KEE LIU,*† DAVID KULICK* and GENEVA H. WILLIAMS†

*Department of Pharmacology, †Specialized Cancer Research Center and

†Department of Obstetrics and Gynecology, The Milton S. Hershey Medical Center,
The Pennsylvania State University College of Medicine, Hershey, PA 17033, U.S.A.

(Received 19 October 1978)

A unique ribonuclease, active only in the presence of Ca²⁺, was present in lactating mammary gland and milk of the rat. This enzyme was absent from virgin-rat mammary gland and non-mammary tissues of lactating rats. The presence of moderate activity in differentiated mammary tumours, together with an increase in activity in normal tissue paralleling development of mammary function, identify this enzyme as a marker of mammary differentiation in the rat.

The activity of a group of RNAases, first reported by one of us (Liu *et al.*, 1977) in the nuclei of lactating rat mammary gland and mammary tumour, exhibits a characteristic Ca²⁺ dependency. The uniqueness of the stimulating effect of Ca²⁺ ions on mammary RNAase, which is very different from other known RNAases in various animal tissues (Roth, 1967; Barnard, 1969), and the fact that the lactating gland secretes a product rich in calcium (see Jenness, 1974; Patton, 1978) led us to study this enzyme further during different stages of the lactational cycle. During pregnancy and early lactation, mammary epithelium expands many-fold, owing to both proliferation (Munford, 1964; Cowie & Tindal, 1971), and cellular hypertrophy accompanying cytodifferentiation (Munford, 1964; Wellings, 1969; Hollmann, 1969; Baldwin, 1969). In addition to increases in proteins normally present in the resting mammary cell, milk proteins and enzymes involved in synthesis of milk lipids and carbohydrates appear and increase in concentration during gestation and continue to increase until the middle of the lactation period. We show here that Ca²⁺-stimulated RNAase activity also increases in parallel with the functional differentiation of the rat mammary gland. The enzyme is also present in rat milk and mammary tumours, but is absent from all other tissues examined.

Experimental

Animals

The animal maintenance, mating and R3230AC mammary-tumour-transplantation procedures used have been described previously (Liu *et al.*, 1975). Normal tissues were taken from female Fischer rats at various stages of the reproductive cycle. Tumours

studied were the R3230AC mammary adenocarcinoma 6–9 weeks after transplantation into female Fischer rats, two spontaneous mammary adenocarcinomas in female Sprague-Dawley rats, one spontaneous mammary fibroadenoma of a male Fischer rat, and Morris hepatoma 3924A transplanted 4 weeks earlier into a male rat of the ACI strain.

Materials

RNAase-free sucrose was obtained from Schwarz/Mann (Orangeburg, NY, U.S.A.). ³H-labelled ribonucleotide homopolymers (sp. radioactivity 27–40 μ Ci/ μ mol of phosphate) and sonicated [³H]DNA of *Escherichia coli* B were from Miles Laboratories (Elkhart, IN, U.S.A.). Non-radioactive ribonucleotide homopolymers were from P-L Biochemicals Inc. (Milwaukee, WI, U.S.A.).

Tissue preparation and enzyme assay

Mammary glands and other organs were removed while the animals were chilled on ice after decapitation. All subsequent procedures were carried out at 4°C. The tissues were cleaned of superficial connective tissue, and the lymph nodes and larger blood vessels were removed from the mammary gland. Abdominal and inguinal mammary glands were pooled for analysis. In the case of tumour tissues, grossly necrotic areas were removed. Tissues were rinsed thoroughly in 0.34M-sucrose.

Total tissue extracts containing the RNAases of all the cell fractions were prepared by a combination of Polytron homogenization, sonication and 'high-salt' extraction, followed by dilution and centrifugation as described by Weil *et al.* (1977). Enzyme activity was assayed by measuring ethanol-soluble nucleotides liberated from ³H-labelled poly(U) under

Abbreviation used: RNAase, ribonuclease.

conditions of 5 mM- Ca^{2+} ions or 5 mM- Mg^{2+} ions or without added ion present. The previously described procedure (Liu *et al.*, 1977) was used except for doubling the amounts of substrate. One unit of enzyme activity represents the conversion of 5% of the poly(U) substrate (0.024 nmol of phosphate) into ethanol-soluble products in 15 min at 37°C. Nuclease activity against DNA was measured by using the same assay system, with 5 mM- Ca^{2+} ions and 0.009 μCi (25 nmol of phosphate) of DNA.

Results and Discussion

For the purpose of answering the questions of tissue specificity and relationship to functional differentiation of mammary cells, we have neglected temporarily the question of the distribution in different parts of the cell and the question of the multiple forms of Ca^{2+} -stimulated RNAase, known to occur at least in the nuclei (Liu *et al.*, 1977).

Activity of total tissue extracts from lactating rat mammary gland was stimulated more than 100-fold by the addition of 5–15 mM- Ca^{2+} ions, whereas Mg^{2+} ions were not stimulatory at concentrations as high as 25 mM (Fig. 1a). The stimulation due to 5 mM- Ca^{2+} ions was blocked completely by a 10 mM concentration of either EDTA or EGTA. The inhibitory effect of 10 mM-EDTA was completely reversed by Ca^{2+} ions at 15 mM or more, but the effect of EGTA was only partially reversed even at 25 mM- Ca^{2+} ions. The difference between EDTA and EGTA was not investigated further because the extracts being assayed at this time are complex mixtures rather than purified enzyme. The curves for EGTA and EDTA in combination with increasing concentrations of Mg^{2+} coincided with that for Mg^{2+} ions alone; they have been omitted from Fig. 1 for the sake of clarity.

As a clue to whether the enzyme originates from the epithelial component of the gland, we measured the activity in rat milk, collected at mid- and late-lactation. In the experiment shown in Fig. 1(b), 5 mM- Ca^{2+} ions, but not 5 mM- Mg^{2+} ions, maximally stimulated milk RNAase activity. The effects of EDTA and EGTA with and without an excess of Ca^{2+} or Mg^{2+} ions paralleled those for extracts of lactating gland. Milk that had been dialysed against 0.34 M-sucrose produced the same results. On a volume basis, activity in milk using ^3H -labelled poly(U) as substrate averaged about 20% of the activity in the mammary tissue from the same animal.

To determine whether Ca^{2+} -stimulated RNAase activity increases as a function of mammary differentiation, tissue extracts were assayed in the presence and absence of 5 mM- Ca^{2+} ions (Table 1). In mammary extracts from virgin and early-pregnant rats, RNAase activity was relatively low and unaffected by the presence or absence of Ca^{2+} ions, indicating that there was no Ca^{2+} -stimulated RNAase activity at

Fig. 1. Ca^{2+} -stimulated RNAase activity in rat mammary gland and milk under various ionic conditions

The milk was collected from a lactating Fischer rat 14 days *post partum* by manual expression under pentobarbital anaesthesia after administration of 2 units of oxytocin. The rat was then decapitated, the mammary glands were removed and a total tissue extract was prepared as described in the Experimental section. Tissue extract (a) and milk (b) were assayed for enzyme activity at the same time. Milk was diluted 500-fold and mammary-tissue extract 100-fold before assay to maintain the linearity of the assay. Concentration of added ion is indicated on the abscissa. ○, Ca^{2+} without chelator; △, Ca^{2+} plus 10 mM-EDTA; □, Ca^{2+} plus 10 mM-EGTA; ●, Mg^{2+} without chelator.

these stages of mammary gland development. At the 20th day of gestation, activity was 10 times that at 6 days of gestation if Ca^{2+} ions were present; assayed in the absence of Ca^{2+} ions, no increase in activity was observed. During lactation, Ca^{2+} -stimulated RNAase activity further increased 15-fold from that at late pregnancy; without Ca^{2+} ions no change in activity was observed. These results, also expressed in ratios of the activity measured with and without Ca^{2+} ions as shown in Table 1, indicate that enzyme appears during late gestation when rapid differentiation and proliferation occur in the gland, and reaches a maximal activity when the gland is actively engaged in milk secretion.

Table 1 also shows the mammary-tissue specificity of Ca^{2+} -stimulated RNAase. Rat kidney, known to have high RNAase activity (de Lamirande & Allard, 1959; Nicholls *et al.*, 1970; Liu & Matrisian, 1977), had the same activity whether or not Ca^{2+} ions were added to the assay, and similar results were obtained with kidney from virgin and lactating animals. In

Table 1. *RNAase activity in whole-tissue extracts assayed in the presence and absence of exogenous Ca^{2+} ions*
Activity is expressed as the mean $\pm$ s.e.m. for the number of animals indicated in parentheses.

	$10^{-4} \times \text{Activity (units/g of tissue)}$		Ratio of activities in the presence and absence of Ca^{2+} ions
	$+\text{Ca}^{2+}$	$-\text{Ca}^{2+}$	
Mammary gland			
Virgin	0.75 ± 0.15 (4)	0.70 ± 0.25 (4)	1.1
Pregnancy 6 days	0.55 ± 0.10 (3)	0.40 ± 0.05 (3)	1.4
20 days	5.60 ± 1.15 (3)	0.10 ± 0.02 (3)	56
Lactation 2-5 days	82.9 ± 12.0 (4)	0.25 ± 0.05 (4)	332
17-20 days	78.7 ± 8.7 (7)	0.19 ± 0.02 (7)	414
Mammary tumour			
R3230AC adenocarcinoma	8.80 ± 1.35 (3)	0.55 ± 0.05 (3)	16
Spontaneous adenocarcinoma	4.00 (1)	0.20 (1)	20
	0.90 (1)	0.10 (1)	9
Fibroadenoma	5.50 (1)	0.30 (1)	18
Hepatoma			
3924A	0.10 (1)	0.10 (1)	1.0
Liver			
Virgin	0.12 ± 0.05 (3)	0.20 ± 0.10 (3)	0.6
Lactation 15 days	0.17 (1)	0.18 (1)	0.9
Kidney			
Virgin	7.95 ± 0.40 (3)	8.65 ± 0.95 (3)	0.9
Lactation 15 days	7.40 (1)	8.83 (1)	0.8
Lung			
Lactation 15 days	0.54 (1)	0.55 (1)	1.0
Uterus			
Lactation 15 days	0.96 (1)	0.99 (1)	1.0
Spleen			
Lactation 15 days	1.45 (1)	1.56 (1)	0.9

liver extracts of either virgin or lactating rats, with or without Ca^{2+} ions, enzyme activity was low. Similar results were obtained from lung, uterus and spleen extracts from the lactating animal. No Ca^{2+} -stimulated enzyme activity was present in the rapidly growing 3924A hepatoma, indicating that the activity in mammary tissues was not due simply to rapid proliferation in the differentiating gland. On the other hand, relatively well-differentiated spontaneous mammary tumours and the transplantable R3230AC mammary tumour (Hilf *et al.*, 1965; Hilf, 1973) possess appreciable activity, which suggests that the expression of Ca^{2+} -stimulated RNAase activity by mammary cells parallels the overall degree of cellular differentiation. The predominantly epithelial R3230AC tumour (Hilf *et al.*, 1965), when grown in virgin rats, has been shown to contain small numbers of mRNA molecules coding for milk proteins (Nardacci & McGuire, 1977) and to make casein and lactose synthetase (McGuire, 1969; Turkington, 1970).

The possibility of an extraglandular origin of the enzyme, with transfer from plasma into mammary cells and milk enhanced by the increased blood flow

in the lactating gland (Hanwell & Peaker, 1977), had to be considered. However, RNAase activity in serum from virgin, lactating and tumour-bearing rats was actually lower in the presence of 5 mM- Ca^{2+} ions than in the absence of added Ca^{2+} ions. Similar results were obtained with urine. In both serum and urine the ratio of activity assayed with added Ca^{2+} ions to that assayed without Ca^{2+} ions was in the range of 0.4 to 0.7.

The Ca^{2+} -stimulated RNAase from milk, lactating gland and tumour demonstrated highest activity toward poly(U), about half as much activity toward poly(C) and some activity toward poly(A), but was totally inactive toward poly(G). We reported previously similar relative activities toward the four polynucleotides for the partially purified nuclear Ca^{2+} -stimulated RNAases (Liu *et al.*, 1977). Deoxyribonuclease activity was not detectable in either gland extracts or milk under the assay conditions used.

A small number of experiments using milk of cow, mouse, goat and dog have revealed disparate RNAase activities, but no evidence of stimulation of activity by Ca^{2+} ions.

Although the biological function of the RNAase activity stimulated by Ca^{2+} ions remains unknown, the enzyme is an organ-specific marker that is dependent on hormonally induced differentiation in normal mammary tissue, but may appear in neoplastic mammary tissue in the absence of hormonal stimulation. The presence of substantial activity in milk suggests that the enzyme is synthesized in mammary epithelial cells.

We thank Dr. K. M. Rose and Dr. S. T. Jacob for providing 3924A rat hepatoma and Dr. Russell Hilf for giving us the R3230AC mammary tumour. We also thank Dr. C. Hill, Dr. S. Patton and Dr. E. S. Vesell for helpful comments. This investigation was supported by grants R01 CA 18200 and P30 CA 18450, awarded by the National Cancer Institute, DHEW.

References

- Baldwin, R. L. (1969) in *Lactogenesis: The Initiation of Milk Secretion at Parturition* (Reynolds, M. & Folley, S. J., eds.), pp. 85–95, University of Pennsylvania Press, Philadelphia
- Barnard, E. A. (1969) *Annu. Rev. Biochem.* **38**, 677–732
- Cowie, A. T. & Tindal, J. S. (1971) *The Physiology of Lactation*, p. 107, Williams and Wilkins Co., Baltimore
- de Lamirande, G. & Allard, C. (1959) *Ann. N.Y. Acad. Sci.* **81**, 570–584
- Hanwell, A. & Peaker, M. (1977) in *Comparative Aspects of Lactation* (Peaker, M., ed.), pp. 297–312, Academic Press, New York
- Hilf, R. (1973) *Methods Cancer Res.* **7**, 55–114
- Hilf, R., Michel, I., Bell, C., Freeman, J. J. & Borman, A. (1965) *Cancer Res.* **25**, 286–299
- Hollmann, K. H. (1969) in *Lactogenesis: The Initiation of Milk Secretion at Parturition* (Reynolds, M. & Folley, S. J., eds.), pp. 27–41, University of Pennsylvania Press, Philadelphia
- Jenness, R. (1974) *J. Invest. Dermatol.* **63**, 109–118
- Liu, D. K. & Matrisian, P. E. (1977) *Biochem. J.* **164**, 371–377
- Liu, D. K., Williams, G. H. & Fritz, P. J. (1975) *Biochem. J.* **148**, 67–76
- Liu, D. K., Liao, W. S. L. & Fritz, P. J. (1977) *Biochemistry* **16**, 3361–3369
- McGuire, W. L. (1969) *Science* **165**, 1013–1014
- Munford, R. E. (1964) *Dairy Sci. Abstr.* **26**, 293–304
- Nardacci, N. J. & McGuire, W. L. (1977) *Cancer Res.* **37**, 1186–1190
- Nicholls, D. M., Ryan, M. P., Miall, S. H. & Cappon, I. D. (1970) *Can. J. Biochem.* **48**, 105–112
- Patton, S. (1978) *J. Dairy Sci.* **61**, 643–650
- Roth, J. S. (1967) *Methods Cancer Res.* **3**, 153–242
- Turkington, R. W. (1970) *Cancer Res.* **30**, 1841–1845
- Weil, P. A., Sidikaro, J., Stancel, G. M. & Blatti, S. P. (1977) *J. Biol. Chem.* **252**, 1092–1098
- Wellings, S. R. (1969) in *Lactogenesis: The Initiation of Milk Secretion at Parturition* (Reynolds, M. & Folley, S. J., eds.), pp. 5–25, University of Pennsylvania Press, Philadelphia