CALMODULIN AND CYCLIC NUCLEOTIDE-PHOSPHODIESTERASE ACTIVITIES IN RAT MAMMARY GLAND DURING THE LACTOGENIC CYCLE

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1. Introduction

Numerous studies [1–4] have documented that cyclic AMP plays a central role in the hormonal regulation of cell growth and function and that its content in the mammary gland increases progressively during the growth stage of pregnancy and then, at parturition, decreases rapidly to the low levels seen during the lactational period. These findings suggest that cyclic AMP is a negative controlling factor for lactogenesis. It has also been demonstrated that 3′,5′-nucleotide phosphodiesterase, the enzyme which hydrolyses cyclic nucleotides, is activated by calmodulin, a thermostable low M_r protein already studied in several tissues, but not in mammary gland [5]. Calmodulin is an intracellular calcium receptor protein that binds to Ca^{2+} when their concentration increases in response to stimulus. This binding induces a distinct change in the shape of the calmodulin molecule which in turn is capable of binding to any of several enzymes, activating them and so setting in motion the biochemical changes that produce directly the response to the stimulus. Calmodulin can also indirectly modify cell activation by affecting the concentration of calcium itself and that of other important cellular regulators, including cyclic AMP. The finding that Ca^{2+}, in cooperation with calmodulin, can alter cyclic nucleotides concentration indicates one way by which the actions of the 2 regulatory agents may be integrated in regulating the lactogenic cycle in mammary gland.

Here, we have analysed the involvement of calmodulin in the control of the lactogenic cycle of the rat and its relation to cyclic nucleotide phosphodiesterase activity in the mammary gland. We present evidence for the existence of calmodulin in the gland and its activating capacity for the enzyme, both from bovine brain and mammary gland, and analyse its kinetic properties. Our results support the view that calmodulin plays a regulatory role during lactogenesis.

2. Materials and methods

Virgin albino rats of the Sprague-Dawley strain, aged ~3 months (180–230 g) were used. Mated animals were taken at different stages of pregnancy and lactation. The animals were killed by decapitation and the abdominal mammary glands rapidly excised and homogenized at 4°C.

Cyclic [8-^{3}H]AMP was obtained from New England Nuclear (1 mCi/ml and 32.3 Ci/mmol). *Crotalus atrox* venom 5′-nucleotidase, cyclic AMP, cyclic GMP, DEAE–Sephadex A-50 and DEAE–cellulose were purchased from Sigma (St Louis MD). All other reagents were from Merck (Darmstadt).

2.1. *Cyclic nucleotide phosphodiesterase purification*

The enzyme was purified both from bovine brain and rat lactating mammary tissue (50 g) by ammonium sulphate precipitation followed by DEAE–Sephadex A-50 chromatography according to [6]. This provided an enzyme preparation free from the activator calmodulin. Active fractions were assayed for phosphodiesterase activity by two different methods.

2.2. *Cyclic AMP-phosphodiesterase activity assay*

Two methods, each of 2 stages, were used to determine phosphodiesterase activity:

Method I was a modified procedure of that in [7] in which P_i was determined by the method in [8] after its release from 5′-AMP via the action of snake venom 5′-nucleotidase.

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Method II, an isotopic assay, was essentially as in [1].

The reaction mixture in each assay method contained 40 mM Tris–HCl (pH 8.0), 2 mM cyclic AMP (with which total phosphodiesterase activity, low and high K_m-values, was determined) and 2 mM $MnCl_2$. The high K_m cyclic AMP enzyme was assayed as in [1]. Specific activity was expressed as μmol cyclic AMP hydrolyzed . mg protein^{-1} . min^{-1}.

2.3. *Calmodulin assay*

The activator protein was assayed for its ability to stimulate the activity of partially purified phosphodiesterase from bovine brain [9] and mammary gland. Mammary gland tissue was homogenized with 5 vol. 20 mM Tris–HCl (pH 7.5) in an Ultra-Turrax blendor (Janke and Kunkel, Staufen) for 2 min. The homogenate was heated in a boiling water bath for 3 min. Boiling inactivates phosphodiesterase but not the activator. After centrifuging for 10 min at 750 × *g*, the supernatant was diluted by the addition of an equal volume of Tris buffer and 0.08 ml this diluted material was taken and added to the phosphodiesterase assay mixture as before. Calcium was present in this mixture at a final concentration of 0.01 mM. The basal activity of the purified phosphodiesterase was subtracted from the total activity and the corrected value was defined as stimulated activity. The amount of protein (μg) required to produce 50% activation of partially purified phosphodiesterase was defined as 1 unit of calmodulin.

Proteins were determined as in [10] using bovine serum albumin as a standard.

3. Results

Fig.1 shows the activities of partially purified cyclic nucleotide phosphodiesterase from lactating rat mammary gland in the presence of different amounts of activator in the crude extract also obtained from rat mammary gland. It is apparent that the activation kinetics are sigmoid, with the enzyme activity attaining a plateau which corresponds to 50-times the activity of the enzyme in the absence of activator. A similar pattern was obtained when the partially purified enzyme from bovine brain was activated by the extract of mammary gland containing the activator (not shown) although the degree of activation was lower than with the mammary enzyme.

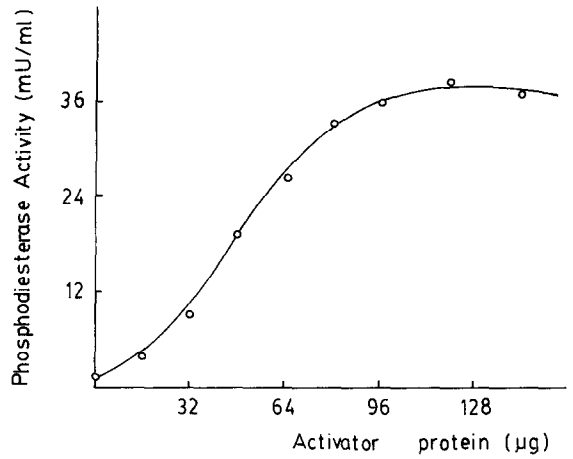

Fig.1. Activation of partially purified cyclic nucleotide phosphodiesterase from lactating mammary gland by crude rat mammary gland activator protein (calmodulin). All values are in duplicate.

Trifluoperazine, a drug which inhibits the calmodulin activation of bovine brain phosphodiesterase, also inhibits the effect of the activator protein on the mammary gland enzyme (fig.2), possibly indicating the identity of the activator and calmodulin.

Results in fig.3 show the activity levels for both activator and cyclic AMP-phosphodiesterase in rat mammary gland during the lactogenic cycle. It can be seen that the activator activity remains low, as in the virgin gland, until day 15 of pregnancy. Then it starts increasing and attains its maximum activity near parturition, maintaining higher levels in lactation than

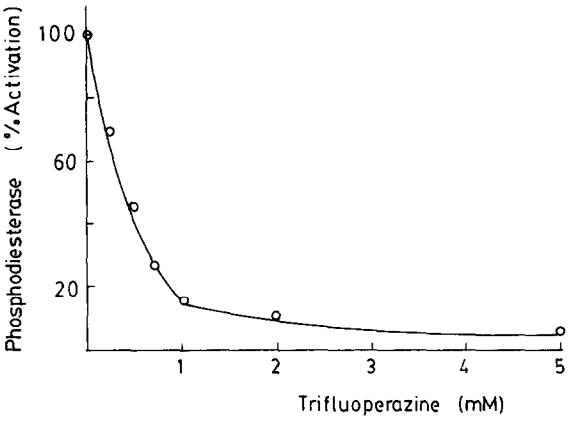

Fig.2. Inhibition by trifluoperazine of calmodulin mammary gland phosphodiesterase activation. All values are in duplicate.

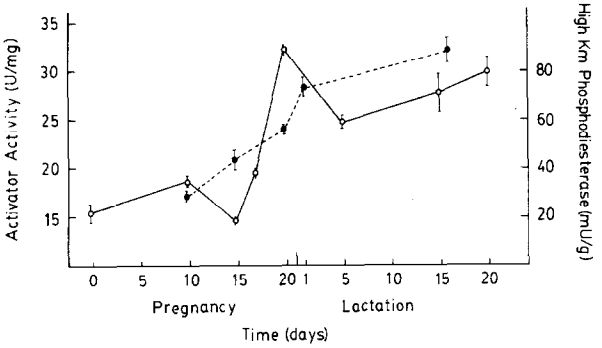

Fig.3. Activator (calmodulin) and high K_m cyclic AMP phosphodiesterase activities in rat mammary gland during the lactogenic cycle. Results are given as the mean values ± SEM; each value represents the mean of 5 animals: (o——o) activator activity; (•——•) high K_m cyclic AMP phosphodiesterase (from [12]).

in pregnancy. This pattern is analogous to that for the high K_m phosphodiesterase, an enzyme which starts increasing during pregnancy but presents its highest activity during lactation.

Table 1 shows some kinetic data obtained with the partially purified cyclic nucleotide phosphodiesterase from rat mammary gland with and without activator present. It can be seen that the activator increases the V_{max} for both cyclic nucleotides, cAMP and cGMP, while it only increases the K_m for cyclic GMP without any change in K_m-value for cyclic AMP.

4. Discussion

Although it is not established that the activator in the mammary gland extracts is, in fact, calmodulin,

Table 1
Kinetic data for partially purified cyclic nucleotide phosphodiesterase from rat mammary gland with and without calmodulin (CaM)

	Cyclic AMP		Cyclic GMP	
	−CaM	+CaM (100 μg)	−CaM	+CaM (100 μg)
K_m (μM)	50	50	30	140
V_{max} (mU/mg protein)	12	28	30	61
V_{max}/K_m	0.2	0.6	1.0	0.4

the mode of extraction used in the preparation of these extracts, the activation characteristics of the extracts on phosphodiesterase activity and the inhibition of this activation by trifluoperazine, make it a reasonable assumption that these 2 substances are identical. Until this identity is firmly established, probably by the use of calmodulin-specific antibody, this discussion will assume, as a working hypothesis, that the activator present in the mammary gland extracts is calmodulin.

This study shows that calmodulin from rat mammary gland is able to activate the cyclic nucleotide phosphodiesterase of the gland in a sigmoidal fashion, similar to that exhibited by the enzyme from bovine brain. The activation of both enzymes is selectively inhibited by 60 μM trifluoperazine, a drug which also inhibits the activation of brain phosphodiesterase by calmodulin from the same tissue. These results confirm the universal action of calmodulin and its lack of tissue specificity [11].

On studying calmodulin levels in mammary gland through the lactogenic cycle, the maximal level was achieved at parturition with the amount present during lactation also being higher than that present during pregnancy. This pattern is analogous to that for the high K_m cyclic AMP-phosphodiesterase, an enzyme which starts increasing during pregnancy but only reaches its maximal level during lactation. This pattern is especially important if we consider that the high K_m phosphodiesterase is much more abundant than the low K_m isoenzyme in mammary gland [12]. Although the distribution of the activities of the 2 proteins, calmodulin and phosphodiesterase, do not parallel one another in various other tissues of the rat during development, they do behave so in isolated rat parenchymal cells and in human blood platelets [5]. Calmodulin is active only as a calmodulin–Ca^{2+} complex [5,13] and this suggests that the higher activity of this modulator during lactogenesis, with respect to the virgin mammary gland, is due to the higher Ca^{2+} content of the lactogenic tissue.

In spite of the many kinetic studies, in several laboratories [14–18], it is still not clear that modification of the kinetic parameters of phosphodiesterase by calmodulin plays a physiological role in cell regulation.

The high K_m phosphodiesterase has been purified from pig heart and bovine brain [14,17] and it has been demonstrated that this same enzyme exists in both tissues. This enzyme, which is sensitive to the

presence of calmodulin, catalyzes the hydrolysis both of cyclic AMP and cyclic GMP, one nucleotide inhibiting competitively the hydrolysis of the other [14,18]. Kinetic studies with these enzyme preparations have shown that calmodulin decreases the K_m of the enzyme for cyclic AMP and increases V_{max} [15] while other authors have also found an increase in V_{max} but without any change in K_m for cyclic AMP as well as for cyclic GMP [14,19]. Still others report an increase in V_{max} without change in K_m [15]. These discrepancies can be ascribed to the different calmodulin concentrations employed by the different authors and to different enzyme preparations or experimental conditions. For example, it is well known that, at low concentrations, cGMP acts as a better substrate than does cAMP while the reverse is observed at high substrate concentration. With our partially purified cyclic nucleotide phosphodiesterase from rat mammary gland we observed that calmodulin produces an increase in V_{max} for both cyclic nucleotides but a K_m increase only for cyclic GMP. Calmodulin increased the catalytic efficiency (V_{max}/K_m) of the enzyme for cyclic AMP and decreased that for cyclic GMP, suggesting a regulatory role for calmodulin in mammary gland in that it allows the predominance of cyclic GMP over cyclic AMP during lactogenesis. These results are in agreement with the physiological role attributed to both cyclic nucleotides during the lactogenic cycle [2–4,20] and open a new perspective for understanding the molecular mechanisms which control lactogenesis.

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