THE EFFECTS OF DI-BUTYRYL cAMP ON ENZYMATIC AND METABOLIC CHANGES IN EXPLANTS OF RAT MAMMARY TISSUE

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Summary The effect of cAMP on the activities of some enzymes and on the rates of synthesis of DNA, RNA, casein and fatty acids in rat mammary gland explants grown in culture has been examined. cAMP inhibits the increase of enzyme activities associated with the development of the tissue under the culture conditions and, in particular, the activities of those enzymes involved in lipogenesis. It also markedly depresses the synthesis of DNA, RNA and fatty acids. Casein synthesis is slightly increased. These results are discussed in relation to the possible role(s) of cAMP as a regulator of mammary gland development and function.

Introduction The onset of lactation in the rat is accompanied by an increase in the number of nuclei present and in the level of DNA in the mammary gland [see 1] and this is followed by a sharp increase in the activities of many enzymes [2,3]. These changes represent an extensive modification of the overall activity of the tissue and of the prevailing metabolic pattern in response to the stress of lactation which may be related to changes in the hormonal stimulation of the tissue at this time. The mechanisms by which changes in the hormonal environment of the tissue effect these responses are not clearly understood. Evidence has been adduced for the involvement of cAMP in the growth and metabolic activities of the gland by the demonstration that the tissue content of the nucleotide is maximal at the end of pregnancy and falls progressively to its lowest level by the sixteenth day of lactation [4]. The present study is an attempt to clarify the role of cAMP in the adaptive changes that occur around parturition. Explants from the mammary glands of rats in mid-pregnancy have been used as it has been shown that these can be maintained in organ culture for 96 hours [5] and that lobular-alveolar formation, which normally precedes lactational development, does not appear to be a strict requirement for cellular function [6]. The present results show that cAMP is a potent inhibitor of nucleic acid and lipid synthesis in mammary tissue and that the activities of almost all of the enzymes studied are markedly lower when cAMP is present in the incubation medium. These results are interpreted as suggesting that cAMP plays a repressive role in pregnancy and that the decrease in the tissue content of the nucleotide concurrent with the onset of parturition may be related to the initiation of galactopoiesis and the concommitant rise in

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the biosynthetic activity of the gland associated with milk formation.

METHODS

Animals Virgin albino females of the Wistar strain, weighing between 200-220 g were mated and then taken on either the 10th or 20th day of pregnancy. The 10-day pregnant rats were killed by CO_2 overdosage and the lower abdominal mammary glands removed. The glands were transferred to 5 ml of medium 199 before being diced into explants. In addition, 10-day and 20-day pregnant rats were killed by cervical dislocation and the abdominal glands removed for the direct preparation of homogenates for enzyme assay.

Explant culture The mammary glands from 2 or 3 rats were diced into explants each weighing about 1 mg and these were maintained for up to 48 hours in organ culture [5].

Determination of enzyme activities About 250 mg of explant tissue were cultured for 44 hours in medium 199 containing insulin, corticosterone and prolactin (5μg/ml of each) with, and without, dibutyryl cAMP (10^{-3}M). After this time, the explants were collected, blotted, weighed and then homogenized in an Ultra-Turrax (Janke and Kunkel, Stauffen, Germany) blender for 8 seconds with 15 volumes of cold medium (150 mM KCl; 5 mM EDTA; 5 mM $MgCl_2$; 10 mM mercaptoethanol adjusted to pH 7.4 with $NaHCO_3$). The homogenates were centrifuged at 15,000 g for 4 minutes and the supernatant fraction collected and dialysed 2 x 1 hour against 400 volumes of the homogenizing medium in the cold. The dialysed preparation was used for the enzyme assays. The same procedure was used for tissues taken directly from 10-day and 20-day pregnant rats.

The procedures used for the measurement of enzyme activities have been previously described [7-10]. Additional procedures were those for hexokinase and glucokinase [11]. Enzyme activities are expressed as units/g tissue (corrected for milk content [12] where necessary), the unit of activity being defined as that amount of enzyme which converts 1 μmole of substrate or forms 1 μmole of product x min^{-1} at 25°.

Determination of the rate of casein, DNA, RNA and fatty acid synthesis The incorporation of radioactive precursors into casein, DNA, RNA and fatty acids was measured as previously described [5,13] using about 20-25 mg of explants for each determination. Incubations were carried out either in medium 199 alone, or with (a) insulin, (b) insulin and corticosterone or (c) insulin, corticosterone and prolactin (5 μg/ml of each) in the presence or absence of cAMP (10^{-3}M). In each case the radioactive product was isolated and counted, the results being presented as c.p.m. x mg^{-1} of wet tissue and represent the means of triplicate cultures.

RESULTS AND DISCUSSION

Table 1 shows the effect of dibutyryl cyclic AMP on the activity of a number of enzymes in mammary explants grown under conditions which lead to an acclerated

Table 1. The effect of dibutyryl cAMP on changes of enzyme activities of mammary gland explants.

ENZYME	EXPLANTS[1] No cAMP	EXPLANTS[1] $+10^{-3}$M cAMP	EXPLANTS[1] $\frac{-cAMP}{+cAMP}$	INTACT TISSUE[4] 10-day pregnant[2]	INTACT TISSUE[4] 20-day pregnant[3]	INTACT TISSUE[4] $\frac{20\text{-day}}{10\text{-day}}$
	Units/g tissue					
Hexokinase	0.28	0.25	1.12	0.26±0.005	0.29±0.07	1.11
Glucokinase	0.59	0.28	2.10	0.22±0.012	0.56±0.05	2.54
Phosphofructokinase	0.61	0.24	2.18	0.18±0.033	0.56±0.12	2.58
Glyceraldehyde phosphate dehydrogenase	0.86	0.73	1.18	-	3.90±0.72	-
α-glycerophosphate dehydrogenase	4.60	3.47	1.33	2.36±0.066	5.60±0.70	2.37
3-phosphoglycerate kinase	0.23	0.16	1.44	0.25-0.019	-	-
Pyruvate kinase	8.54	6.42	1.33	8.30±0.30	10.85±0.80	1.30
Lactate dehydrogenase	44.61	33.83	1.32	26.72±1.0	34.00±3.0	1.27
Isocitrate dehydrogenase	2.32	1.58	1.47	2.11±0.09	2.58±0.20	1.22
Malate dehydrogenase	86.4	53.0	1.63	25.90±2.30	47.30±8.20	1.90
Glucose 6-phosphate dehydrogenase	1.02	0.67	1.52	1.02±0.09	1.22±0.15	1.19
6-phosphogluconate dehydrogenase	1.00	0.63	1.59	0.88±0.04	1.10±0.09	1.25
'Malic enzyme'	2.11	0.60	3.51	1.02±0.15	2.90±0.90	2.83
Citrate cleavage enzyme	0.77	0.44	1.61	0.32±0.08	0.70±0.07	2.19
Fatty acid synthase	0.20	0.05	4.00	0.033±0.001	0.27±0.03	8.18
Glutamate-oxalo-acetate aminotransferase	6.66	5.51	1.21	5.38±0.13	6.00±0.80	1.11

[1] Mean of two determinations, each containing pooled material from 2 or 3 animals
[2] The values are the mean ± S.E.M. from 3 rats
[3] Values taken from references 2 and 3
[4] Values have been corrected per g milk tissue
The explants were grown in medium 199 supplemented by insulin, corticosterone, and prolactin (5 μg ml of each)

development of the tissue such that explants from the mammary glands of rats taken on the 10th day of pregnancy attain, after culture in a fortified 199 medium, the morphological and physiological status of tissue taken from a 20-day pregnant rat [5]. That this rapid growth is not confined to morphological appearance can be seen from a comparison of some of the enzyme activities shown in Table 1. With the exception of glyceraldehyde phosphate dehydrogenase, all enzyme activities in the explants cultured in the absence of added cAMP approximate closely to those found in the glands taken directly from rats on the 20th day of pregnancy. Explants cultured in the presence of 10^{-3} cAMP, however, fail to show this increasing trend and the activities measured in such explants correspond more nearly to those found in the glands taken directly from 10-day pregnant animals. It is noteworthy that the effect of cAMP was most marked on those enzymes (fatty acid synthetase, malic enzyme, glucokinase and phosphofructokinase) which show the greatest rate of change in normal development and which appear to play a special role in the mammary gland metabolism in that they are all specific proportion enzymes in this tissue [14,15]. Indeed, inspection of Table 1 reveals that an approximate inverse proportionality appears to exist between the rate of the increase of enzyme activity in explants cultured in the presence of cAMP and the rate at which the enzymes change in the normal growth of the gland in vivo, i.e. cAMP inhibition is greatest where enzymes in vivo increase most rapidly. On this basis, the enzymes most affected by cAMP are those associated with the lipogenic pathway in the explants, as measured by pulse-labelling with ^{14}C-acetate, which are shown in Table 2, where it may be seen that the presence of cAMP in the medium inhibits the incorporation by 60%.

These findings are consistent with the observations of Bricker and Levey [16] who found a significant inhibition of lipogenesis and cholesterologenesis in rat liver slices after treatment with cAMP and with the results of Allred and Roehrig [17] which showed that cAMP inhibits lipogenesis and acetyl CoA carboxylase in rat liver to about the same extent. The recent report by Carlson and Kim [18] that acetyl CoA carboxylase can be interconverted from an active to an inactive form by phosphorylation-dephosphorylation reactions which are cAMP-dependent supports the idea that control of lipogenesis by cAMP may be localized at this step. Further, the fact that hepatic lipogenesis from glucose is decreased by 74% after glucagon treatment (a hormone which acts through cAMP as second messenger) [19], emphasized the possibility that cAMP may act as a regulator of fatty acid synthesis in vivo.

It should be noted, however, that the concentration of cAMP used here greatly exceeds that found in vivo, although the fact that no theophylline was included probably

Table 2. The effect of dibutyryl cAMP on the rates of incorporation of radioactive precursors into DNA, RNA, fatty acids and casein.

	Medium 199	Medium 199 + Insulin	Medium 199 + Insulin + Corticosterone	Medium 199 + Insulin + Corticosterone + Prolactin
	Counts/ mg wet tissue/ min.			
DNA				
No cAMP	1,565 ± 32	6,990 ± 137	10,142 ± 485	9,712 ± 174
$+10^{-3}$M cAMP	992 ± 115	1,366 ± 77	3,561 ± 856	4,151 ± 476
RNA				
No cAMP	825 ± 79	1,652 ± 132	1,717 ± 67	2,135 ± 102
$+10^{-3}$M cAMP	466 ± 57	553 ± 35	780 ± 111	1,016 ± 89
Fatty Acids				
No cAMP	937 ± 52	5,022 ± 92	5,142 ± 479	7,248 ± 180
$+10^{-3}$M cAMP	997 ± 42	1,216 ± 110	915 ± 137	2,792 ± 598
Casein				
No cAMP	-	-	248 ± 54	764 ± 28
$+10^{-3}$M cAMP	-	-	367 ± 22	968 ± 126

The results are derived from two experiments. Two animals were used for each experiment and the determinations were done in triplicate. The values are given as means ± S.E.M. The explants were cultued as in ref. [5]. Where hormones have been added, these were at a concentration of 5 µg/ml of each.

means that the effective intracellular concentration in the explants would be much lower than that initially in the medium. It is, nevertheless, probable that the results reported here are an exaggeration of the normally occurring cAMP-dependent effect. For instance, in the present experiments cAMP almost completely blocks the increase of enzyme activities whereas, in vivo, there is a pronounced rise in such activities between the 10th and 20th days of pregnancy [2,3] despite a rising tissue content of cAMP [4]. This does not conflict

with the view [4] that cAMP acts as an enzyme repressor since it is reasonable to conclude that, at the low concentration of the nucleotide obtaining in vivo, only a partial repression occurs. Thus, a restricted increase of enzyme activities occurs during the latter half of pregnancy when the intracellular cAMP level is 7×10^{-7}M [4] but the major increases [2,3] coincide with the fall of intracellular cAMP immediately after parturition [4] and its continuing decline as lactation advances.

Cyclic AMP has an effect on the rates of synthesis of both general cell events, DNA and RNA synthesis, and on lactation-specific events, fatty acid and casein synthesis. The synthesis of DNA and RNA is increased by specific hormones and thus agrees with previous studies [5] but the addition of cAMP partially, or completely, abolishes the hormone effect (Table 2). The reduction is greatest in medium that contains insulin alone and the reduction produced by cAMP to the other hormone-containing media would follow as insulin is the premissive hormone in this system [20].

The rate of fatty acid synthesis is increased by the addition of hormones to medium 199 and these fatty acids are predominantly of milk-specific types [13]. The synthesis is reduced by the addition of cAMP, but there is no significant change in the basal rate in medium 199 alone. Although the rate of casein synthesis is increased by culture in medium containing insulin, corticosterone and prolactin, the addition of cAMP caused a slight rise in P^{32} incorporation ($p = 0.05$). This is unexpected as all previous studies have shown that active DNA and RNA synthesis must precede prolactin-stimulated casein synthesis. The present study clearly indicates that cAMP is able to repress both DNA and RNA synthesis while casein synthesis continues.

The inhibition of DNA synthesis in mammary gland explants by cAMP (Table 2) indicates that the nucleotide may play a significant role in the growth of the gland and may provide some explanation for the fact that DNA synthesis occurs at a low level in the latter half of pregnancy, when the intracellular cAMP level is high, whereas it is raised earlier in pregnancy and in lactation, when the tissue content of cAMP is low. The DNA content of the mammary gland approximately doubles soon after parturition see [1,21] and an increase in the number of secretory cells present at this time has also been reported [2,22]. The present results are in keeping with the extensive evidence already available that shows an inverse relationship between the intracellular content of cAMP and the rate of DNA synthesis and that the nucleotide exerts a precise control and does not act as a toxin producing general cell death [23]. The nature of this cAMP-dependent control process is not clear. It could act by inhibiting some membrane transport processes, slowing the rates of RNA or protein syntheses, or by stimulating protein degradation by

analogy with the pleiotypic response observed for other cells grown in culture [24] or the nucleotide may be more directly involved in the control of DNA synthesis. Evidence already exists linking the inhibition of DNA synthesis by cAMP to an inhibition of thymidine kinase [25] and of CDP-ribonucleotide reductase [26].

It is conceivable that the wave of mitosis which follows parturition is significant in that, in addition to increasing the number of secretory cells present, cell division of mammary epithelial cells may, as suggested by Stockdale and Topper [27] make these cells especially susceptible to environmental factors capable of modifying the metabolic pattern.

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