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PROPERTIES OF ADENYLATE CYCLASE OF MURINE MAMMARY CARCINOMA

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SUMMARY

We have investigated the adenylate cyclase activity of a murine mammary carcinoma induced by mammary tumor virus. The adenylate cyclase activity was identified mainly within the 1000 × *g* fraction of the whole homogenate. The optimal concentrations for maximal enzyme activity appeared to be: 1 mM ATP, 6 mM Mg^{2+} or higher; however, optimal Mg^{2+} concentration was 8 mM in the presence of 10 mM NaF. The optimum pH of enzyme activity was 8.2. The adenylate cyclase responded to epinephrine and NaF, and to some degree also to prostaglandins E_1 and E_2. The stimulation by epinephrine was completely inhibited by propranolol, but almost unaffected by phentolamine. Essentially no enzyme activity was found in the 105 000 × *g* fraction. We also failed to demonstrate significant adenylate cyclase activity in normal lactating mammary tissue.

INTRODUCTION

Adenylate cyclase (EC 4.6.1.1) activity has been universally demonstrated in practically all organs and tissues of the animal kingdom. The sole exception reported involved erythrocytes which have lost their nuclei [1]. This enzymatic activity has also been demonstrated in all neoplastic tissues so far subjected to study. We became interested in the properties of the enzyme in mammary carcinoma, because of its possible role in the hormone dependence of the tumor. We found only one relevant communication that could be traced, a preliminary note by Brown et al. [2], of their examination of the enzyme in a mammary carcinoma of the rat induced by dimethyl amino biphenyl. They also investigated this enzyme activity in the normal lactating mammary tissue of the rat. In addition, there is a publication by Majumder and Turkington [3] in which the adenylate cyclase of murine lactating mammary tissue is briefly described.

It was, therefore, decided to investigate the properties of the enzyme in both the mammary carcinoma and the normal lactating tissue of BALBc mice infected through suckling by C3H mammary tumor virus carrying mice. Initial results of our investigation have been published [4].

MATERIALS AND METHODS

Tissue preparation

Mammary carcinoma from a BALBc female mouse was maintained by transplantation into 5–6-weeks-old female mice of the same strain. Normal lactating mammary tissue was obtained from BALBc primiparous lactating mice 5–10 days after delivery. The tissues were homogenized at a concentration of approx. 400 mg/ml in a buffer composed of 62.2 mM Tris and 15.5 mM theophylline at pH 8.2. The homogenate was centrifuged at 1000 × *g* for 10 min. The sediment, resuspended in the buffer, was then used for the enzyme assay. This 1000 × *g* particulate fraction has subsequently been shown by us to possess the highest specific enzyme activity of all the fractions derived from differential centrifugation of tumor homogenate. In initial experiments whole homogenate was used as the enzyme source. In several experiments the 1000 × *g* supernatant was respun at 10 000 × *g* for 10 min and the 10 000 × *g* pellet resuspended in buffer. The 10 000 × *g* supernatant was then respun at 105 000 × *g* for 1 h in a Spinco ultracentrifuge. The 105 000 × *g* pellet was similarly resuspended, the 105 000 × *g* supernatant being also saved. The three particulate fractions and 105 000 × *g* supernatant were then synchronously assayed for their adenylate cyclase activity. The tissue preparations were maintained at 4 °C throughout these steps.

Adenylate cyclase assay

The enzymatic assay was based on the conversion of [α-^{32}P]ATP to cyclic 3′,5′-[^{32}P]AMP. Cyclic 3′,5′-[8-^{3}H]AMP was added at the beginning of the incubation to allow correction for product breakdown and loss during incubation and purification. Particulate fractions suspended in a volume of 40 μl were added to the remainder of the components of the assay made up in a volume of 30 μl. The final concentrations of components in the assay mixture were 1 mM ATP, 6 mM $MgSO_4$, 2 mM cyclic 3′, 5′-AMP, 40 mM Tris and 10 mM theophylline. 8 mM phosphoenolpyruvate and 0.6 units of pyruvate kinase per incubate were included as an ATP-generating system. The incubations were carried out at pH 8.2 for 10 min, usually in a water bath at 37 °C with shaking. The blank tube was boiled for 3 min before incubation. After the termination of the incubation and boiling of the assay tubes, all tubes were centrifuged at 27 000 × *g*. 50 μl of the supernatant was put onto an aluminium oxide column and rinsed following the method of Ramachandran [5]. Scintillation fluid was added to the eluate and the specimens counted. The amount of cyclic 3′,5′-AMP produced was corrected for losses on the basis of recovery of cyclic 3′,5′-[8-^{3}H]AMP. The amount of cyclic 3′,5′-AMP in each blank was subtracted from the corresponding incubate. Adenylate cyclase activity is expressed as pmoles cyclic 3′,5′-AMP formed per mg protein per min.

Protein concentrations were determined by the method of Lowry et al. [6].

Materials

Thyroid-stimulating hormone, luteinizing hormone, follicle-stimulating hormone, prolactin and growth hormone were prepared by Dr L. Reichert of Emory University and provided by the National Institutes of Health. Vasopressin was from Parke, Davis and Co., parathormone from Wilson, glucagon, insulin, adrenocorticotropic hormone and epinephrine were from Sigma Chemical Co. Prostaglandins E_1

and E_2 were a gift of Dr John Pike of Upjohn Company. The following adrenergic antagonists were also tested: propranolol hydrochloride (Inderal, Ayerst) and phentolamine mesylate (Regitine, Ciba). [α-^{32}P]ATP was purchased from Amersham and cyclic 3′,5′-[8-^{3}H]AMP from Schwarz Bio Research.

RESULTS

Optimal ATP concentration for adenylate cyclase activity

The effect of the ATP concentration in the range 0.04–6 mM on the enzyme activity was tested (Fig. 1). Optimal activity was obtained at 1–2 mM ATP, above this concentration substrate inhibition occurred. A K_m value of 1.2 mM, based on a single experiment, was calculated.

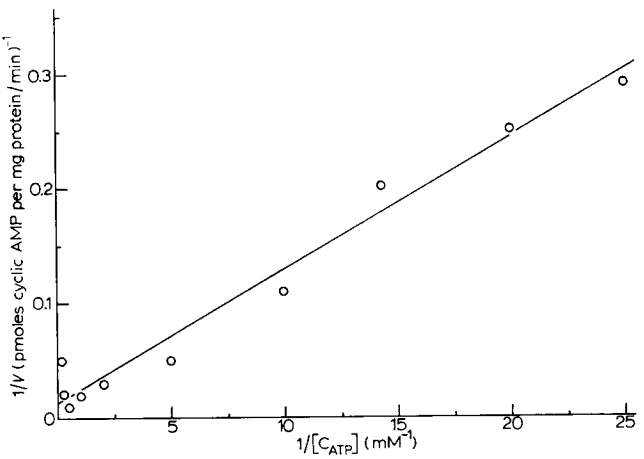


Fig. 1. Lineweaver–Burk plot. For slope and intersection of line only concentrations of 0.04–2 mM ATP were used. The 4–6 mM ATP results of a previous experiment were also recorded.

Optimal incubation period for the enzymatic reaction

The peak production of cyclic 3′,5′-AMP was evident after 5 min of incubation. Further strengthening of the regenerating system by doubling phosphoenolpyruvate and pyruvate kinase concentrations did not increase the amount of product. In contrast, the active agonists epinephrine and NaF increased cyclic 3′,5′-AMP when longer incubation periods up to 15 min were put to test (Fig. 2).

Optimal Mg^{2+} concentration for adenylate cyclase activity

Enzyme activity reached plateau level at 6 mM Mg^{2+}. However, when optimum Mg^{2+} concentrations for enzymatic activity were tested in the presence of 10 mM NaF, peak activity was observed at 8 mM Mg^{2+}. At higher Mg^{2+} concentrations, a marked inhibitory effect supervened (Fig. 3). The authors emphasize that the two curves represent two different experiments, since it is very unusual that the adenylate cyclase activity is lower in the presence of NaF than in its absence.

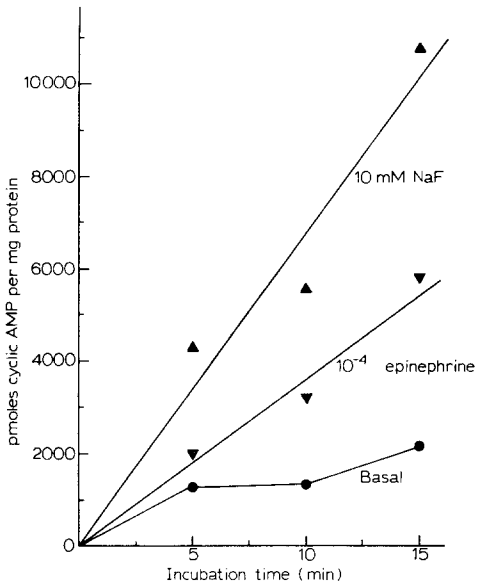


Fig. 2. Effects of 10^{-4} M epinephrine and 10 mM NaF upon optimum incubation period: 1000 × *g* fraction of mammary carcinoma homogenate was used as enzyme source. ATP concentration was 2 mM.

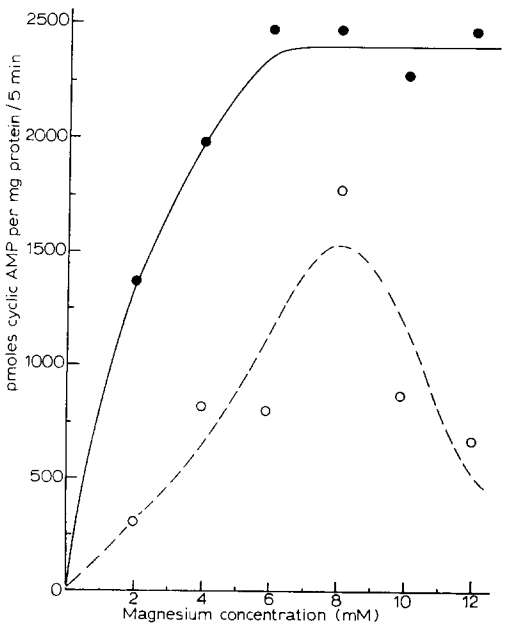


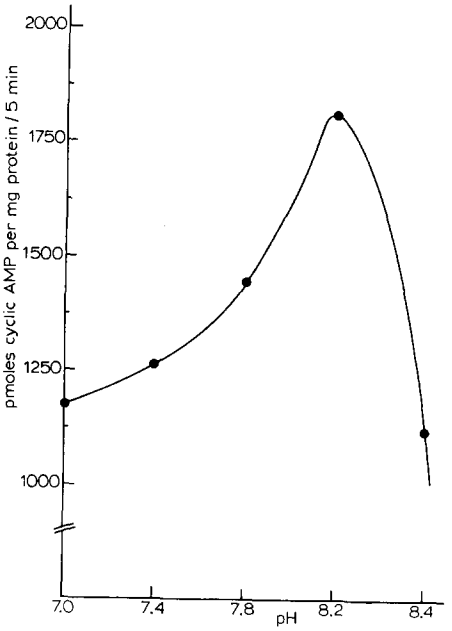
Fig. 3. Effect of Mg^{2+} concentration on cyclic 3′,5′-AMP production: the 1000 × *g* fraction of mammary carcinoma homogenate was used as enzyme source. ATP concentration was 2 mM. ●—●, no addition; ○---○, in the presence of 10 mM NaF. Respective incubation times 5 and 10 min. Results are of two different experiments.

Optimal pH for enzymatic activity

Enzymatic activity peaked at pH 8.2 and subsequently the inhibitory influence of a more alkaline medium was observed (Fig. 4).

Effect of protein concentration upon adenylate cyclase activity

Enzyme activity was proportional to protein concentration ranging from 0.23 to 1.86 mg/ml. The range remained unchanged when epinephrine and NaF stimulation was superimposed. However, at higher protein concentrations a strong inhibitory effect became apparent (Fig. 5).



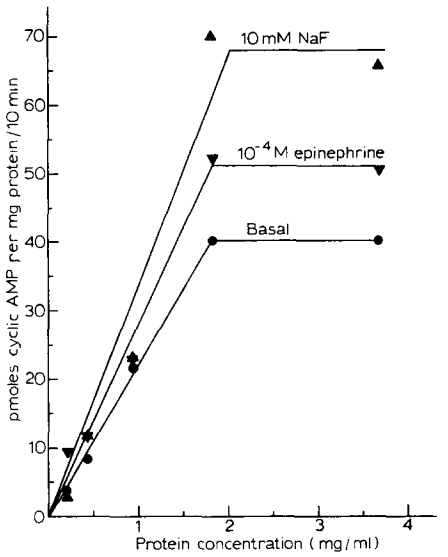


Fig. 4. Effect of pH on cyclic 3′,5′-AMP production: a 5-min incubation was employed. The 1000 × *g* fraction of mammary carcinoma homogenate was used as enzyme source. ATP concentration was 1 mM.

Fig. 5. Effect of protein concentration upon adenylate cyclase activity. Incubation time was 10 min. The 1000 × *g* fraction of homogenate was used as enzyme source. ATP concentration was 1 mM.

Subcellular distribution of adenylate cyclase activity

The three particulate and supernatant fractions of the homogenate clearly showed the 1000 × *g* fraction to possess the highest specific enzyme activity. It is also obvious that the 1000 × *g* fraction contains the bulk of total adenylate cyclase activity (Table I).

Responsiveness to hormones and antagonists of adenylate cyclase

The following hormones were repeatedly subjected to test: thyroid-stimulating hormone, luteinizing hormone, follicle-stimulating hormone, prolactin, growth hormone, adrenocorticotrophic hormone, vasopressin, parathormone, glucagon, insulin and epinephrine. Of these, only epinephrine consistently stimulated this enzymatic

TABLE I

SUBCELLULAR DISTRIBUTION OF ADENYLATE CYCLASE ACTIVITY
Mammary carcinoma homogenate was fractionated as described under Materials and Methods. ATP concentration: 1 mM.

Addition	pmoles cyclic 3′,5′-AMP/mg protein per 10 min			
	1000 × *g*	10 000 × *g*	105 000 × *g*	105 000 × *g* supernatant
Basal	490	141	3	0
10 mM NaF	725	181	28	8
10^{-4} M epinephrine	687	136	0	0
Percentage of tumor protein	62	6.6	4.4	27

activity. Stimulation by epinephrine was completely inhibited by propranolol, but was almost unaffected by phentolamine. All the other hormones tested failed to stimulate the enzyme. Prostaglandins E_1 and E_2 revealed small but consistent stimulatory effects upon the enzyme activity. The only other agonist was NaF (Table II).

Adenylate cyclase activity in normal murine lactating mammary tissue

No cyclase activity could be demonstrated in the 1000 × *g* fraction of normal lactating murine mammary tissue. Neither could a response be defined when NaF, epinephrine, adrenocorticotropic hormone, thyroid-stimulating hormone and luteinizing hormone were added.

DISCUSSION

Our inability to demonstrate adenylate cyclase activity in the 1000 × *g* fraction of the whole homogenate of normal murine lacting mammary tissue was unexpected. This finding differs, for instance, from that in the preliminary communication of Brown et al. [2] and also to some extent from that of Majumder and Turkington [3], who cite low adenylate cyclase activity in the mammary tissues of both lactating rats and mice. However, these investigators used highly purified preparations of the en-

TABLE II

EFFECTS OF EPINEPHRINE, ADRENERGIC BLOCKING AGENTS AND PROSTAGLANDINS E_1 AND E_2 UPON CYCLIC 3′,5′-AMP PRODUCTION
Enzyme source: 1000 × *g* fraction of mammary carcinoma homogenate. ATP concentration: 1 mM.

Addition (10^{-4} M)	pmoles cyclic 3′,5′-AMP/mg protein per 10 min	
	Expt 1	Expt 2
Basal	312	88
10 mM NaF	765	392
Epinephrine	500	139
Epinephrine + propranolol	264	77
Epinephrine + phentolamine	369	120
Prostaglandin E_1	381	113
Prostaglandin E_2	432	99

zyme whereas we used the unwashed 1000 × *g* fraction. In our experiments, moreover, no adenylate cyclase activity could be demonstrated when NaF, epinephrine, adrenocorticotrophic hormone, thyroid-stimulating hormone and luteinizing hormone were added. All these compounds might have activated fat tissue cyclase had any significant contamination of the 1000 × *g* fraction by fat cell membranes existed. The fat from the homogenized fat tissue, in which the murine mammary tissue was originally embedded, floated over the 1000 × *g* supernatant. It is possible that the basal adenylate cyclase in BALBc mice is so low that it cannot be detected by the method of Ramachandran [5] as adapted by us. However, this method consistently detected adenylate cyclase activity in the mammary tumorous tissue. It is also possible that an agonist able to stimulate normal lactating murine mammary tissue enzyme exists, but remains unknown at present.

Contrary to the findings of Brown et al. [2] who used the 100 000 × *g* fraction of a rat mammary carcinoma homogenate as a source of adenylate cyclase, we found that the bulk of adenylate cyclase activity is present in the 1000 × *g* fraction. This was found to be true both in regard to total activity and to highest specific activity (Table I). It is possible that the subcellular distribution of adenylate cyclase in the rat and mouse tumors is different. Thus the discrepancy in these observations could be due to species differences, although it might also be a consequence of the different basis of cancer induction employed. The rat mammary carcinoma was chemically induced whilst the murine tumor is a product of the mammary tumor virus.

Optimal incubation time for adenylate cyclase activity under basal conditions was only 5 min. In the presence of the agonists NaF or epinephrine, however, the optimal incubation time was prolonged (Fig. 2). Additional strengthening of the ATP-regenerating system did not prolong optimal incubation time. Therefore, substrate concentration was probably stable during the incubation process. The ATP concentration used was in the optimal range for the enzymatic reaction. The concentration of enzyme (protein) used in our experiment showed positive relationship to its activity. This correlation is linear in the concentration range used in our experiments (Fig. 5). The optimal Mg^{2+} concentration reaches a plateau at 6 mM, but in the presence of 10 mM NaF, Mg^{2+} exercise an optimal effect upon cyclase activity at a 8 mM concentration (Fig. 3). The optimal pH is in the alkaline range. From one of the early reports [7] it is already known that the pH range within which the enzymatic reaction takes place is relatively wide (Fig. 4).

Both rat and mouse mammary carcinoma possess adenylate cyclase which is active under remarkably similar conditions and is stimulated by the same agonists, NaF and epinephrine [2]. As already indicated therefore, epinephrine was the only hormone found to consistently activate the tumor cyclase. In other words, our experiments could define no stimulatory effect upon the adenylate cyclase activity of murine mammary carcinoma of a range of hormones with a known capacity to stimulate the cyclase of several different tissues other than mammary.

The regulatory component of the adenylate cyclase, which solely shows responsiveness to epinephrine, appears to mainly manifest β-adrenergic properties. Its activity is thus completely blocked by propranolol, a β-adrenergic blocking agent whereas conversely it is only slightly influenced by phentolamine, an α-adrenergic blocking agent (Table II).

We have also consistently observed weak responses of the mammary carcinoma

enzyme to prostaglandins E_1 and E_2 (Table II). At this stage of our work it is difficult to ascribe physiological significance to the prostaglandin effect upon tumor cyclase.

ADDENDUM

Bär [8] has recently reported about the occurrence of an epinephrine- and prostaglandin-sensitive adenylate cyclase in normal rat and rabbit mammary gland. Adenylate cyclase from mouse mammary cells was also shown to be stimulated by epinephrine and prostaglandins.

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