Comparison of Bone and Osteosarcoma Adenylate Cyclase

PARTIAL PURIFICATION OF MEMBRANES AND KINETIC PROPERTIES OF ENZYME

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(Received 23 August 1979)

The purpose of this study was to compare the adenylate cyclase of a tumour (rat osteosarcoma) growing *in vivo* with that of fast-growing embryonic bone. In the tumour the enzyme activity per total protein or DNA (under the same assay conditions) was 6–10-fold lower than in embryonic bone. To characterize this difference, we examined the kinetic properties of the enzyme in partially purified plasma membranes from the two tissues. A purification procedure based on differential centrifugation and discontinuous-sucrose-gradient centrifugation yielded a 10-fold increase in the specific activities of adenylate cyclase and 5'-nucleotidase in bone. The same procedure yielded an enriched membrane preparation from the tumour, but, relative to 5'-nucleotidase, a loss of 30% in adenylate cyclase occurred, which could not be recovered from another fraction. Kinetic analysis revealed that the lower adenylate cyclase activity in the tumour was due to a decrease in $V_{\max}$. There was no significant difference in K_s (approx. 0.15 mM), and in the K_m for GTP and p[NH]ppG. There were marked differences, however, in the extent of stimulation by p[NH]ppG, GTP and hormone, which was greater in tumour, and in the K_i for adenosine inhibition, which was 140 μ M in bone and 500 μ M in tumour. Under maximum stimulatory conditions, the enzyme activity in the tumour approached that in bone. The kinetic differences between bone and tumour enzyme were decreased by detergent solubilization, suggesting that the membrane environment plays a role in the generation of the observed differences.

The observation that exogenously added cyclic AMP inhibits cell division led to a series of investigations on the role of this regulatory molecule in the control of growth and proliferation. Numerous reports showed a negative correlation between the concentration of cyclic AMP and cell proliferation (Anderson *et al.*, 1973b; Otten *et al.*, 1972; Burger *et al.*, 1972; Ryan & Heidrick, 1974; Rudland *et al.*, 1974; Carchman *et al.*, 1974). A certain ambiguity persists, however, with respect to the generality of this relationship, since agents that enhance cyclic AMP accumulation, such as glucagon and lectins, stimulate cell proliferation in liver cells and lymphocytes (Chlapowski *et al.*, 1975; Friedman, 1976).

Studies focused on the cyclic AMP-producing

enzyme adenylate cyclase have shown that malignant transformed cultured fibroblasts had low activity of this enzyme (Burk, 1968; Anderson *et al.*, 1973a; Anderson & Pastan, 1975). This led to the hypothesis that a decrease in the activity of adenylate cyclase, or the failure of the enzyme to be stimulated by signals that normally inhibit proliferation, such as cell density, is an important feature of malignancy. Changes in surface properties of malignant cells have been repeatedly recognized (Brady *et al.*, 1969; Inbar & Sachs, 1969; Burger, 1969; Wu *et al.*, 1969; Bussell & Robinson, 1973; Hynes, 1973; Furcht & Scott, 1975) and are now considered to play an important role in the behaviour of cancer cells (Wallach, 1975). It was postulated that the reduction in adenylate cyclase activity observed in transformed cells was caused by an abnormal membrane environment (Gidwitz *et al.*, 1976), also reflected in membrane micro-viscosity (Inbar *et al.*, 1974) and probably due to a difference in lipid composition. Since these findings were made on cell lines in culture and their relevance to solid tumours was

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Abbreviations used: SDS, sodium dodecyl sulphate; p[NH]ppG, guanosine 5'-[β -imidotriphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

questioned, we examined if the same properties are found in a differentiating tumour growing *in vivo*, rat osteosarcoma. For comparison we analysed the fast-growing normal embryonic rat bone. The kinetic properties of the adenylate cyclase activity of the two tissues and the effect of various modulating agents were examined on partially purified plasma membranes.

Materials

Creatine phosphate, creatine phosphokinase, Tris/HCl, dithiothreitol, ATP (GTP-free, 2383), cyclic AMP, imidazole, neutral alumina, AMP, β -glycerophosphate, cacodylic acid, glucose 6-phosphate, deoxycholic acid, diphenylamine, DNA (type I from calf thymus), succinic acid, 2,6-dichlorophenol-indophenol, Hepes, human serum albumin (fraction V) and sucrose were obtained from Sigma Chemical Co. EGTA was obtained from Eastman Kodak Co., Rochester, NY, U.S.A., and HClO_4 was purchased from Fisher Scientific, Boston, MA, U.S.A. Trichloroacetic acid was obtained from Mallinckrodt, St. Louis, MO, U.S.A. [^{32}P]ATP, cyclic [^3H]AMP, 5'-[^3H]AMP and Bray's Solution (Bray, 1960) were obtained from New England Nuclear, Boston, MA, U.S.A. Dowex cation-exchange resin AG-50W (X4) was obtained from Bio-Rad, Richmond, CA, U.S.A. Aqueous counting scintillant (ACS) was obtained from Amersham-Searle, Arlington Heights, IL, U.S.A., and p[NH]ppG was purchased from ICN, Irvine, CA, U.S.A. Parathyroid hormone (1-84) was a gift from Dr. Henry Keutmann (Massachusetts General Hospital, Boston, MA, U.S.A.).

Methods

Membrane preparation

A transplantable rat osteosarcoma was obtained courtesy of Dr. W. F. Dunning from the Papanicolaou Cancer Research Institute, Miami, FL, U.S.A. The tumour was maintained by subcutaneous transplantation to the dorsal hind-quarters of ACI rats, obtained from Microbiological Associates, Walkersville, MD, U.S.A. Periodic histological examination (Drury *et al.*, 1967) during the past 30 months confirmed the osteogenic properties of the tumour.

Some 18-30 days after implantation, when the tumour had reached a diameter of 3-4 cm, it was excised and immediately immersed in buffer [0.25 M-sucrose/10 mM-Tris/HCl (pH 7.7 at 25°C)/1 mM-EGTA/1 mM-dithiothreitol]. All procedures were carried out at 4°C. The outer vascularized membrane was removed and the tissue was disrupted in a Virtis '23' mill (model 7-109-AF) at full speed (23 000 rev./min) for 45 s. The tissue was then homogenized with a Potter-Elvehjem homogenizer, in a

vessel obtained from A. H. Thomas, Philadelphia, PA, U.S.A., for 20 s at 3000 rev./min at a volume/mass ratio of 10 ml/g wet wt. of tissue. The homogenate was filtered through two layers of cheesecloth and centrifuged for 30 min at 47 000 g (Beckman J21-B; fixed-angle rotor JA-20). The pellet was suspended in an equal volume of buffer [10 mM-Tris/HCl (pH 7.7 at 25°C)/1 mM-dithiothreitol] hereafter referred to as Tris/dithiothreitol and homogenized with a Dounce homogenizer on ice with the loose-fitting glass pestle B for three strokes. This suspension was diluted (1:10) in 20 ml of sucrose (density 1.195 g/ml subsequently adjusted to a density of 1.175 g/ml with buffer, with the aid of a refractometer). On top of this mixture, 17 ml of sucrose at a density of 1.155 g/ml was layered and the sample was centrifuged for 2 h at 100 000 g (IEC B-60, swinging-bucket rotor SB-110). The fractions floating on top of the 1.155 g/ml (F_1) and the 1.155-1.175 g/ml interface (F_2) were carefully removed and diluted with 6 times their volume of Tris/dithiothreitol and centrifuged at 47 000 g for 15 min. The F_1 fraction was precipitated as a creamy-white pellet and the F_2 interface as a tan-white pellet. These pellets were then resuspended in Tris/dithiothreitol as was the 100 000 g sucrose pellet. For the study of recovery and balance of materials the remaining sucrose was pooled, diluted 1:6 in Tris/dithiothreitol and concentrated on an Amicon PM-30 Diaflo filter. Samples for future determination of adenylate cyclase, 5'-nucleotidase and DNA were taken and frozen in liquid N_2 , and stored at -80°C. Succinate dehydrogenase and glucose 6-phosphate were measured on fresh samples.

Partial purification of embryonic rat calvaria adenylate cyclase entailed a similar procedure. Approx. 120 (19-20-day-old) calvaria, dissected free of skin, were minced at 4°C in Krebs-Ringer glucose, pH 7.7, (Krebs, 1950) and were homogenized in 40 ml of a buffer [0.25 M-sucrose/10 mM-Tris/HCl (pH 7.7 at 25°C)/1 mM-EGTA/1 mM-dithiothreitol], followed by filtration through two layers of cheesecloth. The 47 000 g pellet was suspended in Tris/dithiothreitol and was mixed with 20 ml of sucrose (density 1.195 g/ml) re-adjusted to density 1.175 g/ml. This mixture was then layered with sucrose density 1.165 g/ml and centrifuged as above. Fractions were collected and frozen as described for the tumour. Adenylate cyclase was solubilized by agitating the membranes on a Vortex-mixer for 5 s with 10 mM-Tris/HCl (pH 7.6)/1 mM-dithiothreitol/0.5% Lubrol PX (a gift from Dr. L. Chan, University of Connecticut) and then incubating them in an ice bath for 15 min. The mixture was then centrifuged at 38 000 rev./min (IEC B-20 centrifuge, A-321 rotor; approx. 120 000 g) for 30 min. Adenylate cyclase was assayed on the supernatant.

Enzyme assays

Adenylate cyclase was assayed by measuring the production of 3',5'-cyclic AMP from [α - 32 P]ATP by the method of Salomon *et al.* (1974). The reaction mixture (0.1 ml) contained 6.2 units of phosphocreatine kinase, 5 mM-creatine phosphate, 25 mM-Tris/HCl, pH 7.6 at 30°C, 1 mM-dithiothreitol, 1 mM-cyclic AMP and about 2×10^6 c.p.m. of [α - 32 P]ATP. The concentrations of MgATP^{2-} and Mg^{2+} (added as MgCl_2) calculated as described in the following paper (Rodan *et al.*, 1980) are given in the legends to the Figures and Tables. The reaction was started by the addition of protein (5–13 μg /assay). After incubation at 30°C for 3–10 min the reaction was stopped with 100 μl of stopping solution containing 2% SDS, 4 mM-ATP and 1.4 mM-cyclic AMP. Cyclic [^3H]AMP (50 μl) was added for the measurement of recovery. Purification of cyclic AMP was carried out as described by Salomon *et al.* (1974). Eluates were counted for radioactivity in 10 ml of Bray's (1960) solution in a liquid-scintillation counter. All determinations were done in triplicate and the precision was 5% (mean coefficient of variation).

5'-Nucleotidase was assayed as described by Gentry & Olsson (1975). Glucose 6-phosphatase was assayed as described by Gunderson & Nordlie (1975). P_i was measured by the method of Ames (1966) in 0.3 ml of a 5000 rev./min (4000 g) supernatant.

Succinate dehydrogenase activity was measured spectrophotometrically by following the reduction of 2,6-dichlorophenol-indophenol at 38°C as described by Green *et al.* (1955). DNA was measured as described by Burton (1968). Protein was deter-

mined by the method of Lowry *et al.* (1951), with human serum albumin standards in the same medium as samples.

Computation of various parameters, such as K_m , K_s , K_i and V_{max} , was done by a numerical curve-fitting procedure (Hooke & Jeeves, 1961). It is based on a directed iterative search, which for a given expression $y = f(x)$, adjusts the values of the fixed parameters to minimize $\Sigma(y_e - y_c)^2$, where y_e is the experimental and y_c the calculated value of the function.

Results

The subcellular fractionation of calcified connective tissues presented several difficulties, owing to the abundance of dense matrix, the presence of hard calcium hydroxyapatite crystals and the high concentration of Ca^{2+} in the medium. Trials with various separation schemes showed that the following factors had a pronounced effect on the purification and preservation of plasma-membrane enzyme activities: (i) the initial tissue disruption, particularly the volume/solid ratio, the clearance of the homogenizer and the time of homogenization; (ii) precise adjustment of the sucrose density (by refractometer) for gradient separation; (iii) the presence of dithiothreitol throughout the procedure.

The procedure described in the Methods section was adopted and specific markers were followed in all fractions: DNA for nuclei, glucose 6-phosphatase for endoplasmic reticulum; succinate dehydrogenase for mitochondria; 5'-nucleotidase and adenylate cyclase for plasma membrane.

Table 1 shows the specific activity and recovery of

Table 1. *Partial purification of plasma membrane from rat embryonic calvaria*

The fractions were prepared as described in the Methods section from about 120 calvaria. The 47000 g pellet was further fractionated on a sucrose gradient. Results are expressed as means $\pm$ S.E.M. for five experiments, except for 'pooled sucrose' data which are from one representative experiment. After removal of fractions F_1 and F_2 , the remaining sucrose of the gradient was diluted 1:6 in Tris/dithiothreitol and was centrifuged at 47000 g to yield the 'pooled sucrose' pellet and supernatant.

Fraction	Adenylate cyclase					5'-Nucleotidase		
	Protein		Specific activity (pmol/min per mg of protein)	Total (pmol/ min)	(%)	Specific activity (nmol/min per mg of protein)	Total (nmol/ min)	(%)
	(mg)	(%)						
Homogenate	53.7 $\pm$ 3.7	100.00	14.70 $\pm$ 1.10	793.00	100.00	3.61 $\pm$ 0.61	194.00	100.00
47000 g supernatant	23.4 $\pm$ 1.1	43.50	3.76 $\pm$ 0.47	88.00	11.10	0.79 $\pm$ 0.10	18.50	9.50
47000 g pellet	38.3 $\pm$ 8.3	71.20	16.80 $\pm$ 0.43	643.00	81.10	5.69 $\pm$ 0.13	218.00	112.00
Pellet from sucrose gradient	13.4 $\pm$ 1.4	25.00	5.07 $\pm$ 2.45	68.20	8.60	1.18 $\pm$ 0.16	15.90	8.20
1.165–1.175 g/ml interface (F_2)	0.3 $\pm$ 0.06	0.58	71.00 $\pm$ 2.00	22.00	2.80	16.30 $\pm$ 1.83	5.05	2.60
'Pooled sucrose' pellet	3.5	6.50	60.70	212.00	26.70	13.50	32.60	16.80
'Pooled sucrose' supernatant	5.5	10.20	46.40	254.00	32.10	16.30	89.30	46.00
1.165 g/ml float (F_1)	0.5 $\pm$ 0.16	1.00	157.00 $\pm$ 23.50	83.20	10.50	39.40 $\pm$ 5.98	20.90	10.80

Table 2. *Distribution of markers for subcellular fractions in rat embryonic calvaria*
The fractions were prepared as described in the Methods section from about 120 calvaria. The 47 000 g pellet was further fractionated on a sucrose gradient. Data are means of two representative experiments.

Fraction	Protein			DNA			Succinate dehydrogenase		Glucose 6-phosphatase	
	(mg)	(%)	(%)	(μg)	(%)	(%)	Specific activity (nmol/min per mg of protein)	Total (nmol/min)	Specific activity (μmol/min per mg of protein)	Total (μmol/min)
Homogenate	49.8	100.00	703.00	100.00	0.703	35.00	100.00	1.860	0.0374	1.860
47 000 g supernatant	18.2	36.50	0.048	0.007	<0.013	<0.007	<0.02	0.104	0.0057	0.104
47 000 g pellet	33.3	66.90	683.00	97.10	0.990	33.00	94.30	1.580	0.0474	1.580
Pellet of sucrose gradient	13.5	27.10	677.00	96.20	2.78	37.50	107.00	0.591	0.0438	0.591
'Pooled sucrose' pellet	3.5	7.05	<0.015	<0.002	<0.013	<0.007	<0.02	0.214	0.061	0.214
'Pooled sucrose' supernatant	10.0	20.10	<0.015	<0.002	<0.013	<0.007	<0.02	0.716	0.072	0.716
1.165 g/ml float (F ₁)	0.7	1.39	<0.015	<0.002	<0.013	<0.007	<0.02	0.014	0.020	0.014

adenylate cyclase and 5'-nucleotidase in the various cellular fractions of rat calvaria. A 10-fold purification of the two enzymes (per mg of protein) was found in the material that floated on 38% (w/v) sucrose (F₁). This fraction contained about 10% of the total activity of the two plasma-membrane enzymes. The constant ratio between the two enzymes observed in all fractions suggests that they are associated with the same subcellular structures, and that the enzyme activities are not modified by activators or inhibitors during the preparation procedure.

Table 2 shows the distribution of three markers of subcellular particles among the fractions of Table 1. Within the limits of the methods used fraction F₁ was free of DNA and succinate dehydrogenase. Glucose 6-phosphatase was present in fraction F₁, but

Fig. 1. *Time course of adenylate cyclase reaction catalysed by partially purified bone plasma membranes*
Enzyme activity was assayed on 7 μg of protein, as described in the Methods section, under the following conditions; ●, 30 μM-MgATP²⁻ and 5 mM-Mg²⁺; ○, membranes preincubated in the presence of 10 μM-p[NH]ppG then assayed in the presence of 50 μM-MgATP²⁻ and 0.3 mM-Mg²⁺; ▲, 100 μM-MgATP²⁻ and 5 mM-Mg²⁺. Bars represent S.E.M. values for triplicate determinations.

Table 3. *Partial purification of plasma membranes from rat osteosarcoma*

The fractions were prepared as described in the Methods section from an 18–30-day implanted tumour. The 47000 g pellet was further fractionated on a sucrose gradient. Results are expressed as means $\pm$ S.E.M. for five experiments, except for the 'pooled sucrose' data, which are from one representative experiment. After removal of fractions, F_1 and F_2 , the remaining sucrose of the gradient was diluted 1:6 in Tris/dithiothreitol and was centrifuged at 47000 g to yield the 'pooled sucrose' pellet and supernatant. p[NH]ppG, which stimulates the enzyme activity 6–9-fold, was present (0.01 mM) in all assays of adenylate cyclase. Abbreviation: ND, not detectable.

Fraction	Protein		Adenylate cyclase			5'-Nucleotidase		
			Specific activity (pmol/min per mg of protein)	Total (pmol/min)	Specific activity (nmol/min per mg of protein)	Total (nmol/min)	Specific activity (nmol/min per mg of protein)	Total (nmol/min)
	(mg)	(%)						
Homogenate	960.0 $\pm$ 85.9	100.0	17.30 $\pm$ 1.26	16600.0	100.0	6.29 $\pm$ 0.75	6040.00	100.00
47000 g supernatant	553.0 $\pm$ 91.9	57.0	1.14 $\pm$ 0.14	630.0	3.8	1.46 $\pm$ 0.50	807.00	13.40
47000 g pellet	369.0 $\pm$ 46.5	44.0	37.60 $\pm$ 5.39	13900.0	83.0	15.70 $\pm$ 2.20	5800.00	96.10
Pellet of sucrose gradient	251.0 $\pm$ 17.2	26.2	13.20 $\pm$ 5.19	3310.0	19.9	5.00 $\pm$ 1.40	1260.00	20.20
1.155–1.175 g/ml interface (F_2)	5.4 $\pm$ 1.2	0.5	97.10 $\pm$ 3.25	527.0	3.2	20.10 $\pm$ 1.81	109.00	1.81
'Pooled sucrose' pellet	23.6	2.0	61.20 $\pm$	1440.0	8.7	17.90	422.00	7.00
'Pooled sucrose' supernatant	39.4	4.1	ND			ND		
1.155 g/ml float (F_1)	4.0 $\pm$ 0.4	0.4	106.40 $\pm$ 22.8	424.0	2.56	54.70 $\pm$ 14.70	218.00	3.61

Fig. 2. Time course of adenylate cyclase reaction catalysed by partially purified osteosarcoma plasma membranes

Enzyme activity was assayed on 10 μ g of protein, as described in the Methods section, under the following conditions: ●, 30 μ M-MgATP²⁻ and 1.0 mM-Mg²⁺; ○, 30 μ M-MgATP²⁻ and 15 mM-Mg²⁺; □, 10 μ M-p[NH]ppG added with 30 μ M-MgATP²⁻ and 15 mM-Mg²⁺; ▲, membranes preincubated for 5 min at 30°C with 10 μ M-p[NH]ppG then assayed with 100 μ M-MgATP²⁻ and 1 mM-Mg²⁺; △, membranes preincubated with 10 μ M-p[NH]ppG and assayed in

relative to this enzyme, it had a 20-fold enrichment in 5'-nucleotidase and adenylate cyclase. Electron microscopic examination of fraction F_1 revealed vesicular structures of trilaminar membranes characteristic of plasma membranes or smooth endoplasmic reticulum and some contamination by rough endoplasmic reticulum. The preparation was free of mitochondria and nuclei.

Tables 3 and 4 show similar data for the partial purification of tumour plasma membrane. It was less able to be purified by the same procedure. An 8–10-fold purification was achieved by floating the membranes on 36% (w/v) sucrose, but the yield was one-third of that of calvaria, and the glucose 6-phosphatase contamination was higher. Consistently, adenylate cyclase activity was decreased by about 30% during purification, relative to 5'-nucleotidase, owing to the loss of a cytosol activating factor (Egan *et al.*, 1978).

By comparison with the enzyme in embryonic bone, the adenylate cyclase activity in the tumour was about 10-fold lower. The difference was apparent when the denominator was homogenate protein, membrane protein, DNA or 5'-nucleotidase activity.

To examine the nature of this difference, we studied it as a function of substrate concentration, p[NH]ppG, GTP, parathyroid hormone and adenosine concentrations, enzyme solubilization and bi-valent-cation concentration (Rodan *et al.*, 1980).

the presence of 300 μ M-MgATP²⁻ and 1 mM-Mg²⁺. Bars represent S.E.M. values for triplicate determinations.

Table 4. *Distribution of markers for subcellular fractions in rat osteosarcoma*

The fractions were prepared as described in the Methods section from an 18–30-day implanted tumour. The 47000g pellet was further fractionated on a sucrose gradient. Data are means of two representative experiments.

Fraction	Protein			DNA			Succinate dehydrogenase			Glucose 6-phosphatase		
	(mg)	(%)	(μg)	(%)	(nmol/ min)	Total (nmol/ min)	Specific activity (nmol/min per mg of protein)	Total (nmol/ min)	(%)	Specific activity (μmol/min per mg of protein)	Total (μmol/ min)	Total (%)
Homogenate	346.0	100.00	5520.00	100.00	1050.00	1050.00	3.03	1050.00	100.00	0.022	7.61	100.00
47000g supernatant	198.0	57.30	596.00	10.80	<0.013	<0.18	<0.013	<0.18	<0.16	0.009	1.78	23.40
47000g pellet	155.0	45.00	4210.00	76.20	6.67	1040.00	6.67	1040.00	99.00	0.037	5.75	75.60
Pellet of sucrose gradient	88.7	25.70	3320.00	60.10	6.66	591.00	6.66	591.00	56.40	0.038	3.37	44.30
1.155–1.175 g/ml interface (F ₂)	8.6	2.50	9.29	0.17	28.20	211.00	28.20	211.00	20.20	0.050	0.43	5.65
Interface supernatant	13.9	4.01	<0.15	<0.003	<0.013	<0.18	<0.013	<0.18	<0.16	0.086	1.19	15.60
'Pooled sucrose' pellet	13.4	3.88	129.00	2.33	12.00	161.00	12.00	161.00	15.40	0.049	0.658	8.65
'Pooled sucrose' supernatant	19.8	5.76	<0.15	<0.003	<0.013	<0.18	<0.013	<0.18	<0.16	0.004	0.079	0.01
1.155 g/ml float (F ₁)	2.2	0.62	<0.15	<0.003	<0.013	<0.18	<0.013	<0.18	<0.16	0.085	0.183	0.024

Fig. 3. *Double-reciprocal plots of adenylate cyclase activity dependence on substrate in bone membranes*

Adenylate cyclase was measured as described in the Methods section on 7 μg of protein at the indicated concentrations of MgATP²⁻ for 5 min in the presence of 5 mM-Mg²⁺. The computed *K_s* for MgATP²⁻ is 182 μM and the *V_{max}* is 147 pmol/min per mg of protein. Data points are means of triplicate determinations. This is one of several similar experiments conducted on different membrane preparations and yielding similar results.

Figs. 1 and 2 show time-dependence curves under various experimental conditions used in this study. Time-dependence was tested for a range of MgATP²⁻ concentrations, for low Mg²⁺ concentrations and for several p[NH]ppG and parathyroid hormone concentrations. As previously reported (Londos *et al.*, 1974), p[NH]ppG stimulation showed a 2–3 min time lag. To obtain linear conditions, membranes were preincubated for 5 min at 30°C with p[NH]ppG, when used. In osteosarcoma the adenylate cyclase activity tapered off after a few minutes at low MgATP²⁻ concentrations because of high phosphohydrolase activity. Reactions were therefore stopped after 3 min in experiments that included MgATP²⁻ concentrations below 100 μM.

Attempts to carry out kinetic analysis under the ionically defined assay conditions described in the following paper (Rodan *et al.*, 1980) revealed that, at appropriate Mg²⁺ concentrations (5 mM for bone and 15 mM for tumour), the bone and tumour enzymes exhibited Michaelis–Menten behaviour with respect to substrate. Figs. 3 and 4 present double-reciprocal plots of adenylate cyclase activity as a function of MgATP²⁻ concentrations. The decreased enzyme activity observed in the tumour membranes reflects a decrease in *V_{max}* (25.7 compared with 147 pmol/min per mg of protein), the *K_s* being approximately the same, about 150 μM.

Both bone and tumour enzymes were stimulated by p[NH]ppG non-competitively relative to

Fig. 4. Double-reciprocal plots of adenylate cyclase activity dependence on substrate in osteosarcoma membranes

Adenylate cyclase was measured as described in the Methods section on 11.5 μ g of protein for 3 min in the presence of 15 mM- Mg^{2+} . The K_s for MgATP^{2-} was calculated to be 140 μ M and the V_{max} was 25.7 pmol/min per mg of protein. Data points represent means of triplicate determinations. This is one of several similar experiments conducted on different membrane preparations, yielding similar results.

Fig. 5. Effect of p[NH]ppG on osteosarcoma adenylate cyclase activity

Adenylate cyclase was measured for 3 min, as described in the Methods section, on 12 μ g of osteosarcoma membrane protein after preincubation with 0.1 μ M-p[NH]ppG (▲) and 50 μ M-p[NH]ppG (●) for 5 min at 30°C. The assay was started by the addition of assay mixture containing the MgATP^{2-} concentrations shown in the graph and 5 mM- Mg^{2+} . Data points represent means of triplicate determinations.

MgATP^{2-} (Fig. 5). More detailed examination of the p[NH]ppG-concentration-dependence revealed two apparent affinities (Figs. 6a and 6b) with K_m values of 0.25 and 1.8 μ M. This two-site-binding behaviour was independent of MgATP^{2-} concentrations and was qualitatively and quantitatively identical in bone and tumour. However, the extent of stimulation,

Fig. 6. Eadie-Hofstee plots of p[NH]ppG stimulation of bone (a) and osteosarcoma (b) adenylate cyclase

Enzyme activity was assayed as described in the Methods section on 10 μ g of osteosarcoma and 5 μ g of bone membrane protein after preincubation with p[NH]ppG at the indicated concentrations, each in the presence of eight concentrations of MgATP^{2-} ranging between 50 and 100 μ M. The data points are V_{max} values for each p[NH]ppG concentration.

Table 5. Comparison of bone and osteosarcoma adenylate cyclase under standard assay conditions and at saturating Mg^{2+} and p[NH]ppG concentrations

Bone and tumour membranes were prepared as described in the Methods section. Membranes were preincubated in p[NH]ppG, when present as described. MgATP^{2-} concentration was 0.5 mM; incubation time was 5 min; conditions were selected on the basis of Mg^{2+} and p[NH]ppG concentration-dependence curves presented in this study. Data are means $\pm$ S.E.M. for triplicate determinations.

	Adenylate cyclase (pmol/min per mg of protein)	
	Bone	Tumour
5 mM- Mg^{2+} , no p[NH]ppG	146.0 $\pm$ 3.5	17.3 $\pm$ 1.5
15 mM- Mg^{2+} , 50 μ M-p[NH]ppG	459.0 $\pm$ 7.8	218.0 $\pm$ 6.9

relative to basal activity, was much larger in tumour: 8.8 ($\pm$ 0.7)-fold in tumour (24 independent measurements) compared with 3.9 ($\pm$ 0.1)-fold in bone (19 measurements) [results are means ($\pm$ S.E.M.)]. This

Table 6. *Effects of detergent solubilization on kinetic parameters of bone and osteosarcoma adenylate cyclase*
Solubilization of bone and osteosarcoma membranes is described in the Methods section. The assay mixture contained 10mM-Mg²⁺ and MgATP²⁻ concentrations ranging from 30 to 300μM. p[NH]ppG was 10μM. *K_s* is expressed as μM and *V_{max}* is pmol/min per mg of protein.

	Adenylate cyclase			
	Osteosarcoma		Bone	
	Basal	+ p[NH]ppG	Basal	+ p[NH]ppG
Particulate				
<i>K_s</i>	199	232	176	171
<i>V_{max}</i>	19.3	107.0	185.0	623
Solubilized				
<i>K_s</i>	49	34	31.7	39
<i>V_{max}</i>	3.2	17.3	21.3	115.0

Fig. 7. *Stimulation of bone adenylate cyclase by GTP and parathyroid hormone*

Partially purified bone membranes (5.5μg per assay tube) were preincubated with GTP (concentrations as indicated in the graph) and assayed for adenylate cyclase in the presence of 200μM-MgATP²⁻, 3mM-Mg²⁺, with (O) or without (●) 0.1μM-parathyroid hormone for 5 min at 30°C as described in the Methods section. Values represent the means of triplicate determinations.

suggests a regulatory rather than a constitutive difference, a fact supported by Table 5 showing that, under saturating p[NH]ppG and Mg²⁺ concentra-

Fig. 8. *Stimulation of osteosarcoma adenylate cyclase by GTP and parathyroid hormone*

Partially purified osteosarcoma membranes (9μg per assay tube) were preincubated with GTP (concentrations as indicated in the graph) and assayed for adenylate cyclase activity in the presence of 200μM-MgATP²⁻, 6mM-Mg²⁺, with (O) or without (●) 0.1μM-parathyroid hormone for 5 min at 30°C as described in the Methods section. Values represent the means of triplicate determinations.

tions, the 8-fold difference between bone and tumour enzyme activities was decreased to 2-fold.

The findings presented in Table 6 further suggest that the altered response to p[NH]ppG is caused by a change in the membrane environment. Solubilization in non-ionic detergent reduced the catalytic activity, enhanced the affinity for substrate, did not abolish the susceptibility to p[NH]ppG and ap-

Fig. 9. Adenosine inhibition of bone and osteosarcoma adenylate cyclase

Bone (8.2 μg) and osteosarcoma (10.6 μg) membranes were assayed for adenylate cyclase activity in the presence of 500 μM-MgATP²⁻, 5 mM-Mg²⁺ and adenosine as indicated for 10 min as described in the Methods section. Values represent the means of two determinations. The experiment was repeated three times and yielded similar results.

peared to equalize the extent of stimulation by this nucleotide.

Both enzymes were responsive to parathyroid hormone and GTP, and again the extent of stimulation was larger in tumour (Figs. 7 and 8). Like p[NH]ppG, GTP exhibited two apparent affinities on Eadie-Hofstee plots, with apparent K_m values of 0.1 and 0.2 μM for bone, and 0.4 and 0.02 μM for tumour. In both cases parathyroid hormone reduced the higher K_m by about 50% (left shift seen in Figs. 7 and 8).

Both enzymes were inhibited by adenosine, with significantly different K_i values (Fig. 9). In bone, 50% inhibition was seen at 140 μM and in tumour at about 500 μM. Low adenosine concentrations of 1–10 μM were mildly stimulatory (10–20%) in bone and tumour membranes.

Discussion

A reduction in adenylate cyclase activity has been observed in a number of malignant tumours (DeRubertis *et al.*, 1976; Stevens *et al.*, 1976) and transformed cells (Burk, 1968; Anderson *et al.*, 1973a; Anderson & Pastan, 1975). In view of the putative inhibitory role of cyclic AMP in cell proliferation (Anderson *et al.*, 1973b; Otten *et al.*, 1972; Burger *et al.*, 1972; Ryan & Heidrick, 1974; Rudland *et al.*, 1974; Carchman *et al.*, 1974), the reduction in adenylate cyclase was proposed to be inherent to the malignant state (Anderson & Pastan, 1975). Several exceptions to this generalization were reported (reviewed by Chlapowski *et al.*, 1975) and since the mechanism for cyclic AMP involvement in proliferation control is unknown, this issue is still open.

In the present study we examined the adenylate cyclase activity of a tumor growing *in vivo* (rat osteosarcoma) and compared it with that of rapidly growing embryonic bone. We found a substantially lower enzyme activity in the tumour, which was not due to differences in membrane purification, since it was seen when the results were based on tissue DNA, membrane protein or membrane-associated 5'-nucleotidase. Kinetically, the lower activity measured under identical assay conditions was almost entirely due to a reduction in V_{max} . Usually a finding such as this suggests less enzyme, but could also be due to changes in the regulatory influence of modifiers or the membrane environment. The findings of Gidwitz *et al.* (1976), who showed that solubilization equalized the specific activities of adenylate cyclase from normal and transformed cells, are consistent with both possibilities: less enzyme/membrane protein or modification of enzyme activity by the membrane environment. Our findings offer support for the latter alternative and differentiate between kinetic properties that are affected by the presumed 'environmental' change and those that are not.

The apparent affinity for substrate at the catalytic site was virtually the same in bone and tumour, approx. 0.15 mM, a value very similar to that reported in many systems (Perkins, 1973; Glynn *et al.*, 1979).

The apparent affinities for p[NH]ppG stimulation were also similar. Examination of the effects of p[NH]ppG provided kinetic evidence for two nucleotide sites. Previous studies have described two functionally different guanine nucleotide sites (Lad *et al.*, 1977; Welton *et al.*, 1977): one, with an affinity similar to that estimated in this study at 2.5 μM⁻¹, stimulated the catalytic unit directly, whereas the other one mediated hormone stimulation. In bone and tumour membranes exogenous guanine nucleotides were not required for parathyroid hormone stimulation of adenylate cyclase (Figs.

7 and 8), although GTP-free ATP was used as substrate. Similar results were obtained by Crawford *et al.* (1978) in their osteogenic sarcoma membrane and by Hunt *et al.* (1976) in chick kidney membranes. However, the presence of hormone increased slightly the affinity for nucleotide (Figs. 8 and 9), and GTP increased the affinity for hormone (results not shown) along with the extent of stimulation. This reciprocal relationship was also seen for Mg^{2+} activation of the enzyme in the presence of p[NH]ppG (Rodan *et al.*, 1980). We thus favour a model whereby enhancement of adenylate cyclase activity by one modulating agent, such as hormone, guanine nucleotide, Mg^{2+} or 'cytosol factor' (Egan *et al.*, 1978), is amplified by the increased affinity or effectiveness of another stimulatory agent. It seems that the 'coupling efficiency' in (or to) the adenylate cyclase complex increases with enzyme activity.

A major difference between bone and tumour enzyme was the effectiveness of p[NH]ppG stimulation, along with the greater stimulation by GTP and parathyroid hormone. This strongly suggests that changes in regulatory features are the primary reason for the lower enzyme activity in tumour, rather than a decrease in the amount of enzyme or changes in its catalytic moiety. The lower affinity for adenosine inhibition, which was shown to act via specific receptors (Londos & Wolff, 1977), also points to a regulatory difference.

A likely molecular basis for these findings is a change in membrane environment, which was shown to affect the coupling of the adenylate cyclase catalytic moiety to regulatory moieties. Thus experimental elevation of membrane fluidity was found to raise the coupling rate of β -adrenergic agonists to adenylate cyclase in turkey erythrocytes and Chang liver cells (Levitzki & Helmreich, 1979).

Increased membrane fluidity was indeed found in malignantly transformed cells by micro-viscosity measurements (Inbar *et al.*, 1974) and is consistent with the increased activation of the tumour enzyme by guanine nucleotides and parathyroid hormone. The decrease in kinetic differences caused by solubilization also supports the membrane-environment hypothesis and concurs with the interpretation of Gidwitz *et al.* (1976) for their findings in transformed cells. Definite proof, however, could only follow extraction, purification and characterization of the enzyme from the two tissues.

This study did not address questions related to the role of adenylate cyclase in proliferative control, but it shows that plasma membrane from an osteosarcoma exhibits substantially lower adenylate cyclase activity than that from embryonic bone. Kinetic evidence indicates that this difference is not due to the amount of enzyme or its intrinsic properties, but to regulatory features probably associated with the membrane environment.

We thank Dr. L. Cutler for the electron-microscopic examination of the membrane fraction. This work was supported by grants AM17848 from the National Institute of Arthritis and Metabolic Diseases and DE04327 from the National Institute of Dental Research.

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