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Comparison of Bone and Osteosarcoma Adenylate Cyclase

EFFECTS OF Mg^{2+}, Ca^{2+}, ATP^{4-} AND $HATP^{3-}$ IN THE ASSAY MIXTURE

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The effects of Mg^{2+} and Ca^{2+} on bone and osteosarcoma adenylate cyclase were investigated. The concentrations of the cations and other ionic species in the assay mixture were calculated by solving the simultaneous equations describing the relevant ionic interactions (multiple equilibria). We re-examined the effects of $HATP^{3-}$ and ATP^{4-} on enzyme activity and found that: (i) the concentration of the minor ATP species is less than 1% of that of $MgATP^{2-}$, and their ratio to $MgATP^{2-}$ is constant if Mg^{2+} and H^+ concentrations are unchanged; (ii) Mg^{2+} addition decreased the ratio of the minor species to $MgATP^{2-}$ and increased the enzyme activity, but no meaningful kinetic model could attribute this effect of $HATP^{3-}$ or ATP^{4-}. On the other hand, kinetic analysis of Mg^{2+} effects showed: (i) stimulation via two metal sites, separate from the catalytic ($MgATP^{2-}$) site, with apparent K_m values of approximately 1 and 8 mM; (ii) that the low affinity increased towards the higher one when the enzyme activity rose as a result of increased substrate or guanine nucleotide concentrations, this effect being less pronounced in tumour; (iii) conversely, that two apparent affinities for $MgATP^{2-}$ merged into one at high Mg^{2+} concentration; (iv) kinetically, that this relationship is of the mixed con-competitive type, which is consistent with a role for Mg^{2+} as a requisite activator, and binding occurring in non-ordered sequence. Analysis of the Ca^{2+} effects showed: (i) competition with Mg^{2+} at the metal site (K_i 20 μM for bone and 40 μM for tumour); (ii) that relative to the substrate the inhibition was uncompetitive, i.e. velocity decreased and affinity increased proportionally, which is consistent with Ca^{2+} binding after substrate binding. These findings support the existence of interacting enzyme complexes, losing co-operativity at increased enzyme activity. They also indicate a potential physiological role for Ca^{2+} in enzyme regulation and point to quantitative differences between bone and tumour with regard to these properties.

We have shown in the preceding paper (Rodan *et al.*, 1980) that rat osteosarcoma membranes possess significantly lower adenylate cyclase activity, probably as the result of differences in enzyme regulation. One of the major modulators of enzyme activity is Mg^{2+}, which, in addition to its participation in substrate formation ($MgATP^{2-}$), controls enzyme activity by acting as an obligatory activator at a separate site (Drummond & Duncan, 1970; Garbers & Johnson, 1975; Londos & Preston, 1977; Johnson *et al.*, 1979). This function of Mg^{2+} was subject to debate, some investigators attributing the Mg^{2+} effects to a change in the concentration of free ATP anions (deHaen, 1974; Rendell *et al.*, 1975). Another regulatory cation of interest is Ca^{2+}, which has been found to inhibit most adenylate cyclase preparations (Perkins, 1973; Rodan, 1979) and may bear a special relationship to cyclic AMP in the regulation of many cellular functions, including proliferation (Rasmussen, 1970; Berridge, 1975). In the present study we examined, and kinetically characterized, the effects of Mg^{2+} and Ca^{2+} on bone and osteosarcoma adenylate cyclase, in order to evaluate if the regulatory differences observed in the tumour extend to these functions. To define and control the concentrations of Mg^{2+} and Ca^{2+} we solved the mul-

Abbreviation used: p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate.

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tiple equilibria equations describing the relevant interactions of cations and anions in the assay mixture. We could thus quantitatively re-evaluate the $ATP^{4-}/HATP^{3-}$ enzyme-inhibition hypothesis.

Methods

Plasma membranes were prepared from bone and osteosarcoma and protein concentrations were determined as described (Rodan *et al.*, 1980).

Adenylate cyclase was assayed in a reaction mixture of 0.1 ml containing about 2×10^6 c.p.m. of [α-^{32}P]ATP, 1 mM-cyclic AMP, 1 mM-dithiothreitol, 5 mM-creatine phosphate, 62 units of creatine kinase/ml, 25 mM-Tris/HCl (pH 7.8 at 30°C) and ATP, EGTA, $MgCl_2$, NaCl and $CaCl_2$ in amounts calculated to produce the final ionic composition described in the legends to the Tables and Figures. Guanine nucleotides, when present, were preincubated with the membranes for 5 min at 30°C. Reactions were initiated by addition of membranes (5–13 μg of protein) to the temperature-equilibrated (30°C) assay mixtures. After 3–10 min at 30°C, the reaction was terminated and cyclic [^{32}P]AMP isolated as described by Salomon *et al.* (1974).

Determination of the individual ionic species in the assay mixture

Under conditions of minimum requirements the following ions are present in the assay mixture: ATP dissociated into four ionic species as a function of pH; Mg^{2+} and the respective complexes of the latter with the former; ADP and phosphate and their dissociated forms: all the complexes of Mg^{2+} with those anions. Creatine phosphate or phosphoenolpyruvate may also be present as part of an ATP-regenerating system, as well as other cations such as Mn^{2+}, added to test their effect on enzyme activity. When the effects of Ca^{2+} are investigated, EGTA is frequently added as a calcium buffer. EGTA also dissociates into four species as a function of pH. Since each cation interacts with each anion according to a known stability constant, the concentration of all the species in this complex mixture at equilibrium can be calculated by solving the set of simultaneous equations describing the law of mass action and the balance of material for the various species. Using this approach, desired combinations of ionic concentrations of individual species can be obtained experimentally. This is essential for kinetic analyses of the effects of ions with similar ionic properties, such as Ca^{2+} and Mg^{2+} for example.

Since the number of interactions is very large, even for computer calculation, we focused on those that control the species relevant for adenylate cyclase: $MgATP^{2-}$, $HATP^{3-}$, ATP^{4-}, Mg^{2+}, Ca^{2+}, $CaATP^{2-}$, $EGTA^{4-}$ and $HEGTA^{3-}$. We excluded interactions that would affect the concentration of these species by less than 2%, either because of limited availability in the assay mixture (enzyme concentration for example is less than 10 pM assuming a turnover number of $1000\,s^{-1}$), or low affinity. Interactions with association constants of less than $120\,M^{-1}$ have not been included.

Table 1 lists the interactions included in our calculation and the stability constants used. Where possible, we have selected from the literature (Burton, 1959; Taqui Khan & Martell, 1962; O'Sullivan & Perrin, 1964; Hammes & Levison, 1964; Phillips *et al.*, 1966, 1969; Alberty, 1969; Mohan & Rechnitz, 1970; Martell, 1971; Fogt & Rechnitz, 1974; Rechnitz, 1975) constants determined under conditions similar to those of the adenylate cyclase activity (I 0.1 and 30°C). Since it was reported that buffers affect the association of Ca^{2+} and EGTA (Ogawa, 1968), we measured the interaction of these ions in the presence of 25 mM-Tris/HCl at pH 7.6 and I 0.1 by the method of Chelex (Biorad, Richmond, CA, U.S.A.) competitive binding (Briggs & Fleishman, 1965). We found an apparent association constant of $4.4 \times 10^6\,M^{-1}$, which was not significantly different from that predicted from the values determined by titration (Schwarzenbach *et al.*, 1957).

Two computational approaches were used to define the ionic composition of the assay mixture. We started with either the desired ionic concentrations of certain species and, by substitution, solved for the total concentrations of ATP, Mg, EGTA and Ca, or with the total concentrations of ATP, Mg, EDTA and Ca and used a Newton–Raphson iterative procedure (Korn & Korn, 1967) to find the ionic concentrations that satisfy the multiple equilibria. Application of the two procedures to the same data verified the computational accuracy.

Illustrations of the results of such computations are presented in Tables 2 and 3, which list the concentrations of assay-mixture constituents for given

Table 1. *Association constants* (M^{-1}) *used for solution of multiple equilibria equations*

$Mg^{2+} + ATP^{4-} \rightleftarrows MgATP^{2-}$	1.78×10^4	$Mg^{2+} + HATP^{3-} \rightleftarrows MgHATP^-$	1.86×10^2
$Ca^{2+} + ATP^{4-} \rightleftarrows CaATP^{2-}$	8.91×10^3	$Ca^{2+} + HATP^{3-} \rightleftarrows CaHATP^-$	1.35×10^2
$Mg^{2+} + EGTA^{4-} \rightleftarrows MgEGTA^{2-}$	1.58×10^5	$Mg^{2+} + HEGTA^{3-} \rightleftarrows MgHEGTA$	2.51×10^3
$Ca^{2+} + EGTA^{4-} \rightleftarrows CaEGTA^{2-}$	1.0×10^{11}	$Ca^{2+} + HEGTA^{3-} \rightleftarrows CaHEGTA^-$	2.0×10^5
$H^+ + HATP^{3-} \rightleftarrows H_2ATP^{2-}$	6.76×10^3	$H^+ + ATP^{4-} \rightleftarrows HATP^{3-}$	3.3×10^6
$H^+ + HEGTA^{3-} \rightleftarrows H_2EGTA^{2-}$	5.90×10^8	$H^+ + EGTA^{4-} \rightleftarrows HEGTA^{3-}$	2.4×10^9

Table 2. *Effect of changes in the concentrations of $MgATP^{2-}$ and other ATP species on adenylate cyclase activity*
The concentrations of the ATP species were calculated as described in the text. Partially purified membranes from bone were assayed for adenylate cyclase activity as described (Rodan *et al.*, 1980). In all samples Mg^{2+} concentration was 5 mM, Ca^{2+} 10 μM and p[NH]ppG 10 μM.

$[MgATP^{2-}]$ (μM)	$[CaATP^{2-}]$ (μM)	$[HATP^{3-}]$ (nM)	$[ATP^{4-}]$ (μM)	$[MgHATP^{1-}]$ (nM)	$[CaHATP^{1-}]$ (nM)	v (pmol/min per mg of protein)
10	0.1	10	0.10	10	0.10	10.5
20	0.2	20	0.20	17	0.25	18.4
30	0.3	28	0.34	26	0.38	33.5
40	0.4	37	0.45	35	0.50	38.1
50	0.5	47	0.56	43	0.63	50.5
60	0.6	56	0.67	52	0.75	50.9
80	0.8	75	0.90	69	1.00	80.2
100	1.0	93	1.10	87	1.30	100.0
200	2.0	190	2.20	170	2.50	125.7

Table 3. *Effects of Mg^{2+}, $MgATP^{2-}$, $HATP^{3-}$ and ATP^{4-} on adenylate cyclase activity*
The concentrations of the ATP species were calculated as described in the text. Adenylate cyclase was measured in a partially purified membrane fraction from bone as described (Rodan *et al.*, 1980). All samples contained 100 μM-EGTA and 1 μM-Ca^{2+}. Apparent K_i value for ATP^{4-} calculated from the intersection of consecutive $1/v$ versus $[ATP^{4-}]$ lines gave inconsistent values: 7 μM, 2 μM and −4 μM. Kinetic analysis was consistent with Mg^{2+} stimulation with a K_m of 1.4 mM. Range of duplicate measurements is given in parentheses.

$[Mg^{2+}]$ (mM)	$[MgATP^{2-}]$ (μM)	$[HATP^{3-}]$ (nM)	$[ATP^{4-}]$ (μM)	v (pmol/min per mg of protein)
0.1	100	4700	56.0	21.0 (3.2)
1.0	100	470	5.6	99.9 (5.0)
5.0	100	93	1.1	126.0 (1.6)
10.0	100	47	0.56	176.0 (6.0)
0.1	300	14000	170.0	33.5 (1.8)
1.0	300	1400	17.0	216.0 (8.1)
5.0	300	280	3.4	401.0 (34.4)
10.0	300	140	1.7	471.0 (26.5)
0.1	600	28000	340.0	33.6 (0.1)
1.0	600	2800	34.0	207.0 (21.6)
5.0	600	560	6.7	461.0 (60.7)
10.0	600	280	3.4	526.0 (50.3)
0.1	1000	47000	560.0	171.0 (4.0)
1.0	1000	4700	56.0	199.0 (2.4)
5.0	1000	930	11.0	392.0 (82.0)
10.0	1000	470	5.6	422.0 (26.0)

sets of conditions. Computation of kinetic parameters was done by numerical curve fitting as described (Rodan *et al.*, 1980).

Results

Effects of EGTA, ATP^{4-} and $HATP^{3-}$ on enzyme activity

It was now possible to examine independently the effects of changes in substrate ($MgATP^{2-}$) or metal (Mg^{2+}, Ca^{2+}) concentrations on enzyme activity and re-evaluate the role of ATP^{4-} and $HATP^{3-}$. EGTA as such had no effect on enzyme activity when calcium and magnesium concentrations were adjusted to keep Ca^{2+} and Mg^{2+} constant. When the pH and the free-metal concentrations are kept constant, the changes in all ATP species are directly proportional to the changes in $MgATP^{2-}$ (Table 2). Under these conditions the enzyme velocity increased hyperbolically with the rise in $MgATP^{2-}$ concentration between 10 and 200 μM, notwithstanding the parallel increase in all other ATP species. None of the other ATP species exceeded 1.1% of the $MgATP^{2-}$ concentration. For the expression of inhibitory effects the affinity of any of those species would have to be over 100-fold larger than that of $MgATP^{2-}$ and the concentration ratios would have to exceed those in Table 2. The ratio between the various ATP species can be altered by changes in the concentration of bivalent metals and the stimulatory effects of Mg^{2+} had been attributed to this effect (deHaen, 1974). Table 3 presents an experiment in which this hypo-

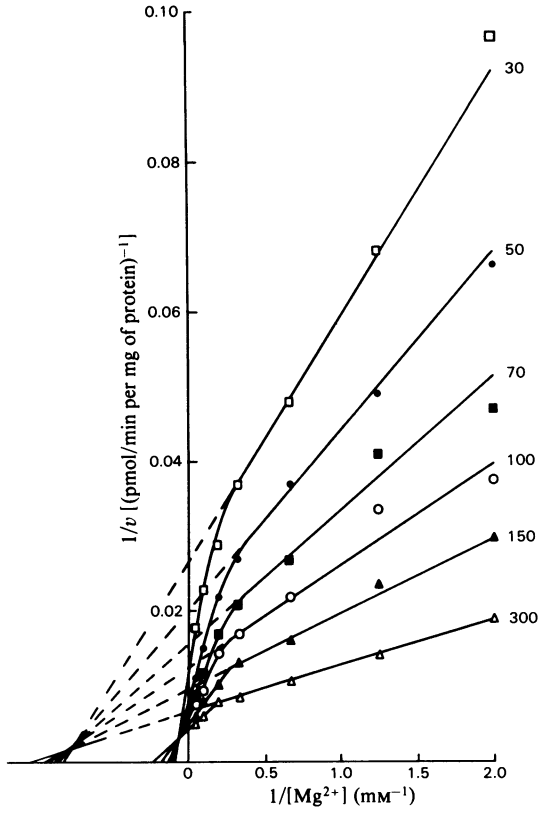

Fig. 1. *Bone adenylate cyclase activity as a function of Mg^{2+} concentration*

Adenylate cyclase activity was assayed on 5 μg of bone-membrane protein in the presence of the indicated concentrations of $MgATP^{2-}$ (μM) and Mg^{2+}, for 5 min as described in the Methods section. The EDTA concentration in the assay mixture was 40 μM, and I was adjusted to 0.1 with NaCl. The values represent means of duplicate measurements.

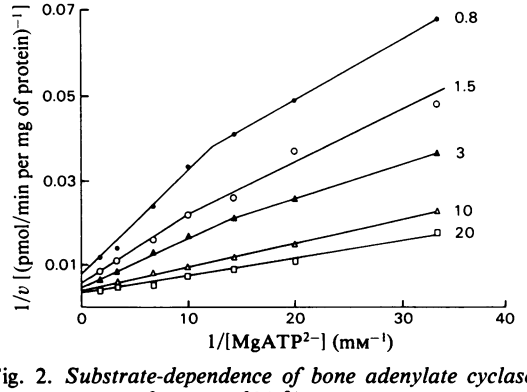

Fig. 2. *Substrate-dependence of bone adenylate cyclase as a function of Mg^{2+} concentration*

Data from Fig. 1 were used for these double-reciprocal plots of the indicated Mg^{2+} concentrations (mM).

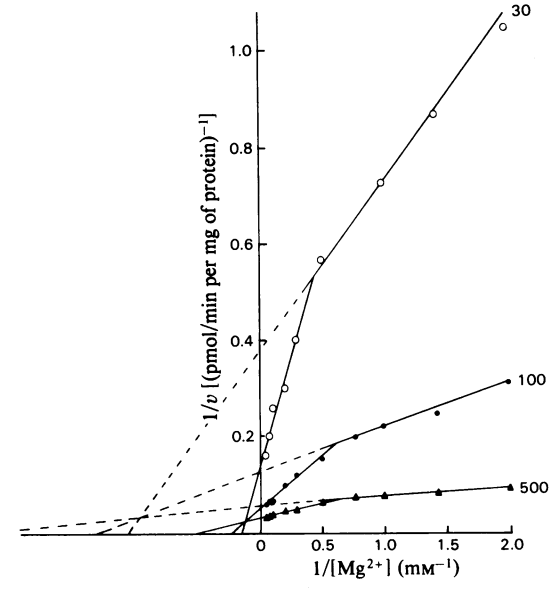

Fig. 3. *Osteosarcoma adenylate cyclase activity as a function of Mg^{2+} concentration*

Adenylate cyclase activity was assayed on 7 μg of osteosarcoma-membrane protein in the presence of the indicated concentrations of $MgATP^{2+}$ (μM) and Mg^{2+} for 3 min as described in the Methods section. The EDTA concentration in the assay mixture was 40 μM, and I was adjusted to 0.1 with NaCl. The values represent the means of triplicate determinations.

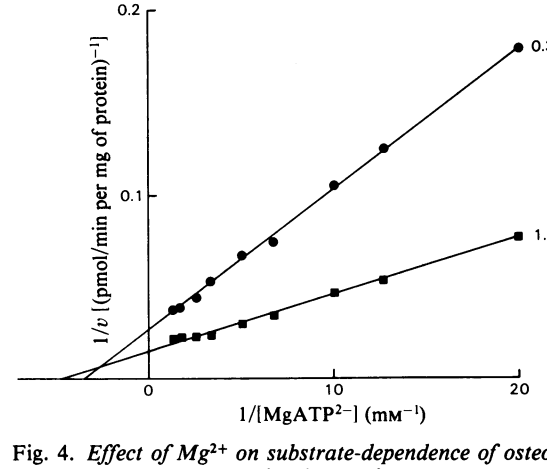

Fig. 4. *Effect of Mg^{2+} on substrate-dependence of osteosarcoma adenylate cyclase*

Adenylate cyclase activity was assayed on 12.5 μg of osteosarcoma-membrane protein in the presence of the indicated concentrations of $MgATP^{2-}$ and Mg^{2+} (mM) for 3 min as described in the Methods section. The concentration of p[NH]ppG was 10 μM, EDTA was 40 μM and I was adjusted to 0.1 with NaCl. The values represent the means of triplicate determinations.

thesis was tested by following the effect of a hundredfold increase in Mg^{2+} concentration on enzyme velocity at different $MgATP^{2-}$ concentrations. The changes in enzyme activity could not be attributed by kinetic analysis to $HATP^{3-}$ or ATP^{4-} inhibition, Dixon plots intercepting on the right of the y-axis or K_i values varying in a haphazard fashion. On the other hand plots of $1/v$ against $1/[Mg^{2+}]$ were consistent with a direct Mg^{2+} effect and are considered in detail below.

Effects of Mg^{2+} on enzyme activity

Fig. 1 presents a double-reciprocal plot of enzyme velocity as a function of Mg^{2+} concentration at various $MgATP^{2-}$ concentrations. All lines bend downwards (concave towards abscissa), which is consistent with either negative co-operativity or more than one class of Mg^{2+} sites. If one considers the linear portions of the Lineweaver–Burk plots separately, the kinetic findings are consistent with two Mg^{2+} sites with K_m values of 1.3 and 16.6 mM respectively. In either case, the lines for the various $MgATP^{2-}$ concentrations meet on the left of the ordinate, above the abscissa, a situation defined as mixed non-competitive enzyme activation (Wong, 1975). This relationship to substrate should be mutual. The double-reciprocal plots of velocity against $MgATP^{2-}$ concentration at various Mg^{2+} concentrations also meet on the left of the ordinate above the abscissa and exhibit downward-concavity (Fig. 2). Interestingly, Mg^{2+} linearized substrate-dependence, eliminating an apparent second lower-affinity site (or negative co-operativity). Above 10 mM-Mg^{2+}, v versus s was hyperbolic, with a K_s of about 0.15 mM.

The osteosarcoma enzyme exhibited, qualitatively, the same behaviour. Two apparent affinities for Mg^{2+} could be detected with K_m values of 1.0 and 7.7 mM (Fig. 3). The mixed non-competitive effect of Mg^{2+} relative to $MgATP^{2-}$ is shown in Fig. 4. The guanine nucleotide p[NH]ppG, which was present in this experiment, linearized the v versus s function, as well as v versus $[Mg^{2+}]$ as shown below.

Effect of guanine nucleotide on Mg^{2+} affinity

It has been previously reported that guanine nucleotides linearize adenylate cyclase dependence on substrate concentration. Since the v versus s function was dependent on Mg^{2+}, we examined the effect of guanine nucleotides on Mg^{2+} stimulation of adenylate cyclase. Fig. 5 presents a Lineweaver–Burk plot of enzyme velocity as a function of Mg^{2+} concentration in the absence and presence of p[NH]-ppG. In bone the low-affinity Mg^{2+} site virtually disappeared in the presence of guanine nucleotide, two sites now having very close K_m values of 0.7 and 0.3 mM (by curve fitting), at saturating p[NH]ppG concentrations. In osteosarcoma the effect of

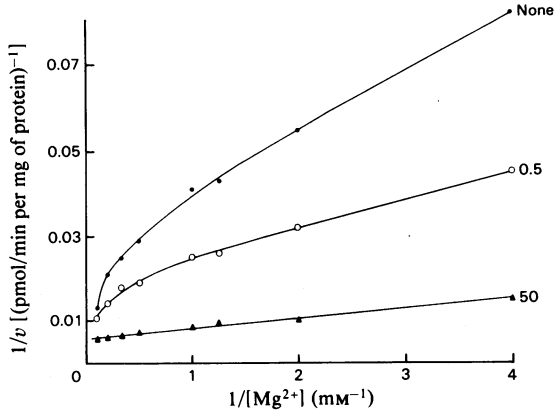

Fig. 5. *Effect of p[NH]ppG on Mg^{2+}-dependence of bone adenylate cyclase*

Adenylate cyclase activity was assayed on 5 μg of bone-membrane protein in the presence of the indicated concentrations of p[NH]ppG (μM) and Mg^{2+} for 5 min as described in the Methods section. The $MgATP^{2-}$ concentration in the assay mixture was 100 μM, EDTA was 40 μM and I was adjusted to 0.1 with NaCl. The values represent the means of triplicate determinations.

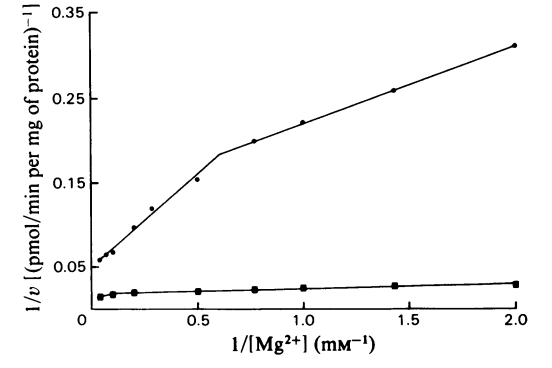

Fig. 6. *Effect of p[NH]ppG on Mg^{2+}-dependence of osteosarcoma membranes*

Adenylate cyclase activity was assayed on 7 μg of osteosarcoma-membrane protein with (■, 50 μM) or without (●) p[NH]ppG in the presence of the indicated concentrations of Mg^{2+} for 5 min as described in the Methods section. The $MgATP^{2+}$ concentration in the assay mixture was 100 μM, EDTA was 40 μM and I was adjusted to 0.1 with NaCl. The values represent the means of triplicate determinations.

p[NH]ppG was similar, but less dramatic (Fig. 6). The lower-affinity K_m shifted from about 8 mM to 2.2 mM, which is clearly distinguishable from the site with a K_m value of 0.26 mM at saturating p[NH]-ppG concentrations. Thus, in the presence of p[NH]-

ppG, Mg^{2+} above 2mM exhibited a significantly lower apparent affinity for adenylate cyclase stimulation in osteosarcoma than in bone. This may be another manifestation of the differences in the coupling of the guanine nucleotide effect in the tumour.

Effects of Ca^{2+} on adenylate cyclase

Calcium and cyclic AMP are jointly implicated in cell-membrane-mediated communication, and Ca^{2+} was found to inhibit most adenylate cyclase preparations. It was therefore of particular interest to examine this effect quantitatively in bone and tumour. Fig. 7 presents a Dixon plot of Ca^{2+} inhibition of adenylate cyclase at various $MgATP^{2-}$ concentrations. Kinetically the calcium inhibitory pattern is consistent with uncompetitive inhibition, i.e. decrease in v proportional to an increase in K_s. This pattern can result from the production of an abortive complex after the binding of Ca^{2+} to the enzyme–substrate complex at a separate site.

The same pattern was observed in osteosarcoma, as shown in Fig. 8. Since Mg^{2+} was found to be an obligatory 'non-competitive' activator of adenylate cyclase, it was possible that Ca^{2+} acted as a competitive inhibitor of Mg^{2+}. As illustrated in Figs. 9 and 10, this was indeed the case. The kinetic find-

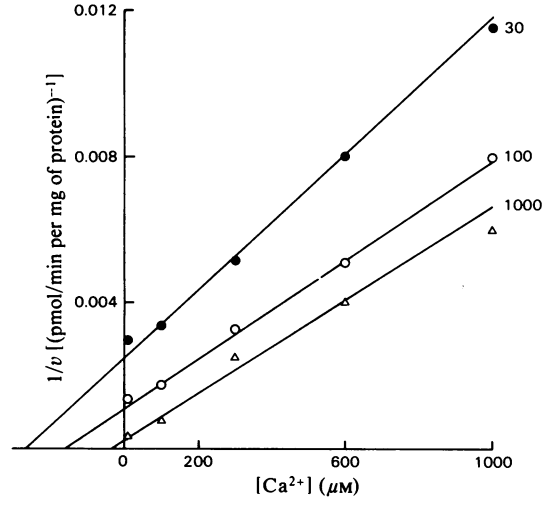

Fig. 7. *Effect of Ca^{2+} on bone adenylate cyclase at various $MgATP^{2-}$ concentrations*

Adenylate cyclase activity was assayed on 7μg of bone-membrane protein in the presence of the indicated concentrations of $MgATP^{2-}$ (μM) and Ca^{2+} for 5min as described in the Methods section. EDTA and p[NH]ppG concentrations were 100μM each, Mg^{2+} was 5mM and I was adjusted to 0.1 with NaCl. Values represent the means of duplicate measurements.

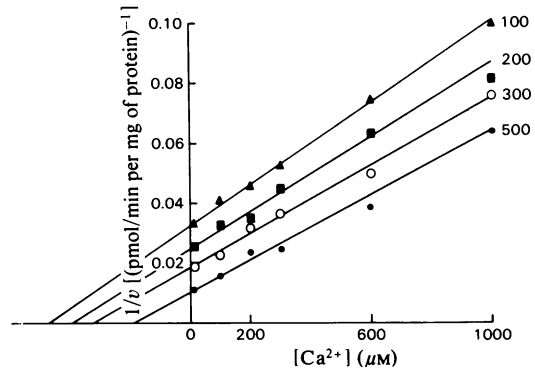

Fig. 8. *Effect of Ca^{2+} on osteosarcoma adenylate cyclase at various $MgATP^{2-}$ concentrations*

Adenylate cyclase activity was assayed on 10μg of osteosarcoma-membrane protein in the presence of the indicated concentration of $MgATP^{2-}$ (μM) and Ca^{2+} for 5min as described in the Methods section. The Mg^{2+} concentration was 5mM, EGTA and p[NH]ppG were 100μM each, and I was adjusted to 0.1 with NaCl. Values represent means of duplicate measurements.

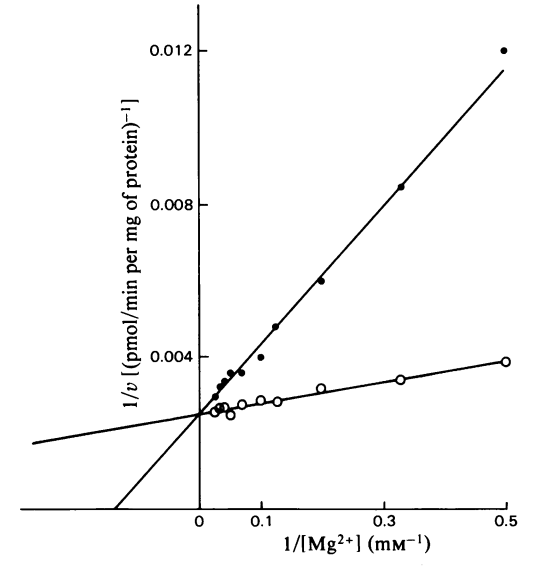

Fig. 9. *Effect of Ca^{2+} on bone adenylate cyclase at various Mg^{2+} concentrations*

Adenylate cyclase activity was assayed on 7.3μg of bone-membrane protein with (●) or without (○) 200μM-Ca^{2+} in the presence of the indicated concentrations of Mg^{2+} for 11min as described in the Methods section. The $MgATP^{2-}$ concentration in the assay mixture was 500μM, p[NH]ppG was 10μM, EGTA was 100μM and I was adjusted to 0.1 with NaCl. Values represent means of duplicate measurements.

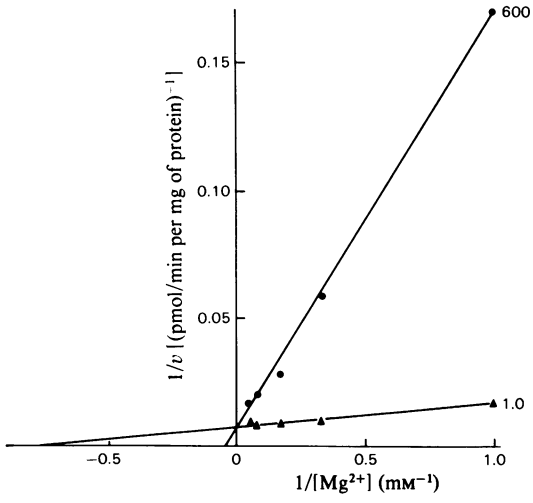

Fig. 10. *Effect of Ca^{2+} on osteosarcoma adenylate cyclase activity at various Mg^{2+} concentrations*
Adenylate cyclase activity was assayed on 15 μg of osteosarcoma-membrane protein in the presence of the indicated concentration of Mg^{2+} and Ca^{2+} (μM). The $MgATP^{2-}$ concentration was 300 μM, p[NH]ppG was 10 μM, EGTA was 100 μM and I was adjusted to 0.1 with NaCl. Values represent the means of duplicate determinations.

ings are consistent with Ca^{2+} competing for the Mg^{2+} site(s), with an apparent affinity of about 25 and 40 μM (K_i) in bone and tumour respectively. Under conditions in which the high-affinity Mg^{2+} site was dominant (in the presence of the guanine nucleotide or over 200 μM-$MgATP^{2-}$) the Ca^{2+}-dependence was hyperbolic.

Discussion

Regulation by cations seems to be a general feature of membrane-associated ATP phosphohydrolases, as illustrated by $(Na^+ + K^+)$-stimulated ATPases and Ca^{2+}-stimulated ATPases.

Adenylate cyclase, the 'messenger'-generating ATP phosphohydrolase, was reported to be sensitive to Mg^{2+} and Ca^{2+}, with which it seems to share the function of membrane-mediated cellular communication. It was therefore decided to examine in detail the effect of these cations on enzyme activity, and compare their regulatory influence quantitatively in bone and tumour.

A precise definition of the concentration of these cations in reaction mixtures containing ATP and EGTA (or EDTA) by solving the multiple equilibria equations has been attempted previously in studies of other enzymes (Storer & Cornish-Bowden, 1976) and investigations of muscle and nerve function (Godt, 1974; Donaldson & Kerrik, 1975). This approach assumes that all ions in solution are at equilibrium and that the association constants used for computation are accurate. The equilibrium assumption is most probably valid. The constants were selected from the literature among reported values with consideration for conditions and methods of determination. Errors in the estimates of the constants would introduce a systematic quantitative error, but would not invalidate general conclusions. Our findings are consistent with the accepted evidence that $MgATP^{2-}$ is the substrate of adenylate cyclase. However, we found no support for the hypothesis that other ATP species in equilibrium with $MgATP^{2-}$ inhibit the enzyme activity (deHaen, 1974; Rendell *et al.*, 1975). These findings confirm the conclusions of Garbers & Johnson (1975) and Londos & Preston (1977).

With the precise definition of the Mg^{2+} concentration and the ability to vary it experimentally over a continuous range, it became possible to examine in detail the effects of Mg^{2+}. Kinetic analysis is consistent with Mg^{2+} enhancement of catalytic activity via two metal sites, distinct from its participation in the formation of $MgATP^{2-}$, which was previously recognized to serve as substrate (Sutherland *et al.*, 1962; Robison *et al.*, 1968; Drummond & Duncan, 1970; Pohl *et al.*, 1971). In the absence of guanine nucleotides, the respective apparent affinities at the two sites were higher than 10 and 1.3 mM in bone and 8 and 1 mM in tumour.

Mg^{2+} caused an increase primarily in enzyme velocity, but it also increased substrate affinity, acting as a 'mixed non-competitive' activator (Wong, 1975). These kinetic findings are consistent with Mg^{2+} being a requisite activator of the enzyme without specifying binding sequence (Birnbaumer *et al.*, 1969; Drummond & Duncan, 1970; Johnson *et al.*, 1979).

At Mg^{2+} concentrations sufficient to saturate the high-affinity site, the velocity versus substrate function became hyperbolic, whereas below that concentration double-reciprocal plots of velocity versus substrate were non-linear, suggesting at least two interacting substrate sites. This effect of Mg^{2+} on $MgATP^{2-}$ was reciprocal, as expected in a mixed non-competitive mechanism. Thus, at $MgATP^{2-}$ concentrations approaching saturation, the velocity versus $[Mg^{2+}]$ function became hyperbolic (one site or non-interacting sites), and the Mg^{2+} apparent affinity was that of the high-affinity site. A 'linearizing' effect on the Mg^{2+}-dependence was also produced by p[NH]ppG, in the presence of which the high-affinity site was again dominant. This effect was more pronounced in bone than in tumour. Similar effects of p[NH]ppG on Mg^{2+} affinity had also been observed in liver (Londos & Preston, 1977) and in cardiac sarcolemma (Narayanan & Sulakhe, 1977; Alvarez & Bruno, 1977). Interpretation of the

'conditional' kinetic manifestation of more than one site for guanine nucleotide (Pfeuffer & Helmreich, 1975; Lad *et al.*, 1977; Welton *et al.*, 1977), substrate (Stolc, 1977) and Mg^{2+} (Steer & Levitzki, 1975; Londos & Preston, 1977; Birnbaumer *et al.*, 1969) is not clear. It seems to indicate that interaction between adenylate cyclase complexes, including the regulatory subunits, is diminished when the enzyme activity increases. The quantitative difference between bone and tumour, on the effect of p[NH]ppG on 'linearization' (one-site behaviour) of the Mg^{2+}-dependence is probably related to the difference in p[NH]ppG enhancement of $V_{max.}$ and again implicates the membrane environment. This phenomenon may reflect different degrees of interaction between enzyme complexes and subunits in the membrane at different states of enzyme activity, as illustrated by the studies of Schlegel *et al.* (1979) and Limbird *et al.* (1979).

Ca^{2+} inhibition is a general property of adenylate cyclases (Perkins, 1973), but few studies have dealt with the mechanism of this effect, which may be of physiological importance (Rasmussen *et al.*, 1975). The reported K_i values measured in the presence of Mg^{2+} concentrations of a few millimolar are approx. 0.5 mM. Birnbaumer *et al.* (1969) found a K_i of 2 mM in fat-cell membrane at 2.5 mM-Mg^{2+}, shifting to 0.75 mM in the presence of corticotropin, which increases Mg^{2+} affinity (Glynn *et al.*, 1979). Severson *et al.* (1972) found a K_i of 0.5 mM in skeletal muscle at 10 mM-Mg^{2+} (approx. $4 \times K_m$). Blume & Foster (1976) found that $CaCl_2$ inhibited neuroblastoma-cell adenylate cyclase non-competitively with respect to ATP or 2-chloroadenosine, with a K_i of 0.5 mM and a Hill coefficient of 2. Mn^{2+} at 8 mM (approx. $8 \times K_m$) competed successfully against the inhibition of 2 mM-Ca^{2+} (approx. $4 \times K_i$), but Mg^{2+} at 6 mM (approx. $3 \times K_m$) did not. Blume & Foster (1976) concluded that Mn^{2+} and Ca^{2+} act at a common site different from that of Mg^{2+}. Several of their findings, however, are not explained by this assumption: the Mg^{2+}-like effect of Mn^{2+} on the affinity for 2-chloroadenosine and the interference of Ca^{2+} with the interaction between Mg^{2+} and chloroadenosine. Steer & Levitzki (1975) and Hanski *et al.* (1977) studied the inhibitory effect of Ca^{2+} on turkey erythrocyte adenylate cyclase and also concluded that it acted on a different site from Mg^{2+} or $MgATP^{2-}$, and exhibited positive co-operativity. Stolc (1977) found that, for polymorphonuclear-leucocytes adenylate cyclase, Ca^{2+} decreased $V_{max.}$ non-competitively relative to substrate and allosteric effectors, with a K_i of 0.4 mM and a Hill coefficient of 1. Ca^{2+} at 1 mM, however, decreased the Hill coefficient for Mg^{2+} from 1.9 to 1.1. These reports, albeit on different tissues, are non-contradictory if one considers the possibility of functional transitions between interacting and non-interacting Mg^{2+} sites, and Mg^{2+} effects on substrate affinity and competition between Ca^{2+} and Mg^{2+}, as suggested by this study.

We found that in the presence of Mg^{2+} concentrations sufficient to maintain hyperbolic substrate-dependence, Ca^{2+} inhibition was approximately uncompetitive relative to $MgATP^{2-}$ and purely competitive relative to Mg^{2+}. This would mean that the enzyme would not bind Ca^{2+} and Mg^{2+} at the same time and that, unlike Mg^{2+}, Ca^{2+} binding occurred only after $MgATP^{2-}$, to yield a non-catalytic (ESI) complex. The metal-binding sequence is analogous to that in sarcoplasmic-reticulum ATPase (Racker, 1976; Tada *et al.*, 1978) with the important difference that in the latter Ca^{2+} enhances the phosphohydrolase activity.

The K_i values for Ca^{2+}, computed on the assumption of competition with Mg^{2+}, were 25 and 40 μM for bone and osteosarcoma respectively, which parallels the lower Mg^{2+} affinity in the tumour.

The physiological implications of Ca^{2+} inhibition would depend on the orientation and accessibility of the Ca^{2+} site. If it faced the cytoplasm, it would hardly be occupied, if one assumes an intracellular Ca^{2+} concentration of approx. 1 μM. If it faced the extracellular fluid, it would obviously be saturated. This situation offers interesting regulatory possibilities for an enzyme that mediates communication across the active extracellular/cytoplasmic interface. For example, membrane rearrangements after hormone binding can lead to Ca^{2+} inhibition of the activated enzyme, resulting in cyclic AMP spikes (or oscillations), whose effect may differ from that of a steady-state elevation.

The quantitative differences between bone and osteosarcoma adenylate cyclase affinities for bivalent cations could generate altered cyclic AMP responses as part of the membrane-related features pertaining to the malignant state.

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