Properties of a Factor in Cytosol that Enhances Hormone-Stimulated Adenylate Cyclase Activity

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1. The addition of 50 000 g cytosol preparations of isolated human platelets, cultured rat osteogenic sarcoma or cultured bone cells to particulate preparations of adenylate cyclase, from the same or unrelated tissues, caused marked enhancement of the hormone-stimulated enzyme activities. 2. The degree of enhancement obtained by addition of the cytosol preparations was similar to that observed on addition of GTP. 3. The enhancing activity of the three cytosol types was found to be sensitive to digestion by trypsin and alkaline phosphatase, partially heat-labile and partially inactivated by exposure to charcoal. 4. Gel filtration studies indicated an apparent molecular weight of 20 000–30 000. Further, the 20 000–30 000-mol.wt. fractions obtained by gel filtration could enhance the adenylate cyclase activity of particulate preparations derived from unrelated cell types. 5. The results suggest a common or similar adenylate-cyclase-enhancing factor or factors, protein in nature, present in the three cytosol types.

In the preceding paper (MacNeil *et al.*, 1980) we established that the supernatant (cytosol) obtained by centrifugation at 50 000 g of various isolated or cultured cell-types and whole tissues contained a factor, or factors, that could activate the adenylate cyclase [EC 4.6.11; ATP pyrophosphate-lyase (cyclizing)] of crude membrane preparations. Further, the pattern of response of adenylate cyclase to the cytosolic factor(s) was very similar to the response to GTP, particularly in its marked synergistic effect upon the hormone-stimulated activity. The aim of this study was to elucidate the nature of the cytosolic factor(s) in cytosols derived from isolated human platelets and from cultured rat osteogenic sarcoma and normal bone-derived cells, and in particular to establish whether such a factor(s) is or is not free GTP in the cytosol.

Materials and Methods

Chemicals

[8-³H]GTP (14 Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. The following were obtained from Boehringer: disodium salts of ATP and GTP, cyclic AMP (free acid). Bovine serum albumin was obtained from Armour Pharmaceutical Co., Eastbourne, U.K. Soya-bean trypsin inhibitor (1 mg inhibited 10 000

units of trypsin), trypsin (EC 3.4.21.4, from bovine pancreas, 10 000 units/mg), alkaline phosphatase (EC 3.13.1, from calf intestinal mucosa, immobilized on agarose beads, 7600 units/g) and 3-isobutyl-1-methylxanthine were obtained from Sigma. Norit GSX charcoal was obtained from Norit-Clydesdale Co., Glasgow, Scotland, U.K., and Dextran T70 from Pharmacia. Prostaglandin E₁ was a gift from Dr. John Pike of the Upjohn Co., Kalamazoo, MI, U.S.A.

Particulate preparations

Particulate preparations of adenylate cyclase were prepared from the following cell types according to the methods given in the previous paper (MacNeil *et al.*, 1980): cultured rat osteogenic sarcoma cells; cultured bone cells derived from rat calvaria; isolated human platelets.

Cytosol preparations

50 000 g cytosol preparations were prepared according to the method described in the previous paper (MacNeil *et al.*, 1980).

Adenylate cyclase assay

The assay carried out was based on that of Salomon *et al.* (1974). Details are given in the previous paper (MacNeil *et al.*, 1980).

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Treatment of cytosol preparations

Dialysis. Cytosols (500 μ l) were dialysed over a 24 h period against three changes, each of 250 ml, of 0.25 M-sucrose/15 mM-Tris/HCl/1 mM-MgSO₄, pH 7.4 at 4°C.

Heat. Cytosols (volume 1 ml) were heated to 95°C for 10 min. Denatured material was removed by centrifugation at 50 000 g for 10 min.

Trypsin. MgSO₄ was added to cytosols to give a final concentration of 5 mM and the pH was adjusted with 1 M-Tris base to give a pH of 7.4 at 37°C. Samples of sucrose buffer and 10⁻⁵ M GTP (in sucrose buffer) were treated in the same manner. Samples (900 μ l) of cytosol, buffer or GTP were incubated for 20 min at 37°C with 100 μ l of trypsin (final concentration 1 mg/ml). The reaction was terminated by addition of excess soya-bean trypsin inhibitor. Control incubations at 37°C with GTP or cytosol *per se* were performed, at the end of which excess soya-bean trypsin inhibitor and trypsin were added.

Alkaline phosphatase. MgSO₄ was added to cytosol preparations to give a final concentration of 5 mM and the pH was adjusted with 1 M-Tris base to give a pH of 10.0 at 37°C. Samples of sucrose buffer and 10⁻⁵ M-GTP (in sucrose buffer) were treated identically. Cytosol, GTP solution or buffer (900 μ l) were incubated at 37°C for 30 min with 100 μ l (0.76 units) of alkaline phosphatase immobilized on agarose beads. The reaction was terminated by centrifugation (1300 g for 10 min, r_{av} 22 cm) and removal of the supernatant. Control incubations at 37°C with GTP or cytosol *per se* were carried out, at the end of which alkaline phosphatase was added and the samples were centrifuged immediately.

Charcoal. A mixture of 1 g of Norit GSX charcoal, 100 mg of bovine serum albumin and 100 mg of Dextran T70 was prepared in 10 ml of sucrose buffer at 4°C. Portions (375 μ l) of the charcoal suspension were centrifuged at 50 000 g for 10 min (r_{av} 11.02 cm). The resultant supernatants were removed and 1 ml samples of cytosol or GTP were added to the charcoal pellets and mixed thoroughly. The samples were then immediately recentrifuged in the same manner to separate the charcoal from the treated samples. The treated cytosol or GTP supernatants were then removed.

Gel filtration over Sephadex G-75. Preparations (5 ml) of cytosol were fractionated using a column of Sephadex G-75 (84 cm $\times$ 2 cm) equilibrated with 150 mM-ammonium acetate, pH 7.4 at 4°C, containing 0.02% (w/v) sodium azide. Fractions (2 ml volume) were collected. Column fractions were dialysed overnight against 50 mM-ammonium acetate, pH 7.8 at 4°C, lyophilized and redissolved in 1 ml of sucrose buffer.

Efficiency of ATP regeneration

Adenylate cyclase assays were performed as described above. After termination (by heating only) samples of incubation products were spotted in 10 μ l together with authentic carrier nucleotides (ATP, ADP or AMP) at a concentration of 3×10^{-3} M on polyethyleneimine cellulose plates. After ascending chromatography in 0.75 M-LiCl in 1 M-acetic acid (Randerath & Randerath, 1964) the plates were dried, the marker nucleotides visualized under ultraviolet light and the appropriate areas were cut out and counted for radioactivity in a liquid-scintillation counter.

Expression of results

Results are expressed as the means $\pm$ S.E.M. for three determinations. The results given are of individual experiments representative of data obtained on a number of occasions. The formulae for the comparison of means of small samples with unknown variances not assumed to be equal was used. The probability of statistical difference between two means was then determined from Students *t*-tables.

Results

The ability of 50 000 g cytosol preparations to enhance the hormone-stimulated adenylate cyclase was neither tissue- nor species-specific, i.e. the activity of particular preparations could be enhanced by cytosols derived from the same or unrelated tissues (Fig. 1). Such results raise the possibility of a common adenylate-cyclase-activating factor or factors in the various cytosols. One such common component of the cytosol preparations is GTP, which is well-documented in many systems *in vitro* to have a synergistic effect upon hormone-stimulated adenylate cyclase activities. The GTP concentrations of the cytosol preparations were not determined by experiment. However, theoretical estimations (MacNeil *et al.*, 1980) suggested that the adenylate-cyclase-enhancing activity could not be explained only by the presence of free GTP in the cytosol preparations. Therefore, the behaviour of the cytosol factor or factors was compared with that of GTP by the following experimental procedures. Dialysis of cytosol preparations (0.8–1.8 mg of protein/ml) derived from osteogenic sarcoma and bone cells caused a 20–30% reduction in the enhancing capability. No changes in enhancing activity occurred during dialysis of platelet-derived cytosol preparations. However, under the same conditions, 98% of [8-³H]GTP was removed from a solution of 10⁻⁵ M-GTP containing 1.5 mg bovine serum albumin/ml to estimate non-specific binding to protein. Heating to 95°C decreased the enhancing activity of all three cytosol preparations. The degree

Fig. 1. Enhancement of hormone-stimulated adenylate cyclase of particulate preparations by unrelated cytosol preparations

(a) Rat osteogenic-sarcoma particulate preparation; (b) rat renal-cortex particulate preparation. Additions were: control (no addition); A, rat osteogenic-sarcoma cytosol; B, rat renal-tubule cytosol; C, human-platelet cytosol; D, rat hepatocyte cytosol; 10⁻⁵M-GTP. Incubations with rat-renal cortex particulate preparations contained 1 × 10⁻⁶M-parathyroid hormone while those with rat osteogenic sarcoma particulate preparations contained 5 × 10⁻⁵M-prostaglandin E₁. A typical experiment is shown; results are means ± S.E.M. for three determinations of activity.

to which the enhancing activity was decreased was variable between experiments but within the range 20–60% for each cytosol type. GTP, however, was stable under these conditions. The enhancing activity of all three cytosol types was sensitive to trypsin digestion (Table 1), leading to the conclusion that protein is an essential component of the activating factor or factors.

Exposure of cytosols derived from osteogenic sarcoma and bone cells to Norit GSX charcoal reduced the enhancing activity of both cytosol preparations (Table 2). The effect of charcoal upon the platelet-derived cytosols was not determined. Initial exposure to charcoal resulted in a 20–40% decrease in the enhancing activity of both cytosols. Sequential charcoal treatments under the conditions described could cause further removal of adenylate cyclase enhancing activity (Table 2) but total removal could not be achieved. A single exposure to charcoal of 10⁻⁵M-GTP resulted in total removal of its enhancement of the prostaglandin E₁-stimulated adenylate cyclase of osteogenic sarcoma. When osteogenic-sarcoma-cytosol preparations were subjected to combined treatments of heat followed by exposure to charcoal, 90–100% of enhancing activity was removed.

The adenylate-cyclase-enhancing activity of all three cytosol types was sensitive to alkaline phosphatase digestion. Exposure under the conditions described caused a total abolition of enhancing activity in platelet- and sarcoma-derived cytosols and approximately 50% decrease in cytosol preparations obtained from bone-derived cells (Table 3). The stimulatory effect of 10⁻⁵M-GTP upon the hormone-activated adenylate cyclase was reduced 50–100% by alkaline phosphatase. To ascertain whether the removal of cytosolic enhancing activity by alkaline phosphatase was due only to degradation of free cytosol GTP, the effect of the enzyme was determined on osteogenic sarcoma cytosol preparations that had been first subjected to sequen-

Table 1. Effect of trypsin upon the ability of cytosol preparations to enhance prostaglandin-E₁-stimulated adenylate cyclase

The method of trypsin digestion of cytosol preparations is given in the Materials and Methods section. Cytosol protein concentrations; osteogenic sarcoma, 1.6 mg/ml; bone, 1.5 mg/ml; platelet, 1.2 mg/ml. All incubations contained prostaglandin E₁ at concentrations of: osteogenic sarcoma and bone incubations, 5 × 10⁻⁵M; platelet incubations 10⁻⁵M. *, Treated cytosols that are significantly different (P < 0.01) from controls.

Cytosols	Tissue ...	Adenylate cyclase activity (pmol of cyclic AMP · min ⁻¹ · mg of particulate protein ⁻¹)		
		Osteogenic sarcoma	Bone	Platelet
None		51 ± 3	79 ± 6	96 ± 3
Control		439 ± 21	296 ± 11	181 ± 5
Trypsin-treated		240 ± 11*	193 ± 6*	147 ± 5*

Table 2. *Effect of (a) exposure to charcoal and (b) combined exposures to heat and charcoal upon the ability of cytosol preparations to enhance prostaglandin-E₁-stimulated adenylate cyclase*

Details of treatment are given in the Materials and Methods section. Cytosol protein concentrations: bone, 1.5 mg/ml; osteogenic sarcoma, (a) 2.1 mg/ml, (b) 1.7 mg/ml. All incubations contained 5×10^{-5} M prostaglandin E₁. (a) The first and second exposures to charcoal caused a significant decrease in enhancing activity ($P < 0.02$). Further exposures gave no significant decrease in enhancing activity. (b) Heat, charcoal exposure and combined heat followed by exposure to charcoal caused a significant decrease in enhancing activity ($P < 0.001$) when compared to the control cytosol.

Additions	Tissue ...	Adenylate cyclase activity (pmol of cyclic AMP · min ⁻¹ · mg of particulate protein ⁻¹)	
		Osteogenic sarcoma	Bone
(a)			
None		71 ± 5	80 ± 3
Control cytosol		339 ± 12	327 ± 18
Cytosol (1 × charcoal-treated)		275 ± 5	192 ± 1
Cytosol (2 × charcoal-treated)		218 ± 11	
Cytosol (3 × charcoal-treated)		216 ± 11	
10 ⁻⁵ M-GTP		223 ± 7	
10 ⁻⁵ M-GTP (1 × charcoal-treated)		70 ± 7	
(b)			
None		107 ± 4	
Control cytosol		545 ± 3	
Cytosol (1 × charcoal-treated)		382 ± 10	
Cytosol (heat-treated)		400 ± 3	
Cytosol (heat treated then 1 × charcoal-treated)		127 ± 2	

Table 3. *Effect of alkaline phosphatase upon the ability of cytosol preparations to enhance prostaglandin-E₁-stimulated adenylate cyclase*

Details of treatment are given in the Materials and Methods section. (a) Illustrates the effect of alkaline phosphatase upon untreated cytosols and (b) the effect upon an osteogenic sarcoma cytosol preparation that was first exposed twice to charcoal. Cytosol protein concentrations: osteogenic sarcoma, (a) 3.1 mg/ml, bone, 1.2 mg/ml; platelet, 0.9 mg/ml; osteogenic sarcoma (b), 1.0 mg/ml. All incubations contained prostaglandin E₁ at concentrations of: osteogenic sarcoma and bone incubations 5×10^{-5} M; platelet incubations, 10^{-5} M. *, Treated cytosols that are significantly different ($P < 0.01$) from controls.

Additions	Tissue ...	Adenylate cyclase activity (pmol of cyclic AMP · min ⁻¹ · mg of particulate protein ⁻¹)		
		Osteogenic sarcoma	Bone	Platelet
(a)				
None		58 ± 3	105 ± 4	274 ± 5
Control cytosol		110 ± 1	225 ± 10	409 ± 18
Phosphatase-treated cytosol		66 ± 2*	153 ± 4*	271 ± 18*
(b)				
None		22 ± 3		
Charcoal-treated cytosol		99 ± 6		
(Charcoal + phosphatase)-treated cytosol		18 ± 2*		
10 ⁻⁵ M-GTP		150 ± 2		
10 ⁻⁵ M-GTP treated with phosphatase		47 ± 1*		

tial exposures to charcoal (2–4 exposures) as described in the Materials and Methods section. Table 3 shows that the charcoal-treated cytosol preparations still retained sensitivity to alkaline phosphatase.

Fractionation of platelet and osteogenic-sarcoma cytosol preparations on columns of Sephadex G-75 yielded peaks of enhancing activity corresponding to a mol.wt. of 20 000–30 000 in both cases (Fig. 2). Further, the 20 000–30 000-mol.wt. Sephadex G-75

Fig. 2. Elution profiles obtained after fractionation of (a) platelet- and (b) osteogenic-sarcoma-cytosol preparations on Sephadex G-75

Details of fractionation given in the Materials and Methods section. Adenylate cyclase incubations were carried out in the presence of prostaglandin E_1 : 10^{-5} M in platelet incubations and 5×10^{-5} M in osteogenic sarcoma incubations. Bars, adenylate-cyclase-stimulating activity; ---, adenylate cyclase activity in presence of buffer only; ●, A_{280} . Values for stimulating activity are means $\pm$ S.E.M. for three determinations.

Table 4. Enhancement of prostaglandin- E_1 -stimulated adenylate cyclase of human-platelet particulate preparations by the 20000–30000 mol.wt. Sephadex G-75 fraction of rat osteogenic sarcoma

Sephadex G-75 fractions of cytosol (20000–30000 mol.wt.) were prepared as described in the Materials and Methods section. Prostaglandin E_1 (10^{-5} M) was present in all the incubations below. The fractions from platelet cytosol and osteogenic sarcoma cytosol contained 250 and 690 μ g of protein/ml respectively.

Additions	Adenylate cyclase activity (pmol of cyclic AMP · min ⁻¹ · mg particulate of protein ⁻¹)
None	555 $\pm$ 40
Sephadex G-75 fraction of platelet-cytosol preparation	1490 $\pm$ 27
Sephadex G-75 fraction of osteogenic-sarcoma-cytosol preparation	1430 $\pm$ 18

fraction of osteogenic sarcoma was found to enhance the prostaglandin E_1 response of platelet particulate preparations (Table 4).

The cytosolic enhancing factor(s) appeared to be independent of Ca^{2+} ions. Enhancement of prostaglandin- E_1 -stimulated adenylate cyclase activity occurred in the presence of 1mM-EDTA and 1mM-EGTA. The effect of the cytosolic enhancing factor or factors on phosphodiesterase activity and the ATP regenerating system used in adenylate cyclase incubations was investigated. This was

carried out to determine whether the enhancement of hormone-stimulated adenylate cyclase by cytosol preparations was due to an inhibition of phosphodiesterase activity or to an increase in ATP levels available to the enzyme rather than by a direct interaction with adenylate cyclase. The effect of the cytosolic preparations on phosphodiesterase activity was assayed indirectly by the following procedure. All adenylate cyclase incubations contained 1mM-cyclic AMP to prevent degradation of the cyclic [32 P]ATP formed during the incubation.

However, inclusion of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (400 or 800 μM) in addition to 1 mM-cyclic AMP did not result in any change in the magnitude of the enhancement of the prostaglandin- E_1 -stimulated adenylate cyclase by cytosol preparations. To determine whether cytosol preparations enhanced adenylate cyclase activity by increasing the concentration of ATP available to the enzyme, the following procedure was used. Adenylate cyclase incubations using osteogenic sarcoma preparations were carried out in the presence of prostaglandin E_1 with and without osteogenic cytosol preparations and the 20000–30000-mol.wt. Sephadex G-75 fraction. After termination, adenine nucleotides were measured as described in the Materials and Methods section. This method gave levels of $84 \pm 3\%$, $66 \pm 5\%$, and $86 \pm 3\%$ of ATP remaining at the end of incubations respectively without cytosol, with cytosol and with the 20000–30000-mol.wt. Sephadex fraction (results are the means $\pm$ S.E.M. of three determinations). Therefore the cytosolic factor or factors did not increase the amount of ATP available to adenylate cyclase.

Discussion

Most studies have utilized purified or semi-purified plasma membrane preparations to investigate the activation of adenylate cyclase by hormones and the control of this process by the guanine nucleotides, GTP and its analogue 5'-guanylyl imidodiphosphate. Little interest has been focused on the possibility that cytoplasmic factors may play a role in the control of adenylate cyclase. However, there are an increasing number of reports observing effects of cytosolic factors on adenylate cyclase. A heat-stable protease-sensitive stimulatory factor has been reported in liver (Pecker & Hanoune, 1977; Doberska & Martin, 1978); similar heat-labile factors have been reported in liver (Katz *et al.*, 1978) and cardiac muscle (Sanders *et al.*, 1977). Cytoplasmic factors have also been implicated in the activation of adenylate cyclase by cholera toxin (Gill & King, 1975).

We have established the presence of a protease- and heat-sensitive factor or factors in cytosols derived from three cell types that has a marked synergistic effect on the hormone-stimulated adenylate cyclase of particulate preparations. This stimulation of adenylate cyclase by the cytosolic factor or factors is probably the result of direct interaction with the enzyme since the factor did not have any apparent effects on phosphodiesterase activity, nor did the factor or factors change the amount of ATP available to the enzyme.

We have previously reported (Crawford *et al.*, 1979) that the enhancing activity in cytosol pre-

parations of platelets and osteogenic sarcoma cells was retained by an ultrafiltration membrane (Diaflo XM 50) with a nominal molecular weight cut-off of 50000. However, fractionation of the cytosols over Sephadex G-75 gave a mol.wt. of 20000–30000 for the cytosolic enhancing activity. The anomalous results obtained using the ultrafiltration technique may be due to formation of aggregates of the enhancing factor during ultrafiltration. We have shown that the cytosolic enhancing factor is neither tissue- nor species-specific, in that particulate preparations of osteogenic sarcoma and rat renal cortex could be stimulated by cytosols from unrelated tissue and further the 20000–30000 mol.wt. Sephadex G-75 fraction of rat osteogenic sarcoma could enhance the prostaglandin- E_1 -stimulated adenylate cyclase of human platelets. These results give some support to the suggestion raised earlier of a common adenylate-cyclase-enhancing factor in the different cytosol types.

The cytosolic adenylate cyclase enhancing factor appears also to be dependent upon the presence of phosphate groups, as indicated by the sensitivity of the cytosolic enhancing activity to alkaline phosphatase. In this respect the cytosolic factor appears similar to that described by Pecker & Hanoune (1977). However, preliminary studies indicate the cytosolic enhancing factor to be resistant to the action of phospholipases A_2 and C, indicating that there is no involvement of phospholipids. Such phosphate groups could be postulated to exist as phosphorylated amino acid residues in the protein structure or as tightly bound protein-nucleotide complexes.

The factor that we have described does not appear to be simply free GTP because, unlike free GTP, it is heat-labile and does not totally pass through a dialysis membrane. Moreover, significant enhancing activity remained in the cytosol after charcoal treatment, which would have totally removed free GTP. In addition, the factor was partially susceptible to treatment with trypsin and furthermore, gel filtration studies indicate a mol.wt. of 20000–30000 (mol.wt. of GTP is 520). The factor or factors could consist of two components, one of which is GTP, the other a protein to which GTP is bound. Speigel *et al.* (1979) have in fact reported the isolation of a guanine-nucleotide-protein complex (G regulatory protein) from turkey-erythrocyte membranes. This protein is able to restore the adenylate cyclase activity of certain adenylate-cyclase-defective cell lines in a non-tissue-specific manner. The mol.wt. of the G regulatory protein is approx. 50000 whereas gel filtration studies indicated the cytosolic factor has a mol.wt. of 20000–30000. However until the molecular weight of the factor is accurately defined the possibility remains that the cytosolic factor we have described

may be a G regulatory protein similar to that described by Speigel *et al.*

The factor does not appear to be dependent on Ca^{2+} for its enhancing activity and therefore is unlikely to be the calcium-dependent regulator protein (CDR or calmodulin) that is known to modulate various biochemical events including stimulation of adenylate cyclase activity.

It is conceivable that the factor is not truly cytoplasmic in origin, but is loosely bound to the inner aspect of the cell plasma membrane and eluted from it during preparation of plasma membrane fractions. The physiological significance of the factor remains to be established, but it is not unreasonable to speculate that it may have an important role in coupling the receptor and catalytic components of hormone-responsive adenylate cyclase systems.

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