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ADENYLATE CYCLASE ENHANCING FACTOR FROM RAT OSTEOSARCOMA CYTOSOL

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SUMMARY

A cytosol factor from a transplantable rat osteosarcoma stimulates the adenylate cyclase (ATP pyrophosphate-lyase (cyclizing) EC 4.6.1.1) activity of partially purified membranes 1.5 fold at pH 7.6 and over 5 fold at pH 6.5. This effect can also be seen at maximum Gpp(NH)p stimulation of the enzyme. The cytosol factor is non-dialyzable, ultrafiltrable through a 100,000 dalton exclusion membrane, heat labile and trypsin digestible. The stimulation is immediate, is independent of Ca^{2+}, exhibits sygmoidal concentration dependency and is enhanced by GTP. The factor did not bind GTP. The stimulatory activity was fully recovered in two Sephadex G-100 fractions of approximate molecular weights of 55,000 and 29,500. Unlike the starting material the fractions were not stable to freeze-thawing or lyophilization. A similar factor could not be found in embryonic bone, nor did the osteosarcoma factor affect bone adenylate cyclase.

INTRODUCTION

During partial purification of plasma membranes from a transplantable rat osteosarcoma we observed a loss in adenylate cyclase activity which could not be recovered from any other fraction. This observation was consistent with the existence of an adenylate cyclase stimulating factor separated from the membrane during purification. Such factors have been previously described in brain cytosol (1, 2), liver cytosol (3) and in the erythrocyte cell sap, where it was shown to be related to cholera toxin stimulation of the enzyme (4, 5).

We have identified in rat osteosarcoma cytosol a similar factor which differs in its properties from those previously described.

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MATERIALS

Phosphocreatine, creatine phosphokinase, Tris-HCl,magnesium acetate, Dithiothreitol (DTT), cAMP, ATP, imidazole, alumina (chromatographic resin), $MgCl_2$, sucrose and Norit A were obtained from Sigma Chemical Co., St. Louis, Mo; Gpp(NH)p from ICN Pharmaceuticals; Ammonium acetate from J.T. Baker Chemical Co., Phillipsburg, N.J.; EGTA from Eastman Kodak Co., Rochester N.Y.; α^{32}P-ATP, ^{3}H-cAMP, ^{3}H-GTP and Brays Solution from New England Nuclear, Boston, Mass.; ACS from Amersham Searle; Dowex cation exchange resin (AG-50W-X4) from Bio-Rad Laboratory, Richmond, California; Sephadex chromatographic resin (G-100 Superfine) and column from Pharmacia, Inc., New Jersey; Trypsin (0.125%) from GIBCO; Soybean Trypsin Inhibitor from Worthington Biochemical Corp., Freehold, N.H.; Diaflo ultrafiltration membranes from Amicon Corp., Lexington, Mass.; Dialysis tubing from VWR' and Chymotrypsinogen (A-CGC), Ovalbumin, Bovine Albumin and Myoglobin from Mann Research Laboratories, Dickinson and Co., New York, N.Y.

METHODS

Membrane Purification -

A transplantable rat osteosarcoma R3359/52A was obtained courtesy of Dr. W.F. Dunning, Papanicolau Cancer Research Institute, Miami, Florida and was maintained by subcutaneous implantation in ACI rats. At 18-30 days the 3-4 cm diameter tumor was excised, dissected free of vascularized membrane, chopped in buffer (50mM Tris HCl pH 7.6 at 30°C, 1 mM DTT, 0.2 mM Mg) at 4° and was disrupted in a Virtis "23" tissue mill at 23,000 rpm for 45 seconds. The tissue was then homogenized for 20 seconds in a volume/mass ratio of 10 ml buffer/gm weight tissue at 4°C in a Potter-Elvehjem homogenizer with a teflon pestle, driven at 3,000 rpm. The homogenate was filtered through cheesecloth and centrifuged for 1 minute at 12,000 x g followed by centrifugation of the supernatant at 47,000 x g. The pellet was further separated on a discontinuous (refractometer adjusted) sucrose gradient and the material floating on 1.155 density, which showed a ten fold enrichment in a 5'-nucleotidase activity, was the plasma membrane used. Aliquots frozen in liquid nitrogen and stored in buffer at -80°C retained full adenylate cyclase activity for 30-45 days.

For purification of embryonic bone membrane approximately 120 (19-20 day) rat, embryonic calvaria were used as starting material and the same procedure was followed. The membrane fraction was collected off 1.165 density sucrose.

Adenylate Cyclase Assay -

Adenylate cyclase was assayed by the method of Salomon et al (6) on 5-15 mg protein in the presence of 0.2 mM ATP, 6.2 U/100 µl phosphocreatine kinase, 5 mM phosphocreatin, 25 mM Tris-HCl pH 7.6 or Tris Sodium dihydrogen phosphate for pH 6.5, 5 mM Mg acetate, 1 mM DTT, 1 mM cAMP and about 2 x 10^6 cpm α^{32}P-ATP. Modifications of this composition are mentioned in legends to tables. Under the conditions used the reactions were always linear with time and protein concentration.

Separation and Treatments of Cytosol Factor

The 47,000 x g supernatant (see above) was centrifuged for one hour at 100,000 x g to yield a microsome-free supernatant (SUP). SUP was filtered through a Diaflo XM-100A membrane under 15 psi N_2 pressure at 4°C to yield an ultrafiltrate (SUPU). SUPU was lyophilized for 24 hours to yield LSUPU which was reconstituted in 1 ml buffer (20 mM Ammonium acetate, 1 mM DTT,

TABLE 1. The Effect of Osteosarcoma 100,000 x g Supernatant (SUP) on Adenylate Cyclase Activity

Experimental Conditions	pmole/mg protein/min.
Osteosarcoma Membrane (OM) pH 7.6	49.7 ± 0.9 (4,2 exps)
OM + 90 µg SUP, pH 7.6	69.9 ± 2.2 (4,2 exps)
OM pH 6.5	1.25 ± 0.04 (24,6 exps)
OM + 90 µg SUP, pH 6.5	8.70 ± 0.36 (24,6 exps)
OM + 10 µM Gpp(NH)p, pH 6.5	8.01 ± 0.51 (24,6 exps)
OM + 90 µg SUP + 10 µM Gpp(NH)p, pH 6.5	37.20 ± 0.68 (24,6 exps)
Bone Membrane (BM), pH 7.6	332.0 ± 6.5 (3,1 exp)
BM + 28 µg SUP, pH 7.6	335.3 ±12.47 (3,1 exp)
BM pH 6.5	99.5 ± 2.5 (3,1 exp)
BM + 45 µg bone supernatant pH 6.5	99.7 ± 0.4 (3,1 exp)

Osteosarcoma and bone membranes and supernatants were prepared and adenylate cyclase was assayed as described in Methods, on 15 µg OM and 9 µg BM per 100 µl. Data are means ± SEM of the number of determinations and experiments, given in parentheses. BM was assayed in the presence of 10 µM Gpp(NH)p, as well as OM at pH 7.6.

0.2 mM $MgCl_2$) and dialyzed against 500 ml buffer for 2 changes (24 hours each) before assaying or gel filtration. Gel chromatography was carried out on a Sephadex G-100 column (0.9 cm I.D. x 54 cm length) with 20 mM Ammonium acetate, 1 mM DTT and 0.2 mM $MgCl_2$, and collected in 1.2 ml fractions.

Heat treatment of SUPU was carried out at 60°C in polypropylene tubes for 30 min in a controlled temperature heating block.

For trypsinization, 400 µl (1.12 mg protein) supernatant were incubated with 100 µl 0.125% trypsin at 37°C for 15 min, after which 275 µg soybean trypsin inhibitor (Worthington) was added. The mixture was vortexed, incubated at 30°C for 1 min and placed in an ice bath until time of assay. The "trypsinization buffer" used for control was processed identically. The effect of trypsin treated SUP (56µg protein) was measured at pH 6.5 for 30 min in the presence of 10 µM Gpp(NH)p.

RESULTS

As shown in Table 1 addition of SUP to osteosarcoma membrane (OM) enhanced the adenylate cyclase activity by about 50%. To amplify this phenomenon we measured the effect of SUP at pH 6.5 where enzymatic activity is substantially reduced, probably as a result of conformational changes. As seen, SUP increased the enzymatic activity about five to seven fold under these conditions.

TABLE 2. Preliminary Characterization of Adenylate Cyclase Enhancing Factor

Treatments/Experimental Conditions	pmole/mg protein/min.
I. Osteosarcoma Membrane (OM), 15 µg protein	8.37 ± 0.44 (4)
OM + 56µg SUP	23.75 ± 0.25 (4)
OM + trypsinized SUP	9.64 ± 0.33 (4)
OM + trypsin buffer	9.49 ± 0.37 (4)
OM + dialysis retentate	21.46 ± 0.16 (4)
OM + 56 µg SUPU, heated 30 min, 60°C	10.83 ± 0.24 (4)
OM + 0.0056 µg SUP ultrafiltrate (SUPU)	9.36 ± 0.67 (4)
OM + 0.056 µg SUPU	8.74 ± 0.25 (4)
OM + 0.56 µg SUPU	9.67 ± 0.59 (4)
OM + 5.6 µg SUPU	21.58 ± 0.77 (4)
OM + 11.2 µg SUPU	27.31 ± 0.25 (4)
OM + 33.6 µg SUPU	28.54 ± 0.01 (4)
OM + 56 µg SUPU	29.12 ± 0.44 (4)
II. Osteosarcoma Membrane (OM), 20 µg protein	11.88 ± 0.37 (4)
OM + 60 µg lyophilized SUPU	33.18 ± 0.025(4)
OM + 4 µg fresh F55 (V_e/V_o = 1.88)	132.18 ± 0.82 (4)
OM + 4 µg freeze thawed F55 (V_e/V_o = 1.88)	25.6 ± 1.66 (4)
OM + 4 µg freeze thawed F30 (V_e/V_o = 3.0)	21.83 ± 0.53 (4)

Osteosarcoma plasma membranes and supernatants were prepared and adenylate cyclase was assayed as described in Methods on 15 µg OM in (I) and 20 µg OM in (II) in the presence of 10 µM Gpp(NH)p at pH 6.5. Data are means ± SEM of the number of samples given in parentheses from two independent experiments.

SUP also enhanced the Gpp(NH)p-stimulated adenylate cyclase activity, suggesting that the effect was not due to GTP or a GTP binding or regenerating activity Neither osteosarcoma SUP nor bone supernatant had any effect on embryonic bone membrane adenylate cyclase.

Using OM adenylate cyclase stimulation at pH 6.5 as an assay, we proceeded to further characterize SUP. As shown in Table 2, SUP was non-dialyzable, trypsin digestible, heat labile, ultrafiltratable through a 100,000 dalton filter and lyophilizable. The stimulatory effect of lyophilized SUP ultrafiltrate (LSUPU) exhibited sigmoidal concentration dependency and leveled off at approximately 12 µg protein.

Chromatography of LSUPU on a G-100 Sephadex column yielded two active peaks at V_e/V_o = 1.88 and V_e/V_o = 3.0. Calibration of the column with myoglobin M.W. 17,200, chymotrypsinogen (A-CGC) M.W. 25,700, ovalbumin M.W. 43,000 and bovine albumin M.W. 67,000 gave a linear relationship for V_e/V_o vs log M.W., on the basis of which the estimated molecular weights of the active fractions were 55,000 (F55) and 29,500 (F30) respectively.

Fresh F55 had a higher "specific activity" than SUP: 4 µg caused a ten fold stimulation of adenylate cyclase activity, and a concentration dependency curve obtained by serial dilution suggested that the effect of 4 µg was below saturation. Attempts to further concentrate F55 revealed that the stimulatory activity was lost by lyophilization and was reduced by freezing (or thawing) as shown in Table 2.

The effect of LSUPU was not Ca^{2+} dependent since it retained full activity in the presence of 0.2 mM EGTA (Table 3). The adenylate cyclase stimulating factor from liver cytosol was shown by Pecker and Hanoune (3) to increase its activity in the presence of GTP. As seen in Table 3, 10 µM GTP alone did not enhance adenylate cyclase activity but in the presence of Norit-A treated LSUPU it produced a 60% stimulation of the enzyme. However, GTP at 10^{-5}M showed absolutely no binding to LSUPU, as shown in Table 4 (the limit of detection of this procedure was 1 nmole GTP/mg protein). According to our preliminary findings the osteosarcoma factor could be similar to that described by Pecker and Hanoune (3).

DISCUSSION

A few cellular macromolecules have recently been shown to be among the numerous modulators of adenylate cyclase activity: the calcium dependent regulator, CDR, (7, 8, 9, 10), the liver cytosol factor (3), the GTP binding protein (11) and the cholera toxin related guanosine triphosphatase (4, 5). The cytosol factor reported here belongs to this category, and like the factors listed above appears to be a protein. Although the MW estimate is preliminary and a monomer-dimer relationship between the 55,000 and the 29,500

TABLE 3. The Effect of GTP and EGTA on the Stimulation of Adenylate Cyclase by Cytosol Factor

Experimental Conditions	pmole/mg protein/min
Osteosarcoma Membrane (OM)	1.33 ± 0.05
OM + 10 μM GTP	1.35 ± 0.06
OM + 56 μg lyophilized supernatant ultrafiltrate (LSUPU)	7.20 ± 0.11
OM + 56 μg Norit A treated LSUPU (NLSUPU)	6.32 ± 2.2
OM + 56 μg NLSUPU + 1 μM GTP	7.68 ± 0.91
OM + 56 μg NLSUPU + 10 μM GTP	10.75 ± 0.68
OM + 10 μM Gpp(NH)p	11.54 ± 1.06
OM + 10 μM Gpp(NH)p + 0.2 mM EGTA	14.08 ± 0.76
OM + 10 μM Gpp(NH)p + 83 μg LSUPU	56.83 ± 2.27
OM + 10 μM Gpp(NH)p + 83 μg LSUPU + 0.2 mM EGTA	70.65 ± 1.63

Osteosarcoma plasma membranes and supernatant were prepared and adenylate cyclase was assayed as described in Methods on 15 μg OM protein at pH 6.5. Data are means ± SEM of triplicate samples.

dalton fractions could exist, the osteosarcoma factor is probably larger than the CDR and certainly smaller than the GTP binding protein (11). Functionally it is clearly distinct from these factors since it is calcium independent and does not bind GTP. Nonetheless, it enhances the effect of GTP on the enzyme, probably as a result of independent interaction. This effect resembles that of catecholamines in other systems (4).

The lack of a time lag in the action of the osteosarcoma factor, the linear time curve in its presence and the concentration dependency of its effect suggest that this factor is not an enzyme. Assuming a turnover number of 1000 sec^{-1} for adenylate cyclase and a MW of 55,000 for the factor, the latter would be one thousand fold in excess of adenylate cyclase at minimum activating concentration.

The physiological role of this factor is not known. Several possibilities can be envisaged. It may be involved in the hormone stimulation of adenylate cyclase, or in the kinetics of cAMP generation as suggested by the strong cooperativity of the factor dose response curve, or in the cellular

TABLE 4. Equilibrium Dialysis of Norit A Treated Lyophilized Osteosarcoma Supernatant (NLSUPU) Against ^{3}H-GTP

10^{-6} x cpm ^{3}H-GTP/ml				
	0.3 hrs	9 hrs	15 hrs	24 hrs
Dialysate (10 μM GTP)	3.78	3.43	3.43	3.17
1 mg NLSUPU	2.03	2.61	2.97	3.21
4 mg NLSUPU	1.87	2.68	2.90	3.14

Equilibrium dialysis was carried out at 4°C in 2 chamber cells across a dialysis membrane. ^{3}H-GTP was injected at 0 hrs into the larger cell and 20 μl samples were removed for liquid scintillation counting at the indicated times. Results are means of duplicate samples from one of two identical experiments.

control of the membrane adenylate cyclase activity (12). With regard to the latter possibility its presence in osteosarcoma,which like other malignant tissues has low adenylate cyclase activity, may be of significance.

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