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Activation of an S6 kinase from rat astroglial cells by cAMP

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Forskolin and isoproterenol, agonists of adenylate cyclase activity, and dibutyryl cyclic AMP, stimulated an S6 kinase activity in astroglial cells. This activity was insensitive to the thermostable inhibitor of cyclic AMP-dependent protein kinase and had the same behaviour on a DEAE-Sephacel column as the mitogen stimulated S6 kinase. These observations support the idea that the cyclic AMP cascade, as well as various growth factors, can activate S6 kinase.

Astrocyte; cyclic AMP; Mitogen stimulation; Protein S6 kinase; Enzyme activation

1. INTRODUCTION

The small ribosomal subunit protein, S6, is the major ribosomal protein phosphorylated in vivo [1]. S6 is rapidly phosphorylated on several serine residues in cells stimulated by a variety of growth factors or TPA and in cells transformed by oncogenes coding for tyrosine kinases. In recent years it has been shown that this phosphorylation is probably catalyzed by specific protein kinases, the S6 kinases. These enzymes are activated in 3T3 rat cells by EGF [2], in Swiss 3T3 cells by FGF [3], in 3T3L1 cells by insulin and TPA [4], in chicken primary fibroblasts by TPA or by cell transformation with retrovirus carrying the sarc oncogene [5,6], in *Xenopus* oocytes by insulin [7], in primary astroglial cells by insulin, IGF1 and TPA [8], and in PC12 cells by NGF [9]. Except for FGF and NGF, for which the mechanism of action is less well documented, it is generally believed that the first activation step is the stimulation of protein kinase C or a tyrosine kinase. The requirement for protein phosphatase inhibitors to maintain S6 kinase activation also suggests that activated forms of these enzymes are phosphorylated [10].

As cyclic AMP (cAMP) is the activator of a class of protein kinases and as many workers have reported that it modulates cell growth [11,12] we have examined its possible role in the activation pathway of S6 kinases using agents known to increase the level of intracellular cAMP, forskolin and isoproterenol, or the cAMP derivative, dibutyryl cAMP. These substances were used on astroglial cells in the presence or absence of insulin which increases cell multiplication [13], S6 kinase activity [8] and, in the presence of thyroid hormone, affects cell morphology [14].

The present results show that the treatment of these cells with cAMP promoting agents enhanced the activity of an S6 kinase resembling the mitogen-stimulated kinase.

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2. MATERIALS AND METHODS

2.1. *Materials*

Pure porcine insulin was obtained from Novo, forskolin, isoproterenol and *N,O*-dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP) were purchased from Sigma.

2.2. *Cell culture and cell extraction*

Astroglial cells were obtained from the cerebral hemispheres of 2-day-old Sprague Dawley rats, grown to confluence (about

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14 days) in DMEM (Dulbecco's modified Eagle's medium) containing 10% fetal calf serum and then transferred into chemically defined medium as described [8]. 48 h prior to the experiment, the culture medium was deprived of insulin. The test reagents were added for various times, the culture medium was removed and the cells were rapidly rinsed with 3 ml/dish of 80 mM β-glycerophosphate, pH 7.4, 20 mM EGTA and 15 mM $MgCl_2$ (buffer A). All subsequent steps were performed at 4°C. The cells were scraped off into 0.3 ml/dish of buffer A containing 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml antipain, 1 μg/ml trypsin inhibitor, 1 mM benzamidine and 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, broken in this buffer by sonication for 3 s and centrifuged at 100000 × *g* for 1 h. The resulting supernatants were stored at −80°C.

2.3. *Protein kinase assays*

S6 kinase activity was measured by adding aliquots of the supernatants (~10 μg protein) to a solution (final volume = 50 μl) containing 20 mM Hepes, pH 7.1, 3 mM $MgCl_2$, 2 mM dithiothreitol, 10–25 μg 40 S ribosomal protein or histone H2b (1 mg/ml) and 50 μM [γ-^{32}P]ATP (~1–2 μCi/nmol). 40 S ribosomal subunits were isolated from rat liver according to Martin and Wool [15]. All subsequent steps were as described in [8].

2.4. *Chromatography on DEAE-Sephacel*

The supernatants of untreated and 45 min treated cells by dbcAMP, forskolin or isoproterenol were dialyzed for 2 h against 30 mM β-glycerophosphate, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride. They were then treated for 30 min with 5 μM cAMP and applied to the DEAE-Sephacel column (3 ml) equilibrated with 20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EGTA, 2 mM dithiothreitol (buffer B) containing 25 mM NaCl. The columns were washed with 15 ml of this buffer and proteins were eluted with a 60 ml linear gradient from 25 to 350 ml NaCl in buffer B. Fractions (1 ml) were collected and S6 kinase activity was measured in each fraction.

3. RESULTS AND DISCUSSION

The astroglial cells were maintained in chemically defined medium as described in section 2.

In the first experiment, the cells were treated for various times with 10^{-3} M dbcAMP, 10^{-5} M forskolin or 10^{-6} M isoproterenol in the presence or absence of insulin. Fig.1 shows the in vitro ^{32}P-incorporation into S6 catalyzed by the cytosols of these cells. In the absence of insulin, dbcAMP, forskolin and isoproterenol promoted a time-dependent increase in S6 phosphorylation. The greatest effect was obtained with dbcAMP, which increased intracellular cAMP content more than forskolin and isoproterenol [16]. Both these latter agents produced a similar effect on cAMP level.

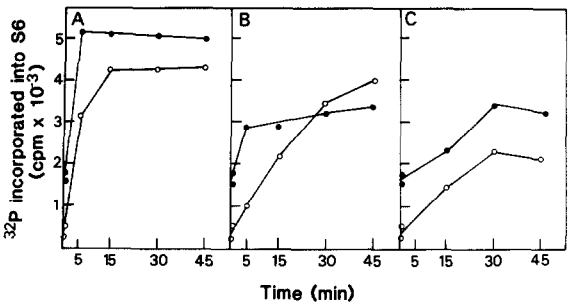

Fig.1. Time course of the ^{32}P incorporation into S6 by cytosols of cells treated with (A) dbcAMP (10^{-3} M), (B) forskolin (10^{-5} M) and (C) isoproterenol (10^{-6} M) in the absence (○—○) or presence (●—●) of insulin (10^{-6} M). The three agents were added to the culture medium at 5, 15, 30 and 45 min prior to the end of the experiments; insulin was present throughout the 45 min.

The presence of insulin enhanced the effects of dbcAMP and isoproterenol on S6 phosphorylation, whereas it exerted a biphasic effect when added with forskolin. A near maximal stimulation was reached at 5 min, but at 45 min there was no increase over the effect of forskolin alone, suggesting a complex relationship between the transduction mechanisms of forskolin and insulin. This observation may be compared to the results obtained by Stadtmauer and Rosen [17] who observed that forskolin treatment reduced the activity of the insulin receptor-tyrosine kinase in IM9 cells. These authors considered that this effect of forskolin was due to an increase in intracellular cAMP. However, this is unlikely to be so for S6 kinase in astroglial cells, since dbcAMP and isoproterenol, which also increase the cAMP content, act additively in the presence of insulin.

The increased ^{32}P-incorporation into S6 described here, could be due either to the cAMP-dependent protein kinase (cAMP-PK), to the mitogen-stimulated S6 kinase or to another unidentified, S6 kinase.

The following experiments were carried out to distinguish between these possibilities.

First, S6 phosphorylation was performed in the presence or absence of the protein inhibitor of cAMP-PK, with cytosols from cells treated for 45 min, with dbcAMP, forskolin or isoproterenol, with or without insulin. Fig.2A shows that the ^{32}P incorporation into S6 with cytosols from untreated, forskolin- and isoproterenol-treated cells

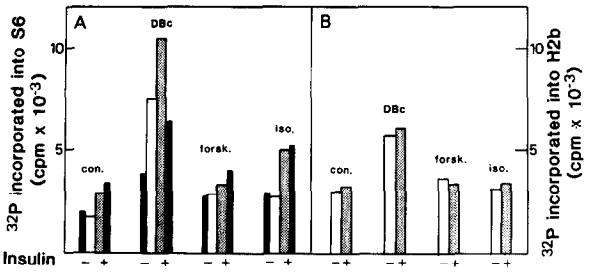

Fig.2. Cyclic AMP-dependent protein kinase activity in the supernatants of cells treated for 45 min with dbcAMP (10^{-3} M), forskolin (10^{-5} M) and isoproterenol (10^{-6} M) in the presence or absence of insulin (10^{-6} M). ^{32}P incorporation into S6 (A) and histone H2b (1 mg/ml) (B) was determined without addition of cAMP. (■) Presence or (□ and ▨) absence of thermostable cAMP-PK inhibitor (to inhibit 15 units of cAMP-PK).

was not affected by the presence of the inhibitor. This indicates that activity measured with these extracts is that of an S6 kinase, which is different from cAMP-PK. In contrast, ^{32}P incorporation into S6 by cytosol from dbcAMP-treated cells was partly (30–50%) blocked by a maximal effective concentration of the cAMP-PK inhibitor, indicating that the activity measured in the cytosol from dbcAMP-treated cells was due, in our test conditions, to both cAMP-PK and S6 kinase. The difference between the effects of cytosols from dbcAMP-treated cells and those from forskolin- or isoproterenol-treated cells might be due to the very high level of cAMP derivative that might be concentrated enough in the test tube to keep the cAMP-PK in its dissociated form. These results were confirmed by assaying in the absence of inhibitor and of added cAMP, protein kinase activities of the different cytosols on histone H2b, which is a good phosphate acceptor for cAMP-PK. The ^{32}P incorporation into this protein by cytosol of forskolin- and isoproterenol-treated cells was similar to that obtained with control extract (fig.2B), showing the absence of free catalytic subunit of cAMP-PK. As expected, only ^{32}P incorporation catalyzed by the cytosol from dbcAMP-treated cells was significantly increased indicating the presence of free catalytic subunit. Fig.2A confirms the additivity of the insulin and the isoproterenol effects and the absence of additivity of the insulin and the forskolin effects. Fig.2B shows the absence of effect of insulin on cAMP-PK activity, measured on its specific substrate histone H2b.

Secondly, to verify that increases in ^{32}P incorporation into S6 promoted by dbcAMP, forskolin and isoproterenol were effectively due to the activation of the mitogen-stimulated S6 kinase, cells deprived of insulin 48 h prior to the experiment were challenged for 45 min with each of the three agents. The cytosols from control and treated cells were chromatographed on DEAE-Sephacel columns. The S6 kinase activities obtained (fig.3) had the same behaviour as the mitogen-stimulated S6 kinase (submitted). This enzyme was retained on the column and was eluted at 0.15 M with a linear

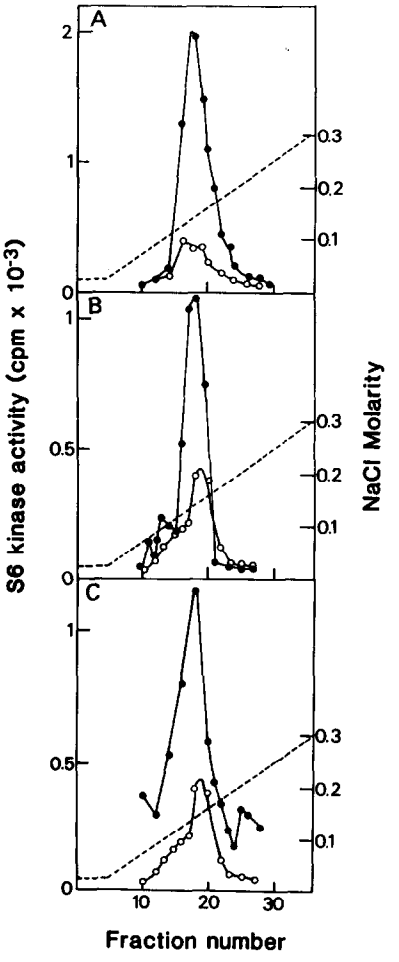

Fig.3. Chromatography on DEAE-Sephacel of S6 kinase activity unstimulated (○—○) and stimulated (●—●) by treatment of cells with (A) dbcAMP (10^{-3} M), (B) forskolin (10^{-5} M) and (C) isoproterenol (10^{-6} M).

gradient of NaCl. The amount of S6 kinase activity eluted at 0.15 M NaCl was low, though variable, in control extracts and higher in the other extracts. cAMP-PK was dissociated before chromatography to avoid binding of its catalytic subunit to the resin, and thus prevent its activity interfering with that of S6 kinase.

These observations strongly suggest that treatment of astroglial cells with agents which increase intracellular cAMP content activated an S6 kinase resembling the mitogen-stimulated kinase. However, as the activated form(s) of the S6 kinase appear(s) difficult to purify, it is not yet possible to provide absolute proof of their identity.

Matsuda and Guroff [18] have also observed that NGF-stimulated S6 kinase in PC12 cells could be activated by dbcAMP treatment. Nevertheless, this protein kinase has properties which distinguish it from other growth factor-stimulated S6 kinases (no protection by EGTA and β-glycerophosphate, no activation by TPA).

As the only known receptor for cAMP is cAMP-PK, it is likely that it mediates, directly or otherwise, the in situ activation on the S6 kinase.

According to Matsuda and Guroff [18] the pretreatment with catalytic subunit of cAMP-PK, of cell-free extracts from control and NGF-treated PC 12 cells, caused an increase in S6 kinase activity. Their results suggest an activation of enzyme by direct phosphorylation. However, similar experiments with crude extracts from astrocytes or with partially purified S6 kinase from the same source, did not provoke the activation of the enzyme (not shown).

The effects of cAMP on S6 kinase reported here, could explain why in in vivo liver, cAMP and glucagon generate at least three phosphorylated derivatives of S6 [19], whereas cAMP-PK in vitro phosphorylates only two residues [20,21].

Two other kinds of protein kinases also seem to be implicated in S6 kinase activation: growth factor-dependent tyrosine kinases and protein kinase C. Since many of these kinases have been purified, it is now possible to test if they can directly phosphorylate the mitogen-stimulated S6 kinase.

Addition of cAMP to cell cultures inhibits cell proliferation in most biological studied systems [11]. Since mitogens generally promote the activation of the S6 kinase, the action of cAMP described in this report is rather paradoxical. If cAMP has this effect in a variety of cells, then, at the same time as S6 kinase is activated, the cAMP-PK may act on other protein targets to inhibit cell multiplication.

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