Activation of S6 kinase activity in astrocytes by insulin, somatomedin C and TPA

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Treatment of cultured astrocytes from 2-day-old rat cerebral hemispheres with insulin or somatomedin C (IGF1) promoted a rapid activation of a cytosolic protein kinase which phosphorylates ribosomal protein S6. Phosphorylation of substrates currently used for protein kinase assays (histone H2B and phosvitin) was not stimulated. Neither the cyclic AMP-dependent protein kinase activity nor that of protein kinase C was modified. Treatment of these astrocytes with TPA also promoted a rapid increase in S6 kinase activity in the cytosolic fraction. Simultaneously, protein kinase C disappeared from the cytosol. Neither cyclic AMP-dependent protein kinase activity nor phosvitin kinase activity was modified. The effects of insulin, IGF1 and TPA were also observed in the presence of cycloheximide. Cycloheximide also potentiated their effects. These data indicate that S6 kinase activity in astrocytes is promoted from a pre-existing molecule via the tyrosine kinase-insulin receptor and suggest that protein kinase C is implicated in the process.

Astrocyte *Insulin* *12-O-Tetradecanoylphorbol 13-acetate* *S6 protein* *Ribosomal protein* *Protein kinase*

1. INTRODUCTION

Multisite phosphorylation of ribosomal protein S6 occurs in response to the addition of hormones [1,2], serum [3,4], growth factors [5–7] and phorbol esters [8,9]. In vitro, S6 is a substrate for a number of protein kinases, e.g. cAMP and cGMP-dependent protein kinases [10], protein kinase C [11,12] and casein kinase I [13]. Only the cAMP-dependent protein kinase has been shown to phosphorylate S6 in vivo [2].

It has recently been reported that S6 kinase activity was increased in 3T3 fibroblasts treated with EGF [14], insulin and TPA [15]. While the present work was in progress it was also reported that insulin stimulates an S6 kinase in *Xenopus* oocytes [16]. As phorbol ester receptors, which are believed to be protein kinase C, and insulin and IGF1 receptors are present in brain [18,19] we decided to look for insulin- and TPA-stimulated S6 kinase(s) in brain cells. The initial phase of the present study involved the testing of these kinase activities in primary cultures of astrocyte-enriched glial cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Histone types V-s and VII-s, phosvitin, heat-stable inhibitor, aprotinin, leupeptin, insulin (24 IU/mg), phosphatidylserine and diolein were from Sigma. Pure porcine insulin was obtained from Novo. Somatomedin C (IGF1) and [γ-^{32}P]ATP were obtained from Amersham, France. 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was from PL Biochemicals. All other chemicals were reagent grade.

2.2. *Cell culture and cell extracts*

Cerebral hemispheres from 2-day-old Sprague Dawley rats were cleaned of meninges and then dissociated into cell suspension by passage through

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a nylon mesh (48 μm pore size). The cells were grown to confluence (about 14 days) as described [20], and then the medium was exchanged for chemically defined medium [21] containing in particular insulin (10 μg/ml), hydrocortisone (36 μg/ml), transferrin (10 μg/ml) and selenium (7.8 ng/ml). The cells were maintained in this medium for 1 week, except that insulin was removed 2 days before experiments.

These cultures showed predominantly glial fibrially acidic protein positive cells, having the morphological appearance of astrocytes and a high level of glutamine synthetase (148.83 ± 19.56 nmol glutamine/min per mg protein).

Insulin, IGF1 or TPA were added to the medium for various periods. When indicated, cycloheximide (2 μg/ml) was added 30 min before hormones or TPA. Following these treatments, media were removed and cells were rapidly washed with 3 ml/dish of 80 mM β-glycerophosphate, pH 7.4, 20 mM EGTA and 15 mM $MgCl_2$ (buffer A). Finally, cells were scraped off in 0.3 ml/dish of buffer A containing 1 mM PMSF, aprotinin (50 μg/ml), leupeptin (4 μg/ml), antipain (4 μg/ml), trypsin inhibitor (1 μg/ml), 1 mM benzamidin and 0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone. Cells were broken in this buffer by sonication for 3 s. The homogenate was centrifuged at 100000 × *g* for 1 h. The supernatant was stored as frozen aliquots at −70°C. As initially reported [14] for a serum- and EGF-activated S6 kinase in 3T3 cells, the insulin-stimulated S6 kinase from astrocytes was stable when cells were homogenized in a β-glycerophosphate-EGTA buffer and when cytosolic and particulate fractions were rapidly separated.

2.3. *Protein kinase assays*

40 S ribosomal subunits were isolated from rat liver according to Martin and Wool [22]. S6 kinase assays were performed by incubation for 20 min at 30°C or 10 min at 37°C in a final volume of 50 μl containing 20 mM Hepes (pH 7.4), 8 mM $MgCl_2$, 2 mM dithiothreitol, 15–25 μg 40 S ribosomal protein and 50 μM ATP (~1–2 μCi/nmol). Reaction was initiated by addition of aliquots of the extracts (5–10 μg protein) and stopped with concentrated electrophoresis sample buffer. Reaction products were separated by electrophoresis in the presence of SDS on 15% polyacrylamide gels [23]. The gels were stained with Coomassie blue, dried and autoradiographed with Kodak XAR5 films. ^{32}P incorporation into 40 S ribosomal proteins took place in a single band (fig.1) corresponding to a component identified as S6 [14–16]. This band was excised from the dried gels and ^{32}P was quantified by counting.

Protein kinase C, cAMP dependent protein kinase and phosvitin kinase activities were assayed as in [24,25].

3. RESULTS AND DISCUSSION

Treatment of confluent primary astrocytes maintained in chemically defined medium with 1 μM insulin for 60 min promoted a 2–4-fold increase in S6 protein kinase activity (fig.1, table 1). No reduction of this activity was observed in the

Fig.1. Presence in astrocytes of an S6 kinase activity stimulated by insulin. Astrocytes were treated with insulin (10^{-6} M) for 1 h. Cytosolic extracts were prepared and incubated with 40 S ribosomal subunits and [γ-^{32}P]ATP. Phosphorylation products were analyzed by gel electrophoresis and autoradiography. Autophosphorylation of 40 S ribosomal subunits (a) and of cytosolic extracts from control cells (c) or insulin-treated cells (e). Phosphorylation of 40 S ribosomal subunit by extract from control cells (b) and insulin-treated cells (d).

Table 1

^{32}P incorporation into various substrates catalyzed by cytosolic extracts from control and stimulated cells

Substrates	Unstimulated cells	Cells stimulated by	
		Insulin	TPA
40 S	0.018	0.038	0.065
Histones H2B			
− cAMP	0.58	0.51	0.61
+ cAMP	0.88	0.89	0.82
Phosvitin	0.3	0.29	0.32

Astrocytes were treated with insulin (10^{-6} M) or TPA (10^{-7} M) for 1 h. Cytosolic extracts were prepared and assayed for ^{32}P incorporation into S6, phosvitin and H2B in the presence or absence of cAMP. Values are expressed as nmol ^{32}P/min per mg protein

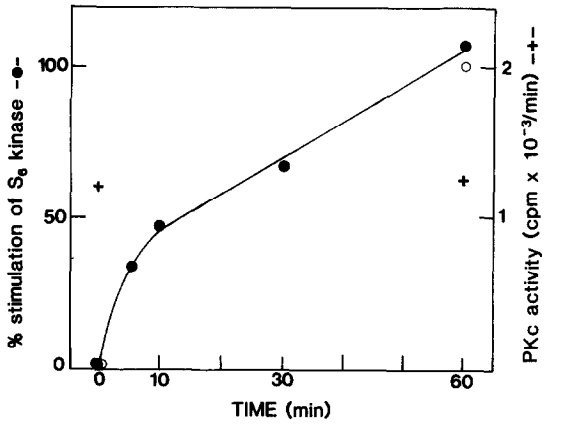

Fig.2. Time course of the stimulation of S6 kinase activity by insulin. Astrocytes were treated with insulin (10^{-6} M) for different time periods. Cytosolic extracts were prepared and S6 kinase assayed in the presence (●) or absence (○) of the heat-stable inhibitor for cAMP-dependent protein kinase. The basal value of S6 kinase activity corresponds to 10^3 cpm ^{32}P incorporated. Protein kinase C activity is shown at 0 and 60 min after insulin addition (+).

presence of the heat-stable inhibitor of cAMP-dependent protein kinase (fig.2). The insulin-stimulated S6 kinase activity was rather specific because no stimulation of protein kinase activity was seen with the substrates currently used for protein kinase assays (histone H2B and phosvitin) (table 1). Moreover, cAMP-dependent protein kinase and protein kinase C activities were unmodified (fig.2, table 1). As changes in the specificity of kinase C have been observed [26] we also assayed kinase C with endogenous substrates (fig.3) but no modification was observed.

The time course for insulin-stimulated S6 kinase activity is shown in fig.2. Activation was detected as early as 5 min after addition of insulin and increased with time for at least 1 h. The dose-response curve for insulin-stimulated S6 kinase is shown in fig.4. Stimulation was detectable with 1–2 nM insulin and appeared to reach a maximum between 0.1 and 1 μM. The addition of cycloheximide 30 min prior to exposure to insulin did not suppress the stimulation of S6 kinase activity but had a potentiating effect (fig.4). Thus, it is likely that the stimulation of S6 kinase activity does not require protein synthesis and that insulin activates a pre-existing molecule.

As it has been reported that somatomedin C (IGF1), a factor with a structure analogous to insulin, acts on glial cells [27], its effect on S6 kinase activity in astrocytes was examined. It was found that IGF1 promoted stimulation of S6 kinase activity similar to that observed with insulin (fig.4, inset).

TPA is known to induce phosphorylation of protein S6 in some cultured cells [8,9] and has been reported to activate S6 kinase in 3T3 cells [15]. We therefore examined the effect of this phorbol ester on S6 kinase activity in the cytosol of astrocytes. TPA stimulated S6 kinase activity more strongly than insulin (fig.4, inset). Fig.5 shows the time course of TPA-stimulated S6 kinase, which is very similar to that observed with insulin. As protein kinase C is known to be the phorbol ester receptor we examined the activity of this kinase in the same experiment. In contrast to the S6 kinase increase, protein kinase C activity decreased rapidly in the cytosol after TPA addition. 70% of the protein kinase activity was lost after 15 min. This decrease may be due to a translocation towards the membrane, as has been previously observed in other cellular systems [28,29]. It therefore appears that protein kinase C, one enzyme phosphorylating S6 in vitro, cannot be the S6 kinase activated in situ by TPA. Similarly, cAMP-dependent protein kinase and phosvitin kinase are excluded as their activities remained unmodified (table 1).

To date, S6 kinase stimulation by growth factors

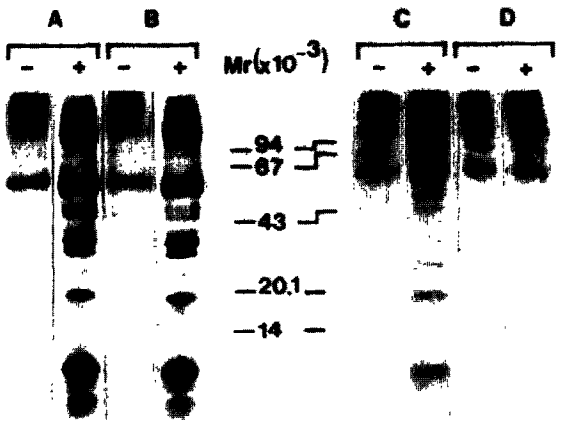

Fig.3. Protein kinase C on endogenous substrates in cytosolic extracts from control and stimulated cells. Astrocytes were treated for 1 h with insulin (10^{-6} M) or TPA (10^{-7} M). 15 μg cytosolic extracts were incubated with [γ-^{32}P]ATP in buffer A in the presence (+) or absence (−) of PL (phosphatidylserine, 10 μg/ml + diolein, 0.5 μg/ml) and Ca^{2+} (0.5 mM above EGTA). After 5 min incubation at 30°C the reaction products were analyzed by polyacrylamide gel electrophoresis and autoradiography. (A,C) Control cells, (B) insulin-treated cells, (D) TPA-treated cells.

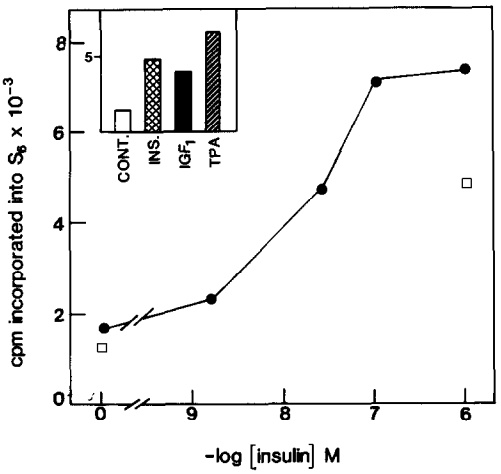

Fig.4. Dependence on insulin concentration of S6 kinase stimulation. Control (□) and cycloheximide-treated (●) astrocytes were treated with increasing concentrations of insulin. Cytosolic extracts were prepared and S6 kinase assayed. The inset compares S6 kinase activity promoted by insulin, IGF1 and TPA. Each agent was added to cell cultures at 10^{-7} M for 1 h.

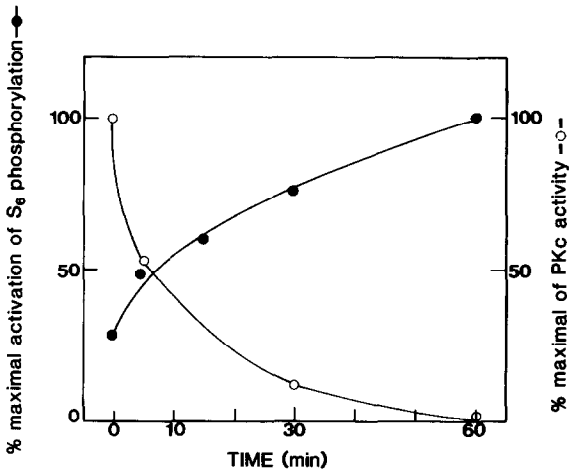

Fig.5. Time course of S6 kinase and protein kinase C activities after TPA addition. Astrocytes were treated with TPA (10^{-7} M) for various times. Cytosolic extracts were prepared and kinases assayed.

activating tyrosine kinases or by cell transformation with oncogenes coding for such an enzyme has been observed in 3T3 fibroblasts [14,30], 3T3 fibroblasts differentiated into adipocytes [15], oocytes [16] and astrocytes (this work). These results suggest that S6 kinase stimulation is linked to tyrosine kinase activation. However, the relationship between tyrosine kinase and S6 kinase remains to be elucidated. Several hypotheses may be considered. Among them activation of S6 kinase by phosphorylation is supported by the observation that phosphatase inhibitors must be present in homogenates to keep the S6 kinase in an activated form [31]. But there is as yet no direct demonstration that tyrosine kinases phosphorylate S6 kinase.

S6 kinase is also stimulated by TPA in 3T3 fibroblasts [15] and astrocytes (this work), indicating the existence of another activation route, probably via the kinase C. Phosphorylation of S6 kinase by kinase C could be possible but has not yet been demonstrated. An attractive explanation, at the present time, would be that TPA treatment of cells generates a proteolyzed form of kinase C which phosphorylates S6. This schema has a certain amount of experimental support: (i) limited proteolysis of kinase C gives rise to kinase M [32], which has been shown to be very active on S6 in the absence of phospholipids and calcium [12]; (ii) treatment of cells with TPA results in increased

kinase M activity [33,34]; (iii) an S6 kinase named PAKII activated by mild trypsin treatment has been purified by Traugh et al. [35,36]. PAKII shows several similarities to kinase M, but proteolysis of kinase C has been shown to generate different active derivatives under different conditions [31]. Thus the question must remain open as to the relationship between kinase M and PAKII.

To test the validity of the different hypotheses (phosphorylation by tyrosine kinase and/or kinase C, limited proteolysis, etc.) the purification of insulin- and TPA-stimulated S6 kinase(s) is currently in progress in our laboratory.

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