Activation of mitogen-activated protein kinase and p70S6 kinase is not correlated with cerebellar granule cell survival

Frank J. GUNN-MOORE*, Alan G. WILLIAMS*, Nicholas J. TOMS† and Jeremy M. TAVARÉ*‡

*Department of Biochemistry, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K., and †Department of Pharmacology, School of Medical Sciences, University of Bristol, Bristol BS8 1TD, U.K.

We have investigated the role of mitogen-activated protein (MAP) kinase in the survival of cerebellar granule cells in primary culture. Brain-derived neurotrophic factor (BDNF) and insulin, but not epidermal growth factor (EGF), promoted the survival of P₆ cerebellar granule neurons. BDNF promoted a sustained activation of MAP kinase, whereas that induced by EGF was only transient. Insulin promoted a small but transient activation of MAP kinase that was completely blocked by PD98059, an inhibitor of MAP kinase kinase activation. PD98059 had no effect on the insulin- or BDNF-induced survival of cerebellar granule cells. We also investigated the role of p70S6

kinase in survival. The activation of p70S6 kinase by EGF was transient, whereas BDNF and insulin promoted a sustained activation of p70S6 kinase. Rapamycin, which blocked p70S6 kinase activation, had no effect on the BDNF- or insulin-induced survival of cerebellar granule cells. We conclude that sustained activation of MAP kinase is not correlated with the survival response of cerebellar granule cells; indeed insulin-mediated survival is independent of MAP kinase. Survival of cerebellar granule cells is also independent of the activation of p70S6 kinase.

INTRODUCTION

The signalling pathways elicited by the neurotrophic factors nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3) have been extensively studied. However, this has largely involved using permanent cell lines such as fibroblasts transfected with the appropriate receptor cDNA species, or the rat chromaffin cell type PC12, which differentiates into sympathetic-like neurons in the presence of NGF [1]. The sustained activation of mitogen-activated protein (MAP) kinase, and its translocation to the nucleus, have been reported to be necessary and sufficient for NGF-dependent differentiation of PC12 cells [2,3]. However, studies with mutant PDGF receptors argue against this simple hypothesis [4]. In contrast, considerably less is known about the signalling pathways initiated in primary neurons.

Cerebellar granule cells of 6–7-day-old rats are a good source of an almost homogeneous neuronal cell culture [5,6]. These cells possess a variety of receptors for neurotrophic factors and growth factors, including TrkB, which binds BDNF, and TrkC, which binds NT-3. Previous studies have shown that BDNF, IGF-I and cAMP are able to promote the survival of these neurons [7], but the role of NT-3 in survival is less clear [7,8].

The stimulation of cerebellar granule cells by BDNF has been shown to promote tyrosine autophosphorylation of TrkB and the subsequent tyrosine phosphorylation of the signalling molecules Shc and phospholipase C γ as well as their association with TrkB [6]. Activation of Ras has also been reported in response to BDNF [6,9] presumably via a Shc $\rightarrow$ Grb2 $\rightarrow$ Ras pathway. Other reports suggest that BDNF activates protein kinase C and promotes an increase in intracellular Ca²⁺ con-

centration [6], though this has not been observed by all groups [10]. There are no reports of the activation of MAP kinase or p70S6 kinase by BDNF, nor are there any studies on the pathways activated by insulin. However, the insulin receptor is abundantly expressed in granule cells [11] and insulin can promote the survival of these neurons [12].

In the present study we demonstrate that BDNF, insulin and epidermal growth factor (EGF) all stimulate the activity of MAP kinase and p70S6 kinase but with markedly differing kinetic profiles. These results, together with the additional use of inhibitors of MAP kinase and p70S6 kinase activation, suggest that the survival of cerebellar granule cells is likely to be independent of the activation of MAP kinase or p70S6 kinase.

MATERIALS AND METHODS

Materials

Anti-p70S6 kinase antibodies were provided by Dr. E. Foulstone (University of Bristol, Bristol, U.K.). Anti-(active MAP kinase) antiserum was generously provided by Dr. E. Schaeffer (Promega, Madison, WI, U.S.A.); this affinity-purified antiserum was raised against a doubly phosphorylated peptide corresponding to the sequence containing phosphothreonine-183 and phosphotyrosine-185 of active Erk2 (the antibody is commercially available). BDNF and NT-3 were gifts from Dr. G. Yancopoulos (Regeneron Pharmaceuticals, New York, U.S.A.). Rapamycin was from Calbiochem and PD98059 from New England Biolabs. Unless otherwise stated, all biochemicals were from Sigma (Poole, Dorset, U.K.), and general-purpose laboratory reagents were of analytical grade and from BDH (Poole, Dorset, U.K.).

Abbreviations used: BDNF, brain-derived neurotrophic factor; EGF, epidermal growth factor; NT-3, neurotrophin 3; MAP kinase, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; TUNEL, TdT-mediated dUTP nick end labelling.

‡ To whom correspondence should be addressed.

Cell cultures

Cerebellar granule cells, from P6-7 neonatal rats, were isolated and cultured by the method of Thangnipon et al. [5]. In brief, cerebella were dissected under sterile conditions and cells were isolated by dissociation with plastic pipettes in versene. Cells were collected by centrifugation at 500 *g* for 5 min and plated on poly-(L-lysine)-coated plates in basal Eagle's medium supplemented with 10 % (v/v) foetal calf serum, 2 mM glutamine and 25 mM glucose, with 50 i.u./ml benzylpenicillin and 50 µg/ml streptomycin. After 24 h the medium was changed to serum-free medium and stimulated with growth factors as described in the figure legends.

Cell survival assay

Cell viability was estimated by use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay [6,13]. Approximately 10⁵ cerebellar granule cells were plated in each well of a 96-well plate precoated with poly-(L-lysine). After 24 h the cells were serum-starved for 2 h and treated as shown in the figure legends; 24 h after treatment, 200 µl of 0.5 mg/ml MTT in PBS was added to the cells and incubated for 3 h. The medium was removed and the cells were washed in PBS and dissolved in 100 µl of DMSO. After 30 min at room temperature the absorbance was measured at 570 nm.

The extent of apoptosis of cerebellar granule cells was determined by the use of the TdT-mediated dUTP nick end labelling (TUNEL) assay. In brief, cells were incubated in the absence or presence of survival factors as indicated in the figure legends. The cells were then washed in PBS, fixed for 20 min in 3 % (v/v) paraformaldehyde and permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 5 min. Incubation with fluorescein-12-dUTP and terminal deoxynucleotidyl transferase was then performed in accordance with the manufacturer's instructions (Promega). Cells were imaged with a Hamamatsu intensified charge-coupled device camera (Hamamatsu Photonics, Hamamatsu, Japan) attached to a Zeiss Axiovert 100TV microscope with an Achroastigmat LD32X/0.4NA phase lens and a High Q FITC filter set (41001; Chroma Technology, Brattleboro, VT, U.S.A.).

MAP kinase assays

Approximately 5 × 10⁶ cerebellar granule cells on dishes 35 mm in diameter were serum-starved for 2 h before incubation with growth factors for the times indicated in the figure legends. Cells were washed and extracted, and MAP kinase activity in the cell lysate was assayed in the presence of [γ -³²P]ATP as described by Young et al. [14] but with 200 µM T669 peptide as substrate. This peptide is based on the sequence surrounding the MAP kinase phosphorylation site in the EGF receptor (KRELVEPLTPSGEAPNQALLR).

Active MAP kinase immunostaining

Cerebellar granule cells were fixed for 20 min in 3 % (v/v) paraformaldehyde and permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 5 min. Cells were blocked in 3 % (w/v) BSA in PBS, and were incubated in a 1:250 dilution of an affinity-purified anti-(active MAP kinase) antibody. After further washing, the cells were incubated in a 1:500 dilution of anti-(rabbit IgG) FITC conjugate (Sigma). The mounted cells were imaged with a Leica confocal laser scanning microscope (inverted IBRE microscope) in the University of Bristol Cell Imaging Facility. Images were collected by using Leica TCS-NT software.

p70S6 kinase assays

From approx. 10⁷ cells plated on dishes 60 mm in diameter, extracts were prepared as for MAP kinase assays above, but p70S6 kinase was immunoprecipitated with an antibody specific for p70S6 kinase. Activity of this kinase was assayed as previously described [15] by using the peptide KEAKEKRQEIAKRRRLSSLRASTSKSESSQK, which is based on the sites phosphorylated by this enzyme in the ribosomal protein S6.

RESULTS AND DISCUSSION

We used cerebellar granule cells from 6–7-day-old rats as a convenient source of neurons supplying an almost homogeneous neuronal population (approx. 95 % of the cells represent cerebellar granule cells). These neurons express TrkB and TrkC but not TrkA [16].

Insulin and BDNF, but not EGF, promote the survival of cerebellar granule cells

Cerebellar granule cells are non-proliferative and their survival can be determined by measuring the extent of conversion of MTT to a colorimetric product by cellular dehydrogenases [13]. With this assay we confirmed that maximally effective concentrations of serum, BDNF, NT-3 and insulin were all able to promote the survival of cerebellar granule cells (Figure 1, upper panel). EGF did not promote survival despite the presence of EGF receptors (see below). Furthermore NGF did not promote survival consistent with the lack of TrkA expression. Using TUNEL assays, which measure the incorporation of fluorescein-12-dUTP incorporation into fragmented DNA ends, we confirmed that serum, BDNF and insulin all blocked the induction of apoptosis induced by serum withdrawal (Figure 1, lower panel, and Table 1).

Although the effects of BDNF and NT-3 were not additive, when the neurotrophins and insulin were added together there seemed to be an additive effect on survival (Figure 1, upper panel). This suggests that insulin and the neurotrophins might use dissimilar signalling pathways to promote granule cell survival.

Stimulation of MAP kinase by BDNF, EGF and insulin and its role in survival

BDNF, EGF and insulin all promoted a rapid stimulation of MAP kinase activity (Figure 2a). However, the kinetics of stimulation varied greatly. BDNF caused a sustained activation of MAP kinase (Figure 2a), which remained elevated for at least 24 h (results not shown). Interestingly, we consistently observed two phases of activation by BDNF, one peaking at 5 min, which then decreased over the next 55 min. This was followed by a steady rise in activity over the next 1 h. MAP kinase activation paralleled that of MAP kinase, also showing two distinct activation phases (F. J. Gunn-Moore, unpublished work). This situation is almost identical with the kinetics of MAP kinase kinase and MAP kinase activation by NT-3 in fibroblasts expressing TrkC [17]. The molecular basis behind these two phases of MAP kinase activation is not known [17].

Compared with BDNF, EGF caused an equally if not more robust initial stimulation of MAP kinase; however, this was short-lived, dropping to unstimulated levels after 1 h. Insulin-activated MAP kinase was also rapid but very transient and lower in magnitude than that observed for either BDNF or EGF.

The stimulation of MAP kinase by BDNF, EGF and insulin in the cerebellar granule cells was confirmed by immunostaining with an affinity-purified antibody specific for active MAP kinase

Figure 1 BDNF, insulin and NT-3 all promote survival of cerebellar granule cells

Cerebellar granule cells were incubated for 24 h in the presence of serum-free medium, either alone or supplemented with various growth factors: foetal calf serum (10%, v/v), BDNF (100 ng/ml), NT-3 (100 ng/ml), NGF (100 ng/ml), insulin (100 nM), or EGF (100 ng/ml) as indicated. Upper panel: survival was determined by the MTT conversion assay and is expressed as a percentage of the conversion observed in the presence of serum. Results shown are means $\pm$ S.E.M. (eight separate wells per treatment) for a representative experiment. Lower panel: the extent of apoptosis was determined with TUNEL assays as described in the Materials and methods section. Shown for each treatment are bright field and corresponding fluorescence images; the latter were taken with an FITC filter set.

(which recognizes the Erk1 and Erk2 isoforms of MAP kinase only when phosphorylated on both the threonine and tyrosine residues in the TEY motif). As shown in Figure 3, after 10 min of stimulation, BDNF (Figure 3b), EGF (Figure 3c) and insulin (Figure 3d) all resulted in an increased immunofluorescence staining compared with the control (Figure 3a). The extent of this increase closely paralleled the degree of MAP kinase activation as determined in the cell lysate assays (Figure 2a). Interestingly, BDNF, EGF and, to a smaller extent, insulin activated MAP kinase in the neurites of these cells as well as the cell bodies. It will be particularly interesting to use this antibody in studies on the distribution of active MAP kinase *in vivo*, where neurotrophic factor receptor activation occurs in the distal

portion of the neurites before retrograde transport to the cell body [18].

The relative effects and kinetics of BDNF-, EGF- and insulin-stimulated MAP kinase in cerebellar granule cells are highly reminiscent of those observed in PC12 cells [2,14]. The various stimuli that promote neurite outgrowth and survival of PC12 cells (e.g. NGF and CPT-cAMP) also promote sustained activation of MAP kinase [2,3,14]. In cerebellar granule cells, however, the differing kinetics of MAP kinase activation by BDNF and insulin suggest that sustained activation of MAP kinase is not well correlated with the survival response. Thus sustained MAP kinase activation might not be a prerequisite for supporting the survival of cerebellar granule cells.

Table 1 BDNF and insulin block apoptosis in cerebellar granule cells

In the experiment shown in Figure 1 (lower panel), eight separate fields of view from each treatment were counted for the presence of apoptotic cells. The results are expressed as percentages of cells undergoing apoptosis (means $\pm$ S.E.M.) and are representative of three independent experiments.

Condition	Cells undergoing apoptosis (%)
Serum	11.4 $\pm$ 2.8
Serum-depleted	42.3 $\pm$ 3.3
BDNF	12.7 $\pm$ 1.2
Insulin	8.5 $\pm$ 1.5

Figure 2 Activation of MAP kinase and p70S6 kinase by BDNF, insulin and EGF

Cerebellar granule cells were stimulated without (■) or with BDNF (100 ng/ml) (□), EGF (100 ng/ml) (●) or insulin (100 nM) (○), extracted at the times shown and then assayed for either MAP kinase (a) or p70S6 kinase activity (b). The results are representative of two separate experiments, each of which was performed in duplicate.

To test this hypothesis we used of an inhibitor of MAP kinase activation, PD98059 [19,20]. The stimulation of MAP kinase by insulin was completely blocked by this compound (Table 2). However, whereas BDNF-stimulated MAP kinase was incompletely inhibited at the concentration used (50 μ M), higher concentrations could not be tested because of insolubility problems. Neither BDNF- nor insulin-stimulated survival were

Figure 3 Distribution of active MAP kinase in cells stimulated with BDNF, insulin or EGF

Cerebellar granule cells were stimulated for 5 min without (a) or with BDNF (100 ng/ml) (b), EGF (100 ng/ml) (c) or insulin (100 nM) (d) and then fixed, permeabilized and stained with an antibody that recognized only the active form of MAP kinase.

Table 2 Effects of PD98059 on MAP kinase activation, and rapamycin on p70S6 kinase activation

Cerebellar granule cells were incubated for 30 min in the presence or absence of 50 μ M PD98059 and/or 20 nM rapamycin before the addition of either BDNF (100 ng/ml) or insulin (100 nM). The cells were extracted after 10 min for MAP kinase activity measurements, and after 30 min for p70S6 kinase activity measurements. The results are expressed as percentages of maximum activity achieved (means $\pm$ S.D.) for two separate experiments, each performed in duplicate. n.d., not determined.

Condition	MAP kinase activity (% of maximum)		p70S6 kinase activity (% of maximum)	
	No inhibitor	+ PD98059	No inhibitor	+ Rapamycin
Control	23.7 $\pm$ 3.7	n.d.	15.5 $\pm$ 3.2	n.d.
BDNF	100	60.9 $\pm$ 7.5	72.3 $\pm$ 19.8	8.5 $\pm$ 1.1
Insulin	37.3 $\pm$ 3.7	16.6 $\pm$ 2.8	100	21.5 $\pm$ 0.4

affected by this inhibitor (Figure 4). Thus we conclude that MAP kinase activation is not sufficient for insulin-stimulated survival. However, because the inhibitor did not completely block the activation of MAP kinase by BDNF, we cannot categorically conclude that MAP kinase is not required for BDNF-stimulated survival.

Stimulation of p70S6 kinase and its role in granule cell survival

Neurotrophic factors have been reported to activate the p70S6 kinase in PC12 cells ([21], and F. J. Gunn-Moore, unpublished work) although this has not been previously reported in primary neurons. BDNF, insulin and EGF all stimulated p70S6 kinase in cerebellar granule cells (Figure 2b). BDNF and insulin promoted a sustained activation over 2 h that contrasted with the transient

Figure 4 Effect of PD98059 and rapamycin on BDNF and insulin stimulated survival of cerebellar granule cells

Cells were preincubated for 30 min with or without the inhibitors (50 μ M PD98059 and/or 20 nM rapamycin) before stimulation with foetal bovine serum (10%, v/v), BDNF (100 ng/ml), EGF (100 ng/ml) or insulin (100 nM), as indicated. After 6 h the medium was changed to fresh medium containing the inhibitors and growth factors, and incubation was continued for a further 16 h. Survival was determined by the MTT assay and the results shown are means $\pm$ S.E.M. (eight separate wells per treatment) for a representative experiment.

activation induced by EGF. Interestingly, the activation of p70S6 kinase was considerably slower than that of MAP kinase (compare Figures 2b and 2a respectively). At face value, therefore, the survival of cerebellar granule cells in response to BDNF and insulin was well correlated with sustained p70S6 kinase activation.

To investigate the role of the p70S6 kinase in granule cell survival we used rapamycin, a potent inhibitor of the activation of this enzyme [22]. Rapamycin blocked both BDNF- and insulin-stimulated p70S6 kinase activation; with BDNF this inhibition was apparently complete, but with insulin there was still a very small stimulation in the presence of inhibitor (Table 2). Rapamycin had no effect on the survival of cerebellar granule cells (Figure 4). This strongly suggests that p70S6 kinase activation is not sufficient for granule cell survival, at least in response to BDNF. Indeed a combination of PD98059 and rapamycin also had no effect on granule cell survival in response to BDNF or insulin (Figure 4).

Concluding remarks

We have shown that BDNF and insulin, but not EGF, promote the survival of cerebellar granule cells. We have additionally demonstrated that BDNF, insulin and EGF all stimulate the activation of MAP kinase and p70S6 kinase in these cells but with markedly differing kinetic profiles. Our results suggest that, unlike the situation with PC12 cell differentiation, sustained MAP kinase activation is not a prerequisite for the survival of cerebellar granule cells. Indeed, at least for insulin, the survival

response is independent of MAP kinase activation. Virdee and Tolkovsky [23] have also concluded that p42 and p44 MAP kinases are not essential for the survival of rat sympathetic neurons although they have reported a requirement for Ras [24,25]. We have also shown that the survival of cerebellar granule cells in response to BDNF, and probably insulin, is independent of the activation of p70S6 kinase. Indeed, at least for insulin, survival occurs even in the absence of appreciable activation of both MAP kinase and p70S6 kinase (Table 2 and Figure 4).

Calphostin C, a protein kinase C inhibitor, has been reported to block BDNF-induced survival at a concentration of 10 ng/ml, suggesting an involvement for protein kinase C [6]. However, the specific effect of this inhibitor on BDNF-stimulated protein kinase C activity was not addressed; furthermore at 30 ng/ml this inhibitor was found to have non-specific toxic effects on the cells [6]. In conclusion, therefore, the signalling pathways involved in insulin and BDNF-induced survival remain to be established.

We thank Dr. George Yancopoulos (Regeneron Pharmaceuticals, New York, U.S.A.) for the BDNF and NT-3 used in this study, and Dr. Erik Schaeffer (Promega) for the anti-(active MAP kinase) antiserum. This work was supported by the Wellcome Trust and Medical Research Council. A.G.W. is supported by a B.B.S.R.C./CASE studentship with Amersham International plc and J.M.T. is a British Diabetic Association Senior Research Fellow. Some of this work was performed with the University of Bristol Cell Imaging Facility, which was funded by an Infrastructure Award from the Medical Research Council.

REFERENCES

- Greene, L. A. and Tishler, A. S. (1982) *Adv. Cell. Neurobiol.* **3**, 373–414
- Traverse, S., Gomez, X., Paterson, H., Marshall, C. and Cohen, P. (1992) *Biochem. J.* **288**, 351–355
- Marshall, C. J. (1995) *Cell* **80**, 179–185
- Vaillancourt, R. R., Heasley, L. E., Zamarripa, J., Storey, B., Valius, M., Kazlauskas, A. and Johnson, G. L. (1995) *Mol. Cell. Biol.* **15**, 3644–3653
- Thangnipon, W., Kingsbury, A., Webb, M. and Balazs, R. (1983) *Develop. Brain Res.* **11**, 177–189
- Zirrgiebel, U., Ohga, Y., Carter, B., Berninger, B., Inagaki, N., Theonen, H. and Lindholm, D. (1995) *J. Neurochem.* **65**, 2241–2250
- D'Mello, S. R., Galli, C., Ciotti, T. and Calissano, P. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10989–10993
- Lindholm, D., Dechant, G., Heisenberg, C. P. and Theonen, H. (1993) *Eur. J. Neurosci.* **5**, 1455–1464
- Carter, B. D., Zirrgiebel, U. and Barde, Y.-A. (1995) *J. Biol. Chem.* **270**, 21751–21757
- Gaiddon, C., Loeffler, J. P. and Larmet, Y. (1996) *J. Neurochem.* **66**, 2279–2286
- Marks, J. L., Porte, D., Stahl, W. L. and Baskin, D. G. (1990) *Endocrinology (Baltimore)* **127**, 3234–3236
- Tanaka, M., Sawada, M., Yoshida, S., Hanaoka, F. and Marunouchi, T. (1995) *Neurosci. Lett.* **199**, 37–40
- Mosmann, T. (1983) *J. Immunol. Meth.* **65**, 55–63
- Young, S. W., Dickens, M. and Tavaré, J. M. (1994) *FEBS Lett.* **338**, 212–216
- Moule, S. K., Edgell, N. J., Welsh, G. I., Diggle, T. A., Foulstone, E. J., Heesom, K. J., Proud, C. G. and Denton, R. M. (1995) *Biochem. J.* **311**, 595–601
- Ernfors, P., Merlio, J.-P. and Persson, H. (1992) *Eur. J. Neurosci.* **4**, 1140–1158
- Gunn-Moore, F. J., Williams, A. G. and Tavaré, J. M. (1997) *Biochem. J.* **322**, 193–198
- Curtis, R. and DiStefano, P. S. (1994) *Trends Cell Biol.* **4**, 383–386
- Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 7686–7689
- Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 27489–27494
- Tang, T., Hirata, Y., Whalin, M. and Guroff, G. (1996) *J. Neurochem.* **66**, 1198–1206
- Chung, J., Koo, C. J., Crabtree, G. R. and Blenis, J. (1992) *Cell* **69**, 1227–1237
- Virdee, K. and Tolkovsky, A. M. (1995) *Eur. J. Neurosci.* **7**, 2159–2169
- Nobes, C. D. and Tolkovsky, A. M. (1995) *Eur. J. Neurosci.* **7**, 344–350
- Nobes, C. D., Reppas, J. B., Markus, A. and Tolkovsky, A. M. (1996) *Neuroscience* **70**, 1067–1079