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Differentiation of PC12 cells in response to a cAMP analogue is accompanied by sustained activation of mitogen-activated protein kinase

Comparison with the effects of insulin, growth factors and phorbol esters

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Abstract

It has been proposed previously that the sustained activation of mitogen-activated protein kinase may be necessary for the differentiation of PC12 cells. Differentiation of PC12 cells is induced by many extracellular agonists including nerve growth factor (NGF) and cyclicAMP analogues, but not epidermal growth factor (EGF), insulin or phorbol esters. Our results demonstrate that: (i) 8-(4-chlorophenylthio)-cyclicAMP (CPT-cAMP) activates MAP kinase; this raises the possibility that the MAP kinase pathway may be activated by agents that act through adenylate cyclase; (ii) NGF and CPT-cAMP as well as phorbol esters promote sustained activation of MAP kinase. This suggests that while sustained MAP kinase activation may be associated with differentiation it may not be sufficient, and that other as yet unidentified parallel pathways may be involved.

Key words: MAP kinase; Cyclic AMP; Nerve growth factor; Phorbol ester; Insulin; PC12 cell

1. Introduction

The mitogen-activated protein (MAP) kinases are stimulated by a wide variety of extracellular agents including growth factors (e.g. EGF and PDGF), neurotrophins (e.g. NGF), insulin and phorbol esters (reviewed in [1–3]). The pathways by which these enzymes (including ERK1 and ERK2) are activated have become increasingly well characterised in the past few years. Several MAP kinase kinases have now been identified that can phosphorylate and activate the MAP kinases (see [3] for recent review) and at least three MAP kinase kinase kinases have been identified that phosphorylate and activate MAP kinase kinases e.g. Raf1 [4–6], Mos [7] and MEKK [8]. Recently, several groups have demonstrated that Raf1 associates with Ras [9–12] but it is not known how this simple association could lead to Raf1 activation.

Growth factors, insulin and neurotrophins promote widely differing cellular responses, and thus it remains to be firmly established how the MAP kinase cascade could play a central role in the actions of this diverse array of extracellular agonists. A good example is the rat phaeochromocytoma (PC12) cell line in which NGF, but not EGF, insulin or phorbol esters, promotes differentiation into a cell type resembling sympathetic neurones [13,14].

Although both NGF and EGF promote a rapid activation of MAP kinase in PC12 cells, the activation in response to EGF is transient while that in response to NGF is sustained over a period of several hours and this is accompanied by parallel changes in MAP kinase kinase activation [15–17]. Furthermore, NGF, but not EGF, was reported to promote the translocation of MAP kinase immunoreactivity into the nucleus [16,17]. It was proposed on the basis of these results that a sustained activation of the MAP kinase cascade might be required for the differentiation of PC12 cells [15,16]. If such a phenomenon is central to the differentiation response then it should also apply to other agents which promote PC12 cell differentiation, including cAMP analogues such as dibutyryl cyclicAMP [18], interleukin 6 [19] and fibroblast growth factors [18,20]. As cAMP is well known to activate the cAMP-dependent protein kinase and had not previously been reported to regulate the activity of MAP kinase, we examined whether a more membrane permeant cAMP analogue, 8-(4-chlorophenylthio)-cyclicAMP (CPT-cAMP) would promote PC12

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Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated protein kinase; PMA, phorbol 12-myristate 13-acetate; CPT-cAMP, 8-(4-chlorophenylthio)-cyclic AMP.

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cell differentiation and concomitant activation of MAP kinase. In addition we investigated the time course of activation of MAP kinase by insulin and PMA, agents which do not promote PC12 cell differentiation.

2. Materials and methods

2.1. Materials

All reagents were as previously described [21] except tissue culture media and serum which were from Gibco BRL (Paisley, UK), and 8-(4-chlorophenylthio)-cyclicAMP which was from Sigma (Poole, UK). An antiserum (pan-ERK1/2) reactive towards both ERK1 and ERK2 was raised essentially as described by Boulton and Cobb [22] towards the peptide CAIFQETARFQPGAPEAP (corresponding to the C-terminal 15 amino acids of ERK1) coupled to keyhole limpet haemocyanin. For Western blot analysis the serum was affinity purified by chromatography on a Sepharose column containing covalently coupled ERK1 C-terminal peptide; bound antibodies were eluted with 0.2 M glycine (pH 2.5) followed by extensive dialysis against phosphate buffered saline. PC12 cells were maintained in collagen coated plates with DMEM medium supplemented with 10% foetal calf serum and 5% horse serum.

2.2. Assay of MAP kinase in anti-ERK immunoprecipitates

Approximately 2×10^6 cells in 60-mm dishes were serum starved by incubation at 37°C for 120 min in 2 ml of serum-free medium. After incubation with ligands for the times and at the concentrations indicated in the figure legends, the cells were washed with 2 ml of ice-cold phosphate-buffered saline and rapidly extracted by scraping into 0.5 ml of ice-cold extraction buffer (50 mM β-glycerophosphate (pH 7.4), 1.5 mM EGTA, 1 mM benzamidine, 1 mM dithiothreitol, 0.5 mM Na_3VO_4, 0.1 mM PMSF, 1 μM microcystin, 1 μg/ml each of pepstatin, antipain and leupeptin). After centrifugation at $10,000 \times g$ for 15 min at 4°C the supernatants were incubated with 5 μl of pan-ERK1/2 antiserum and 2.5 mg protein A-Sepharose (Sigma, Poole, UK) by tumbling for 2 h at 4°C. The beads were washed three times with extraction buffer without microcystin and resuspended in 50 μl extraction buffer. Pellets (20 μl) were assayed in duplicate incubations (50 μl final volume) containing 20 mM MOPS (pH 7.4), 3 mM EGTA, 10 mM $MgCl_2$, 1 μM microcystin, 1 μM-cAMP-dependent protein kinase inhibitor peptide and 0.5 mg/ml myelin basic protein. Reactions were initiated by the addition of [γ-^{32}P]ATP (50 μM; ~ 500 cpm/pmol) and after 15 min at 30°C terminated by spotting a 30 μl sample onto P81 phosphocellulose paper squares. Papers were washed four times with 150 mM H_3PO_4 and counted in 10 ml of water by the Cerenkov method.

2.3. Western blot analysis

A PC12 cell lysate (500 ml) was prepared as described above and solubilized by addition of 100 μl of SDS sample buffer. Proteins were separated on a 7% polacrylamide gel, transfered to Immobilon P membrane (Millipore Corporation, Bedford, USA) and Western blotted with affinity-purified pan-ERK1/2 (75 μg/ml) followed by chemiluminescent detection as described [23].

2.4. Fractionation of PC12 extracts by MonoQ FPLC

Cell extracts (approx. 3 mg of protein) prepared as described above were loaded onto a MonoQ HR 5/5 column (Pharmacia) at a flow rate of 0.5 ml/min, pre-equilibrated with buffer A (50 mM β-glycerophosphate (pH 7.4), 1 mM EGTA, 1 mM dithiothreitol). The column flow-through was collected and the column washed with buffer A until the absorbance at 280 nm returned to baseline. The column was developed, at a flow rate of 0.5 ml/min, with a linear gradient to 0.5 M NaCl in buffer A. Fractions (0.5 ml) were collected into tubes containing 50 ml of 50 mM β-glycerophosphate (pH 7.4), 2 mM Na_3VO_4. Fractions (20 μl) were assayed for MAP kinase activity using myelin basic protein as a substrate as described above except that the reactions were performed in 96-well microtitre plates. Reactions were terminated with 20 μl 2 M HCl and applied to an 8 cm × 12 cm P81 paper square using an 8 × 12 Hybridot manifold (BRL, Cambridge, UK). The P81 paper was then washed four times with 500 ml 150 mM H_3PO_4, dried and ^{32}P-incorporation determined using a PhophorImager (Molecular Dynamics, USA). Standard samples of known amounts of [γ-^{32}P]ATP were included to allow conversion of spot intensity to cpm. This method allowed the rapid and sensitive assay of several hundred samples with relative ease, and gave results which were essentially identical to those obtained using the standard assay method described previously.

3. Results and discussion

We first assessed the ability of the 8-(4-chlorophenylthio) analogue of cAMP to induce neurite outgrowth in PC12 cells. As shown in Fig. 1, after 24 h treatment, CPT-cAMP promoted neurite outgrowth in PC12 cells to an extent which was almost indistiguishable to that induced by NGF. Identical results were observed in serum starved cells (data not shown). This confirms, therefore, the observations of Togari et al. [18] who used the less permeant dibutyryl analogue of cAMP. In addition, we confirmed that EGF (100 nM), insulin (100 nM) and PMA (1.6 μM) did not induce any detectable neurite outgrowth in these cells (data not shown). These concentrations of agents were chosen to give maximal levels of response for each agonist.

PC12 cells were then treated with various agents and extracted in the presence of protease and phosphatase inhibitors. MAP kinase was immunoprecipitated from these cell extracts using a pan-ERK1/2 antiserum, and the activity in the immunoprecipitates assayed using myelin basic protein as substrate. This serum is highly specific for the ERK1 and ERK2 isoforms of MAP kinase as determined by Western blot analysis of PC12 cell lysates (Fig. 2b).

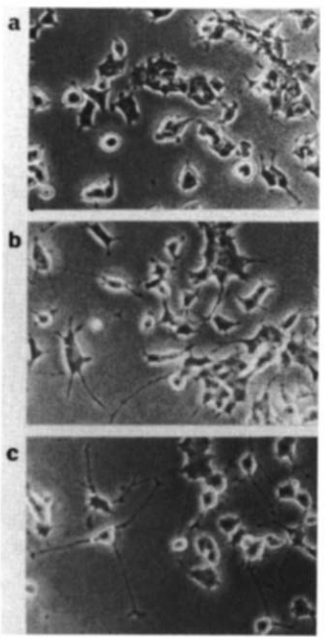

Fig. 1. Induction of neurite outgrowth in PC12 cells treated with NGF and CPTcAMP. PC12 cells in collagen coated dishes were treated without (a) or with 50 ng/ml NGF for 24 h (b) or with 0.5 mM CPT-cAMP for 24 h (c).

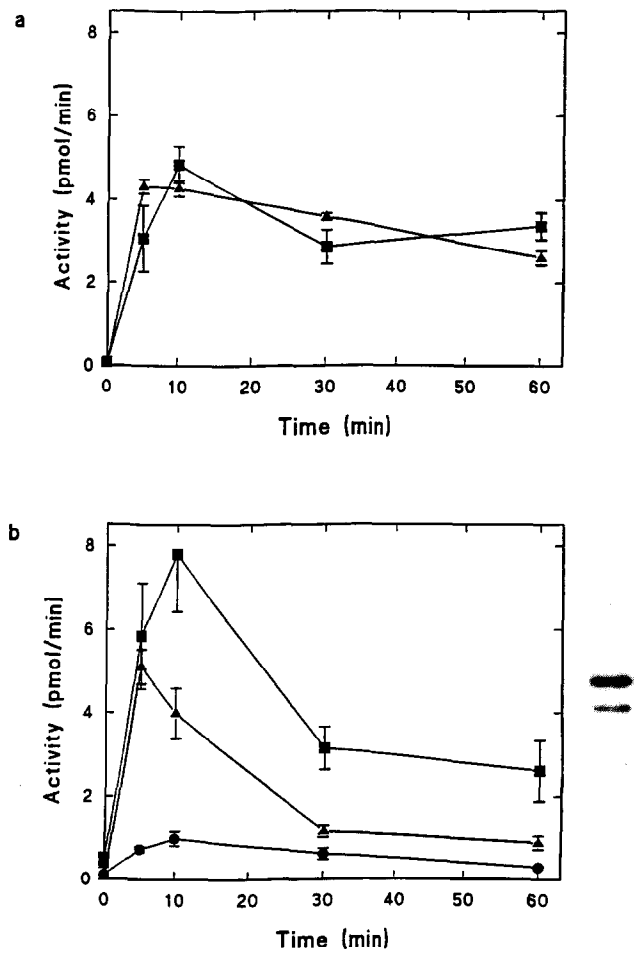

Fig. 2. Time course of activation of MAP kinase in PC12 cells PC12 cells were incubated for the indicated times with (a) agents that promote neurite outgrowth such as 50 ng/ml NGF (■) or 0.5 mM CPT-cAMP (▲) and (b) with agents that do not promote neurite outgrowth such as 1.6 μM PMA (■), 100 nM EGF (▲) or 100 nM insulin (●). Cell extracts were prepared and MAP kinase immunoprecipitated with pan-ERK1/2 antiserum and assayed for its ability to phosphorylate myelin basic protein as described in section 2. Each data point represents the mean ± S.E.M. of at least three separate observations. Panel b, inset: PC12 cell lysate was Western blotted with affinity purified pan-ERK1/2 antiserum followed by ECL detection as described in section 2. The apparent molecular weights of the isoforms in our hands are 44 kDa (open triangle) and 51 kDa (closed triangle) (see also Dickens et al. [21]).

We first examined the ability of two agents that promote neurite outgrowth to activate MAP kinase, namely NGF and CPT-cAMP. Both promoted a sustained activation of MAP kinase (Fig. 2a). The NGF response was consistently biphasic with activation peaking at 10 min, dropping slightly over the next 20 min and followed by a small slower rise such that the activity at 60 min was approx. 40-fold above basal (Fig. 2a; it should be noted that it is difficult to quantitate accurately the extent of activation as the basal activities observed using our assay method were very low). CPT-cAMP promoted a consistently more rapid rise in MAP kinase activity which peaked at 5 min (apparently ~100-fold over basal) and fell only slowly over the next hour (Fig. 2a).

We next examined the time course of activation of MAP kinase by agents that do not promote neurite outgrowth but which, nevertheless, are well known MAP kinase activators. As expected, EGF induced a transient activation of MAP kinase (maximal at 5 min; Fig. 2b). Insulin also promoted a transient activation of MAP kinase although the extent of activation, while still ~ 9-fold above basal at 10 min, was considerably lower than that induced by EGF (Fig. 2b). In several experiments we have been unable to demonstrate neurite outgrowth in our PC12 cells in response to FGF (in contrast with Togari et al. [18] and Rydel and Greene [20]). The cells do, however, respond to FGF as we see a small and transient activation of MAP kinase rather similar to that observed with insulin (S.W.Y., unpublished observations).

Thus far the data is clearly consistent with the hypothesis that only a sustained activation of MAP kinase is associated with neurite outgrowth in PC12 cells (see introduction). Surprisingly, therefore, we found that phorbol esters, which do not promote neurite outgrowth, also promoted a sustained activation of MAP kinase

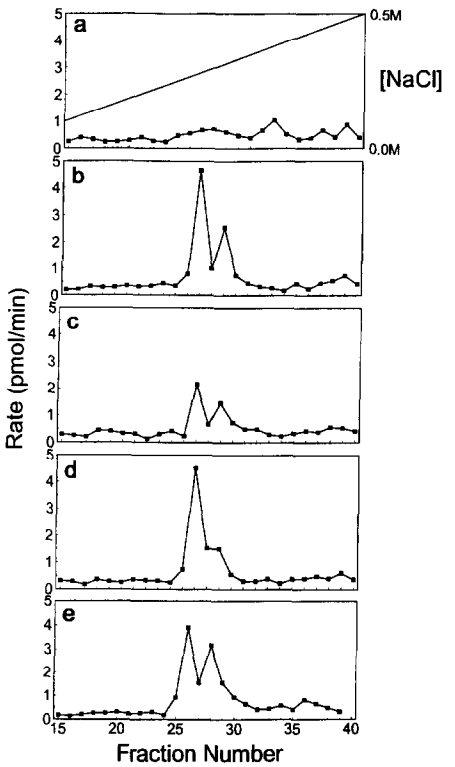

Fig. 3. MonoQ separation of MAP kinases after activation by NGF, PMA or CPT-cAMP. PC12 cells were incubated in the absence (a) or presence of 50 ng/ml NGF for 10 min (b), 0.5 mM CPT-cAMP for 10 min (c), 1.6 μM PMA for 10 min (d) or 1.6 μM PMA for 120 min (e). Subsequently prepared extracts (equal amounts of proteins) were separated by MonoQ FPLC and fractions assayed for myelin basic protein kinase activity as described in section 2.

(Fig. 2b). The initial magnitude of the PMA effect was generally greater than that of NGF, peaking at 10 min and falling back to a level at 60 min which was not significantly different to that observed with either NGF or CPT-cAMP (compare Fig. 2b with 2a). The activation of MAP kinase in response to phorbol esters and NGF remained elevated for at least a further two hours (data not shown).

We obtained very similar results to those illustrated in Fig. 2 when we performed assays either in immunoprecipitates using a distinct antiserum, A249 (specific for ERK2; a gift from Dr M.H. Cobb, Department of Pharmacology, Southwestern Medical Center, Dallas) or in crude cell lysates (data not shown).

We further confirmed that the myelin basic protein kinase activities seen in the pan-ERK1/2 immunoprecipitates were indeed MAP kinase by separating cell extracts by MonoQ chromatography (Fig. 3). Two peaks of myelin basic protein kinase activity were observed after 10 min treatment with NGF and CPT-cAMP. These were confirmed as ERK2 and ERK1 (eluting in fractions 26 and 28, respectively), by Western blotting with antisera specific for these isoforms (S.W.Y., M.D. and J.M.T., unpublished observations). Interestingly, ERK2 was the predominant MAP kinase isoform activated in response to PMA after 10 min, while ERK1 was stimulated more slowly, only becoming significantly activated at 120 min (Fig. 3). Clearly the mechanism of this differential activation of ERK1 and ERK2 by phorbol esters is of further interest.

Phorbol esters do not promote neurite outgrowth, thus the sustained activation of MAP kinase, while possibly necessary, is not sufficient for neurite outgrowth in PC12 cells. Perhaps NGF and CPT-cAMP, but not PMA, activate other parallel pathways that are required for the differentiation response. However, we cannot rule out the possibility that PMA (but not NGF or CPT-cAMP) causes the down-regulation of a component(s) of the differentiation machinery, although this is rather unlikely as phorbol esters do not block NGF-induced neurite outgrowth [13].

Others have demonstrated that MAP kinases translocate to the nucleus in reponse to NGF but not EGF [16,17]. Despite the fact that our pan-ERK1/2 antiserum demonstrates considerable immunoreactivity with cytosolic and nuclear ERK1 and ERK2 by Western blotting of subcellular fractions and by immunofluorescence confocal microscopy, we have been unable to detect any effect on translocation upon treatment of PC12 cells with any agonist. The reasons for this are not known and thus we have been unable to investigate the effect of CPT-cAMP or PMA on MAP kinase translocation.

The effect of CPT-cAMP is clearly of considerable interest. There are several reports that in some cell types cAMP antagonises growth factor-activated MAP kinase [24–26]. By contrast, in dog thyroid epithelial cells, MAP kinase is unaffected by cAMP [27]. The interaction between the MAP kinase pathway and the cAMP-dependent protein kinase pathway must depend, therefore, on the cell type involved and thus the mechanisms involved are likely to be complex.

How may CPT-cAMP activate MAP kinase in PC12 cells? The ability of CPT-cAMP to activate other members of the MAP kinase cascade (e.g. Raf1 and MAP kinase kinase) now warrants investigation. Perhaps CPT-cAMP activates the recently identified MEKK, the murine homologue of *Saccharomyces cerevisiae* Ste11, which phosphorylates and activates MAP kinase kinase, and has been shown to be expressed in PC12 cells [8]. In *Saccharomyces cerevisiae*, Ste11 is activated via a G-protein-dependent pathway from the 'serpentine receptors' and this has lead Lange-Carter et al. [8] to propose that MEKK might play a similar role in mammalian cells (see also Gardner et al. [28]). Alternatively perhaps, by promoting inhibitor-1 phosphorylation and thus inhibition of protein phosphatase-1 activity [29], the dephosphorylation and thus activation of Raf1 or MAP kinase kinase could be achieved. This would be unlikely as a mechanism of direct regulation of MAP kinase itself as one would have to invoke parallel increases in Thr-183 and Tyr-185 phosphorylation to achieve activation [1]. This possibility awaits identification of the physiologically relevant phosphatases involved in the regulation of the MAP kinase pathway.

In conclusion, therefore, we have shown that MAP kinase can be activated by a cAMP analogue through a mechanism that remains to be established. This activation is sustained and associated with neurite outgrowth. However, our data demonstrate that while sustained MAP kinase activation is associated with, it may not be sufficient for, neurite outgrowth and that other as yet unidentified parallel pathways may be involved.

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