Distinct signalling pathways mediate the cAMP response element (CRE)-dependent activation of the calcitonin gene-related peptide gene promoter by cAMP and nerve growth factor

Karen FREELAND, Yu-Zhen LIU[1] and David S. LATCHMAN[2]

Department of Molecular Pathology, The Windeyer Institute of Medical Sciences, University College London, The Windeyer Building, 46 Cleveland Street, London W1P 6DB, U.K.

The gene encoding the calcitonin gene-related peptide (CGRP) is activated in neuronal cells by treatment with cAMP and nerve growth factor (NGF). Both stimuli induce the phosphorylation of the cAMP response element (CRE)-binding protein (CREB) transcription factor on Ser-133 and require the CRE in the CGRP promoter to stimulate transcription. However, whereas the CRE is necessary and sufficient for promoter activation by cAMP, it is necessary but not sufficient for activation by NGF. We show that this difference is paralleled by a difference in the signalling pathways which are required for each stimulus to activate the CGRP promoter. Thus whilst cAMP-mediated activation requires the protein kinase A pathway, NGF-mediated stimulation requires the Ras/Raf mitogen-activated protein kinase kinase-1 (MEK-1)/p42/p44 mitogen-activated protein kinase (MAPK) pathway. Although NGF can activate the protein kinase C, p38 MAPK and c-Jun N-terminal kinase (JNK) pathways, these pathways are not involved in its effect on the CGRP promoter. The effect of the p42/p44 MAPK pathway on CREB and associated transcription factors, and the manner in which this results in activation of the CGRP promoter is discussed.

Key words: CREB transcription factor, gene regulation, p42/p44 MAPK pathway, protein kinase A, signal transduction.

INTRODUCTION

The single gene which encodes the peptide hormones calcitonin and calcitonin gene-related peptide (CGRP) is transcribed in cells of both the endocrine and nervous systems [1,2]. Following transcription, a tissue-specific alternative splicing event results in the production of the mRNA encoding calcitonin in thyroid C cells, and a distinct mRNA encoding CGRP is generated in specific neuronal cells in the central and peripheral nervous systems [3–5]. As well as these cell-type-specific transcriptional and post-transcriptional controls, specific stimuli have been shown to modulate the expression of the calcitonin/CGRP gene in different cell types, and the sequences in the gene promoter responsible for such transcriptional regulation have been identified. Thus, for example, it has been shown that calcitonin/CGRP gene expression is reduced in thyroid C cells treated with either glucocorticoid hormone [6] or retinoic acid [7], and this effect has been shown to be dependent upon a decrease in the activity of an enhancer element (located between −1127 and −957 bases relative to the start site of transcription in the rat gene and between −941 and −898 in the human gene), which normally strongly increases promoter activity in thyroid C cells [8,9]. Similarly, a downstream region between bases −132 and −252 mediates the stimulation of calcitonin/CGRP gene expression by cAMP in a thyroid C cell line but not in a small-cell carcinoma cell line [10].

In addition to these studies in thyroid cells, we have previously studied the response of the calcitonin/CGRP gene to different stimuli in neuronal cells [11,12]. Thus we showed that a sequence located at bases −103 to −109, which showed a close relationship to the consensus sequence of a cAMP response element (CRE), was essential for the response of the calcitonin/CGRP gene promoter to cAMP in transfected dorsal root ganglion neurons, since site-directed mutagenesis of this element rendered the construct non-responsive to cAMP [12]. Moreover, transfer of this element to a heterologous promoter was sufficient to confer cAMP inducibility on the test promoter, indicating that the CRE in the CGRP promoter is both necessary and sufficient for its response to cAMP in neuronal cells [12]. This effect is dependent upon the phosphorylation of the CRE-binding protein (CREB) transcription factor on Ser-133 after cAMP treatment of the neuronal cells [12], allowing it to stimulate transcription following binding to the CRE.

In addition to its response to cAMP, the levels of both CGRP and its corresponding mRNA have been shown to increase up to 15-fold in cultured adult sensory neurons treated with nerve growth factor (NGF), even though NGF has no effect on the survival of adult sensory neurons [13,14]. As in the case of cAMP inducibility in neuronal cells, the inducibility by NGF in adult sensory neurons is abolished by mutational inactivation of the CRE [11,12]. However, in this case, transfer of the CRE to a heterologous promoter does not render this promoter NGF inducible. Hence, the CRE in the CGRP promoter is necessary and sufficient for its response to cAMP, but is necessary and not sufficient for its response to NGF. Despite this difference between the two stimuli, NGF induces the phosphorylation of the CREB

Abbreviations used: CAT, chloramphenicol acetyltransferase; CGRP, calcitonin gene-related peptide; CRE, cAMP response element; CREB, CRE-binding protein; CBP, CREB-binding protein; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEK-1, MAPK kinase-1; NGF, nerve growth factor; PKA, protein kinase A; PKC, protein kinase C; PKI, PKA inhibitor.

[1] Present address: Pfizer Ltd., Ramsgate Road, Sandwich, Kent, U.K.

[2] To whom correspondence should be addressed at: The Institute of Child Health, University College London, 30 Guilford Street, London WC1N 1EH, U.K. (e-mail d.latchman@ich.ucl.ac.uk).

transcription factor on Ser-133 in the same manner as cAMP [12].

In view of the similarities and differences between the activation of the calcitonin/CGRP gene promoter by cAMP and NGF we have further characterized the signalling pathways which are involved in the activation of the promoter by these two different stimuli.

MATERIALS AND METHODS

Cell culture

PC12 cells were grown in Leibowitz L-15 medium supplemented with 10% (v/v) foetal-bovine serum, 0.37% (w/v) $NaHCO_3$, 2 mM L-glutamine, 0.35% (w/v) glucose, 100 units/ml penicillin and 100 μg/ml streptomycin for $\leqslant$ 15 passages. For passaging, cells were incubated in Hanks balanced salt solution for 5 min, in order to bring them into suspension, centrifuged at 145 ***g*** for 5 min and resuspended in growth medium. NGF/cAMP-treated cells were deprived of serum overnight and then resuspended in L-15 culture medium supplemented with 1% (v/v) horse serum, 0.37% (w/v) $NaHCO_3$, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. 2.5 S NGF (Sigma) 25 ng/ml and 1 mM dibutyryl cAMP (Sigma) (final concentrations) were added. Chemical inhibitors, PD98059 (New England Biolabs) at a final concentration of 50 μM and SB203580 (Calbiochem), H89 (Calbiochem) and H7 (Tocris) each at a final concentration of 20 μM, were added to cells 1 h before NGF/cAMP treatment.

Plasmid DNA

The calgcat 1 plasmid contains the region of the calcitonin/CGRP gene promoter from -1670 to $+9$ relative to the transcriptional start site [15]. The expression vectors for the constitutively active p42/p44 MEK-1 mutant (SS/DD) and the dominant negative p42/p44 mitogen-activated protein kinase (MAPK) mutants (MAPKTA and MAPKYF) were given by Dr J. Pouyssegur [16]. The Rap1 N17 construct was a gift from Dr P. J. S. Stork [17], and the protein kinase A (PKA) inhibitor (PKI) vector and the constitutively active PKA were generously given by Dr R. Maurer [18]. MAPK kinase (MKK)6 was a gift from Dr R. Davis, and the expression vectors encoding two distinct dominant negative forms of CREB, ACREB and KCREB, were generously given by Dr C. Vinson and Dr R. Goodman respectively.

Transfection

At least 16 hs after plating PC12 cells onto 6 well plates, DNA transfections were carried out with 6 μg plasmid DNA/well using the calcium phosphate procedure. Cells were transfected, for 5 h, at a density of 100000 cells/well in 1 ml of Dulbecco's modified Eagle's medium supplemented with 10% foetal-bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. For each transfection, 1 μg of β-galactosidase expression vector was added as an internal control. Cells were subsequently subjected to glycerol shock for 45 s in 15% (v/v) glycerol, 2 $\times$ Hepes buffered saline, washed twice with Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal-bovine serum and left for 16 h in medium containing 1% (v/v) horse serum before treatment. Cells were harvested 48 h after transfection, assayed for chloramphenicol acetyltransferase (CAT) activity and the results were subsequently normalized to β-galactosidase activity.

RESULTS

In the case of cAMP, it has been shown previously that phosphorylation of the CREB transcription factor is mediated via PKA (for a review see [19]). To investigate the role of PKA in mediating the effects of NGF or cAMP on the CGRP promoter, the construct calgcat-1 containing the CGRP promoter from -1680 to $+9$ was transfected into the PC12 neuronal cell line, which is responsive to both cAMP and NGF [20]. The cells were treated with either NGF or cAMP, or left unstimulated, in either the presence or absence of the PKI H89. As indicated in Figure 1 (top panel), in the absence of H89, both cAMP and NGF stimulated the promoter with a much greater effect being observed with cAMP. Upon treatment with H89, as expected,

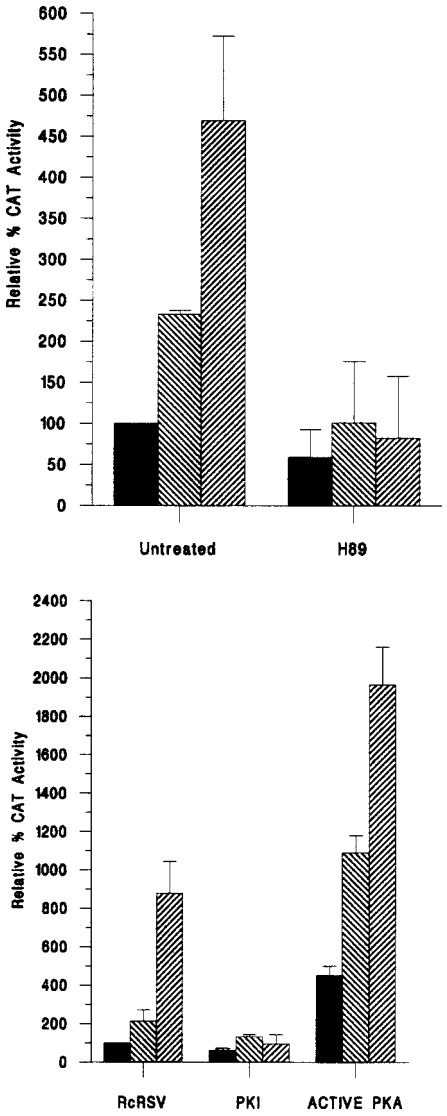


Figure 1 Effect of inhibiting the PKA pathway on activation of the CGRP promoter by cAMP or NGF

Top panel: results (shown as relative percentages of CAT activity) of transfecting PC12 cells with the calgcat 1 construct in the presence or absence of the H89 PKI. Bottom panel: effects of transfecting calgcat with either parental expression vector (RcRSV) or the same vector expressing the PKI or a constitutively active form of PKA. In each case the cells were unstimulated (■), treated with NGF (▧) or with cAMP(▨). Values represent the means $\pm$ S.D. of three independent experiments.

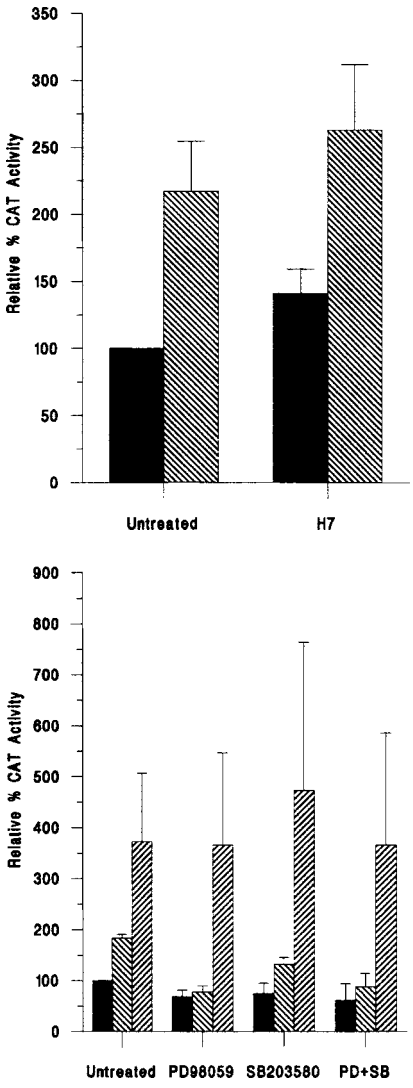


Figure 2 Effect of chemical inhibitors of PKC, p42/p44 MAPK or p38 MAPK on the expression of calgcat 1 in transfected PC12 cells

Top panel: effect of H7 (PKC inhibitor) on the expression of calgcat 1 in transfected PC12 cells, either unstimulated (■) or treated with NGF (▧). Bottom panel: effects of PD98059 (inhibitor of p42/p44 MAPK), SB203580 (inhibitor of p38 MAPK) and a combination (PD + SB) on the expression of calgcat 1 in transfected PC12 cells, either unstimulated (■), treated with NGF (▧) or cAMP (▨). The results, presented as relative percentages of CAT activity, are the means ± S.D. of three independent experiments.

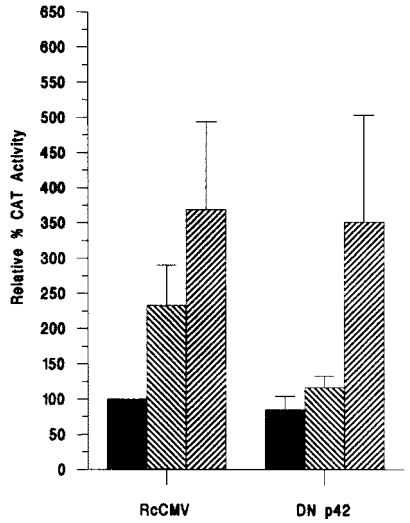
stimulation by cAMP was dramatically reduced. A decrease in promoter stimulation by NGF was also observed in the presence of H89, although this was much smaller than that observed with cAMP. Thus the activity of the promoter in the presence of NGF and H89 was not significantly different from that with NGF alone ($P = 0.08$), whereas a significant difference was observed with cAMP and H89 compared with cAMP alone ($P = 0.01$).

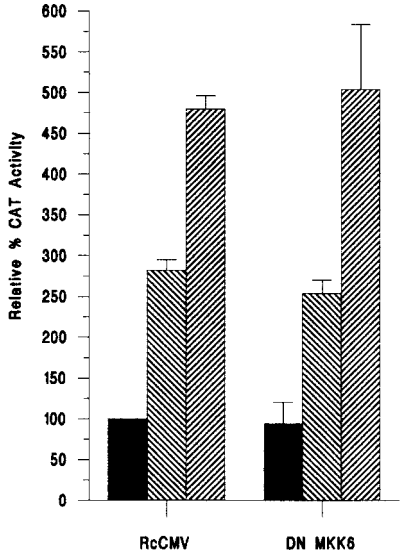
Similar results were obtained when the calgcat-1 construct was transfected with a construct encoding PKI, which binds with high affinity to the active sub-unit of PKA and inhibits its activity [21]. Thus stimulation by cAMP was virtually abolished in the presence of PKI but a less dramatic decrease in NGF inducibility was observed (Figure 1, bottom panel). As with H89, the activity of the promoter with NGF and PKI was not significantly different from that observed with NGF alone ($P = 0.88$), whereas a significant difference was observed for cAMP with PKI compared with cAMP alone ($P = 0.01$). As expected, transfection of a constitutively active form of PKA with the promoter construct resulted in enhanced activity both in the absence of any stimulation and in the presence of NGF or cAMP (Figure 1, bottom panel).

These results suggested therefore that, whereas in the case of cAMP, PKA-mediated activation of CREB was likely to be responsible for virtually all of the stimulatory effect on the promoter, this was not the case with NGF treatment, suggesting that other signalling pathways were also involved. We therefore tested the effect on promoter activation of various different chemical inhibitors that have been shown previously to inhibit specific signalling pathways which can be activated by NGF. In these experiments, the protein kinase C (PKC) inhibitor H7 had no effect on the ability of NGF to stimulate the CGRP promoter (Figure 2, top panel), despite previous findings that NGF treatment of PC12 cells can activate the PKC pathway [22]. Similarly, SB203580, a specific inhibitor of the p38 MAPK pathway, had only a minimal effect on inducibility of the promoter by NGF and had no effect on its inducibility by cAMP (Figure 2, bottom panel). In contrast, however, activation of the promoter by NGF was completely abolished by treatment with PD98059, which is a specific inhibitor of the p42/p44 MAPK pathway [23], although this inhibitor had no effect on the ability of cAMP to stimulate the promoter. Interestingly, the PD and SB compounds in combination did not produce enhanced repression of the NGF effect compared with the PD compound alone (Figure 2, bottom), again indicating that the p38 MAPK pathway is unlikely to be involved in the NGF inducibility of the CGRP promoter.

These findings suggested, therefore, that the p42/p44 MAPK pathway was important in activation of the CGRP promoter by NGF and not by cAMP. To confirm these observations, we investigated the effect of co-transfecting the calgcat reporter construct with a plasmid encoding a dominant negative form of the p42 MAPK enzyme. For comparison, we carried out similar co-transfections with a plasmid encoding a dominant negative form of MKK6, an upstream activator of the p38 MAPK pathway, as well as with a dominant negative form of the third MAPK enzyme, the c-Jun N-terminal kinase (JNK). In these experiments, the dominant negative form of p42 MAPK strongly decreased the activation of the promoter by NGF ($P < 0.02$) but had no effect on its activation by cAMP ($P = 0.74$) (Figure 3, top panel), confirming the involvement of the p42/p44 MAPK pathway. In contrast, no effect on stimulation by cAMP or NGF was observed with the dominant negative form of MKK6 (Figure 3, middle panel), which confirms the result with SB203580 and indicates that the p38 pathway was not involved in stimulation of the CGRP promoter by either stimulus. Similarly, no effect was observed with the dominant negative JNK mutant, indicating that the JNK pathway is unlikely to be involved in the effects we observed (Figure 3, bottom panel). The lack of effect of the dominant negative JNK mutant was not due to the inactivity of this construct, since it inhibited both the basal activity of the tyrosine hydroxylase promoter and its activation by anisomycin, which is known to be dependent on the JNK pathway (results not shown).

Activation of the p42/p44 MAPK pathway normally involves the initial activation of the ras G-protein, which leads to activation of the upstream kinases, raf and MEK-1, leading in turn to phosphorylation and activation of p42 MAPK [extracellular regulated kinase (ERK2)] and p44 MAPK (ERK1) [24,25]. To further investigate the involvement of this system in





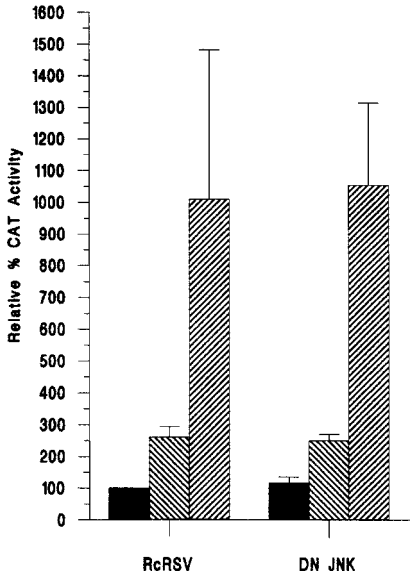


Figure 3 Effect on the activity of the calgcat 1 construct of dominant negative forms of the p42 MAPK enzyme (DN p42; top panel), the MKK6 activator of the p38 MAPK enzyme (DN MKK6; middle panel) and the JNK enzyme (DN JNK; bottom panel)

In each case the activity of the promoter is shown in unstimulated cells (■) or cells treated with NGF (▧) or cAMP (▨). In each panel, the effect of the appropriate expression vector lacking any insert (RcCMV) is shown for comparison. The results, presented as relative percentages of CAT activity, are the means ± S.D. of at least three independent experiments.

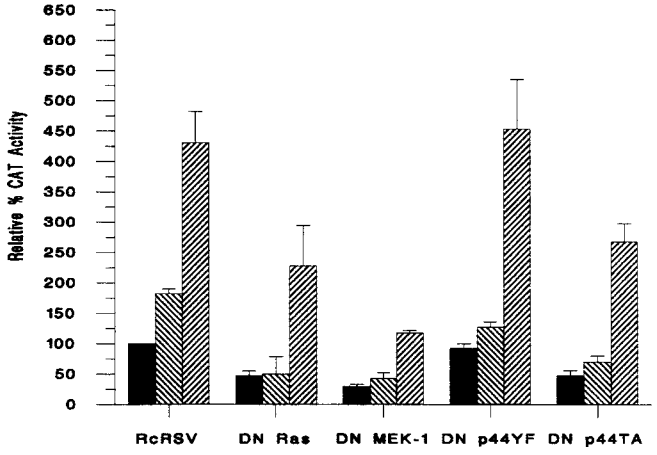


Figure 4 Effect of various dominant negative mutants of components of the p42/p44 MAPK pathway on the activity of the calgcat1 construct in PC12 cells

Results of using dominant negative (DN) forms of the Ras G-protein, the MEK-1 upstream activator and two dominant negative forms of the p44 MAPK enzyme (p44YF and p44TA) are indicated. The activity of the promoter is shown in unstimulated cells (■) or cells treated with NGF (▧) or cAMP (▨). The effect of the appropriate expression vector lacking any insert (RcRSV) is shown for comparison. The results, presented as relative percentages of CAT activity, are the means ± S.D. of three independent experiments.

NGF activation, we therefore carried out transfections of calgcat1, together with plasmids encoding dominant negative forms of Ras, MEK-1 or p44 itself. In these experiments (Figure 4), all of the mutants strongly reduced stimulation of the promoter by NGF, with a virtual abolition of promoter activation being observed both with dominant negative Ras or MEK-1. Similarly, both of the dominant negative forms of p44 MAPK produced a reduction in promoter stimulation by NGF, although this was not complete, suggesting that both p42 and p44 MAPK may be able to independently mediate stimulation of the promoter by NGF (see also Figure 3, top panel). In all cases, a statistically significant decrease in promoter activity was observed when the activity in the presence of NGF was compared in the presence or absence of the mutant construct ($P < 0.05$ in all cases). In contrast, stimulation of the promoter by cAMP was observed with each of the dominant negative mutants, suggesting that this stimulation can occur independently of the p42/p44 MAPK pathway. Promoter stimulation was reduced, however, in some cases, suggesting that this pathway may play some role in cAMP stimulation of the CGRP promoter.

We have shown previously that activation of the CGRP gene promoter by NGF required the CRE in the promoter, and was associated with phosphorylation of the CREB transcription factor on Ser-133 [12]. Moreover, the weaker activation of the CGRP promoter by cAMP compared with NGF (see Figure 1) correlated with a lower level of CREB phosphorylation induced by NGF (results not shown). To determine whether CREB was involved in activation of the CGRP promoter by the p42/p44 MAPK pathway, we co-transfected the clagcat1 construct with a constitutively active form of the MEK-1 factor, which is an

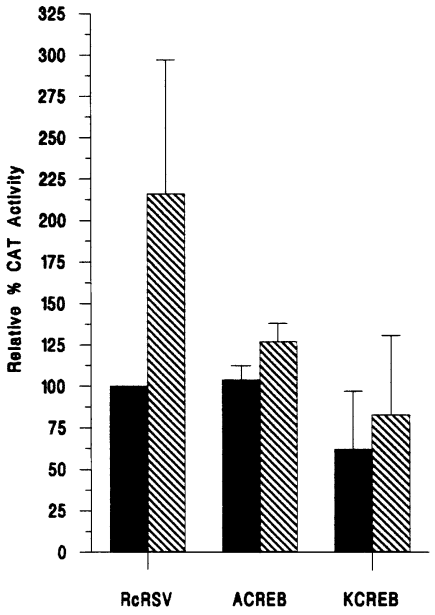


Figure 5 Effect of two dominant negative forms of the CREB factor (ACREB or KCREB) on the activity of the calgcat construct when transfected with (▨) or without (■) a plasmid encoding a constitutively active form of the MEK-1 activator (SS/DD) of the p42/p44 MAPK pathway

The effect of the appropriate expression vector lacking any insert (RcRSV) is shown for comparison. The results, presented as relative percentages of CAT activity, are the means ± S.D. of three independent experiments.

upstream activator of the p42/p44 MAPK pathway. We then included in the transfection either a control expression vector or expression vectors encoding two distinct dominant negative forms of CREB (ACREB and KCREB). In these experiments (Figure 5), the CGRP gene promoter was, as expected, activated by the constitutively active MEK-1 enzyme, which further confirmed the response of this promoter to activation of the p42/p44 MAPK pathway. However, this activation was greatly reduced by inclusion of the expression vectors for ACREB and KCREB, confirming that the CREB transcription factor was indeed involved in activation of the CGRP promoter by the p42/p44 MAPK pathway.

DISCUSSION

Previous studies have shown that NGF treatment of PC12 cells results in the activation of a number of different signalling pathways, including those involving PKC [22], protein kinase A [26], the p38 MAPK [27,28] and the p42/p44 MAPKs [28,29]. In this report, by using both chemical inhibitors and dominant negative gene mutants of each of these pathways, we have demonstrated that the p42/p44 MAPK pathway is critical for the activation of the CGRP promoter by NGF. Other studies have indicated that the p42/p44 MAPK enzymes are capable of phosphorylating the pp90 ribosomal S6 kinase (RSK) family, which in turn can phosphorylate CREB on Ser-133 [21,28,30]. It is likely, therefore, that this pathway is responsible for activating the CREB transcription factor following NGF treatment, which then enables the CREB to activate the CGRP promoter.

It has also been suggested that a similar stimulation of CREB phosphorylation can be achieved via activation of the p38 MAPK pathway, resulting in phosphorylation of MAPK-activated protein kinase 2 (MAPKAP kinase 2) and that inhibition of both the p42/p44 MAPK pathway and the p38 MAPK pathway by the chemical inhibitors PD90859 and SB203580 is necessary for the full inhibition of CREB phosphorylation following NGF treatment [28]. Similarly, it has been suggested that NGF may mediate its effects on CREB phosphorylation via a novel mitogen and stress-activated protein kinase (MSK1), which can be activated by both p42/p44 MAPK and p38 MAPK, again requiring inhibition of both these pathways to prevent phosphorylation of CREB following NGF treatment [27]. Our data, however, suggest that, in the case of the CGRP gene promoter, signalling via the p42/p44 MAPK pathway is essential for promoter activation, since inhibition of the p38 MAPK pathway by SB203580 produced only a very weak effect on promoter activation and the effect of this drug in combination with PD98059 was no stronger than the effect observed with PD98059 alone.

Hence, the p42/p44 MAPK pathway is vital for activation of the CGRP promoter by NGF. This is in contrast to the essential role of PKA in activation of the promoter by cAMP. Interestingly, we did detect a weak effect of both genetic and chemical inhibitors of the PKA pathway on CGRP gene activation by NGF, although this was much weaker than the effect observed upon cAMP inducibility. Such weak inhibition may be dependent upon cross-talk between the PKA and p42/p44 MAPK pathways, since sustained activation of the p42/p44 MAPK pathway by NGF has been shown to require PKA [30], with PKA being required for translocation of p42/p44 MAPK into the nucleus [31].

Despite the involvement of different signalling pathways, it is clear that both cAMP via activation of PKA and NGF via activation of p42/p44 MAPK lead to the phosphorylation of CREB on Ser-133. Moreover, CREB is required for activation of the CGRP promoter by these stimuli. However, as has been shown previously [12], binding of CREB to the CRE is necessary and sufficient for activation of the CGRP promoter by cAMP but is necessary and not sufficient for activation by NGF. Thus activation by NGF requires a 68 bp fragment of the CGRP promoter and cannot be produced with the 18 bp of the promoter which contain the CRE [12]. These studies suggest, therefore, that an additional transcription factor, which binds to the 68 bp region, is required for activation by NGF. The requirement for this additional factor may be due to differences in the kinetics of phosphorylation of CREB, mediated by the two different kinases [31–33]. Alternatively, it has been demonstrated that calcium/calmodulin-dependent protein kinase type II phosphorylates CREB on Ser-133 and Ser-142 and does not activate transcription via a CRE, whereas calcium/calmodulin-dependent protein kinase IV phosphorylates only Ser-133 and activates transcription [34]. Hence, it is possible that NGF and cAMP may differ in their ability to produce other post-translational modifications of CREB, which, for example, inhibit its ability to activate transcription in the absence of another co-operating transcription factor.

Interestingly, in the case of the activation of the c-fos promoter, the CRE has also been shown to be necessary, but not sufficient, for the NGF response [35,36]. In this case, it has been shown that the serum-response factor binds to a site in the c-fos promoter adjacent to the CRE and is necessary for its activation by NGF [35,36]. Interestingly, activation of the serum-response factor also requires its phosphorylation by NGF, which is mediated by the p42/p44 MAPK pathway.

In the case of the CGRP promoter, the factor which co-operates with CREB to activate the promoter following NGF

treatment has not yet been identified. Although this factor may be constitutively active in the presence or absence of NGF, with activation of CREB being the key signalling event, it is evidently also possible that, as in the case of the c-fos promoter, this additional factor is also activated following NGF treatment. Hence, it is possible that the critical role for the p42/p44 MAPK pathway which we have identified may reflect its actions not only on CREB but also on another transcription factor that can bind to the region of the CGRP promoter which is critical for activation by NGF.

As well as being involved in the activation of CREB, and potentially of other DNA binding transcription factors, it is also clear that the p42/p44 MAPK pathway can stimulate the activity of the CREB-binding protein (CBP) transcription factor. This factor was initially identified as a transcriptional co-activator, which binds to CREB only following phosphorylation on Ser-133 and then stimulates transcription, so explaining why Ser-133 phosphorylation is necessary for CREB to activate transcription [37,38]. However, we have recently shown that the ability of CBP itself to stimulate transcription is enhanced by NGF treatment with this effect occurring even when CBP is recruited to the DNA via a heterologous DNA-binding domain [39]. Such activation is dependent upon the p42/p44 MAPK pathway and can therefore be inhibited by PD98059 as well as dominant negative forms of the factors in this pathway [39]. Hence, the effect of p42/p44 MAPK on activation of the CGRP promoter may also involve a need for CBP to be activated in order to stimulate transcription following its interaction with CREB, which has been phosphorylated by p42/p44 MAPK rather than by PKA.

A number of different targets, including CREB, CBP and other, as yet unidentified, transcription factors may therefore be involved in the effect of p42/p44 MAPK on activation of the CGRP promoter by NGF. It is already clear, however, from the present studies that such activation by p42/p44 MAPK is required for activation of the promoter by NGF, whereas this does not occur following cAMP stimulation, even though the effect of both stimuli on the CGRP promoter requires the CREB transcription factor and an intact CRE in the promoter.

This work was supported by the Biotechnology and Biological Sciences Research Council (BBSRC). K.F. is supported by a BBSRC Research Studentship.

REFERENCES

1 Rosenfeld, M. G., Amara, S. G. and Evans, R. M. (1984) Science **225**, 1315–1320
2 Rosenfeld, M. G., Mermod, J. J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. and Evans, R. M. (1983) Nature (London) **304**, 129–135
3 Amara, S. G., Jonas, V., Rosenfeld, M. G., Ong, E. S. and Evans, R. M. (1982) Nature (London) **298**, 240–244
4 Crenshaw, E. B., Russo, A. F., Swanson, L. W. and Rosenfeld, M. G. (1987) Cell **49**, 389–398
5 Leff, S. E., Evans, R. M. and Rosenfeld, M. G. (1987) Cell **48**, 517–524
6 Tverberg, L. A. and Russo, A. F. (1992) J. Biol. Chem. **267**, 17567–17573
7 Lanigan, T. M., Tverberg, L. A. and Russo, A. F. (1993) Mol. Cell. Biol. **13**, 6079–6088
8 Stolarsky-Freedman, L., Leff, S. E., Klein, E. S., Crenshaw, E. B., Yeakley, J. and Rosenfeld, M. G. (1990) Mol. Endocrinol. **4**, 497–504
9 Ball, D. B., Compton, D., Nelkin, B. D., Baylin, S. B. and de Bustros, A. (1992) Nucleic Acids Res. **20**, 117–123
10 de Bustros, A., Lee, R. Y., Compton, D., Tsong, T. Y., Baylin, S. B. and Nelkin, B. D. (1990) Mol. Cell. Biol. **10**, 1773–1778
11 Watson, A., Ensor, E., Symes, A., Winter, J., Kendall, G. and Latchman, D. (1995) Eur. J. Neurosci. **7**, 394–400
12 Watson, A. and Latchman, D. S. (1995) J. Biol. Chem. **270**, 9655–9660
13 Lindsay, R. M. (1988) J. Neurosci. **8**, 2394–2405
14 Lindsay, R. M. and Harmar, A. J. (1989) Nature (London) **337**, 362–364
15 Symes, A. J., Craig, R. K. and Brickell, P. M. (1992) FEBS Lett. **306**, 229–233
16 Lavoie, J. N., L'Allemain, G., Brunet, A., Muller, R. and Pouyssegur, J. (1996) J. Biol. Chem. **271**, 20608–20616
17 York, R. D., Yao, C. H., Dillon, T, Ellig, C. L., Eckert, S. P., McClesky, E. W. and Stork, P. J. (1998) Nature (London) **392**, 622–626
18 Maurer, R. A. (1989) J. Biol. Chem. **264**, 6870–6873
19 Lalli, E. and Sassone-Corsi, P. (1994) J. Biol. Chem. **269**, 17359–17362
20 Greene, L. A. and Tischler, S. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2424–2428
21 Ginty, D. D., Glowacka, D., DeFranco, C. and Wagner, J. A. (1991) J. Biol. Chem. **266**, 15325–15333
22 Hama, T., Huang, K.-P. and Guroff, G. (1986) Proc. Natl. Acad. Sci. U.S.A. **86**, 1756–1760
23 Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J. and Saltiel, A. R. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7689–7689
24 Marshall, C. J. (1995) Cell **80**, 179–185
25 Lenormand, P., Sardet, C., Pages, G., L'Allemain, G., Brunet, A. and Pouyssegur, J. (1993) J. Cell. Biol. **122**, 1079–1088
26 Schubert, D, Heinemann, S. and Kidokoro, Y. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 2579–2583
27 Deak, M., Clifton, A. D., Lucocq, J. M. and Alessi, D. R. (1998) EMBO J. **17**, 4426–4441
28 Xing, J., Ginty, D. D. and Greenberg, M. E. (1996) Science **273**, 959–963
29 Cowley, S., Paterson, H., Kemp, P. and Marshall, C. J. (1994) Cell **77**, 841–852
30 Xu, A., Kornhauser, J. M., Xia, Z, Thiele, E. A. and Greenberg, M. E. (1998) Mol. Cell. Biol. **18**, 1946–1955
31 Yao, H, York, R. D., Misra-Press, A., Carr, D. W. and Stork, P. J. S. (1998) J. Biol. Chem. **273**, 8240–8247
32 Impey, S., Obrietan, K., Wong, S. T., Poser, S., Yano, S., Wayman, G., Deloulme, J. C., Chan, G. and Storm, D. R. (1998) Neuron **21**, 869–883
33 Nguyen, T. T., Scimeca, J.-C., Filloux, C., Peraldi, P., Carpentier, J.-L. and van Obberghen, E. (1993) J. Biol. Chem. **268**, 9803–9810
34 Sun, P., Enslen, H., Myung, P. S. and Maurer, R. A. (1994) Genes Dev. **8**, 2527–2539
35 Ginty, D. D., Bonni, A. and Greenberg, M. E. (1994) Cell **77**, 713–725
36 Bonni, A., Ginty, D. D., Dudek, H. and Greenberg, M. E. (1995) Mol. Cell. Neurosci. **6**, 168–183
37 Chriva, J. C., Kwok, R. P. S., Lamb, N., Hagiwara, M., Montminy, M. R. and Goodman, R. (1993) Nature (London) **365**, 855–859
38 Kwok, R. P. S., Lundblad, J. R., Chriva, J. C., Richards, J. P., Bachinger, H. P., Brennan, R. G., Roberts, S. G. E, Green, M. R. and Goodman, R. H. (1994) Nature (London) **370**, 223–226
39 Liu, Y.-Z., Chirivia, J. C. and Latchman, D. S. (1998) J. Biol. Chem. **273**, 32400–32407

Received 18 June 1999/17 September 1999; accepted 10 November 1999