British Journal of Pharmacology (2003) 138, 751–756

www.nature.com/bjp

Identification of MEK1 as a novel target for the treatment of neuropathic pain

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1 In the present study we have attempted to identify changes in gene expression which are associated with neuropathic pain using subtractive suppression hybridization analysis of the lumbar spinal cord of animals suffering streptozocin induced diabetic neuropathy.

2 Using this approach, we found a significant up-regulation of several key components of the extracellular signal-regulated kinase (ERK) cascade. These findings were confirmed by Western blot analysis, which demonstrated that the levels of active ERK1 and 2 correlated with the onset of streptozocin-induced hyperalgesia.

3 Intrathecal administration of the selective MAPK/ERK-kinase (MEK) inhibitor PD 198306 dose-dependently (1–30 μg) blocked static allodynia in both the streptozocin and the chronic constriction injury (CCI) models of neuropathic pain.

4 The antihyperalgesic effects of PD 198306, in both the streptozocin and CCI models of neuropathic pain, correlated with a reduction in the elevated levels of active ERK1 and 2 in lumbar spinal cord.

5 Intraplantar administration of PD 198306 had no effect in either model of hyperalgesia, indicating that changes in the activation of ERKs and the effect of MEK inhibition are localized to the central nervous system.

6 In summary, we have demonstrated for the first time that the development of neuropathic pain is associated with an increase in the activity of the MAPK/ERK-kinase cascade within the spinal cord and that enzymes in this pathway represent potential targets for the treatment of this condition.
British Journal of Pharmacology (2003) **138,** 751–756. doi:10.1038/sj.bjp.0705103

Keywords: Gene expression; ERK; hyperalgesia; neuropathic pain; allodynia

Abbreviations: BL, baseline; CCI, chronic constriction injury; CREB-IR, immuno-reactive cyclic AMP response element-binding protein; ERK, extracellular signal-regulated kinase; Ipl, intraplantar; i.t., intrathecal; MAPK, mitogen-activated protein kinase; MED, minimum effective doses; MEK, MAPK/ERK-kinase; ROD, relative optical density

Introduction

A common and severely debilitating symptom of neuropathic pain conditions is so-called hyperalgesia, a heightened pain response generated by a painful stimulus that is often resistant to conventional analgesics. The general failure of analgesics to treat these conditions may be a consequence of long term changes in neuronal processing in the spinal cord (Iadarola & Caudle, 1997). Such changes may stem from 'central sensitization' caused by persistent C/Aβ fibre input to the spinal cord (Basbaum & Woolf, 1999). This process can increase the excitability of neurones present in the dorsal horn *via* a number of mechanisms including sprouting of primary sensory neurones and the altered transcription of numerous genes (Yaksh, 1999). Indeed changes in expression of a variety of neurotransmitters, their receptors and other genes in both the spinal cord and the dorsal root ganglia have been shown to be associated with hyperalgesia (Woolf & Costigan, 1999).

Despite the wealth of information that exists detailing the physiological and neuroanatomical changes that are associated with the development of neuropathic pain (Zimmermann, 2001; Bridges *et al*., 2001), the precise molecular mechanisms that underlie its propagation and maintenance still remain poorly understood. Much of the cellular plasticity related to the hyperalgesic state may ultimately be regulated by temporal and spatial alterations in gene expression (Woolf & Salter, 2000). In view of this, in the present study we initially attempted to investigate the global changes in gene expression which are associated with this condition using the unbiased approach of subtractive suppression hybridization (Diatchenko *et al*., 1996). During the course of these studies we identified elements of the MAPK/ERK pathway as key components in the generation of hyperalgesia. In view of this, our studies were extended to clarify the therapeutic potential of this enzymic system for the treatment of neuropathic pain. Some of these results have been communicated to the Physiological Society (Ciruela *et al*., 2001).

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Methods

Surgery

Neuropathic pain was induced in male Sprague Dawley rats (250–300 g), obtained from Charles River, (Margate, U.K.). All procedures were performed according to the Home Office Animals Scientific Procedures Act 1986. In the streptozocin-induced diabetic model, diabetes was induced in rats by a single i.p. injection of 50 mg kg^{-1} streptozocin (Aldrich, U.K.), dissolved in 0.9% w v^{-1} NaCl as described previously (Courteix *et al*., 1993). The Chronic Constriction Injury (CCI) model was generated as previously described (Bennett & Xie, 1988). Briefly, four loose ligatures of 4-0 chromic gut suture were placed 1 mm apart above the trifurcation of the sciatic nerve on one side of the animal whilst under anaesthesia.

Intrathecal injections

Compounds were administered intrathecally in a volume of 10 μl using a 100 μl Hamilton syringe by exposing the spine of the rats under brief isoflurane anaesthesia. A single injection was made into the intrathecal space between lumbar region 5–6 with a 10 mm long 27 gauge needle. Penetrations were judged successful if there was a tail flick response. The wound was sealed with an autoclip and rats appeared fully awake within 2–3 min following injection.

Behavioural studies

Paw withdrawal threshold to mechanical stimulation was assessed prior to induction of neuropathy and tactile allodynia was assessed 2 weeks later by measuring the force required in grams to elicit paw withdrawal using von Frey hairs. The paw withdrawal threshold in neuropathic animals was significantly reduced compared to the measurements taken before induction of neuropathy (data not shown). Baseline (BL) measurements were taken before treatment. Animals received single doses of intrathecal (i.t.) or intraplantar (ipl) of PD 198306 (1–30 μg per 10 μl and 3 mg per 100 μl respectively, suspended in cremophor:ethanol:water, 1 : 1 : 8; synthesized at Parke-Davis, Ann Arbor, MI, U.S.A.), and withdrawal thresholds were re-assessed at 30 min, 1 h and 2 h after treatment. All experiments were carried out by an observer blind to drug treatments. All studies were performed using parallel positive (pregabalin, synthesized at Parke-Davis, Ann Arbor, MI, U.S.A.) and negative (saline) controls.

Analysis of differential gene expression

After demonstrating the presence of mechanical allodynia 2 weeks after induction of the model only in the streptozocin treated group, animals from both control and treated groups were sacrificed by an approved schedule 1 procedure, stunning and decapitation. The lumbar spinal cords (L4–L5) from each group were rapidly dissected and total RNA extracted using Trizol reagent to manufacturer's specifications (Invitrogen, Paisley, U.K.). These samples were then further enriched for mRNA using the message maker kit (Invitrogen). The PCR-select methodology (Clontech, Palo Alto, U.S.A.) was used to generate a subtracted cDNA library containing partial fragments of genes potentially up-regulated in the lumbar spinal cord of hyperalgesic rats. Five hundred clones were selected at random and sequenced on an Applied Biosystems 310 genetic analyser (Perkin Elmer, Connecticut, U.S.A.).

Broad scale expression profiling was carried out using an Atlas cDNA expression array (Clontech). Trizol reagent was used to extract total RNA from the lumbar spinal cord of four control and four streptozocin treated animals (Invitrogen). RNA was pooled from animals in the same treatment group and 1 μg reverse transcribed in the presence of [α-^{32}P]-dATP, to generate labelled cDNA for probing of membranes. Following hybridization membranes were washed and exposed to X-ray film at -70°C. Analysis of autoradiographs generated was carried out using an MCID image analyser (model M4, Imageworks). The image was scanned in and converted into a digitized image of 256 shades of grey. Measurements were made by selecting the gene of interest and using a template of a fixed size over the area. Measurements were then taken over the linear range of relative optical density (ROD) levels. The ROD levels of nine housekeeping genes which were used as standards for normalization of the signal thus enabling comparisons to be made between membranes.

Western blot analysis

Lumbar spinal cords were dissected from animals behaviourally tested at 1, 2 and 3 weeks after induction of the model. In the case of the CCI model the spinal cord was hemisected into the ipsilateral and contralateral sides. The spinal cords were homogenized in lysis buffer (in mM Tris (pH 7.5) 20, NaCl 150, EDTA 1, EGTA 1, sodium pyrophosphate 2.5, β-Glycerolphosphate 1, Na_3VO_4 1, PMSF 1, Triton X-100 1%, Leupeptin 1 μg ml^{-1}). Samples containing 20 μg of protein were separated in 10% SDS-polyacrylamide gels and electrophretically transferred to nitrocellulose membranes (Novex) using a wet transfer apparatus (Novex). Membranes were incubated in a blocking buffer containing 4% (w v^{-1}) non-fat dry milk in TBS with 0.1% Tween 20. Membranes were probed with phospho-p42/44 MAPK in blocking buffer for 1 h at room temperature at 1 : 2000 dilution, washed three times in TBS-T, and then detected using a horseradish peorxidase-conjugated secondary antibody and LumiGlo reagent (New England Biolabs). *p*Elk-1 was detected with the p44/42 MAP Kinase Assay Kit. (New England Biolabs).

Results

Gene expression studies

To confirm that the technique of subtractive suppression hybridization was successful, we analysed the levels of the housekeeper genes β-Actin and glyceraldehyde-3-phosphate in forward and back subtracted libraries. Semi quantitative PCR revealed that these genes were more than 500 fold downregulated in each library demonstrating that the subtraction procedure had been performed efficiently (not shown). Sequencing and bioinformatical analysis of 500 potentially up-regulated clones from the forward subtracted

library revealed that several members of the p44/42 mitogen-activated protein kinase (MAPK) pathway, also referred to as the extracellular signal-regulated kinase (ERK) cascade, were present. Multiple clones were identified as ERK1 and ERK2. In addition multiple clones in the subtracted library were identified as Ras and Raf, genes upstream of ERK1 and 2 in the MAPK signalling cascade.

Subsequent to this observation, broad scale expression profiling was carried out using the Atlas stress toxicology cDNA expression array (Clontech). This revealed that levels of ERK1 and ERK2 mRNA were indeed significantly increased in the lumbar spinal cord of STZ-treated rats compared to saline-treated control animals (Figure 1). Relative optical densities (ROD) measured for each gene were normalized to those of nine housekeeping genes. ROD for ERK1: control = 0.82 ± 0.08, STZ = 1.75 ± 0.05 for ERK2: control = 0.18 ± 0.02, STZ = 0.24 ± 0.01 ($n = 3$; $P < 0.05$). Having confirmed that the level of expression of ERK1 and 2 mRNA increased in the lumbar spinal cord following the induction of neuropathic pain, we endeavoured to determine if this correlated with an increase in the level of the active protein.

Western blot analysis

ERKs may become doubly phosphorylated by an upstream MAPK/ERK-kinase (MEK) on both the threonine and tyrosine residues in the catalytic core of the protein, thus rendering them active (Derkinderen *et al*., 1999). Active ERKs may then phosphorylate a wide range of proteins, including other enzymes, constituents of the cellular cytoskeleton and specific transcription factors, like Elk-1, which can therefore be used as a measure of ERK activity (Sugden & Clerk, 1997).

Having confirmed the presence or absence of static allodynia, the levels of phosphorylated Elk in the lumbar spinal cords of streptozocin-treated and control rats were compared by Western blot analysis. Three rats were used for each treatment group, within each group protein extracted from the lumbar spinal cord was pooled. This revealed that streptozocin induced a time-dependent increase in the activity of both ERK1 and ERK2 (Figure 2). The increase in ERK activity was first observed 1 week after streptozocin treatment and was maximal at week 3. Significantly this correlated directly with the observed hyperalgesia, even in the control saline treated animals that spontaneously exhibited hyperalgesia.

Behavioural analysis

Having confirmed that the increase in expression levels of the ERKs mRNAs correlated with an increase in the level of the active protein, the antihyperalgesic effects of the MEK inhibitor PD 198306 was investigated in two animal models of neuropathic pain.

Intrathecal administration of PD 198306 dose-dependently (1 – 30 μg) blocked static allodynia the streptozocin model of neuropathic pain (Figure 3a; $P < 0.001$). The minimum effective doses (MED) of 3 μg significantly blocked static allodynia 30 min after treatment. Both 10 μg and the highest dose used (30 μg) totally blocked the maintenance of static allodynia, for up to 1 h (Figure 3a). Western blot analysis was subsequently carried out on the lumbar spinal cord of the control and PD 198306 treated animals (30 μg). This demonstrated that PD 198306 treatment also resulted in an observable reduction in the streptozocin induced increase in the level of active ERK1 and 2.

Having confirmed that the activity of ERK1 and 2 increases in the lumbar spinal cord following streptozocin induced hyperalgesia, and the resultant static allodynia can be blocked by PD 198306, we investigated these findings in an additional model of neuropathic pain, the CCI model. Intrathecal administration of PD 198306 dose-dependently (1 – 30 μg) blocked the static allodynia associated with this condition (Figure 3c; $P < 0.001$). The MED associated with this response was 10 μg and as observed with the streptozocin treated animals, this effect lasted 1 – 2 h after administration (Figure 3c). Subsequent Western blot analysis revealed that treatment with PD 198306 resulted in a reduction in the level of active ERK1 and ERK2 (Figure 3d).

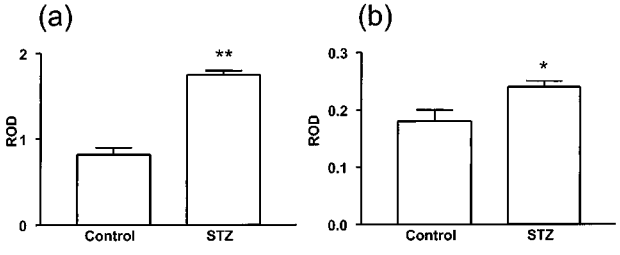

Figure 1 Relative gene expression levels of ERK 1 (a) and 2 (b) in the rat spinal cord of control and streptozocin treated rats. Broad scale expression profiling was carried out using an Atlas cDNA expression array (Clontech) to compare the level of ERK 1 and 2 gene expression in the lumbar spinal cord of four control and four streptozocin treated animals. The presence or absence of static allodynia was determined in each animal, RNA was pooled from animals in the same treatment group. Measurements were then taken over the linear range of relative optical density (ROD) levels and the levels of nine housekeeping genes were used as standards for normalization. It was evident from the measurements made that the expression of ERK1 (a) and ERK 2 (b), were significantly increased in the rat spinal cord following streptozocin treatment (*$P < 0.05$, **$P < 0.01$; *t*-test, $n = 3$).

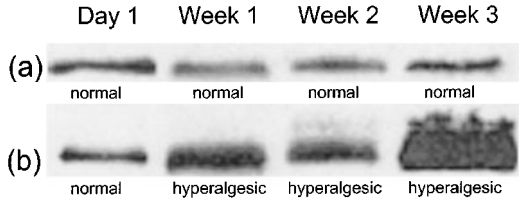

Figure 2 Western blot analysis of ERK1 and ERK2 activity in the lumbar spinal cord of control (a) and streptozocin (b) treated animals. The size and intensity of bands is directly proportional to the level of *p*Elk-1, this being a direct measurement of ERK activity. Where stated 'hyperalgesia' indicates that the animal was exhibiting static allodynia as determined by the use of Von Frey hairs, whilst normal denotes the animals were not showing any form of hyperalgesia. Three rats were used for each treatment group, within each group protein extracted from the lumbar spinal cord was pooled. It is clearly evident that an increase in the level of *p*Elk-1 is directly linked to the on-set of static allodynia 1 week after streptozocin treatment.

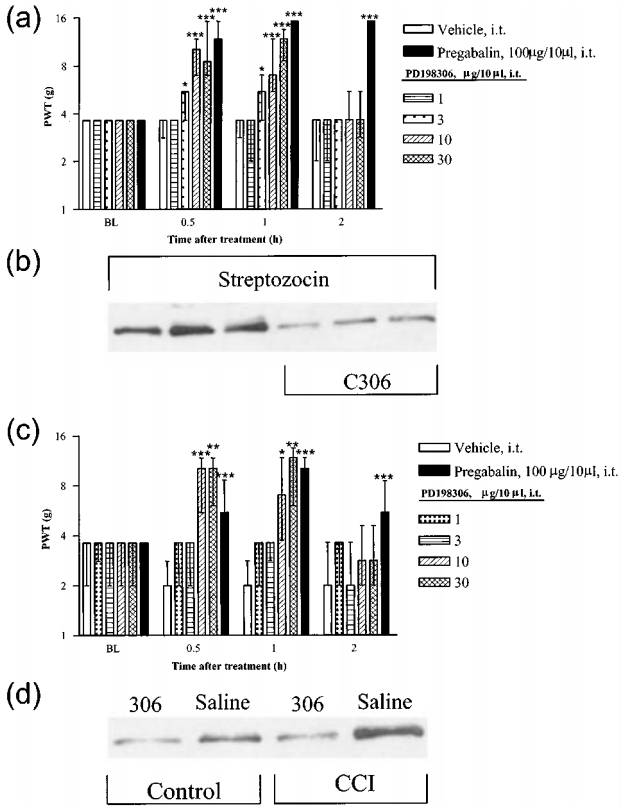

Figure 3 Effect of intrathecal (i.t.) administration of PD 198306 on streptozocin-induced (a) and CCI-induced (c) static allodynia. PD 198306 significantly blocks the onset of static allodynia in both the streptozocin and CCI models of neuropathic pain. In both instances the antihyperalgesic effects of PD 198306 were detected 30 min after administration and were effective for 1 h. The minimal effective dose for PD 198306 was 3 μg and 10 μg for streptozocin and CCI treatment respectively. Following each experiment the level of ERK1 and 2 activity the lumbar spinal cord was analysed by Western blotting. A significant reduction in the level of *p*Elk-1 can be seen in both instances following PD 198306 administration (b and d). All experiments were carried out by an observer blind to drug treatments. Penetrations were judged successful if there was a tail flick response. Results are expressed median $\pm$ first and third quartiles. ($^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$; Kruskall–Wallis ANOVA for non-parametric results, followed by Mann–Whitney *t*-test *vs* vehicle group; $n=6-9$).

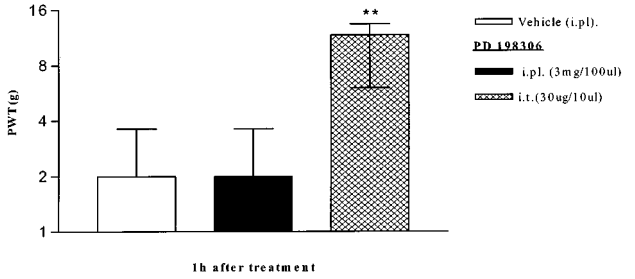

Figure 4 Comparison of the effect of intraplantar (ipl) and intrathecal (i.t.) administration of PD 198306 on CCI induced static allodynia. Intraplantar PD 198306 treatment (3 g per 100 μl) had no discernible effect, whilst intrathecal administration (3 μg per 10 μl) significantly blocked CCI induced static allodynia). All experiments were carried out by an observer blind to drug treatments. Penetrations were judged successful if there was a tail flick response. Results are expressed median $\pm$ first and third quartiles. ($^{**}P<0.01$, Kruskall–Wallis ANOVA for non-parametric results, followed by Mann–Whitney *t*-test *vs* vehicle group; $n=6-9$).

In an attempt to gain more information on the site of action of PD 198306 we used a peripheral route of administration, injecting into the ipsilateral hind paw. However, intraplantar administration of PD 198306 had no significant effect on CCI induced hyperalgesia (Figure 4).

Discussion

The results obtained in this study demonstrate for the first time a role for ERK signalling in animal models of neuropathic pain. These molecules have been previously implicated in nociception, however these observations have only ever been made in inflammatory models of pain (Ji *et al*., 1999; Aley *et al*., 2001; Karim *et al*., 2001).

Pain is a multi-faceted disorder, and can be generally compartmentalized into physiological, inflammatory, or neuropathic pain. Underlying each of these, there are believed to be complex and distinct molecular mechanisms that contribute to their generation and propagation (Woolf & Salter, 2000).

Inflammatory pain is generated by the action of inflammatory mediators on primary afferents. Whereas chronic or neuropathic pain often arises from damage to peripheral nerves, which may be mechanical or the result of diabetes or viral infection (Payne, 1986). In contrast to inflammatory pain, the mechanisms associated with neuropathic pain are poorly understood and hence the observations made in the present study are of particular interest.

At this stage it is difficult to establish a defined role for spinal ERKs in neuropathic pain. However, it is important to note that the role ERKs play in this process is central to, and apparently identical in both STZ and CCI induced neuropathic pain. This is an important point since it demonstrates that the initial observation of ERKs in the STZ model was not an effect resulting from the induced diabetes, but was directly linked to the induced neuropathy.

Activation of ERKs affects many cellular and physiological processes, including apoptosis, cell proliferation, development and differentiation (Lewis *et al*., 1998). However, one of the primary ways in which ERK activation may influence the generation and propagation of neuropathic pain is *via* the regulation of gene expression. It has been well documented that ERKs regulate transcription *via* the activation of Rsk and the subsequent phosphorylation of cyclic AMP response element binding protein (CREB) (Impey *et al*., 1998). Therefore, it is of particular interest to note that in the Seltzer model of neuropathic pain the number of phosphorylated CREB immunoreactive cells is significantly increased in the dorsal horn ipsilateral to the injury (Ma & Quirion, 2001). In addition, Ma & Quirion demonstrated that the majority of the CREB-IR cells also co-expressed protein kinase Cγ, directly implicating them in the processing of neuropathic pain (Malmberg *et al*., 1997). In the present study the methods employed do not identify the cells in which ERK activity is up-regulated. However if the observed up-regulation in spinal ERK activity is directly linked to the

observations of Ma & Quirion it is likely that these changes are also occurring in the cells of the dorsal horn.

Such an increase in ERK activity would directly influence transcriptional activity in these cells. Indeed, the expression levels of a number of genes have been reported to alter following the induction of neuropathic pain. For example, the level of gene expression for the sodium channel auxiliary subunits $\beta 1$, $\beta 2$ and $\beta 3$ have all been shown to alter following both CCI and streptozocin treatment (Blackburn-Munro & Fleetwood-Walker, 1999; Shah *et al*., 2001). In addition an increase in both interleukin-6 and c-*fos* mRNA levels have been observed in the dorsal horn following peripheral nerve injury (Arruda *et al*., 1998; Huang & Simpson, 1999). It is possible that some or all of these observed alterations in gene expression could result from transcriptional regulation by ERKs in the dorsal horn. However the observed antihyperalgesic effects of PD 198306 are too rapid to influence any ERK mediated transcriptional regulation. It is more likely that any ERK-mediated alterations in gene expression contribute the propagation and chronic maintenance of neuropathic pain. The immediacy PD 198306 effects on static allodynia are probably the result of inhibiting ERKs phosphorylation of ion channels and receptors that are responsible for altered neuronal excitability.

Such changes in gene expression represent an important mechanism by which neurones are able to translate transient electrical events into more permanent functional and morphological alterations. It has been demonstrated that calcium entry in neurones activates transcriptional activity *via* the ERK signalling cascade (Johnson *et al*., 1997). These events may well underlie the neural plasticity that results in the characteristic central sensitization associated with chronic neuropathies. The importance of the ERKs in long-term potentiation, particularly with respect to memory and learning, is well established (Chang & Karin, 2001). Here we demonstrate a role for the ERKs in the 'rewiring' responsible for long term central sensitization, resulting in the development of both hyperalgesia and allodynia. Indeed, the central nature of this phenomenon is demonstrated by the inability of intraplantar administered PD 198306 to alleviate CCI induced hyperalgesia. It has also been previously demonstrated that induction of ERK phosphorylation occurs in the dorsal horn following intraplantar capsaicin injection (Ji *et al*., 1999).

In summary, we have identified the involvement of the ERK signalling cascade in neuropathic pain and demonstrated that MEK1 represents a novel and effective target for treatment of this complex and debilitating disorder.

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(Received October 9, 2002
Accepted November 14, 2002)