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European Journal of Pharmacology 469 (2003) 29–36
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Characterization of bradykinin-induced prostaglandin E_2 release from cultured rat trigeminal ganglion neurones

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Received 30 January 2003; received in revised form 7 April 2003; accepted 15 April 2003

Abstract

Bradykinin and prostaglandins are both local mediators strongly implicated in pain and inflammation. Here, we have investigated the effects of bradykinin on the release of prostaglandin E_2 from cultured neurones derived from adult rat trigeminal ganglia. Bradykinin was a potent inducer of prostaglandin E_2 release, an effect that was likely mediated by bradykinin B_2 receptors, as the bradykinin-induced prostaglandin E_2 release was attenuated by the bradykinin B_2 receptor-selective antagonist, arginyl-L-prolyl-trans-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α, 3β, 7aβ)-octahydro-1*H*-indole-2-carbonyl-L-arginine (HOE 140), but not by the bradykinin B_1 receptor-selective antagonist, des-Arg^9,[Leu^8]-bradykinin. Furthermore, bradykinin-induced prostaglandin E_2 release was inhibited following treatment with the phospholipase A_2 inhibitor, arachidonyltrifluoromethyl ketone ($AACOCF_3$), the nonselective cyclooxygenase inhibitor, piroxicam, the mitogen-activated protein kinase kinase-1 (MEK1) inhibitor, 2′-amino-3′-methoxyflavone (PD98059), and the protein kinase C inhibitor, bisindolylmaleimide XI (Ro320432). Taken together, these data suggest that bradykinin, acting via bradykinin B_2 receptors, induces prostaglandin E_2 release from trigeminal neurones through the protein kinase C and mitogen-activated protein kinase-dependent activation of phospholipase A_2 and consequent stimulation of cyclooxygenases.

Keywords: Bradykinin; Prostaglandin E_2; Trigeminal; Migraine; Primary culture

1. Introduction

Prostaglandins and bradykinin are both local mediators that have been strongly implicated in inflammation and pain. The role of prostaglandins, particularly those of the E-series, in these processes is well established, partly because of the antiinflammatory and antinociceptive effects of nonsteroidal antiinflammatory drugs (NSAIDS), but also because of observations that exogenous administration of prostaglandins can cause hyperalgesia and allodynia (Vane, 1971; Bley et al., 1998). Similarly, the nonapeptide, bradykinin, also induces nociceptive responses in animal models of pain and causes pain in humans (Dray and Perkins, 1993).

Bradykinin is a kininogen derivative and a known activator of sensory neurones (see Calixto et al., 2000; Dray and Perkins, 1997). In addition, it has been shown to activate systems that have been implicated in pain transmission during migrainous headache. For example, bradykinin stimulates primary afferent neurones and also increases the firing rate of second order neurones of the trigeminal nucleus caudalis (Ebersberger et al., 1997), which is innervated by the trigeminal fifth cranial nerve, the cell bodies of which reside in the trigeminal ganglia. In addition, bradykinin has also been shown to enhance the release of the vasodilator neuropeptide, calcitonin gene-related peptide (CGRP) from cultured dorsal root and trigeminal ganglion neurones (Vasko et al., 1994; Hingtgen et al., 1995; Jenkins et al., 2001b). Bradykinin exerts its effects through the activation of seven transmembrane G protein coupled receptors, termed B_1 and B_2 receptors, respectively. Bradykinin B_1 receptors appear to largely be

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doi:10.1016/S0014-2999(03)01732-1

up-regulated in response to inflammation and tissue damage, whereas bradykinin B_2 receptors exhibit a more widespread constitutive distribution pattern (Marceau and Bachvarov, 1998). However, despite these differences, both these receptors are expressed constitutively in both dorsal root and trigeminal ganglia (Ma et al., 2000; Seabrook et al., 1997).

In addition to their accepted role in inflammatory pain, E-series prostaglandins have also been implicated in migraine headache. The cyclooxygenase inhibitor, aspirin, is an effective antimigraine agent, particularly when administered intravenously (see Limmroth et al., 1999) and the infusion of E-series prostaglandins also causes migraine-like symptoms in human volunteers (see Coleman et al., 1990). Furthermore, prostaglandin E_2 levels are raised in the saliva and jugular venous blood of patients undergoing migraine attacks (Tuca et al., 1989; Sarchielli et al., 2000). It has also been shown that prostaglandin E_2 is released from rat dura mater encephali following stimulation of the trigeminal ganglion either electrically or chemically with an ''inflammatory soup'' comprising 5-hydroxytryptamine, histamine and bradykinin (Ebersberger et al., 1999). Finally, studies from our laboratory have recently shown that prostaglandin E_2 can induce the release of CGRP from cultured trigeminal neurones (Jenkins et al., 2001a).

In light of this evidence, coupled with the potential involvement of prostaglandin E_2 in the peripheral neurogenic inflammation that may accompany a migraine attack, we have used cultured trigeminal neurones to investigate the receptor types and signalling mechanisms involved in mediating bradykinin-induced prostaglandin E_2 release from cultured rat trigeminal neurones.

2. Materials and methods

2.1. Preparation of primary cultures of adult rat trigeminal neurones

Cultures of adult rat trigeminal neurones were prepared as previously described (Jenkins et al., 2001a). Briefly, adult Wistar rats (175–250 g; either sex) were killed by CO_2 inhalation in accordance with UK Home Office regulations. Trigeminal ganglia were dissected out and placed in icecold Ca^{2+}-, Mg^{2+}- and bicarbonate-free Hanks' balanced salt solution (CMF-Hanks'; Gibco, Paisley, UK) before being chopped and incubated for 17 min at 37 °C in 3 ml CMF-Hank's containing 20 U ml^{-1} papain. Cells were pelleted by centrifugation at 250*g* for 3 min and the supernatant was replaced with 3 ml CMF-Hank's supplemented with 0.3% (w/v) collagenase (Worthington) and 0.4% (w/v) dispase II (Worthington). After a further incubation at 37 °C for 20 min, the cells were re-spun at 250*g* and the pellet resuspended in 3 ml CMF-Hank's and 2 ml Liebowitz's L-15 medium (Gibco) supplemented with 5 mM Na^+HEPES, 5 mM D-Glucose (both from Sigma, Poole, UK), 10% (v/v) heat-inactivated foetal bovine serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (all from Gibco) and 0.15% (w/v) deoxyribonuclease I (Sigma). Clumps of cells were dissociated via trituration through a graded series of fire-polished Pasteur pipettes. After centrifugation at 250*g* for 3 min, the cell pellet was resuspended in culture medium [Ham's F-12 (GlutaMAX-I) containing 10% heat-inactivated foetal bovine serum, 100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (all from Gibco) and nerve growth factor (m2.5S NGF; 50 ng ml^{-1}; Alomone)] before being plated down on Poly-D-lysine (150 K+; 0.1 mg ml^{-1}; Sigma) and laminin (20 μg ml^{-1}; Sigma) pretreated 12-well plates (Costar). Cells (200–500 per well) were incubated at 37 °C in a 5% CO_2/humidified air atmosphere for 4–6 days. After 24 h and every other day thereafter, the culture medium was replaced with F-12 medium further supplemented with 20 μM cytosine-β-D-arabinofuranoside (Ara-C). After treatment of the cells for 4–5 days with Ara-C, the cultures were enriched with neurones (estimated to be >90%) based on visual morphological examination and expression of a neuron-specific 160-kDa neurofilament subunit protein.

2.2. Prostaglandin E_2 release from trigeminal neuronal cultures

After 4–6 days in culture, the medium was gently aspirated and replaced with 1 ml prostaglandin E_2 release buffer (Hingtgen et al., 1995) (composition: 22.5 mM Na^+HEPES, 135 mM NaCl, 3.5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$ 3.3 mM D-glucose, 0.1% (w/v) bovine serum albumin, 0.003% (w/v) bacitracin, 1 μM phosphoramidon (all Sigma), pH 7.4 at 37 °C). Cells were incubated for 30 min at 37 °C in release buffer before this was replaced with 1 ml test agonist or vehicle (dimethylsulfoxide (DMSO), maximal concentration 0.01%) for a further 30 min. Following each incubation, 0.8-ml samples were removed and assayed for prostaglandin E_2 content. A commercial enzyme immunometric assay (Cayman Chemicals, Ann Arbor, MI) was used for quantitative analysis of the prostaglandin E_2 content of the eluates. Prostaglandin E_2 levels were determined photometrically at 405 nm using a microplate reader (Packard SpectraCount™). None of the compounds used in this study were found to cross-react nonspecifically with the assay at the concentrations indicated.

2.3. Data analysis

The prostaglandin E_2 concentrations in the samples were quantified in picograms per milliliter. In order to account for differences in neuronal numbers and baseline prostaglandin E_2 concentrations between preparations and between individual wells, each well acted as its own control. In some experiments, the increase in immunoreactive prostaglandin

E_2 concentrations following 30-min drug incubation was calculated as a percentage increase over the baseline concentration obtained in the 30 min prior to drug administration [(prostaglandin E_2 concentration after drug treatment − basal)/basal × 100]. Only one drug treatment was given to any single well. Unless otherwise stated, values from individual experiments were pooled and expressed as a means ± S.E.M. Statistical analysis between treatments was by one-way analysis of variance followed by the Tukey's post hoc test, and P values of less than 0.05 were considered statistically significant.

2.4. Western blotting

At the end of the prostaglandin E_2 release experiments, reactions were terminated by the removal of the remaining media and the addition of 75 μl of 3 × strength Laemmli sample buffer. Following solubilization, the well contents were transferred to Eppendorf tubes and the wells were washed with 75 μl of deionised water. Equivalent amounts of protein were electrophoretically resolved on 10% polyacrylamide gels. Following electrophoretic transfer onto nitrocellulose (0.22 μm) using a semidry blotter, the membrane was washed briefly in Tris-buffered saline (TBS) and saturated overnight in TBS supplemented with 0.1% (v/v) Tween 20 and 5% (w/v) dried milk. Antibodies recognizing the phosphorylated and nonphosphorylated forms of extracellular regulated kinases (ERKs) 1 and 2 were used at dilutions of 1:800 and 1:1000, respectively. All primary incubations were for 1 h at 22 °C in TBS containing 0.1% (v/v) Tween 20 (TBST) followed by washing five times for 10 min each in TBST. Membranes were incubated for 1 h at 22 °C with a 1:2000 dilution of the appropriate horseradish peroxidase-conjugated secondary antibody in TBST containing 5% (w/v) dried milk. Excess antibody was removed by washing as above and immunocomplexes were visualized using enhanced chemiluminescence detection, according to the manufacturer's instructions (Amersham Life Science). The Western blots shown are representative of three separate experiments and each panel is taken from a single immunoblot.

2.5. Materials

All cell culture media was purchased from Gibco BRL and 12-well plates were from Corning Costar (High Wycombe, UK). Collagenase (Type 2) and papain were from Worthington (Reading, UK) and dispase II was purchased from Roche (Lewes, UK). Apyrase, bovine serum albumin (fraction V, protease-free), bovine pancreas crude DNase I, bradykinin, Ara-C, Lys-des-Arg^9-bradykinin, des-Arg^9,[Leu^8]-bradykinin, arginyl-L-prolyl-trans-4-hydroxy-L-prolylglycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2α, 3β, 7aβ)-octahydro-1*H*-indole-2-carbonyl-L-arginine (HOE 140), *N*-nitro-L-arginine (L-NAME), murine Engelbroth swarm laminin, piroxicam, and poly-D-lysine (MW 150 K+) were obtained from Sigma. Anti p44/p42 mitogen-activated protein (MAP) kinase antibodies were purchase from Santa Cruz (Wiltshire, UK) and anti phospho-p44/p42 MAP kinase antibodies were from New England Biolabs (Hitchin, UK). The horseradish peroxidase-conjugated secondary antibodies were purchased from Biorad (Hemel Hempstead, UK). Nerve growth factor was from Alomone Labs (Botolph Clayton, UK). Arachidonyltrifluoromethyl ketone ($AACOCF_3$), 2′-amino-3′-methoxyflavone (PD98059) and bisindolylmaleimide XI (Ro320432) were from Calbiochem (Nottingham, UK).

3. Results

3.1. Initial characterization of prostaglandin E_2 release from trigeminal neurones

To investigate the characteristics of immunoreactive prostaglandin E_2 release from cultured trigeminal neurones, we initially studied the effects of several different neuronal activators. Incubation of the cultures in release buffer resulted in an average baseline prostaglandin E_2 concentration of 32 ± 3 pg ml^{-1} (range: 11–68 pg ml^{-1}; n = 33, independent culture preparations). When stimulated with bradykinin (1 μM), prostaglandin E_2 concentrations were significantly increased from 29 ± 4 to 85 ± 31 pg ml^{-1} (n = 4; P < 0.001 compared with control wells). In contrast, in untreated wells, the prostaglandin E_2 concentration fell from a baseline value of 44 ± 7 to 9 ± 3 pg ml^{-1} (n = 7). This decrease in immunoreactive prostaglandin E_2 release was also seen when cells were depolarized with 30 mM KCl (Fig. 1). However, increases in immunoreactive PGE_2 release were also seen in response to ATP [30 μM; prostaglandin E_2 concentrations before and after stimulation were 47 ± 5 and 127 ± 11 pg ml^{-1}, respectively; Fig. 1 (n = 3)].

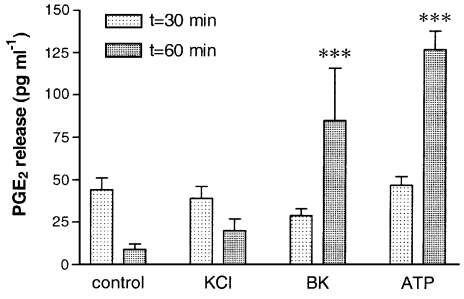

Fig. 1. Effects of KCl, bradykinin and ATP on prostaglandin E_2 release. Following 4–6 days in culture, adult trigeminal ganglion cells were stimulated with KCl (30 mM), bradykinin (1 μM) or ATP (30 μM). Data are expressed as immunoreactive prostaglandin E_2 concentrations (pg ml^{-1}) after 30-min equilibration time in release buffer (t = 30 min) and after 30-min treatment with drug (t = 60 min). Data are presented as means ± S.E.M. from three to seven independent experiments. ***P < 0.001, significantly different from control.

3.2. Effects of L-NAME and apyrase on bradykinin-induced prostaglandin E_2 release

To further investigate the nature of the bradykinin-induced immunoreactive prostaglandin E_2 release, the effects of the nonspecific nitric oxide synthase inhibitor, L-NAME (10 μM), and the ATP hydrolyzing enzyme, apyrase (1 U ml^{-1}), were tested. It was found that neither of these agents modified baseline prostaglandin E_2 concentrations and, furthermore, that these agents did not significantly modify immunoreactive prostaglandin E_2 release evoked by 1 μM bradykinin ($n=4$; Fig. 2).

3.3. Effects of bradykinin receptor agonists and antagonists on prostaglandin E_2 release from trigeminal neurones

To further characterize the bradykinin-induced increases in prostaglandin E_2 release, we constructed concentration effect curves to bradykinin and the bradykinin B_1 receptor-selective agonist, Lys-des-Arg^9-bradykinin (Fig. 3A). Concentration-dependent increases in prostaglandin E_2 release were seen in response to bradykinin (pEC_{50}: 8.7 ± 0.32), but not following stimulation with Lys-des-Arg^9-bradykinin, at concentrations as high as 10 μM.

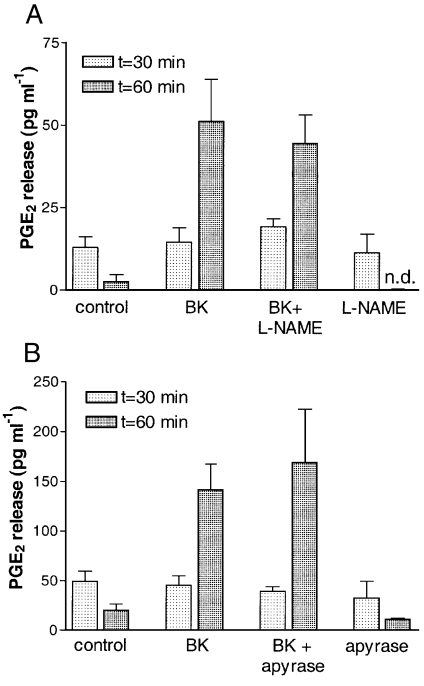

Fig. 2. Effects of L-NAME and apyrase on bradykinin-induced prostaglandin E_2 release from cultured trigeminal neurones. After 4–6 days in culture, cells were incubated in release buffer for 30 min in the presence or absence of either the nonselective nitric oxide synthase inhibitor, L-NAME (10 μM) or the ATP hydrolysing enzyme, apyrase (1 U ml^{-1}), before being stimulated with bradykinin (1 μM) in the continued presence/absence of either of the two inhibitors for a further 30 min. Data are expressed as immunoreactive prostaglandin E_2 (pg ml^{-1}) and expressed as means ± S.E.M. from four independent experiments. n.d. = not detectable.

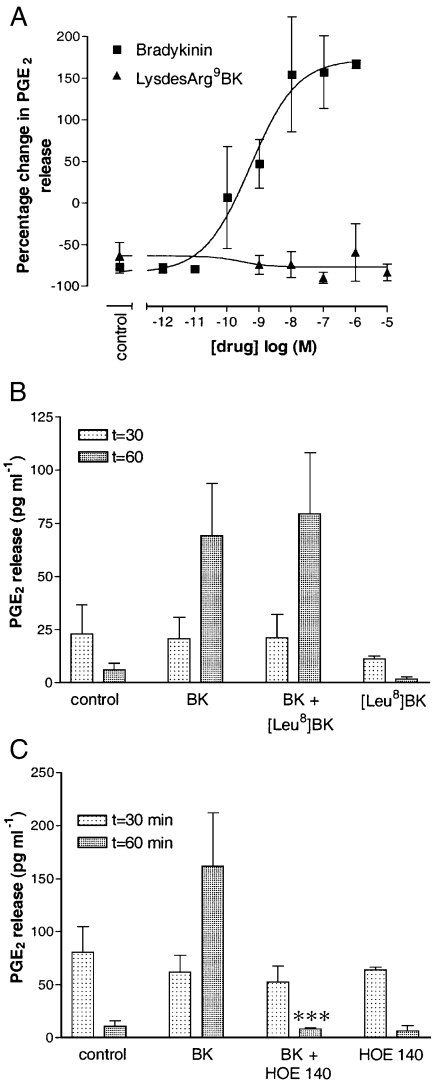

Fig. 3. Effects of bradykinin receptor selective agonists and antagonists on PGE_2 release from trigeminal neurones. (A) Cultured trigeminal ganglion neurones were incubated with release buffer for 30 min and then subsequently challenged with either bradykinin (■) or the B_1 receptor-selective agonist, Lys-des-Arg^9-bradykinin (▲), for a further 30 min. Data are expressed as the percentage change in prostaglandin E_2 release and are expressed as the means ± S.E.M. from four to eight independent experiments. Rat trigeminal ganglion neurones (4–6 days in culture) were incubated with either release buffer for 30 min and then bradykinin alone and either the B_1 receptor antagonist, des-Arg^9,[Leu^8]-bradykinin (B), or the B_2 receptor-selective antagonist, HOE 140 (C, both 1 μM), for another 30 min or antagonist alone for 30 min and then bradykinin and antagonist for a further 30 min. Data are presented as the immunoreactive prostaglandin E_2 (pg ml^{-1}) and represent the means ± S.E.M. of four to five independent experiments. ***$P<0.001$ versus bradykinin alone.

To confirm the apparent lack of bradykinin B_1 receptor involvement in mediating the bradykinin-induced prostaglandin E_2 release, we also tested the effects of bradykinin B_1 and B_2 receptor-selective antagonists on bradykinin-induced prostaglandin E_2 release. In the continuous presence of the bradykinin B_1 receptor-selective antagonist, des-Arg^9,[Leu^8]-bradykinin (1 μM), prostaglandin E_2 release in response to bradykinin (1 μM) was not significantly differ-

ent to the response observed in the presence of bradykinin alone (prostaglandin E_2 concentrations after bradykinin stimulation were 79 ± 28 and 69 ± 24 pg ml^{-1} in the presence and absence of antagonist, respectively) (Fig. 3B). In contrast, the prostaglandin E_2 release observed in response to 1 μM bradykinin was abolished in the presence of the bradykinin B_2 receptor-selective antagonist, HOE 140 (1 μM; prostaglandin E_2 concentrations in the presence and absence of antagonist were 9 ± 1 and 162 ± 50 pg ml^{-1}, respectively) (Fig. 3C). Neither antagonist by itself had any effect on PGE_2 release at the concentrations used in this study (Fig. 3B and C).

3.4. Characterization of the signalling pathways involved in mediating bradykinin-induced prostaglandin E_2 release

In order to examine the dependency of the observed prostaglandin E_2 release on cyclooxygenase activation, experiments were conducted in the continuous presence of different concentrations of the nonselective cyclooxygenase inhibitor, piroxicam (0.01–1 μM). Under these conditions, a concentration-dependent inhibition of bradykinin (1 μM)-induced prostaglandin E_2 release was observed, with the release being abolished in the presence of 1 μM piroxicam (Fig. 4A).

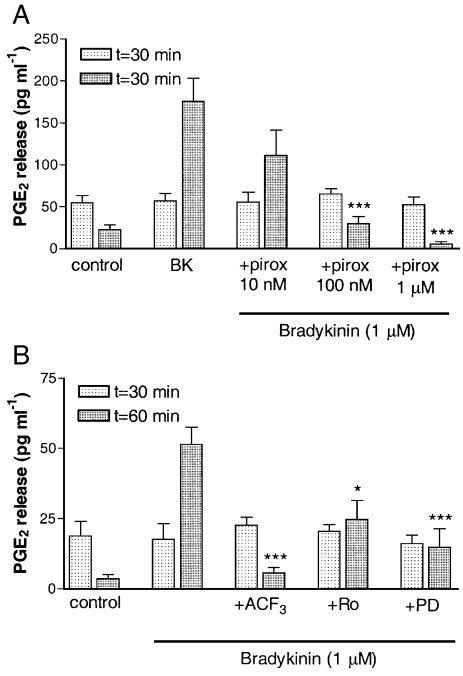

Fig. 4. Effects of piroxicam, PD98059, $AACOCF_3$ and Ro320432 on bradykinin-induced prostaglandin E_2 release. (A) Neuronal cultures were stimulated with bradykinin (BK; 1 μM) in the continuous presence or absence of the nonselective cyclooxygenase inhibitor, piroxicam (pirox; 0.01–1 μM). (B) After 4–6 days in culture, neurones were stimulated with bradykinin in the continuous presence/absence of the MEK1 inhibitor, PD98059 (PD; 20 μM), the phospholipase A_2 inhibitor, $AACOCF_3$ (ACF_3; 30 μM), or the protein kinase C inhibitor, Ro320432 (Ro; 300 nM). Data for (A) and (B) are expressed as immunoreactive prostaglandin E_2 (pg ml^{-1}) and are expressed as the means ± S.E.M. of three to eight experiments. ***$P<0.001$ compared with bradykinin alone, *$P<0.05$ compared with bradykinin alone.

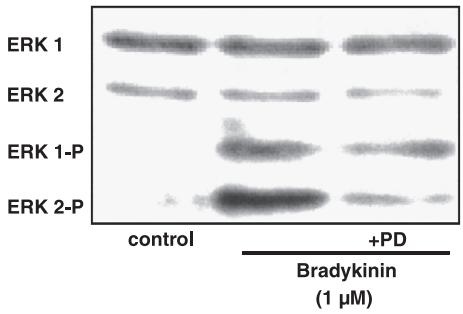

Fig. 5. Western analysis of the phosphorylation state of ERK 1/2 following treatment with bradykinin and the MEK 1 inhibitor, PD98959. Western analysis was performed using samples prepared from whole cell extracts of the trigeminal cultures incubated with either release buffer (control) or with bradykinin in the presence or absence of PD98059 (PD). Detection was made with an anti-ERK1/2 antibody (upper) to validate consistency of protein loading and with a phosphospecific ERK1/2 antibody (lower). The Western blot shown is taken from a single immunoblot and is representative of three separate experiments.

Furthermore, we also investigated the effects of phospholipase A_2, protein kinase C and mitogen-activated protein kinase kinase (MEK) inhibition on the release of prostaglandin E_2 from these cells in response to bradykinin (1 μM). Immunoreactive prostaglandin E_2 release was abolished following treatment with the inhibitor of cytosolic phospholipase A_2, $AACOCF_3$ (30 μM) and significantly attenuated following a 30-min pretreatment with the protein kinase C inhibitor, Ro320432 (300 nM; $P<0.05$). A significant inhibition ($P<0.001$) of immunoreactive prostaglandin E_2 release was also seen when cells were pretreated with the MEK 1 inhibitor, PD98059 (20 μM), before being stimulated with bradykinin (1 μM; Fig. 4B). None of these compounds altered the basal level of prostaglandin E_2 release (data not shown).

To further examine the mechanism of action of PD98059 (20 μM), the activation status of ERK 1/2 was assessed using antibodies specific for the phosphorylated (active) and non-phosphorylated (inactive) forms of this enzyme. Changes in the phosphorylation state of ERK 1/2 could be reliably detected in the small numbers of neurones used for the prostaglandin E_2 release studies (200–500). Over the time course of the experiment, there was no detectable change in the expression of ERK proteins (data not shown), but treatment with bradykinin (1 μM) induced an increase in the phosphorylation of both ERK 1 and ERK 2 that was attenuated by the MEK 1 inhibitor, PD98059 (Fig. 5).

4. Discussion

Although there is considerable debate concerning the underlying pathogenesis of migraine headache, it is well established that activation of the trigeminal nerve is a critical event. It has also been suggested that a peripheral

neurogenic inflammation, initially involving neuropeptide release, may play a further role in trigeminal nerve activation and stimulate the release of inflammatory mediators, such as prostaglandins, histamine and bradykinin (Burstein, 2001). With this in mind, we have used primary cultures of trigeminal neurones to investigate the effects of inflammatory peptide, bradykinin, on the release of another inflammatory agent, prostaglandin E_2.

Initial studies demonstrated that bradykinin and ATP both induced prostaglandin E_2 release from the cultured neurones, an effect that was not mimicked by depolarization with KCl, despite observations that this treatment can cause neuropeptide release and clearly activate trigeminal neurones (Smith and Humphrey, 2001; Carruthers et al., 2001; Durham and Russo, 1999). In agreement with the present study, the cyclooxygenase-dependent release of prostaglandin E_2 from sensory neurones in culture has previously been reported (Vasko et al., 1994), and the expression of cyclooxygenase isoforms in both cultured and acutely excised dorsal root ganglia is well established (Vasko et al., 1994; Chopra et al., 2000). It was also found that the bradykinin-induced prostaglandin E_2 release was unaffected by the nonspecific nitric oxide synthase inhibitor, L-NAME or the ATP metabolising enzyme, apyrase. Hence, it appears that the observed prostaglandin E_2 release is likely a direct consequence of bradykinin receptor activation and the subsequent activation of downstream signalling pathways. The lack of effect of apyrase is particularly important, given that ATP caused prostaglandin E_2 release per se from the cultured trigeminal neurones (see above), an effect that has been previously reported in a number of other experimental systems (Lui et al., 1998; Watanabe-Tomita et al., 1997). Likewise, the lack of effect of L-NAME is significant, as nitric oxide has also been shown to modulate cyclooxygenase activity (Clancy et al., 2000). These observations are of further interest, given the potential involvement of nitric oxide in the pathophysiology of migraine headache (see Thomsen and Olesen, 1998).

Several lines of evidence have suggested that the bradykinin B_1 receptor is not constitutively expressed, but rather up-regulated in response to tissue injury, including within the nervous system (Marceau et al., 1998). Indeed, several behavioural studies have suggested this to be the case. For example, the bradykinin B_1 receptor agonist, des-Arg^9-bradykinin, only induces nociceptive responses when applied to inflamed tissue (Perkins et al., 1993). However, recent evidence suggests a basal expression of bradykinin B_1 receptors at both the mRNA and protein levels in both dorsal root and trigeminal ganglia, raising the possibility that constitutive bradykinin B_1 receptors may play functional roles in primary afferent neurones (Seabrook et al., 1997; Ma et al., 2000; Wotherspoon and Winter, 2000). However, in this study, using primary cultures derived from rat trigeminal ganglia, we found no evidence for bradykinin B_1 receptor functionality as assessed by prostaglandin E_2 release or frank neuronal depolarization (Paul A. Smith, personal communication). Indeed, the evidence presented here suggests that the actions of bradykinin in causing prostaglandin E_2 release are likely exclusively mediated by the bradykinin B_2 receptor. This is consistent with several studies that suggest that bradykinin exerts direct actions on sensory neurones through bradykinin B_2 receptors (Kasai et al., 1998; Dray and Perkins, 1997). However, bearing in mind that bradykinin B_1 receptor knock-out mice display reduced nociceptive responses under noninflammed conditions and that constitutive bradykinin B_1 receptors may play a role in central sensitisation (Pesquero et al., 2000), it is clear that further studies are required to assess the roles of bradykinin B_1 receptors in pain.

The involvement of phospholipase A_2 isoforms, which preferentially cleave phospholipids at the *sn*-2 position, in the liberation of arachidonic acid from the plasma membrane and its consequent conversion to prostaglandins via cyclooxygenase is well documented (Capper and Marshall, 2001). In this study, we used the nonselective cyclooxygenase inhibitor, piroxicam and the type IV phospholipase A_2 inhibitor, $AACOCF_3$ to investigate the significance of these enzymes in mediating the bradykinin-induced prostaglandin E_2 release, demonstrating a significant attenuation of prostaglandin E_2 release with both agents. Furthermore, the inhibitory effect of piroxicam was highly concentration-dependent, effectively abolishing the bradykinin-induced responses at a concentration of 1 μM. Thus, these data further confirm the essential role of cyclooxygenase in prostaglandin E_2 release, although, as piroxicam does not distinguish between the cyclooxygenase isoforms, the relative involvement of cyclooxygenase-1 and cyclooxygenase-2 in this system remains to be evaluated.

The inhibition of the bradykinin-mediated prostaglandin E_2 release observed in the presence of $AACOCF_3$ is consistent with other studies that have demonstrated the importance of this phospholipase A_2 isoform in mediating prostaglandin production (see Scott et al., 1999; Pyne et al., 1997). Indeed, this enzyme displays an almost ubiquitous distribution and contains phosphorylation sites for both protein kinase C and MAP kinase (Capper and Marshall, 2001), although consistent increases in enzyme activity appear to be dependent upon the phosphorylation of serine 505 by MAP kinase family members (Lin et al., 1993). The MAP kinases form a diverse family of serine/threonine protein kinases that are involved in numerous cellular functions (see Cobb, 1999). Their signalling pathways comprise kinase cascades of no fewer than three enzymes. For example, MAP kinases (MAPKs), such as ERK 1 and ERK 2, are phosphorylated and, hence, activated by MAPK kinases (MAPKKs), such as MEK1 and 2, which are in turn phosphorylated by MAPKK kinases (MAPKKK). Thus, the phosphorylation state of ERK1/2 can be used as measure of MAPK activation.

Consistent with presence of phosphorylation sites for protein C and MAPK on Type IV phospholipase A_2 the bradykinin-induced prostaglandin E_2 release was signifi-

cantly attenuated by both the protein kinase C inhibitor, Ro320432 and the MEK1 inhibitor, PD98059, suggesting that the observed prostaglandin E_2 release is dependent upon the activation of protein kinase C and MAPK pathways. Although Ro320432 has been demonstrated to show some selectivity in inhibiting protein kinase Cα over other protein kinase C isoforms (Wilkinson et al., 1993), no changes in the phosphorylation state of this protein kinase C isoform were observed following stimulation with bradykinin (our unpublished observations), suggesting that this isoform may not be involved. In addition, consistent with the activation of the MAPK pathway, the inhibitory effects of PD98059 on bradykinin-induced prostaglandin E_2 release could be effectively correlated with changes in the phosphorylation and hence activation states of the MAP kinases, ERK1/2.

In conclusion, we have shown that bradykinin can, through the activation of bradykinin B_2 receptors, cause prostaglandin E_2 release from cultured trigeminal neurones from adult rats. Furthermore, we have shown that this prostaglandin E_2 release is dependent upon the activation of protein kinase C and MAP kinase pathways, but not on the release of nitric oxide or ATP. Since bradykinin has previously been demonstrated to both activate and sensitize sensory neurones (Calixto et al., 2000) and E-series prostaglandins have been implicated in migrainous headache (see Coleman et al., 1990; Burstein, 2001), bradykinin receptor antagonists may warrant further investigation as potential antimigraine agents.

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