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# Nociceptive sensitization by the secretory protein Bv8

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**1** The small protein Bv8, isolated from amphibian skin, belongs to a novel family of secretory proteins (Bv8-Prokineticin family, SWISS-PROT: Q9PW66) whose orthologues have been conserved throughout evolution, from invertebrates to humans.

**2** When injected intravenously or subcutaneously (from 0.06 to 500 pmol $kg^{-1}$) or intrathecally (from 6 fmol to 250 pmol) in rats, Bv8 produced an intense systemic nociceptive sensitization to mechanical and thermal stimuli applied to the tail and paws.

**3** Topically delivered into one rat paw, 50 fmol of Bv8 decreased by 50% the nociceptive threshold to pressure in the injected paw without affecting the threshold in the contralateral paw.

**4** The two G-protein coupled prokineticin receptors, PK-R1 and PK-R2, were expressed in rat dorsal root ganglia (DRG) and in dorsal quadrants of spinal cord (DSC) and bound Bv8 and the mammalian orthologue, EG-VEGF, with high affinity. In DSC, PK-R1 was more abundant than PK-R2, whereas both receptors were equally expressed in DRG. $IC_{50}$ of Bv8 and EG-VEGF to inhibit [$^{125}$I]-Bv8 binding to rat DRG and DSC were $4.1 \pm 0.4$ nM Bv8 and $76.4 \pm 7.6$ nM EG-VEGF, in DRG; $7.3 \pm 0.9$ nM Bv8 and $330 \pm 41$ nM EG-VEGF, in DSC.

**5** In the small diameter neurons ($<30$ $\mu$m) of rat DRG cultures, Bv8 concentrations, ranging from 0.2 to 10 nM, raised $[Ca^{2+}]_i$ in a dose-dependent manner.

**6** These data suggest that Bv8, through binding to PK receptors of DSC and primary sensitive neurons, results in intense sensitization of peripheral nociceptors to thermal and mechanical stimuli. *British Journal of Pharmacology* (2002) **137,** 1147–1154. doi:10.1038/sj.bjp.0704995

**Keywords:** Bv8; prokineticins; hyperalgesia; nociceptors; DRG; spinal cord; receptors

**Abbreviations:** ACTX-Hvf17, atracotoxin-f17 of *Handronyche versuta*; DRG, dorsal root ganglion; DSC, dorsal spinal cord; EG-VEGF, endocrine-gland-derived vascular endothelial growth factor; GDP-$\beta$-S, guanosine 5′-O-(2-thiodiphosphate) Li salt; MIT1, mamba intestinal toxin 1; PK-R1, prokineticin 1 receptor; PK-R2, prokineticin 2 receptor; PLC, phospholipase C; RT–PCR, reverse transcriptase-polymerase chain reaction; SCN, suprachiasmatic nucleus; U73122, 1-[6-[[(17$\beta$)-3-Methoxyestra-1.3.5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2.5-dione; VPRA, venom protein A

## Introduction

The presence of gene families coding for secretory proteins whose orthologues were found across invertebrates, snakes, amphibians and mammals indicates that the products of these genes are involved in basic cellular communications conserved throughout evolution (Erspamer, 1994). An emerging new family of secretory proteins numbers six members found in spiders (ACTX-Hvf17), snakes (VPRA or MIT1), amphibia (Bv8), rodents (mouse and rat prokineticin1 and prokineticin 2), and humans (human Bv8 or prokineticin 2 and prokineticin 1 or EG-VEGF). The first member of the family to be identified was VPRA (Joubert & Strydom, 1980), also named MIT1 (Schweitz *et al*., 1990), a non-toxic constituent of the venom of the black Mamba snake (*Dendroaspis polylepis polylepis*). More recently, a basic small protein, containing 77 amino acids, has been isolated from the skin secretion of the discoglossid frog *Bombina variegata* (Mollay *et al*., 1999) and named Bv8, to indicate its origin (*Bombina variegata*) and its molecular mass (about 8 kDa). The primary structure of Bv8 resembles that of MIT-1; the two proteins share the same pattern of cysteine distribution in their amino acid sequence and their overall identity is 58%. In addition, both Bv8 and MIT-1 possess significant sequence homology with another secretory protein (ACTX-Hvf17) isolated from the venom of the funnel-web spider *Hadronyche versuta* (Szeto *et al*., 2000). In mice and humans, cDNA cloning identified two mammalian orthologues of amphibian Bv8, named mBv8 and hBv8 (Wechselberger *et al*., 1999). The genomic structure of these murine and human Bv8 genes has been determined, and the chromosomal localization was identified near a synteny breakpoint of mouse chromosome 6 and human 3p21 (Jilek *et al*., 2000). The distribution of murine Bv8 protein and its mRNA has been reported in testis, brain, spinal cord and other peripheral organs of mice and rats (Wechselberger *et al*., 1999; Melchiorri *et al*., 2001). Searching the EST database using the predicted coding of Bv8, Li *et al*. (2001) identified two human EST sequences, one encoding the human protein already described by Wechselberger *et al*. (1999), and the other encoding a slightly different Bv8-like protein. The two proteins were named prokineticin 2 and prokineticin 1. The name

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prokineticin refers to the ability of these peptides to contract guinea-pig ileum, a property shared with amphibian Bv8 (Mollay *et al*., 1999). Screening a library of human secreted proteins for the ability to induce proliferation in capillary endothelial cells, Lecouter *et al*. (2001) identified a protein which induced proliferation, migration and fenestration in the endothelial cells of steroid synthesizing glands (ovary, testis, adrenals) and named it endocrine-gland-derived vascular endothelial growth factor (EG-VEGF). EG-VEGF and prokineticin 1 are the same protein and have an overall identity of 58% and homology of 76% with human prokineticin 2 and murine Bv8 and 43% identity with amphibian Bv8. Rat mRNAs for prokineticin 1 and prokineticn 2 have been cloned and their expression patterns reported in peripheral tissues and central nervous system (Masuda *et al*., 2002). Prokineticin 1 (EF-VEGF) has been isolated and sequenced also from bovine milk (Masuda *et al*., 2002). Thus ACTX-Hvf17, MIT-1 (VPRA), amphibian Bv8, mouse prokineticin 2 (mouse Bv8), rat prokineticins, human prokineticin 1 (EF-VEGF) and prokineticin 2 (human Bv8), belong to the same gene family (Bv8-Prokineticin family, SWISS-PROT: Q9PW66) with orthologues conserved throughout evolution, from invertebrates to humans.

These proteins are ligands for two G-protein-coupled receptors that have been cloned (Lin *et al*., 2002; Masuda *et al*., 2002) and named PK-R1 and PK-R2. These two receptors are most divergent in their N-terminal sequences and show distinct expression patterns in different organs (Lin *et al*., 2002; Masuda *et al*., 2002). Although one of these two receptors (PK-R2) is expressed abundantly in the brain (Cheng *et al*., 2002; Masuda *et al*., 2002), the neurobiology of Bv8 and its mammalian homologues is largely unexplored. In a preliminary report we showed that Bv8 injected into the rat brain produced hyperalgesia (Mollay *et al*., 1999). Recently, Cheng *et al*. (2002) reported that prokineticin 2 is rhythmically expressed in the suprachiasmatic nucleus (SCN) and probably transmits the behavioural circadian rhythm through activation of PK-R2, which is abundantly expressed in the major target nuclei of the SCN output pathway.

Because preliminary results indicated that pituitary and circulating leucocytes express high level of Bv8-like proteins (Lattanzi *et al*., 2001; Cheng *et al*., 2002; Masuda *et al*., 2002), we hypothesized that these proteins might be released into the blood or inflammatory exudates and evoke biological responses by systemic and topical delivery. In this study we therefore assessed the effects of subcutaneous, intravenous, intrathecal and intraplantar injections of Bv8 on rat nociceptive threshold to thermal and mechanical stimuli. To elucidate further the mechanism of Bv8 action in the periphery we investigated the following: (1) the presence of the receptors PK-R1 and PK-R2 in rat dorsal root ganglia (DRG) and posterior horn of spinal cord; (2) the binding of amphibian Bv8 to PK receptors of DRG and spinal cord; and (3) the agonist activity of Bv8 on $[Ca^{2+}]_i$ in cultured DRG neurons.

## Methods

### *Animals*

Adult male Sprague-Dawley rats (280–350 g, b.w.) or 3–7-day-old Sprague-Dawley rats were used for the experiments under protocols approved by the Animal Care and Use Committee of the Italian Ministry of Health according to EC directives. Each adult rat was used only once and immediately killed by $CO_2$ inhalation after the experiment ended. Neonatal rats were used only to remove DRG and were killed by decapitation.

### *Intrapaw injections*

Bv8 was injected into the rat paw with a microsyringe fitted with a 30-gauge needle. A Bv8 dose, dissolved in 40 $\mu$l saline solution (0.9% NaCl), was injected into the plantar (20 $\mu$l) and dorsal (20 $\mu$l) regions of the paw. Control rats were injected with an equal volume of saline solution.

### *Intrathecal injections*

Under ketamine-xylazine anaesthesia ($60+10$ mg kg$^{-1}$, i.p.) chronic lumbar intrathecal catheters were implanted in rats with a modified Yaksh & Rudy (1976) procedure. In brief, an incision was made through the atlanto-occipital membrane and an 8.5-cm PE10 catheter was inserted into the intrathecal space so that the caudal end of the catheter reached the lumbar enlargement. Intrathecal vehicle was artificial cerebrospinal fluid, and each rat received 5 $\mu$l of the vehicle or Bv8 solution in vehicle, followed by a 5 $\mu$l vehicle flush.

### *Systemic injections*

Bv8 dissolved in saline solution was injected in a volume of 2 ml kg$^{-1}$ by the subcutaneous (s.c.) route and 300 $\mu$l kg$^{-1}$ by the intravenous (i.v.) route. Bv8 was injected into the rat tail vein that had been dilated by immersing it in hot (40°C) water just before the injection. Controls were injected with an equal volume of saline solution.

### *Mechanical and thermal nociception*

The pressure-evoked paw withdrawal response was assessed with the Randall-Selitto test (Randall & Selitto, 1957). The nociceptive threshold to thermal stimuli was measured at the tail of restrained rats with the D'Amour & Smith tail-flick test (D'Amour & Smith, 1941) and at the paw of freely moving rats with the Hargreaves' plantar test (Hargreaves *et al*., 1988). Commercially available apparatus (Ugo Basile, Comerio, Italy) were used for all these tests. For measurement of the nociceptive threshold, rats were trained in tail-withdrawal or paw-withdrawal tests during the week preceding the experiment, at 30-min intervals for 3 h daily. This adaptation protocol reduced variability in threshold measurements, giving a more stable baseline and making drug-induced changes easier to detect. In the tail-flick test, the intensity of the light beam focused on the tail tip was preset to obtain a tail-withdrawal latency ranging from 7 to 9 s. On the day of the experiment, nociceptive threshold was measured for 2 h at 30-min intervals before drug injection. The mean of the last three of these threshold measurements was taken as baseline nociceptive threshold ($NT_B$). Nociceptive threshold was then determined three times at 15, 30, 45, 60, 90, 120, 150, 180, 240, 300, and 360 min after saline or Bv8 solution injection. The mean of the three readings at each time point was defined as the nociceptive threshold at

that time in the presence of the test solution ($NT_{ts}$). The effect of the test drug was calculated as the percentage change in nociceptive threshold from baseline threshold (%ΔNT) according to the following equation:

$$\%\Delta NT = 100 \times (NT_{ts} - NT_B)/NT_B$$

### *Primary cultures of rat DRG neurons*

To remove DRG, neonatal (from 3–7-day-old) Sprague-Dawley rats were decapitated. Under aseptic conditions, lumbar DRGs were collected in culture medium ( DMEM-F12 ratio 1:1, BioWhittaker, Bergamo, Italy) on ice, trimmed of nerve roots and desheathed, with the aid of a dissecting microscope. After two washes in DMEM/F12, ganglia were incubated with 0.125% collagenase I (Sigma-Aldrich, Milan, Italy) in the same medium for 45 min at 37°C, and then dissociated into single cells by mechanical trituration through fire-polished Pasteur pipettes of decreasing tip-diameter. After three washes in culture medium, cells were sedimented (5 min, $300 \times g$, 25°C) and resuspended in DMEM/F12 enriched with 10% foetal bovine serum (FBS; Gibco, Milan, Italy ), 200 mM L-glutamine, $N_2$-supplement (Gibco, Milan, Italy), penicillin (100 U $ml^{-1}$) and streptomycin (100 $\mu g\ ml^{-1}$). Cells were plated at a density of approximately 20,000 cells per well onto 4-well plates, precoated with 10 $\mu g\ ml^{-1}$ poly-L-lysine (Sigma-Aldrich, Milan, Italy) and with 10 $\mu g\ ml^{-1}$ EHS laminin (Invitrogen, Milan, Italy). After 1 h at 37°C, cells had attached to the wells and a further 500 $\mu l$ of culture medium containing 50 ng $ml^{-1}$ NGF (Sigma-Aldrich, Milano, Italy) was added. Cells were maintained in a water saturated atmosphere (95% air, 5% $CO_2$) at 37°C for up to 7 days. Medium was exchanged every 3 days.

### *Intracellular calcium imaging*

Optical microfluorometry using Fura-2 as fluorescent indicator was used to evaluate changes in $[Ca^{2+}]_i$. DRG cells grown onto wells were loaded for 30 min at room temperature with 5 $\mu$M Fura-2 acetoxymethyl ester (Fura-2AM, Molecular Probes, Eugene, OR, U.S.A.) in a balanced saline solution containing (mM): NaCl 125, KCl 1.0, $CaCl_2$ 5, $MgCl_2$ 1, glucose 8, and HEPES 20 (pH 7.35). This solution was then replaced with a fresh one without Fura-2AM, and cells were maintained at room temperature for a further 30 min to de-esterify the fluorescent indicator. Plates with culture wells containing 500 $\mu l$ of the balanced saline solution were mounted onto an inverted microscope and illuminated with a xenon lamp to excitate fluorescence. Fluorescence was measured with 10 Hz alternating wavelength time scanning with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. The fluorescence ratio at the two excitation wavelengths ($Ca^{2+}$ bound Fura-2/ free Fura-2) was recorded with a Hamamatsu Argus 50 computerized analysis system. BV8 was added to the culture wells by replacing 250 $\mu l$ of the balanced saline solution with an equal volume of the same solution containing twice the final Bv8 concentrations (from 0.2 to 10 nM). Bv8-evoked $Ca^{2+}$ transients and the number of cells responding to Bv8 were recorded.

### *Receptor binding assay*

Bv8 was radiolabelled with [$^{125}$I]-Bolton-Hunter reagent ([$^{125}$I]-BH, NEX120, NEN) and [$^{125}$I]-BH-Bv8 was purified by high-performance liquid chromatography as previously described (Mollay *et al*., 1999). Sprague-Dawley rats were anaesthetized by i.p. injection of pentobarbitone (100 mg $kg^{-1}$) and DRG were removed along the whole column length using a dissecting microscope. A 2 cm segment of the lumbar enlargement of spinal cord from the same rats was isolated and hemisected longitudinally into lateral halves. These halves were hemisected again and the dorsal quadrants containing posterior horns were retained for receptor binding studies. For membrane preparation, DRG from a total of 25 rats (wet weight ~1 g) and spinal cord dorsal quadrants of the same animals were homogenized in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4), containing 0.3 M sucrose. Homogenates were then centrifuged at low speed ($900 \times g$, 4°C) to remove nuclei and debris, and the pellets resuspended in the same buffer and centrifuged at $100{,}000 \times g$ for 30 min at 4°C. Pellets were then stored at −70°C and resuspended in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4), immediately before binding assay. Protein concentration was determined by the bicinchonic acid protein assay kit (BCA Protein Assay Kit, Pearse, Rockfort, IL, U.S.A.), using bovine albumin as the standard. Membranes from DRG and spinal cord were preincubated in 50 mM Tris-HCl buffer (pH 7.4) containing 200 $\mu$M GDP-$\beta$-S (guanosine 5′-O-(2-thiodiphosphate) Li salt) (Calbiochem-Novabiochem, San Diego, CA, U.S.A.) for 30 min at 35°C to constrain G protein coupled receptors in high affinity state, centrifuged at $33{,}000 \times g$ and resuspended in Tris-HCl buffer, pH 7.4, containing 0.2% albumin and 100 $\mu$M GDP-$\beta$-S, to a concentration of 1 mg protein $ml^{-1}$ (working membrane preparation). Each assay contained 150 $\mu l$ of the working membrane preparation, 150 pM [$^{125}$I]-BH-Bv8, and 350 $\mu l$ of Tris-HCl buffer, pH 7.4, to which graded concentrations of unlabelled Bv8 (from 0.2 to 300 nM) or EG-VEGF (from 1 to 5000 nM) were added. Each concentration was tested in duplicate. After a 90 min incubation period at 35°C, the samples were cooled and membranes were harvested on Whatman GF/B filters presoaked in 0.5% polyethylenimine (Sigma-Aldrich, Milano, Italy), washed three times with 2 ml of cold Tris-HCl buffer and transferred to counting vials. Radioactivity was measured with a $\gamma$-ray counter. Nonspecific binding was determined in the presence of 1 $\mu$M unlabelled Bv8. Displacement curves, and $IC_{50}$ values were calculated with the PRISM software (GraphPad Software, San Diego, CA, U.S.A.).

### *Gene expression analysis of PK-R1 and PK-R2 by RT–PCR*

Cultured DRG neurons from 3–7-day-old rats, dorsal quadrants of spinal cord (DSC) and lumbar DRG of adult rats were used for RNA extraction. Extracted RNA was purified using RNeasy columns (Qiagen, Milano, Italy), and checked for accidental degradation on agarose gel. Two micrograms of purified RNA were used for cDNA synthesis with reverse transcriptase (Promega, Milan, Italy) and the reaction product was diluted to 100 $\mu l$ with deionized water. An aliquot of 2.5 $\mu l$ of cDNA solution was used for PCR

amplifications (Robocycler 40, Stratagene, Florence, Italy). To compensate for variations in RNA quantitation and random tube-to-tube variations a multiplex PCR reaction was performed in which the level of product from the gene of interest was normalized against the product from 18 S ribosomal RNA, assumed as internal control. Primers for 18 S ribosomal RNA amplification were purchased from Ambion (Milan, Italy). Based on the Primer-Competimer™ Technology (Ambion), PCR amplification conditions were the same as for targets. Specific sense and antisense primers were synthesized (Biogen, Rome, Italy) to PCR amplify the rat PK-R1 and PK-R2 cDNA, according to the following sequences: for PK-R1, 5′-GGACCCTGGGCGAGAATA-3′ and 5′-AGATGGGATGGCGATGAG3′; for PK-R2, 5′-TCTCCTTGCTCCCCTTAAAC-3′ and 5′-AGATGGGA-TGGCGATGAG3′. PCR products (PK-R1, size 575 bp; PK-R2, size 500 bp) were separated by agarose gel electrophoresis, stained with ethidium bromide and the resulting fluorescent bands were revealed with the VersaDoc 3000 imaging system (Bio-Rad, Milan, Italy), excised from the gel, eluted (GFX™ PCR kit, Amersham Pharmacia, Italy) and sequenced (Primm s.r.l., Milan Italy) to confirm the expected products.

### *Drugs*

Bv8 was extracted from the skin secretion of electrically stimulated *Bombina variegata* and purified to 98% (HPLC), as previously described (Mollay *et al*., 1999). EG-VEGF was a kind gift of Dr N. Ferrara, Genentech Inc., South San Francisco, CA, U.S.A. Verapamil was purchased from Sigma-Aldrich, Milan, Italy and U73122 (1-[6-[[(17$\beta$)-3-Methoxyestra - 1.3.5(10) - trien-17-yl]amino]hexyl]-1H-pyrrole-2.5-dione) from Tocris Cookson Ltd., Bristol, U.K.

### *Statistical analysis*

The data are presented as mean$\pm$s.e.mean values. Statistical analyses were performed using one-way ANOVA followed by Tukey multiple comparison post test.

## Results

### *Effects of Bv8 on the nociceptive threshold*

When administered by s.c., i.v., i..t. and topical routes, Bv8 decreased the nociceptive threshold to thermal and mechanical stimuli applied to the skin of rat tail and paw. By the s.c. route, a Bv8 dose of 25 pmol kg$^{-1}$ reduced the pressure eliciting paw withdrawal by about 50% (138$\pm$12 g vs 278$\pm$18 g) (Figure 1a). A ten-times higher dose (250 pmol kg$^{-1}$) was required to halve the nociceptive threshold of the tail or paw to thermal stimuli (Figure 1b,c). The time-course of Bv8-induced hyperalgesia showed that the nociceptive threshold to mechanical and thermal stimuli declined in two successive phases. At each dose tested, the largest decrease in the nociceptive threshold was reached 60 min after Bv8 injection and was followed by a recovery that lasted until 180 min, when the nociceptive threshold began to decline again reaching a second minimum around 300 min. At about 360 min after Bv8 injection, the nociceptive threshold recovered to baseline. Bv8 was more potent by the i.v. than by the s.c. route. An i.v. dose of 3 pmol kg$^{-1}$ halved the nociceptive threshold to pressure (Figure 1d), whereas an i.v. dose of 30 pmol kg$^{-1}$ was required to reduce the nociceptive threshold to heat by about 50% (Figure 1e,f). Although hyperalgesia to s.c and i.v. Bv8 showed a similar biphasic pattern, the time course of the two activity peaks differed: hyperalgesia peaked 30 and 60 min earlier after i.v. than after s.c. injections. After a large i.v dose (250 pmol kg$^{-1}$) the nociceptive threshold to pressure fell to 50% of the baseline value within 30 min and remained at this low value for at least 4 h.

Bv8 achieved the highest potency by the i.t. route. An i.t. dose of 60 fmol halved the nociceptive threshold to paw pressure (Figure 2a). The response had a biphasic time-course. The first decrease in the nociceptive threshold began within 2 min after injection, peaked at 30 min, lasted 90 min and was followed by a 1 h-recovery. The second decrease in the threshold peaked at the fourth hour and lasted till the fifth hour. After a large i.t. dose (250 pmol) the nociceptive threshold fell to 40% of the baseline value within 30 min and remained at low values for 4 h. An i.t. dose of about 6 pmol was required to halve the nociceptive threshold to thermal stimuli applied to the rat tail or paw (Figure 2b,c).

When Bv8 was topically injected into the dorsal and plantar region of one rat hindpaw, at doses that left the nociceptive threshold of the contralateral paw unchanged, a dose of 50 fmol decreased the local nociceptive threshold to mechanical stimuli by 45% (Figure 3), producing a monophasic response. The paw pressure eliciting the nocifensive behaviour reached a minimum at 60 min, recovered after 2 h, and thereafter remained unchanged. Bv8 produced no inflammatory signs in the injected paw and plethysmography detected no changes in paw volume (data not shown).

### *Expression of prokineticin receptors (PK-Rs) in DRG and spinal cord*

Both isoforms of PKRs were equally expressed in adult rat DRG, while in the dorsal quadrants of spinal cord, including dorsal horns, PK-R1 was more abundantly expressed than PK-R2 (Figure 4). Both receptor isoforms were present in 3-day cultures of neonatal rat DRG while only PK-R1 transcript was revealed by RT–PCR in DRG neurons surviving in 6-day cultures (Figure 4).

### *Binding of Bv8 to prokineticin receptors of DRG and spinal cord*

To produce monophasic competitive curves, [$^{125}$I]-BH-Bv8, bound to membrane preparations of rat DRG and dorsal quadrants of spinal cord (DSC), was displaced by competitive ligands in presence of 100 $\mu$M GDP-$\beta$-S. The concentrations of unlabelled ligands needed to inhibit 50% [$^{125}$I]-BH-Bv8 binding ($IC_{50}$ ) were 7.3$\pm$0.9 nM for Bv8 and 330$\pm$41 nM for EG-VEGF, in rat DSC; 4.1$\pm$0.4 for Bv8 and 76.4$\pm$7.6 for EG-VEGF, in rat DRG (Figure 5a,b).

### *Effects of Bv8 on $[Ca^{2+}]_i$ of DRG neurons*

Bv8 (from 0.2 to 10 nM) increased $[Ca^{2+}]_i$ in DRG neurons bathed by a balanced saline solution containing 5 mM $CaCl_2$

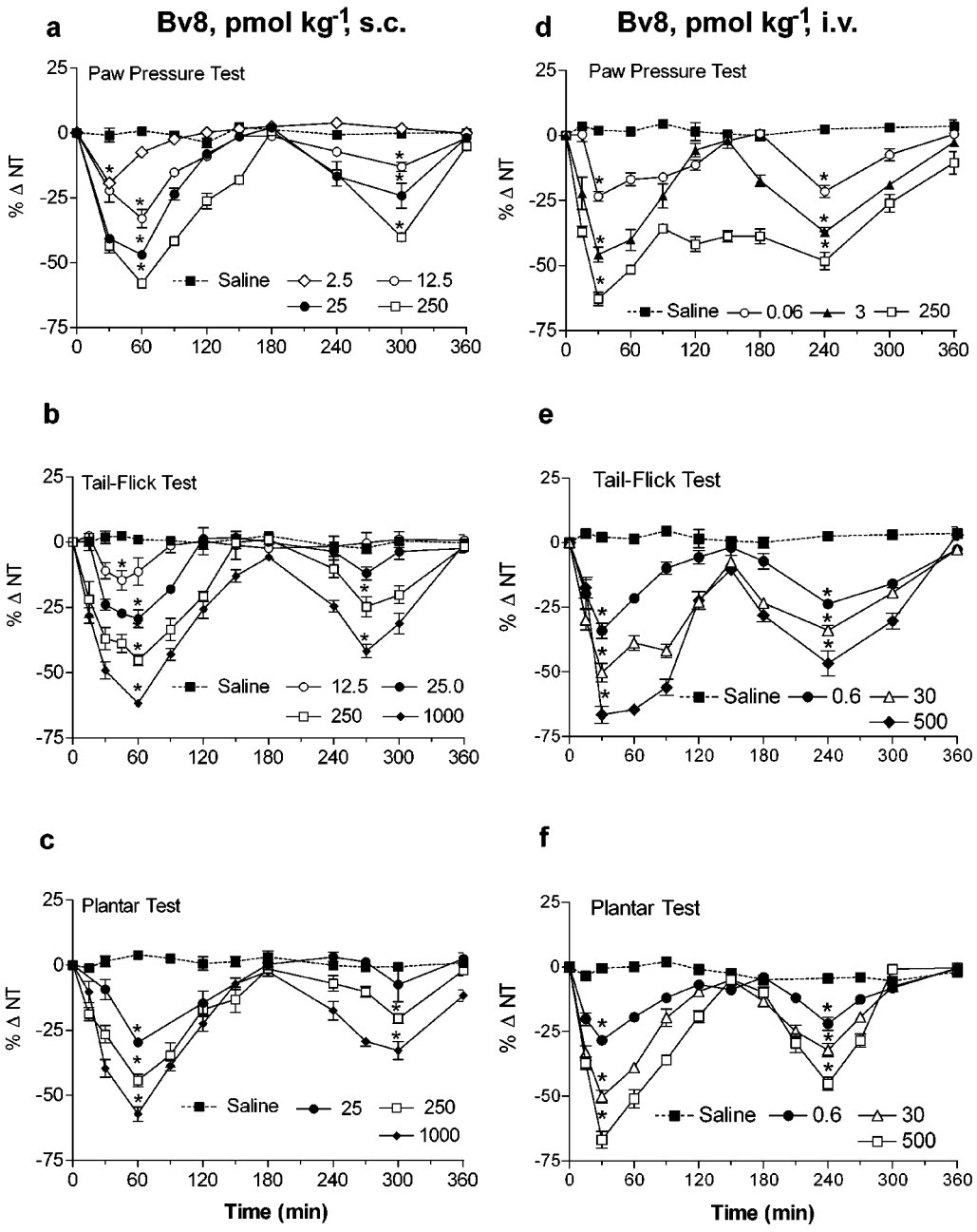


**Figure 1** Time-course of per cent decrease in nociceptive threshold (% ΔNT) elicited in rats by s.c. and i.v. injection of Bv8. Each point represents mean ± s.e.mean of a 10-rat group. In (a) and (d) per cent decrease in the nociceptive threshold to paw pressure, assessed with the Randall-Selitto test. Baseline threshold was 278 ± 12 g ($n = 50$ rats). In (b) and (e) per cent decrease in the nociceptive threshold to heat, assessed with the D'Amour-Smith tail-flick test. Baseline threshold was 8.4 ± 0.7 s ($n = 40$ rats). In (c) and (f) per cent decrease in the nociceptive threshold to heat, assessed with Hargreaves' plantar test. Baseline threshold was 12 ± 2 s ($n = 30$ rats). *$P < 0.01$ compared with saline (one-way ANOVA and Tukey multiple comparison post test).

(Figure 6a). When 0.5 nM Bv8 was applied, $[Ca^{2+}]_i$ rapidly increased in about 50% of small diameter neurons (< 30 $\mu$m): the increase began within 30 s after the addition of Bv8 and lasted 60–80 s. A concentration of 10 nM Bv8 induced $[Ca^{2+}]_i$ transient lasting 120–150 s in about 100% of small neurons. Repeated additions of Bv8 to the same culture well within 90 min after the first application revealed a strong homologous desensitization of the $[Ca^{2+}]_i$ response (Figure 6b). The phospholipase C inhibitor U73122 (3 $\mu$M) did not reduce the $[Ca^{2+}]_i$ response to Bv8, although it prevented the bradykinin-induced $[Ca^{2+}]_i$ increase. On the contrary, the addition of U73122 to DRG cultures reduced the homologous desensitization of the $[Ca^{2+}]_i$ response to Bv8 (Figure 6c). Once extracellular $Ca^{2+}$ was replaced by $Mg^{2+}$, Bv8, even at a 50 nM concentration, failed to increase $[Ca^{2+}]_i$ while bradykinin still produced $[Ca^{2+}]_i$ transients (data not shown). Verapamil (100 $\mu$M) failed to prevent the $[Ca^{2+}]_i$ elevation produced by Bv8 (data not shown).

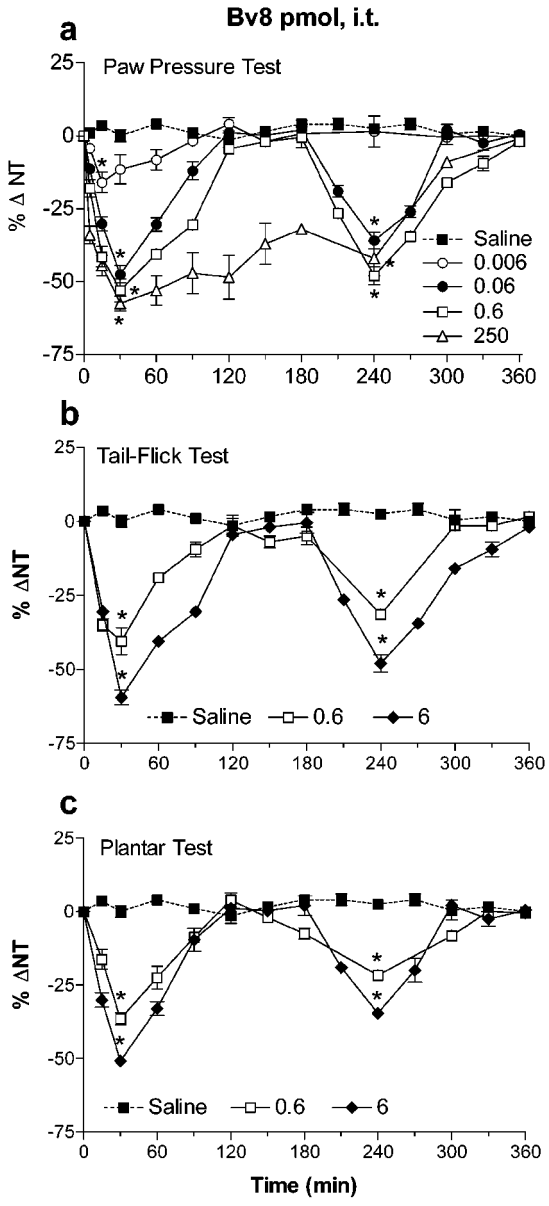


**Figure 2** Time-course of per cent decrease in nociceptive threshold (% ΔNT) elicited in rats by intrathecal injection of Bv8. Each point represents mean ± s.e.mean of a 10-rat group. In (a) per cent decrease in the nociceptive threshold to paw pressure, assessed with the Randall-Selitto test. In (b) per cent decrease in the nociceptive threshold to heat, assessed with the D'Amour-Smith tail-flick test. Baseline threshold was 9.2 ± 0.8 s ($n = 30$ rats). In (c) per cent decrease in the nociceptive threshold to heat, assessed with Hargreaves' plantar test. Baseline threshold was 10 ± 3 s ($n = 30$ rats). $^*P < 0.01$ compared with saline (one-way ANOVA and Tukey multiple comparison post test).

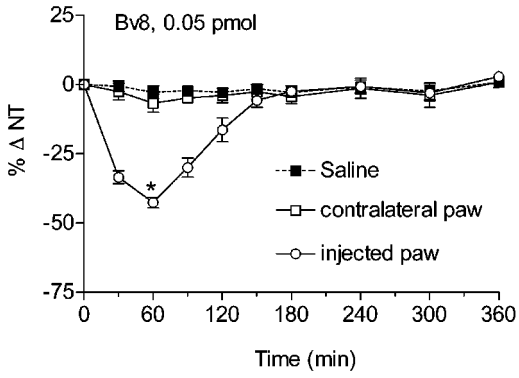


**Figure 3** Time-course of per cent decrease in nociceptive threshold (% ΔNT) to pressure elicited in rats ($n = 5$) injected into one paw with 0.05 pmol Bv8 and with saline into the contralateral paw. A further rat group ($n = 5$) was injected with saline into both hind paws (saline). $^*P < 0.01$, injected paw compared with contalateral paw and with saline injected paws (one-way ANOVA and Tukey multiple comparison post test).

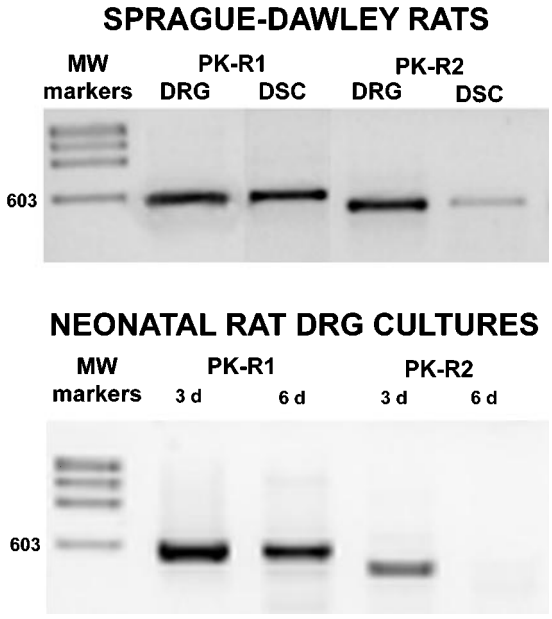


**Figure 4** RT–PCR amplification of mRNAs for prokineticin receptors PK-R1 and PK-R2 in dorsal root ganglia (DRG) and dorsal quadrants of spinal cord (DSC) of adult Sprague-Dawley rats and in 3-day- and 6-day cultures (3-d, 6-d) of DRG neurons from neonatal rats. The left lane indicates the molecular marker for a 603-base pair DNA fragment.

## Discussion

In the rat, i.v. or s.c injections of Bv8 lowered the threshold of skin nociceptors to mechanical pressure and heat, whereas intrapaw injections of Bv8 at doses 200 times lower than s.c. effective doses decreased the nociceptive threshold in the injected paw leaving threshold in the contralateral paw unchanged. Intrathecal doses of Bv8, equal to those active topically in the paw, decreased the nociceptive threshold to mechanical and thermal stimuli applied to rat tail or paws. Conversely, as shown by our previous report (Mollay *et al*., 1999), injection of Bv8 into brain ventricles lowered the nociceptive threshold only at doses more than 10,000 times higher than the intrathecal doses. The most plausible explanation of these findings is that, depending on the route of administration, Bv8 produces nociceptive sensitization by acting at peripheral and central terminals of DRG neurons or at the spinal cord, or at both sites. To corroborate this

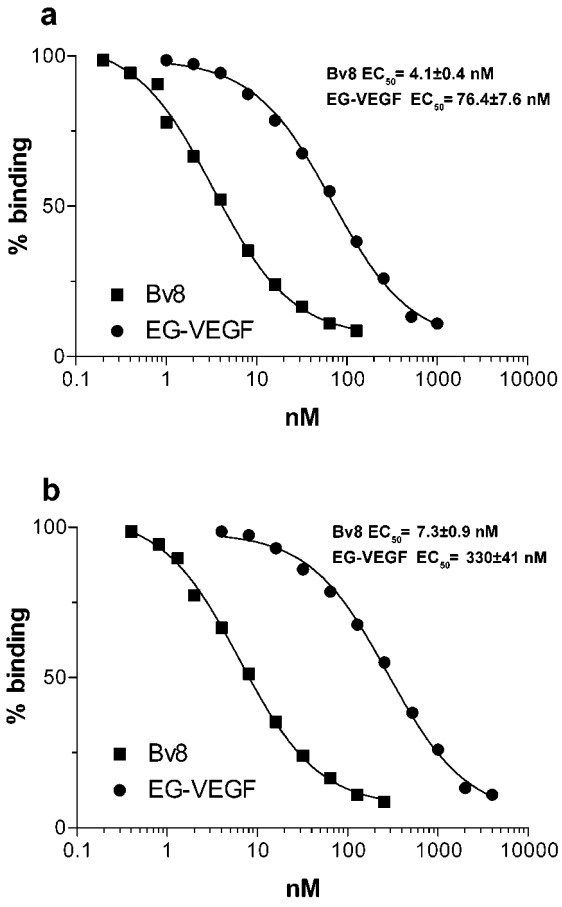


**Figure 5** Displacement curves of [$^{125}$I]-BH-Bv8 from membrane preparations of rat dorsal root ganglion (DRG) (a) and dorsal quadrants of spinal cord (DSC) (b) by unlabelled Bv8 and EG-VEGF (prokineticin 1). Each point represents the mean of three samples. Binding curves were replicated three times to calculate $EC_{50}$ values.

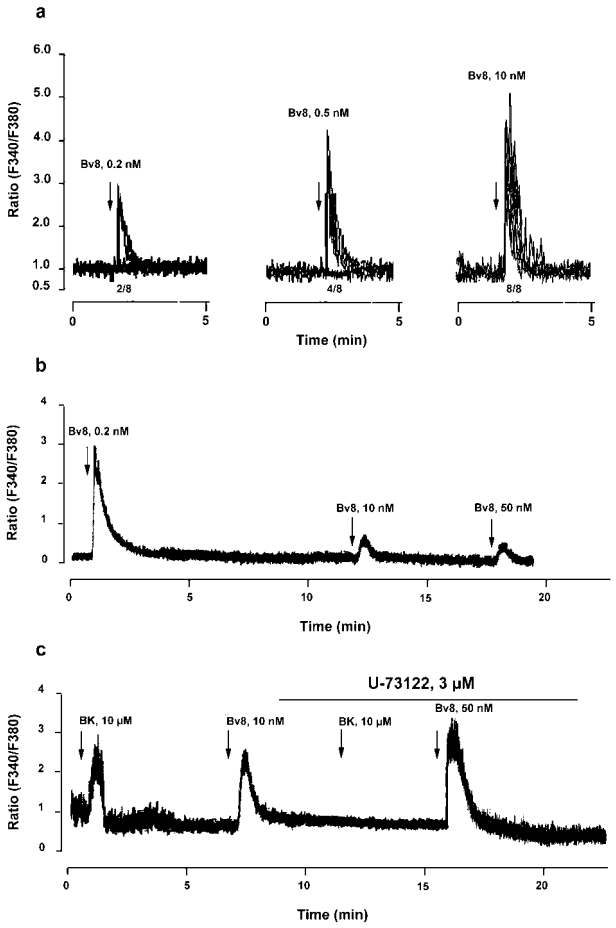


**Figure 6** Intracellular $Ca^{2+}$ concentration in small diameter neurons ($<30$ $\mu$m) of neonatal rat dorsal root ganglion (DRG) cultures measured by microspectrofluorometry of the $Ca^{2+}$ sensitive dye Fura-2. In each culture well, eight neurons were targetted for $[Ca^{2+}]_i$ Fura-2 fluorescence recording. In (a) the fraction of targetted neurons responding to Bv8 is annotated under each record. In (b) repeated application of increasing concentrations of Bv8 induced a strong homologous desensitization of $[Ca^{+2}]_i$ response. In (c) the PLC inhibitor U-73122 prevented bradykinin (BK) released $Ca^{2+}$ from $IP_3$-sensitive $Ca^{2+}$ stores but not Bv8-induced $[Ca^{+2}]_i$ transients. Each experiment was repeated from 4–6 times with similar results.

explanation we attempted to detect Bv8 receptors in DRG and spinal cord and identify them with the recently isolated prokineticin receptors PK-R1 and PK-R2 (Lin *et al*., 2002; Masuda *et al*., 2002). RT–PCR amplification of RNA extracted from DRG and spinal cord of adult and neonatal rats as well as from primary cultures of neonatal rat DRG, revealed two transcripts corresponding to the expected products from PK-R1 and PK-R2. The binding of [$^{125}$I]-BH-Bv8 to membrane preparations of DRG and dorsal horns of spinal cord was inhibited by EG-VEGF in a concentration-dependent manner and with $IC_{50}$ values comparable to those reported for EG-VEGF in PK-R transfected CHO cells (Masuda *et al*., 2002), The results from our binding assay indicate that both Bv8 and EG-VEGF are ligands for the same PK-Rs expressed by DRG and dorsal horns of spinal cord. Accordingly, the competitive displacement of [$^{125}$I]-BH-Bv8 from DRG and spinal cord receptors by graded concentrations of Bv8 and EG-VEGF indicated that the apparent affinity of Bv8 for PK-Rs is slightly higher than that reported for rat prokineticin 2 in PK-R transfected CHO cells (Masuda *et al*., 2002), but from 20 to 40 times higher than EG-VEGF affinity.

Bv8 acted as an agonist on DRG neurons. Added to cultures of neonatal rat DRG, Bv8 increased $[Ca^{2+}]_i$, showing a potent agonist activity on primary sensitive neurons of small diameter ($EC_{50}$ $\approx 0.5$ nM). These $[Ca^{2+}]_i$ transients underwent a rapid desensitization upon repeated application of Bv8. Data available on prokineticin-induced $[Ca^{2+}]_i$ transient in CHO cells expressing PK-Rs indicate that the source of increased $[Ca^{2+}]_i$ is the $IP_3$-sensitive intracellular $Ca^{2+}$ store activated by $G_q$-coupled PK-Rs through the $IP_3$-PLC signalling pathway (Lin *et al*., 2002; Masuda *et al*., 2002). Surprisingly, Bv8 failed to increase $[Ca^{2+}]_i$ in cultured DRG neurons when extracellular $Ca^{2+}$ was removed and the PLC inhibitor U-73122 apparently reduced the homologous desensitization of the $[Ca^{2+}]_i$ response instead of inhibiting the Bv8-induced $[Ca^{2+}]_i$ elevation. Thus PLC signalling pathway in DRG neurons may be implicated in the homologous desensitization rather than in the transduction of Bv8-induced $[Ca^{2+}]_i$ transients. Furthermore, this influx of extracellular $Ca^{2+}$ was not blocked by verapamil, indicating

that L-type channels are not involved in Bv8-induced $Ca^{2+}$ entry. Preliminary experiments appear to indicate that this influx of $Ca^{2+}$ occurs through the activation of non-selective cation channels (M. Colucci, personal communication). The short latency to $[Ca^{2+}]_i$ transient ($\approx$30 s) after the addition of Bv8 to the DRG culture apparently disagrees with the delayed onset of the nociceptive response to topical or s.c. injection of Bv8. After an intrathecal dose, however, hyperalgesia was already detectable 2 min after Bv8 injection, a latency comparable to that observed for $[Ca^{2+}]_i$ transient in the DRG culture. Thus the *in vivo* latency to the nociceptive response appears to depend on the pharmacokinetics of the protein rather than on indirect excitability changes in nociceptors.

Because no selective PK-R antagonists are yet available, we did not attempt to determine why there are two phases in the nociceptive response to Bv8. However, because the second phase of hyperalgesia develops only after intrathecal or systemic but not after plantar administration of the protein, it may be due to the well-known process of central sensitization (Doubell *et al*., 1999). The strong stimulation of a large number of nociceptive primary afferent terminals induced by the systemically delivered Bv8 or the direct activation of dorsal horn neurons evoked by the intrathecal injection of the protein, may trigger a delayed transient state of increased excitability of nociceptive dorsal horn neurons throughout the mechanism of central sensitization. On the contrary, local unilateral hyperalgesia produced by the intraplantar low doses of Bv8 probably did not lead to sufficient afferent activity to trigger the central sensitization.

In conclusion, our results suggest that Bv8, possibly through binding to the two prokineticin receptors, PK-R1 and PK-R2, located in primary sensitive neurons and spinal cord dorsal horn, results in sensitization of cutaneous nociceptors to painful mechanical and thermal stimuli. Further studies will be required to ascertain the exact cellular targets of the Bv8-induced nociceptive sensitization and to dissect and localize Bv8 activities at the molecular level. Clearly, analysing cellular receptor distribution with specific antibodies and detecting intracellular pathways involved in Bv8 signalling will be critical steps in defining the role of this novel secretory protein in the neurobiology of pain.

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