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Biological activities of Bv8 analogues

*,[1]Lucia Negri, [1]Roberta Lattanzi, [1]Elisa Giannini, [1]Maria Antonella Colucci, [2]Giuseppina Mignogna, [2]Donatella Barra, [3]Fabio Grohovaz, [3]Franca Codazzi, [4]Alessandra Kaiser, [4]Guenther Kreil & [1]Pietro Melchiorri

[1]Dipartimento di Fisiologia Umana e Farmacologia 'V. Erspamer', Università 'La Sapienza', 00185 Roma, Italy; [2]Dipartimento di Scienze Biochimiche 'A. Rossi Fanelli', Università 'La Sapienza', P.le A. Moro 5, 00185 Rome, Italy; [3]Dipartmento di Neuroscienze, San Raffaele, 20100 Milano, Italy and [4]Institute of Molecular Biology, Austrian Academy of Sciences, A-5020 Salzburg, Austria

1 The small protein Bv8, secreted by the skin of the frog *Bombina variegata*, belongs to a novel family of secreted proteins whose orthologues have been identified in snakes (MIT) and in mammals (prokineticins (PKs)). A characteristic feature of this protein family is the same N-terminal sequence, AVITGA, and the presence of 10 cysteines with identical spacing in the C-terminal domain. Two closely related G protein-coupled receptors that mediate signal transduction of Bv8/PKs have been cloned (PK-R1 and PK-R2). In mammals, the Bv8/PK protein family is involved in a number of biological activities such as ingestive behaviours, circadian rhythms, angiogenesis and pain sensitization.

2 In an attempt to identify the structural determinants required for the pronociceptive activity of Bv8, we prepared Bv8 derivatives lacking one (*des-Ala*-Bv8) or two (*des-Ala-Val*-Bv8) residues from the N-terminus.

3 *des-Ala*-Bv8 displayed a receptor affinity five times lower than that of Bv8, it was five times less potent in inducing $[Ca^{2+}]_i$ transients and in causing p42/p44 MAPK phosphorylation in CHO-cells expressing PK-R1 and PK-R2. Moreover, dA-Bv8 was about 20 times less potent than Bv8 in inducing hyperalgesia in rats.

4 The deletion of the first two amino acids of Bv8 abolished any biological activity both '*in vitro*' and '*in vivo*'; however, *des-AlaVal*-Bv8 is able to antagonize the Bv8-induced hyperalgesia, binding the PK-Rs on peripheral and central projections of the primary sensitive neurons.
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Abbreviations: dA-Bv8, *des-Ala*-Bv8; dAV-Bv8, *des-Ala-Val*-Bv8; MIT, mamba intestinal toxin; PK1, prokineticin 1; PK2, prokineticin 2; PK-R1 and PK-R2, prokineticin receptors 1 and 2

Introduction

The small protein Bv8, isolated from the skin of the closely related frogs *Bombina variega*ta and *B. bombina* (Mollay *et al*., 1999), belongs to a novel family of secreted proteins of about 80 amino acids. Bv8 homologues have been found in other species of *Bombinae*, *B. orientalis* and *B. maxima* (Chen *et al*., 2005), in the venom of the black mamba *Dendroaspis polylepsis* (VPRA or MIT-1; Joubert & Strydom, 1980; Schweitz *et al*., 1999), in rodents (mouse: mBv8 or prokineticin 2 (PK2); Wechselberger *et al*., 1999; rat: prokineticins, Masuda *et al*., 2002) and humans (EG-VEGF or prokineticin 1 (PK1) and hBv8 or prokineticin 2, LeCouter *et al*., 2001; Li *et al*., 2001; Jilek *et al*, 2000). All these proteins have the same amino-terminal sequence AVITGA, a fact that led us to propose the general name AVIT proteins for this family (Kaser *et al*., 2003). Another feature is the presence of 10 cysteines with identical spacing. A similar Cys motif is also present in mammalian co-lipase and in the carboxy-terminal region of members of the Dickkopf family of extracellular signalling proteins. However, the frog protein does not stimulate the activity of pancreatic lipase and it is also inactive in an assay for Dickkopf functions (Kaser *et al*., 2003). Starting from the 3D structure of mammalian co-lipase, models for MIT from snake venom (Boisbouvier *et al*., 1998) and frog Bv8 could be built, in which the hydrophobic amino-terminal sequence AVITGA forms a 'beak' exposed at the surface of the tightly folded rest of the protein.

In human, rat and mouse tissues, two closely related G protein-coupled receptors termed prokineticin receptors 1 and 2 (PK-R1 and PK-R2) have been identified for this family of proteins (Lin *et al*., 2002). The number of biological activities associated with Bv8 and its mammalian homologues is rapidly increasing. Originally identified as potent agents that contract smooth muscles of the gastrointestinal tract (Mollay *et al*., 1999; Li *et al*., 2001), they have been shown to also modulate complex behaviours, such as feeding and drinking (Negri *et al*., 2004), circadian rhythms (Cheng *et al*., 2002), and have been involved in hypothalamic hormone secretions (Lattanzi *et al*., 2001b), in neuronal survival (Melchiorri *et al*., 2001) and angiogenesis (Ferrara *et al*., 2003). Injections of small amounts of Bv8 (in the order of fmol) into rats and mice produce hyperalgesia and peripheral nociceptive sensitization (Mollay

*Author for correspondence; E-mail: lucia.negri@uniroma1.it

et al., 1999; Negri *et al*., 2002) by activating PK-R1 and PK-R2 in the primary sensitive neurons of dorsal root ganglia. The mammalian Bv8 (or PK2) is highly expressed in circulating and inflammatory leukocytes (Lattanzi *et al*., 2001a), which suggests that Bv8-like proteins released from these cells at the site of inflammation contribute to inflammatory hyperalgesia.

In this report, we present our attempts to identify the structural determinants required for the pronociceptive activity of Bv8. In view of the strict conservation of the amino end mentioned earlier, we prepared Bv8 derivatives lacking one or two residues from this terminus. The binding affinity, the '*in vitro*' biological activity and the hyperalgesic potency '*in vivo*' of these truncated forms were analysed. In addition, their activity as agonists or antagonists was tested.

Methods

Preparation of Bv8 and its derivatives with N-terminal deletions

Bv8 was purified from skin secretions of *B. variegata* as described previously (Mollay *et al*., 1999).

Des-Ala-Bv8 (dA-Bv8) was prepared by digestion of the protein with aminopeptidase M (Sigma-Aldrich; sequencing grade). The dried sample was dissolved in 100 mM sodium phosphate buffer, pH 7.0, and the aminopeptidase was added to a concentration of 5% by weight of the substrate. Following incubation at 37°C for 24 h, the reaction was stopped by lyophilization. Interestingly, cleavage virtually stops after the first amino acid.

Des-Ala-Val-Bv8 (dAV-Bv8) was obtained by digestion of the protein with thermolysin. An aliquot of the dried sample was dissolved in 100 mM NH_4HCO_3, 1 mM $CaCl_2$ and thermolysin (Roche) was added to 6% by weight of the substrate. Following incubation at 37°C for 24 h, the reaction was stopped by lyophilization.

The complete removal of the N-terminal amino acid(s) was tested by automated Edman degradation, using an Applied Biosystems model 476A sequencer (Applied Biosystems, Framingham, MA, U.S.A.), and by MALDI-TOF analysis, using a Voyager-DE™ STR instrument (Applied Biosystems, Framingham, MA, U.S.A.).

Biological activity on GPI

Smooth muscle preparations were obtained from guinea-pig ileum (GPI) using the segment 10–15 cm rostral to the caecum. These were suspended at 37°C in a 10 ml bath containing Krebs solution gassed with 5% CO_2, as described previously (Mollay *et al*., 1999). The motility of the isolated preparations was recorded using an isotonic microdynamometer (Basile, Italy). Adequate and reproducible contractions of the smooth muscle preparations were tested with 6–10 nM acetylcholine.

Culture of CHO cells stably expressing PK-R1 and PK-R2

CHO/PK-R1 and CHO/PK-R2 cells (kind gift of Dr Hui Tian, Tularik, San Francisco, U.S.A.) were plated onto flasks and cultured in DMEM/F12 (1 : 1) enriched with 1% penicillin/streptomycin, 5% foetal bovine serum (FBS) and 1% L-glutamine plus 200 ng ml^{-1} geneticin G418 (Invitrogen srl, Milano, Italy). Every 3 days, confluent cells were removed with phosphate-buffered saline (PBS; Invitrogen) containing 1 mM EDTA (PBS/EDTA), splitted 1 : 10 and plated under the same conditions.

Receptor-binding assay

Affinity of Bv8 proteins for prokineticin receptors was assayed on membrane preparations from CHO cells stably transfected with PK-R1 or PK-R2. Confluent CHO cells (about 20 million cells) were rinsed with PBS/EDTA, detached from the culture flasks and collected by centrifugation. The resulting pellet was homogenized in 10 ml ice-cold homogenizing buffer (50 mM Tris-HCl, pH 7.4) by a Polytron homogenizer (PT3000, Kinematica) at 16,000 r.p.m. for 2 min. The homogenate was centrifuged at low speed ($700 \times g$ for 15 min at 4°C) and the resulting supernatant was centrifuged at $100{,}000 \times g$ for 60 min at 4°C. The resulting pellet was then resuspended in 10 ml 50 mM Tris-HCl, pH 7.4, and stored at −80°C until use. Protein concentration was determined by the BCA Protein Assay Kit (Pierce, Rockfort, IL, U.S.A.). Membranes (20 μg of proteins for PK-R1 and 40 μg of proteins for PK-R2) were incubated with 4 pM [^{125}I]MIT and graded concentrations of Bv8, dA-Bv8 or dAV-Bv8 in a final volume of 1 ml at 37°C for 90 min. Each concentration was tested in duplicate. After incubation the samples were cooled and membranes were harvested on Whatman GF/B filters pre-soaked in 0.5% polyethylenimine (Sigma-Aldrich, Milano, Italy), washed nine times with 2 ml of cold 50 mM Tris-HCl, pH 7.4, and transferred to counting vials. Radioactivity was measured in a γ-counter (Packard, Cobra II auto-gamma). Nonspecific binding was determined in the presence of 0.1 μM Bv8. Displacement curves and IC_{50} values were calculated with the PRISM software (GraphPad Software, San Diego, CA, U.S.A.).

ERK phosphorylation in CHO cells stably expressing PK-R1 and PK-R2

Cells of low passage number (3–6) were used in all studies. About 5×10^5 cells were seeded in 2-cm^2 wells. At confluency, cultures were starved in 0.5 ml serum-free medium for 6 h. Various concentrations of Bv8 and its shortened derivatives were then added in a volume of 5 μl. Where indicated, a 5-μl solution of dAV-Bv8 was added 20 min before Bv8. Cells were lysed in 70 μl lysis buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 50 mM NaF, 5 mM β-glycerophosphate, 2 mM EDTA, 2 mM EGTA, 1 mM Na_3PO_4, 1% NP-40, 1 mM PMSF, 5 μg ml^{-1} leupeptine, 5 μg ml^{-1} pepstatin A) on ice for 10 min. The lysate was centrifuged (10 min, $15{,}000 \times g$, 4°C) and the supernatant stored at −80°C. The samples (10 μg per lane) were separated by SDS–PAGE, on a 12.5% polyacrylamide gel (25 mA per gel), transferred onto a nitrocellulose Hybond C membrane (Amersham Pharmacia) and incubated overnight at 4°C with phospho-p44/42 MAPK antibody (1 : 2000, New England Biolabs). After 1 h incubation at room temperature with a goat anti-rabbit IgG conjugated to horseradish peroxidase (1 : 2000, Amersham Pharmacia), the immunoreactive bands were exposed to chemiluminescent reagents (ECL plus, Amersham Pharmacia) and densitometrically analysed by a Versadoc 3000 (BioRad).

Intracellular calcium imaging

CHO cells were plated onto coverslips precoated with $10\,\mu g\,ml^{-1}$ poly-L-lysine at a density of 1×10^5 cells per coverslip. After 20 h, cells were incubated for 30 min at 37°C with the fluorescent indicator Fura-2 acetoxymethyl ester (2 μM Fura-2AM; Calbiochem, San Diego, CA, U.S.A.), dissolved in the presence of 0.0125% F-127 pluronic acid (Calbiochem). Cells were then washed in the following Krebs-Ringer solution buffered with HEPES (KRH): 125 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 1.2 mM KH_2PO_4; 1.2 mM $MgSO_4$; 25 mM HEPES, pH 7.4. Coverslips were mounted onto an inverted microscope Zeiss Axiovert 135 TV ($\times$63 objective), connected with a double-wavelength CAM-230 microfluorimeter (Jasko). The fluorescent images were recorded by an intensified CCD camera (Photonic Science), transferred to a digital processor, digitalized and integrated, and the fluorescence intensity measured. Various concentrations of Bv8 (0.2–20 nM) and its truncated forms were added in a volume of 100 μl in a final volume of 700 μl. The effect of the compounds was evaluated as (number of responding cells/number of cells in the field) $\times$ 100.

Animals

All experiments were carried out in male Sprague–Dawley rats (250–300 g) under protocols approved by the Animal Care and Use Committee of the Italian Ministry of Health according to EC directives. Each rat was used only once and immediately killed by CO_2 inhalation after the experiment ended.

Drug injections

Bv8, dA-Bv8 and/or dAV-Bv8 were injected by subcutaneous (s.c.), intrathecal (i.t.) or intrapaw (i.paw) routes. For the i.pl. injections, the drugs dissolved in 0.9% NaCl were injected into the plantar (20 μl) and dorsal (20 μl) regions of the paw with a microsyringe fitted with a 30-gauge needle. Control rats were injected with an equal volume of saline solution. For i.t. applications, chronic lumbar i.t. catheters were implanted in rats under ketamine–xylazine anaesthesia ($60\,mg\,kg^{-1}+10\,mg\,kg^{-1}$, i.p.) as described previously (Negri *et al*., 2002). I.t. vehicle was artificial cerebrospinal fluid, and each rat received 5-μl of the vehicle or solution of tested compounds in vehicle, followed by a 5 μl vehicle flush. For systemic administration, compounds dissolved in saline solution were injected in a volume of $2\,ml\,kg^{-1}$ by the s.c. route. Controls were injected with an equal volume of saline solution.

Mechanical nociception

The pressure-evoked paw withdrawal response was assessed with the Randall–Selitto test (Randall & Selitto, 1957) in male Wistar rats (250–300 g), trained to tests during the week preceding the experiment (Negri *et al*., 2002). On the day of the experiment, nociceptive threshold was measured for 1 h at 30-min intervals before drug injection. The mean of the last two of these measurements was taken as baseline nociceptive threshold (NT_B). After injection of test substance, the nociceptive threshold was then determined three times at 15, 30, 45, 60, 90, 120, 150, 180, 240, 300 and 360 min. 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 from baseline threshold (%ΔNT) according to the following equation:

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

Statistical analysis

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

Results

Preparation of Bv8 proteins with deletions at the amino end

By incubation of Bv8 with either aminopeptidase M or thermolysin, truncated forms of the protein could be prepared. Interestingly, the aminopeptidase only cleaved the first amino acid yielding dA-Bv8, while thermolysin only cut after the second residue yielding dAV-Bv8. Yields of hydrolysis were above 90%.

Contraction of the guinea-pig ileum

As previously reported, due to intense tachyphylaxis lasting for about 2 h, it was not possible to obtain dose–response curves for the action of Bv8 or its shortened forms on a single-ileum preparations. Comparisons of equipotent doses demonstrate that, cleavage of the amino-terminal residues of Bv8 reduced its potency. At a concentration of 10 nM, dA-Bv8 induced contractions comparable to those induced by 1.25 nM Bv8, while even very high concentrations of dAV-Bv8 (125 nM) induced only negligible contractions (Table 1).

Binding to prokineticin receptors

Similar concentrations of Bv8 were necessary to displace bound ^{125}I-MIT from human PK-R1 (IC_{50} 0.69$\pm$0. 23 nM) or PK-R2 (IC_{50} 0.71$\pm$0.5 nM) expressed in CHO cells. dA-Bv8 had a somewhat lower affinity for both PK-R1 (about three times) and PK-R2 (about five times). Deletion of the first two amino acids had a dramatic effect. The affinity of dAV-Bv8 for both PK-R1 and PK-R2 was about 200 times lower than that of intact Bv8 (Table 1).

Table 1 Inhibitory potencies of Bv8 and its shortened forms on the specific binding of 4 pM ^{125}I-MIT at PK-R1 and PK-R2 receptors in CHO cell membranes and concentration of Bv8 and dA-Bv8 equiactive on guinea-pig ileum (GPI) contractions

	IC_{50} (^{125}I-MIT, 4 pM)		
	PK-R1	*PK-R2*	*GPI* (nM)
Bv8	0.69$\pm$0.23	0.71$\pm$0.5	1.2
dA-Bv8	1.9$\pm$0.1	4.4$\pm$0.9	10
dAV-Bv8	145$\pm$10	159$\pm$21	—

p44/42 MAP kinase (ERK) phosphorylation

In CHO-PK-R1 and CHO-PK-R2 cells, starved in a serum-free medium for 6 h, Bv8 produced a dose-dependent phosphorylation of p44/42 MAPK. Pilot studies demonstrated that this response peaked around 10 min. The phosphorylation of p44/42 MAPK induced by different doses of Bv8 (from 0.1 to 15 nM) was determined after 10 min. Bv8 was about twice as active in CHO-PK-R1 than in CHO-PK-R2 cells (Figure 1).

In CHO-PKR1 cells, dA-Bv8 at 1 nM induced a p44/42 MAPK phosphorylation comparable to that produced by 0.2 nM Bv8, while dAV-Bv8 up to 20 nM did not produce a signal beyond control values. Interestingly, pretreatment (−20 min) of cells with 20 nM dAV-Bv8 antagonized the phosphorylation induced by 0.2 nM Bv8 (Figure 2).

Intracellular calcium imaging

In CHO-PK-R1 cells, Bv8 at 0.02–1 nM induced transient increases in $[Ca^{2+}]_i$ in a concentration-dependent number of cells (Figure 3a). The analysis of the temporal traces demonstrated that the $[Ca^{2+}]_i$ increased 10–40 s after the application and lasted for 50–100 s (Figure 3b).

Both in PK-R1- and in PK-R2-transfected CHO cells, effects comparable to those of Bv8 were obtained with five times higher concentrations of dA-Bv8. Conversely, dAV-Bv8 up to 100 nM induced an increase in $[Ca^{2+}]_i$ in only a negligible percentage of cells (Figure 4).

Hyperalgesia

As already described (Negri *et al*., 2002), when administered by systemic or i.t. routes, Bv8 decreased the nociceptive threshold to mechanical stimuli applied to the paw with a characteristic biphasic time-course. An i.t. injection of 0.06 pmol of Bv8 halved the nociceptive threshold. Hyperalgesia was evident within 2 min, peaked at 30 min, lasted 90–120 min, and was followed by a 1-h recovery. The second decrease in the threshold peaked at the fourth hour and lasted till the sixth hour. An i.t. dose of 0.06 pmol of dA-Bv8 failed to induce hyperalgesia, but 0.25 pmol dose induced weak hyperalgesia and 1.25 pmol dose induced hyperalgesia comparable to that induced by 0.06 pmol Bv8 (Figure 5a).

An i.paw injection of 0.05 pmol of Bv8 produced a monophasic time-course of hyperalgesia in the injected paw,

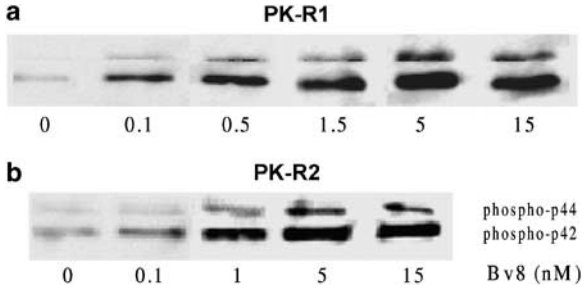

Figure 1 Bv8-induced p44/42 MAPK phosphorylation in PK-R1 (a) and PK-R2 (b) stably transfected CHO cells.

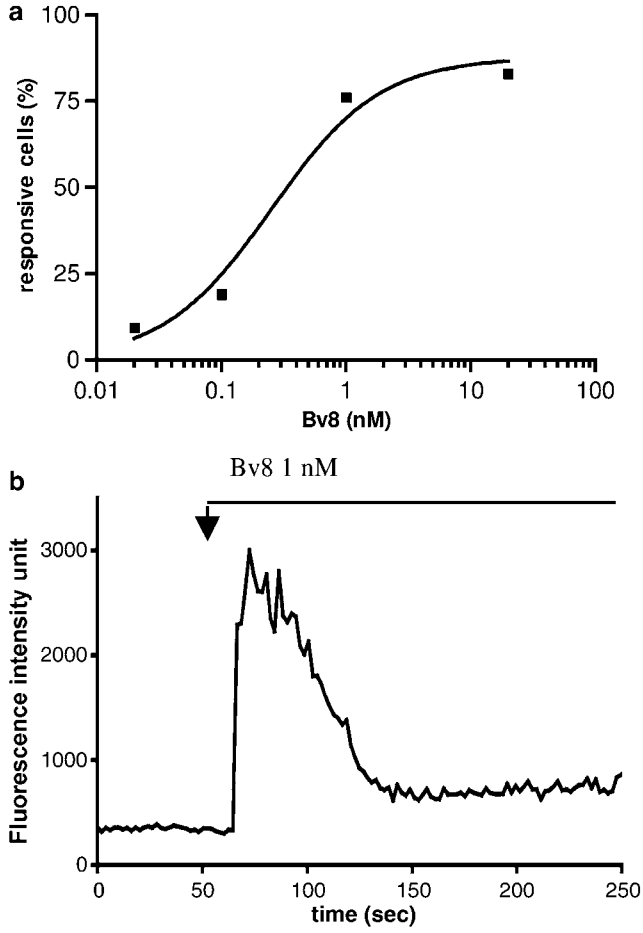

Figure 3 (a) $[Ca^{2+}]_i$ transient induced by different concentrations of Bv8 (0.02–20 nM) in CHO-PK-R1-transfected cells. The responding cells are indicated as % of total cells in the field (70–100 cells). Values are mean ± s.e.m. of four experiments. (b) Time course of $[Ca^{2+}]_i$ transient in a single CHO-PK-R1-transfected cell. The temporal fluorescent trace is expressed as ratio of Fura-2AM excitation wavelength (340/380 nm).

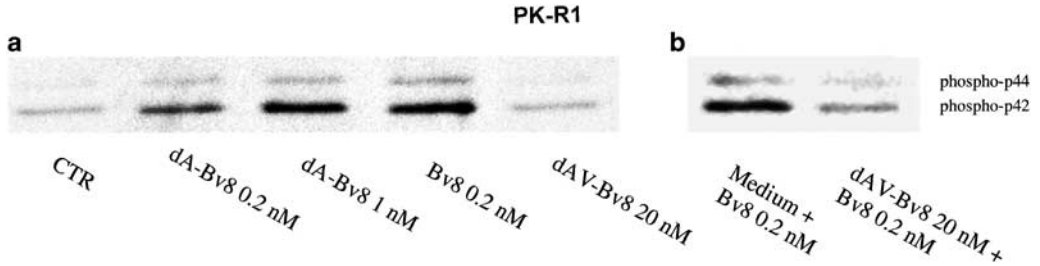

Figure 2 (a) P44/42 MAPK phosphorylation in PK-R1-transfected CHO cells treated for 10 min with Bv8 and its shortened forms; (b) antagonistic effect of dAV-Bv8 (20 nM) added 20 min before the delivery of Bv8 0.2 nM.

but left the nociceptive threshold of the controlateral paw unchanged. An i.paw dose of 0.05 pmol of dA-Bv8 failed to induce hyperalgesia, but 1 pmol of dA-Bv8 induced hyperalgesia comparable to that obtained with 0.05 pmol of Bv8 (5b). A higher dose (2.5 pmol, i.paw) produced a systemic effect, inducing hyperalgesia also in the controlateral paw (data not shown).

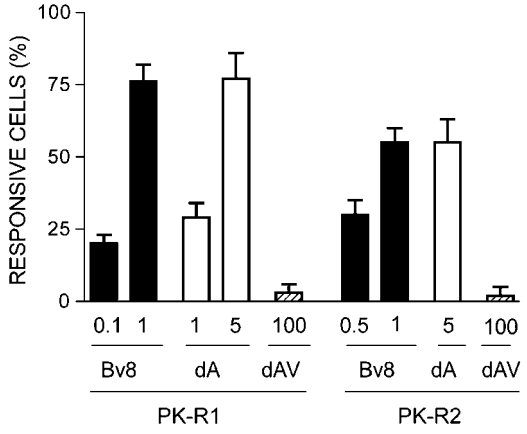

Figure 4 $[Ca^{2+}]_i$ transients induced by Bv8 and its shortened forms in PK-R1- and PK-R2-CHO-transfected cells. The responding cells are indicated as % of total cells in the field (70–100 cells). Values are mean ± s.e.m. of four experiments.

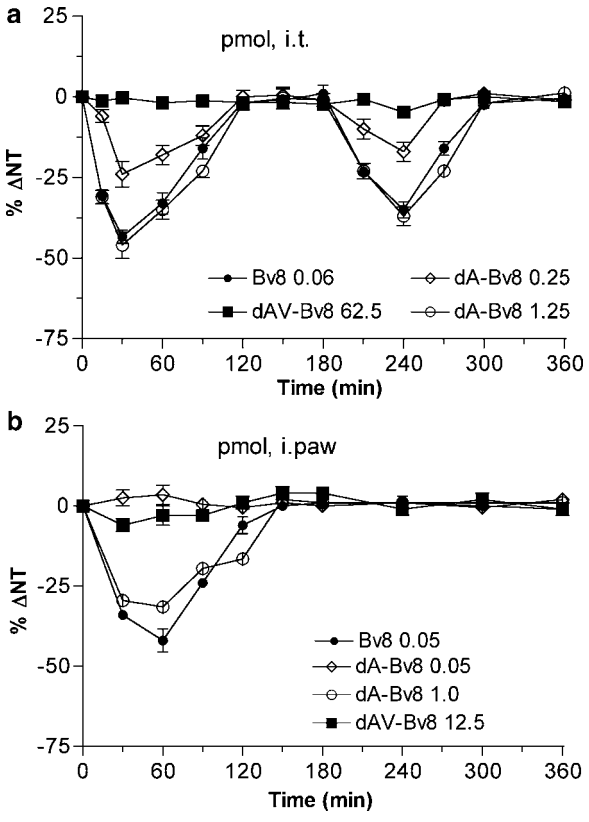

Figure 5 Time course of % decrease in mechanical (Randall–Selitto test) nociceptive threshold (%ΔNT) elicited in rats by i.t. (a) and i.paw (b) injection of Bv8 and its shortened forms (pmol per rat). Each point represents mean ± s.e.m. of six-rat groups. Baseline threshold was 263 ± 15 g ($n = 50$ rats).

Very high doses of dAV-Bv8, both i.t. (up to 62.5 pmol i.t) or i.paw (up to 12.5 pmol), was completely ineffective (Figure 5a and b), but it was able to abolish Bv8-induced hyperalgesia. Indeed, hyperalgesia caused by i.paw injection of 0.05 pmol of Bv8 could be abolished by first injecting dAV-Bv8 (−15 min; 2.5 nmol kg^{-1} s.c. or 12.5 pmol i.paw, Figure 7a). I.t. administration of dAV-Bv8 12.5 pmol, 15 min before i.t. administration of Bv8 0.06 pmol, abolished the first phase of nociceptive threshold decrease, leaving the second phase unchanged. Lower doses (3.75 and 1.25 pmol) significantly reduced, but not completely abolished, the first phase (Figure 6a). In all,

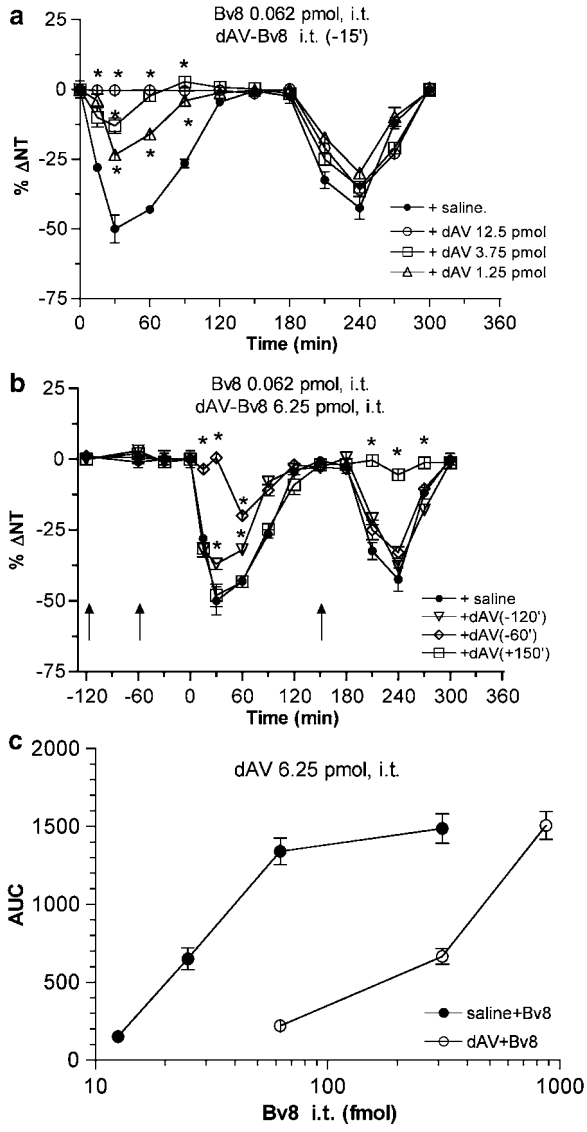

Figure 6 dAV-Bv8 antagonizes the hyperalgesia induced by i.t. injection of Bv8. (a) i.t. injections of different doses of dAV-Bv8 (−15′) abolish (12.5 pmol) or significantly reduce (3.75 and 1.25 pmol) the first phase of hyperalgesia induced by i.t. injection of 62 fmol of Bv8. (b) Antagonistic efficacy of dAV-Bv8 (6.25 pmol i.t.) when administered i.t. 60 or 120 min before, or 150 min after i.t. injection of 62 fmol Bv8. (c) The dose–response curve of hyperalgesic effect induced by i.t. injection of Bv8 is shifted to the right by i.t. preadministration (−15 min) of dAV-Bv8 6.25 pmol. The effect of Bv8 was evaluated as AUC corresponding to the first phase of hyperalgesia. *$P < 0.01$ compared with Bv8.

12.5 pmol of desAV-Bv8 i.t., injected 150 min after Bv8 (i.e. before the beginning of the second phase of hyperlagesia), abolished the second phase of hyperalgesia. The same dose of dAV-Bv8 was still able to antagonize Bv8-induced hyperalgesia when injected i.t., 60 min before Bv8, but was ineffective if administered more than 2 h before Bv8 injection (Figure 6b). The dose–response curve of Bv8 induced hyperalgesia (expressed as area under the response curve (AUC) to the first phase of hyperalgesia) was shifted to the right of one order of magnitude by 6.25 pmol dAV-Bv8 (Figure 6c).

S.c. injection of Bv8 (25 pmol kg^{-1}) induced a biphasic hyperalgesia, as was also observed after i.t. injection. The first peak of hyperalgesia caused by Bv8 could be abolished by first injecting dAV-Bv8 (−15 min; 2.5 nmol kg^{-1}, s.c.; Figure 7b).

We also tested the 'cross-talk' between different routes of administration of Bv8 and of its antagonist dAV-Bv8. I.t. injection of 12.5 pmol of dAV-Bv8 (the dose that abolishes the hyperalgesia produced by i.t. injection of Bv8) did not antagonize the systemic hyperalgesia induced by s.c. injection of Bv8 (25 pmol kg^{-1}; Figure 7b) nor the local hyperalgesia induced by the i.paw injection of Bv8 (0.05 pmol; Figure 7a). However, s.c. injection of dAV-Bv8 (−15 min; 2.5 nmol kg^{-1}, s.c.) was able to antagonize the first hyperalgesic phase induced by i.t. injection of Bv8 (0.062 pmol; Figure 7c) and the hyperalgesia induced by 0.05 pmol of Bv8 i.paw (Figure 7a).

Discussion

It seemed likely that the highly conserved amino-terminal sequence of all members of the AVIT-family, which moreover protrudes from the tightly folded rest of the molecule, is important for the biological activity. Indeed, it could recently be shown that substitutions, deletions and insertions at the N-terminus of human prokineticin1 led to the loss of agonist activity and sometimes yielded antagonists (Bullock *et al*., 2004). In this study, we have analysed the biological activities of Bv8 molecules lacking one or two residues from the amino end. These truncated variants still bind to the prokineticin receptors, but their ability to activate them is changed. Using membranes from CHO cells expressing PK-R1 or PK-R2, it could be shown that the affinity of dA-Bv8 for these receptors was 3–5 times lower than that of Bv8. It also was five times less potent in inducing $[Ca^{2+}]_i$ transients and in causing p42/p44 MAPK phosphorylation in these transfected cells. Moreover, dA-Bv8 was about eight times less potent than Bv8 in stimulating GPI contractions and 20 times less potent than Bv8 in inducing hyperalgesia in rats. The deletion of the first two amino acids of Bv8 abolishes any biological activity, both *in vitro* and *in vivo*, in the different tests used in this study; however, it does not preclude the possibility to bind the receptors. Albeit an affinity 200 times lower than that of Bv8, dAV-Bv8 is still able to bind the receptors and to antagonize the Bv8-induced ERK phosphorylation *in vitro*, and hyperalgesia *in vivo*. *In vivo* experiments demonstrated that dAV-Bv8 shifts to the right the dose–response curve of hyperalgesia, competitively binding the PKRs: it is able to antagonize the Bv8-induced hyperalgesia only when it is administered by the same route as Bv8, or when, after systemic administration, it reaches PKRs on peripheral and central projections of the primary sensitive neurons. The antagonist effect of dAV-Bv8

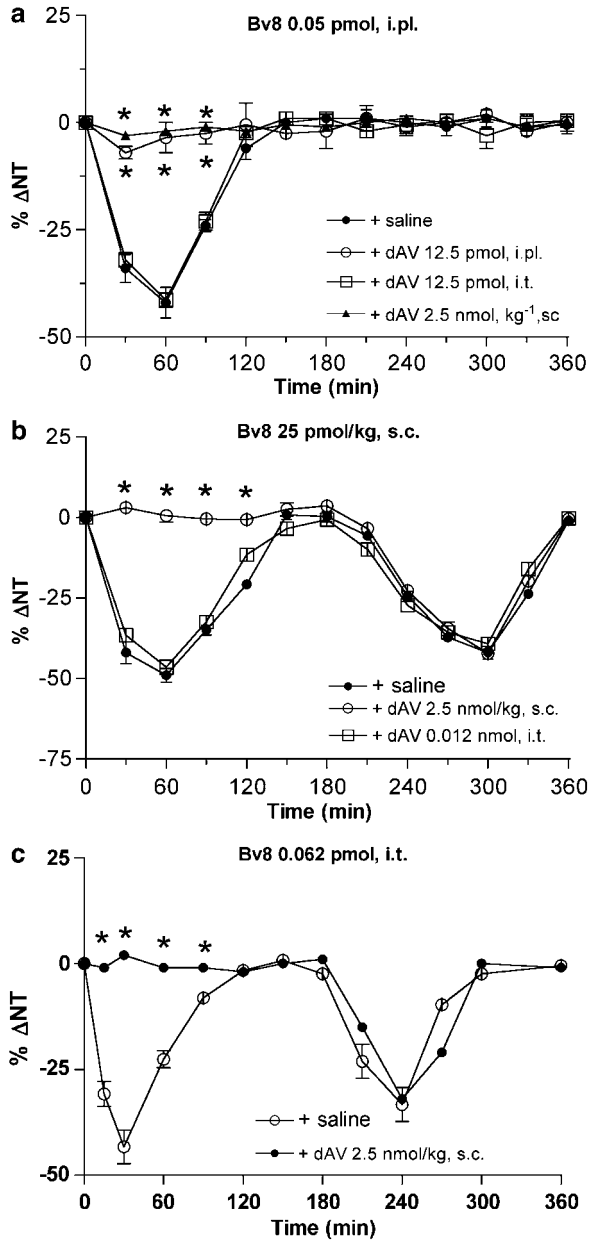

Figure 7 (a) The hyperalgesia induced by i.paw injection of Bv8 is antagonized by i.paw or s.c., but not by i.t. preadministration (−15 min) of dAV-Bv8. (b) s.c. but not i.t. preadministration of dAV-Bv8 abolishes the first phase of hyperalgesia produced by s.c. injection of Bv8. (c) s.c. preadministration of dAV-Bv8 abolishes the first phase of hyperalgesia produced by i.t. injection of Bv8. $^*P<0.01$ compared with Bv8.

lasts for no more than 2 h. It is thus able to abolish the hyperalgesia induced by the topical injection of Bv8 into the paw, while it antagonizes only the first phase of hyperalgesia caused by systemic or i.t. injection of Bv8. Administration of dAV-Bv8 before the beginning of the second phase, that is, about 150 min after the systemic or i.t. administration of Bv8, abolished the second phase of hyperalgesia.

In case of Bv8, deletion of the first amino acid, apparently, has less dramatic effects than in PK1. It has been reported (Bullock *et al*., 2004) that PK1 lacking the amino-terminal alanine has only residual activity at PK-R1, about 150-fold lower than that of PK1. This PK1 variant also had only

negligible activity in tests on $[Ca^{2+}]_i$ release in CHO cells expressing PK-R2 and stimulation of ileum contraction. The fact that the same N-terminal deletion induces only a five-fold loss of biological activity in Bv8, but a more than 150-fold loss of activity in PK1, clearly indicates that, besides the amino-terminal segment, the rest of these molecules also contribute significantly to the activation of the receptors. This notion is supported by comparing the hyperalgesic potency of PK1/EG-VEGF, Bv8 and MIT: we have demonstrated that PK1/EG-VEGF is at least 40 times less potent than Bv8 in inducing hyperalgesia (work in preparation) but the snake analogue, MIT, is at least 20 times more potent than Bv8 (Mollay *et al*., 1999). As most of the differences between the proteins from frog, snake and mammals are found close to the carboxyl end, we could speculate that the C-ends of the molecules are responsible for the different activation of the PK-R1 and PK-R2 localized on peripheral nociceptive neurons.

On the other end, the N-terminal deletion of alanine and valine, in Bv8 molecule, yields a molecule lacking any biological activity but still able to bind the receptors, demonstrating that the C-terminal cysteine-rich domain also contributes to binding. Unfortunately, dAV-Bv8 is a short-acting antagonist *in vivo*. Nevertheless, this could be a starting point for attempts to develop PKR antagonists.

We have demonstrated that Bv8 sensitizes nociceptors to mechanical and thermal stimuli (Negri *et al*., 2002) and activates prokineticin receptors on macrophages to produce chemotaxis and release inflammatory cytokines. Moreover, the mammalian Bv8 (or PK2) is highly expressed in circulating and inflammatory leukocytes (Lattanzi *et al*., 2001a; Le Couter *et al*., 2004). Systemic *in vivo* exposure to Bv8 or PK1/EG-VEGF resulted in significant increases in total leukocyte, neutrophil and monocyte counts (Le Couter *et al*., 2004) and PK1 induces a distinct monocyte-derived cell population, which is primed for release of proinflammatory cytokines (Dorsch *et al*., 2005). We would like to propose that Bv8/prokineticins and their receptors are part of a novel signalling pathway that triggers the development of inflammatory and neuropathic pain. The signalling molecules expressed by peripheral nociceptors and inflammatory cells are potential targets for drugs which block this nociceptive information before it reaches the brain. The identification of the structural determinants required for the receptor binding and the hyperalgesic activity of Bv8 would be a first step in a search for antagonist molecules.

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