ELSEVIER

European Journal of Pharmacology 374 (1999) 189–196

ejp

www.elsevier.nl/locate/ejphar

Bv8, a small protein from frog skin and its homologue from snake venom induce hyperalgesia in rats

Christa Mollay [a], Christian Wechselberger [a], Giuseppina Mignogna [b], Lucia Negri [c], Pietro Melchiorri [c], Donatella Barra [b], Günther Kreil [a,*]

[a] *Institute of Molecular Biology, Austrian Academy of Sciences, Billrothstrasse 11, A-5020 Salzburg, Austria*
[b] *Dipartimento di Scienze Biochimiche and CNR Centro di Biologia, Moleculare, Universita La Sapienza, I-00185 Rome, Italy*
[c] *Istituto di Farmacologia Medica, Universita La Sapienza, I-00185 Rome, Italy*

Received 3 December 1998; received in revised form 23 March 1999; accepted 30 March 1999

Abstract

From skin secretions of *Bombina variegata* and *Bombina bombina*, we isolated a small protein termed Bv8. The sequence of its 77 amino acids was established by peptide analysis and by cDNA cloning of the Bv8 precursor. Bv8 stimulates the contraction of the guinea-pig ileum at nanomolar concentrations. The contraction is not inhibited by a variety of antagonists. Injection of a few micrograms of Bv8 into the brain of rats elicits, as assessed by the tail-flick test and paw pressure threshold, a marked hyperalgesia which lasts for about 1 h. Bv8 is related to protein A, a component of the venom of the black mamba. After i.c.v. injection, protein A is even more active than Bv8 in inducing hyperalgesia.

Keywords: Bv8 (*Bombina variegata* protein 8 kDa); Hyperalgesia, rat; Ileum, guinea pig

1. Introduction

Over the past 30 years, numerous studies have demonstrated that skin secretions of amphibia contain peptides with diverse biological activities (Bevins and Zasloff, 1990; Lazarus and Attila, 1993). Many of these peptides are related to mammalian hormones or neurotransmitters. In addition, in recent years, a large variety of antimicrobial peptides has been isolated from the same source (Barra and Simmaco, 1995).

The peptides produced in the skin of *Bombina* species were among the first to be investigated (Kiss and Michl, 1962). These are mainly bombesin (Erspamer et al., 1970; Taché et al., 1988), a peptide related to gastrin releasing hormone from mammals, and the bombinins (Gibson et al., 1991; Simmaco et al., 1991) and bombinins H (Mignogna et al., 1993), two heterogeneous groups of antimicrobial peptides. In addition, a trypsin inhibitor related to protease inhibitors from nematodes has recently been isolated from this source (Mignogna et al., 1996).

Here, we describe the isolation and characterization of a small protein from *Bombina* skin secretions. This amphibian protein is related to protein A, a non-toxic constituent of the venom of the black mamba (Joubert and Strydom, 1980). The new protein is termed Bv8 (*Bombina variegata* protein with a molecular mass of 8 kDa). Some of the pharmacological activities of Bv8 and protein A are described.

2. Materials and methods

2.1. Materials

Lys-C and Asp-N endoproteinases were purchased from Boehringer, 4-vinylpyridine from Aldrich-Chemie, solvents for high-pressure liquid chromatography (HPLC) from Carlo Erba Reagenti, and other sequence grade chemicals from Perkin-Elmer. Restriction enzymes were obtained from Boehringer Mannheim, Bethesda Research

* Corresponding author. Tel.: +43-662-639-6121; fax: +43-662-639-6129; E-mail: gkreil@oeaw.ac.at

0014-2999/99/$ - see front matter
PII: S0014-2999(99)00229-0

Laboratories and MBI Fermentas; [^{32}P]-dATP was purchased from New England Nuclear; oligonucleotides were synthesized on a Beckman Oligo 1000 synthesizer. IRD-41 labeled sequencing oligonucleotides were obtained from MWG Biotech. Black mamba venom was purchased from ICN. Atropine, pyrilamine (*N*-[(4-methoxyphenyl)methyl]-*N*′,*N*′-dimethyl-*N*-2-pyridinyl-1,2-ethanediamaine), nifedipine (1,4-Dihydroxy-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester) were from Sigma, ICS 205,930 (3-tropanyl-indole-3-carboxylate methiodide) from RBI (Natick, USA), SR 140,333 ((*S*)1-(2-[(3,4-dichlorophenyl)-1-(3-isopropoxy-phenylacetyl)-piperidin-3-yl]ethyl)-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride) from SANOFI (Italy), tetrodotoxin from Tokris Cookson (UK), endothelin-1-(11–21) and endothelin-1-(16–21) from Bachem (Switzerland), [^{3}H](D-Ala^2, *N*-Me-Phe^4, glycinol^5)-enkephalin (DAGO), [^{3}H]nociceptin, [^{3}H]naltrindole, and [^{3}H]U-69,593 [(5α,7α,8β)-(+)-*N*-methyl *N*-7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl)benzenaceta-ide] were from NEN (Milano, Italy).

2.2. Protein purification

About three dozen frogs, mostly *B. variegata*, but as judged from the coloration of the belly, also some *Bombina bombina* and some intermediate forms, were kept in shallow water and fed with tubifex. Skin secretions were collected in physiological saline (Simmaco et al., 1991) and then dialyzed. The secretion was passed over a concanavalin-A-Sepharose column and the flow-through was chromatographed once more through such a column to remove glycoproteins, mucins, etc. The flow-through from the second column was dried and the dry residue was extracted with water. The solution was then extracted three times with *n*-butanol to remove bombinins and bombinins H. The aqueous phase was dried, redissolved in a small volume of water, adjusted to pH 4.5 with acetic acid and chromatographed over SP-Sepharose (Pharmacia). Bound material was eluted with 2 M NaCl and then de-salted by filtration through a 3 kDa centriprep (Amicon). The solution was then lyophilized, dissolved in 0.2% trifluoroacetic acid and finally separated by reversed phase HPLC (see Fig. 1).

Protein A from the venom of the black mamba was isolated as described by Schweitz et al. (1990). The fraction which eluted early from a Sephadex G50 column was further resolved by HPLC.

2.3. Amino acid sequence determination

The purified proteins (0.2 mg) were pyridylethylated with 4-vinylpyridine under denaturing and reducing conditions as described previously (Mignogna et al., 1996). Aliquots of the modified frog protein were digested with Lys-C or Asp-N endoprotease (80–100 μg sample, 3 μg enzyme, in 25 mM Tris–HCl, pH 8.5, 15 h, 37°C). Proteolytic fragments were purified on a Beckman System Gold chromatographer using a macroporous reversed-phase column (RP-300, 4.6 × 250 mm, Perkin-Elmer; 0–30% acetonitrile in 0.2% trifluoroacetic acid). The elution of peptides was monitored at 220 and 280 nm (Beckman 168 detector).

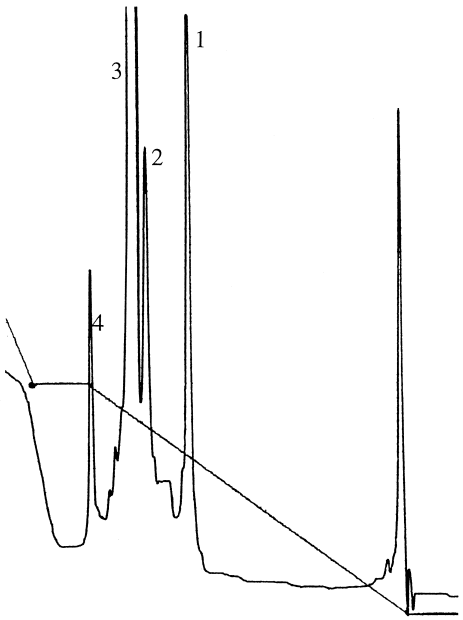

Fig. 1. Reversed phase HPLC of the fraction eluted from the SP-Sepharose column (see Section 2). For the fractionation, we used an Aquapore RP-300 C8 column (Perkin-Elmer, 7 × 250 mm). Solvents were: (A) 0.2% trifluoroacetic acid; (B) acetonitrile:isopropanol 4:1 in 0.2% trifluoroacetic acid. The gradient was from 10 to 40% B in 20 min. The third peak corresponds to Bv8, peak 2 apparently contains nicked forms of Bv8.

The amino acid sequence of the pyridylethylated peptides and its proteolytic fragments was determined by automated Edman degradation using a Perkin-Elmer AB 476A sequencer. Samples were spotted onto ProBlott membranes coated with polybrene (2 μl, 0.1 mg/μl in 50% methanol) and run in a Blott cartridge (Perkin-Elmer) with an optimized fast program.

2.4. cDNA cloning

Total RNA prepared from skin of *B. variegata* was transcribed into cDNA using the adapter primer Ada-3 (5′-GGAATTCTCGAGCTCAAGC-T_{18}-3′) and reverse transcriptase (SuperscriptTM GIBCO/BRL) as recommended by the supplier. With the RACE protocol (Frohman et al., 1988), a degenerated oligonucleotide (5′-GGAATTCGGNGARGAYTGYCAYCCNGC-3′), and an adapter specific primer (Ada-4: 5′-GGAATTCTCGAGCTCAAGC-3′) (R = G,A; Y = T,C; N = G,A,C,T), an amplified fragment was obtained which was digested with *Eco*RI and *Hin*dIII and cloned into the Bluescript II KS-plasmid (Stratagene). The sequence was confirmed using the thermosequenase-kit (Amersham) and IRD-41 fluorescent labelled T3 and T7 primers (MWG Biotech).

Sequencing reactions were analyzed on a LI-COR automated DNA sequencer 4000 (MWG Biotech). The fragment was labeled by the random priming method (Feinberg and Vogelstein, 1984) and used as a probe to screen a plasmid-cDNA library from *B. variegata* skin. This library was prepared by a standard procedure using *Escherichia coli* DNA polymerase I, RNase H and ligase to synthesize the second strand (Gubler and Hoffmann, 1983). *Eco*RI linkers were ligated to the blunt ended double stranded cDNAs. After heat inactivation of the T4-ligase, the reaction mixture was digested with *Eco*RI and *Hin*dIII. Following size selection on a 0.8% agarose gel to remove linkers and smaller cDNA fragments, the cDNA was eluted using the Qiaex II gel extraction kit (Qiagen) and cloned into Bluescript II KS-plasmid. Approximately 30,000 colonies were screened as described previously (Wechselberger et al., 1992). Positive clones were isolated by additional rounds of screening at lower density. Plasmid DNA was isolated by standard procedures and sequenced on both strands.

2.5. Biological activity on isolated organ preparations

Smooth muscle preparations were suspended in a 10 ml bath containing either Krebs or Tyrode solution. The bath was kept at 37°C and gassed with 5% CO_2. The activity of Bv8 was tested on the following isolated preparations: ileum of guinea-pig, rat and mouse; jejunum of guinea-pig, rat and rabbit; colon of guinea-pig, rabbit, rat and mouse; gall bladder of guinea-pig; vas deferens of mouse and guinea-pig; trachea and aorta of guinea-pig; and rat uterus. The motility of the isolated preparations was recorded using an isotonic microdynamometer (Basile, Italy). Protein A was also tested on the guinea-pig ileum using the same experimental conditions.

2.6. Biological activity on the central nervous system

The in vivo activities of Bv8 and protein A were tested by injection into the lateral brain ventricles of rats. A guide cannule (Linka, Tel Aviv) was implanted into the skull above the left lateral ventricle as described previously (Negri et al., 1995). The compounds were dissolved in 5 μl saline and injected i.c.v. with a Hamilton syringe. Each rat was habituated to the procedure by four i.c.v. injections of 5 μl saline each at 10 min intervals before administering the peptide. Control animals only received injections of 5 μl saline. Male Wistar rats (200–250 g) were used to assess the nociceptic responses. The time course of the responses was analyzed using the two-way analysis of variance (ANOVA). The slopes of the curves describing the relationship between thermal stimulus intensity and nociceptive responses of the different groups were analyzed by one-way ANOVA.

In one set of experiments, nociceptive responses were evoked in rats by exposing the tail to radiant heat (D'Amour and Smith, 1941). The latency of tail withdrawal, expressed in s, was taken as a measure of the response to heat exposure. Depending on the voltage applied to the lamp (4.7 to 8.6 V), the baseline reaction time to the thermal stimulus ranged from 8.3 to 3.5 s.

In a second series of tests, a mechanical painful stimulus was applied to the hindpaw (Randall and Smith, 1957). Rats were gently restrained under paper wadding and incremental pressure was applied to a 1.75 mm^2 area of the dorsal surface of the hindpaw via a wedge-shaped blunt piston connected to an automated gauge, designed to generate a linearly increasing mechanical force (Ugo Basile, Comerio, Italy). The pressure required to elicit paw withdrawal, the paw pressure threshold, was determined and expressed in grams. In the absence of a nociceptive response, cutoff pressure was 250 g.

Pressure was applied to normal (left) and to inflamed (right) hindpaws of rats. Inflammation was produced by injecting 0.15 ml of modified Freund's complete adjuvant (Stein et al., 1968) into the plantar region of the right hindfoot under brief ether anaesthesia. In all rats studied, the inflammation remained confined to the inoculated paw and all testing was conducted between 4 and 6 days after inoculation. Three consecutive trials, separated by 10 s intervals, were conducted and the average pressure was determined. The same procedure was applied to the untreated and inflamed hindfoot of each rat. To preclude order effects, the sequence of feet tested was alternated between subjects. Two separate groups of animals (eight rats per group) were used. A further group of eight rats with inflamed hindpaw received vehicle alone and acted as controls. The time course of the nociceptive responses was analyzed using the two-way ANOVA.

Another group of 30 rats received an i.c.v. injection of 5 ml saline each. They were then transferred to a 40×40 cm observation box. After 1 h, the animals received 10 μg Bv8 ($n = 10$), 2 μg protein A ($n = 10$) or 5 μl saline. The overt behaviour of the rats was monitored with a TV camera, tape recorded and finally scored by an independent observer.

3. Results

3.1. Isolation and structural studies

Bv8 was purified from *Bombina* skin secretions as described under Methods. Peak 3 in Fig. 1 is Bv8. The amino acid sequence of the reduced and pyridylethylated peptide could be determined up to residue 49 by automated Edman degradation. Several proteolytic fragments generated by digestion with Lys-C or Asp-N endoprotease were also sequenced (see Fig. 2A). These experiments demonstrated that Bv8 contains 77 amino acids including 10 cysteines. Its molecular mass determined on a Finnigan LCQ ion trap mass spectrometer was found to be 8020 ±

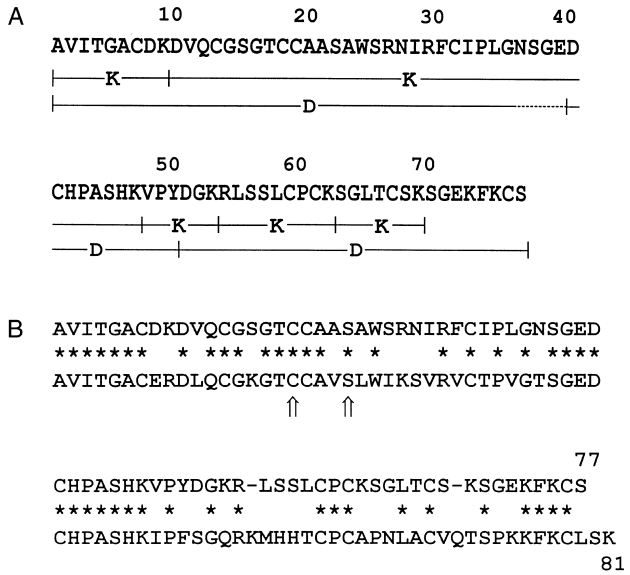

Fig. 2. Amino acid sequence of Bv8. (A) Sequence of Bv8. Residues 1–49 were identified by automated Edman degradation of the pyridylethylated protein. Peptides after digestion with Lys-C protease (K) and Asp-N protease (D) are indicated. Residues not identified by Edman degradation are marked by dots. (B) Comparison of the amino acid sequence of Bv8 and protein A (lower line). Identities are marked (*); two gaps (–) were introduced to maximize homology. Cys in position 18 and Ser in position 22 are marked (⇑) (see Section 3).

0.6 Da (the calculated mass with all cysteines present as disulfides is 8020.18 Da).

The amino acid sequence of Bv8 was confirmed by cDNA cloning. A cDNA library prepared from skin of two *B. variegata* specimen was screened with a suitable probe. A clone with an insert of 413 nucleotides excluding a short poly(A) tail was investigated in detail. After the first ATG codon, an open reading frame is present which codes for a polypeptide consisting of 96 amino acids. The first 19 residues have the characteristics of a signal peptide followed by the sequence of Bv8. The poly(A) tail is preceded by a typical poly-adenylation sequence (Fig. 3).

A search in the data banks revealed that Bv8 is related to protein A from the venom of *Dendroaspis polylepis polylepis*, the black mamba (Joubert and Strydom, 1980). This non-toxic venom constituent contains 81 amino acids, of which 44, including nine of the 10 cysteines, are identical in Bv8 and protein A (see Fig. 1B). We noted that Bv8 contained cysteine in position 18 and serine in position 22, while the opposite was reported for protein A (Joubert and Strydom, 1980). To check for a possible sequencing error, we fractionated a commercial preparation of black mamba venom using a published procedure (Schweitz et al., 1990). Automated Edman degradation of the pyridylethylated protein A indeed demonstrated that residue 18 was cysteine and residue 22 serine (see Fig. 2B). Otherwise, the sequence up to residue 26 was identical to that reported earlier.

3.2. Biological activities on isolated organs

The activity of Bv8 was first tested on the isolated guinea-pig ileum. At a minimal concentration of 5–10 ng/ml (about nanomolar), it stimulated the longitudinal contraction of the smooth muscle in this preparation. Higher amounts gave stronger contractions, but after the first exposure, intense tachyphylaxis developed which lasted for more than 1 h (see Fig. 4A). It was thus not possible to obtain a dose–response curve for the action of Bv8 on a single ileum preparation.

Fig. 3. Nucleotide sequence of a cloned cDNA encoding the presursor of Bv8. The putative polyadenylation signal is underlined, the start of the mature protein is marked by an arrow.

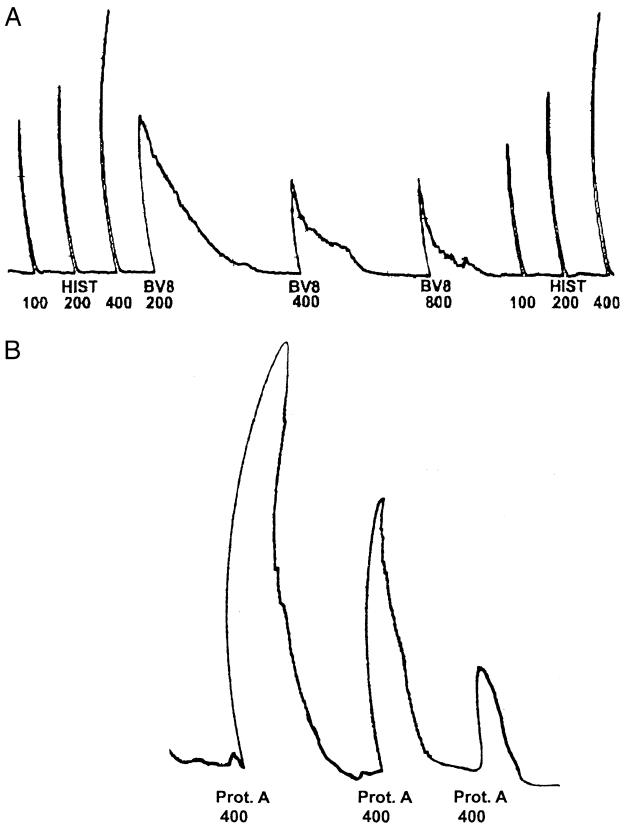

Fig. 4. Action of Bv8 and protein A on isolated guinea pig ileum. (A) Contraction elicited by Bv8. Tachyphylaxis developed after the first addition and subsequent addition of larger amounts yielded less contraction. Control with histamine remains unchanged before and after addition of Bv8. (B) Contraction caused by protein A. The response to the same amount of protein decreases. Numbers refer to ng/10 ml bath. Chart speed: 2 mm/min.

The contraction of guinea-pig ileum caused by Bv8 was not reduced in the presence of the following antagonists: atropine (0.38 μM), pyrilamine (0.35 μM), SR 14033 (200 ng/ml), ICS 205930 (0.95 μM), endothelin-1-(11–21) (9 μM) and endothelin-1-(16–21) (3 μM). This indicated that the action of this protein was not mediated by acetylcholine, histamine, substance P, 5-hydroxy-tryptamine, or endothelin selective receptors, respectively. Contraction of the ileum was also not inhibited by the Ca^{2+} channel blocker nifedipine (0.01 μM) and by 1 mM EGTA. In addition, pretreatment of the isolated organ with tetrodotoxin (0.63 μM) did not antagonize the action of Bv8.

The activity of Bv8 was also tested on several additional smooth muscle preparations. At concentrations of 2.5–25 nM, Bv8 also stimulated the contraction of rat and mouse ileum and increased the tone of the smooth muscle of rabbit jejunum and guinea-pig colon. The height of the rhythmic contractions of the rabbit colon was reduced in the presence of Bv8 (0.25–7.5 nM). Again, it was not possible to obtain a dose–response relationship since tachyphylaxis was also observed with these organs. At concentrations up to 25 nM, Bv8 did not display any effect on all the other smooth muscle preparations tested (see Section 2).

We also tested the action of protein A on guinea-pig ileum. Contraction was rapidly initiated in the presence of protein A (0.35–3.5 nM). Again, tachyphylaxis was observed which, however, was less pronounced than with Bv8 (see Fig. 4B).

3.3. Nociceptive and behavioural effects

Injection of Bv8 into the left lateral ventricle of rat brains produced a dose dependent decrease in the nociceptive reaction time to radiant heat (see Fig. 5A). At a dose of 10 μg of Bv8 per rat, a maximal effect was observed. Hyperalgesia developed within 10 min after injection, peaked at 30 min and disappeared after about 1 h (see Fig. 5A). The reduction of the nociceptive reaction time after Bv8 injection was inversely related to the intensity of the

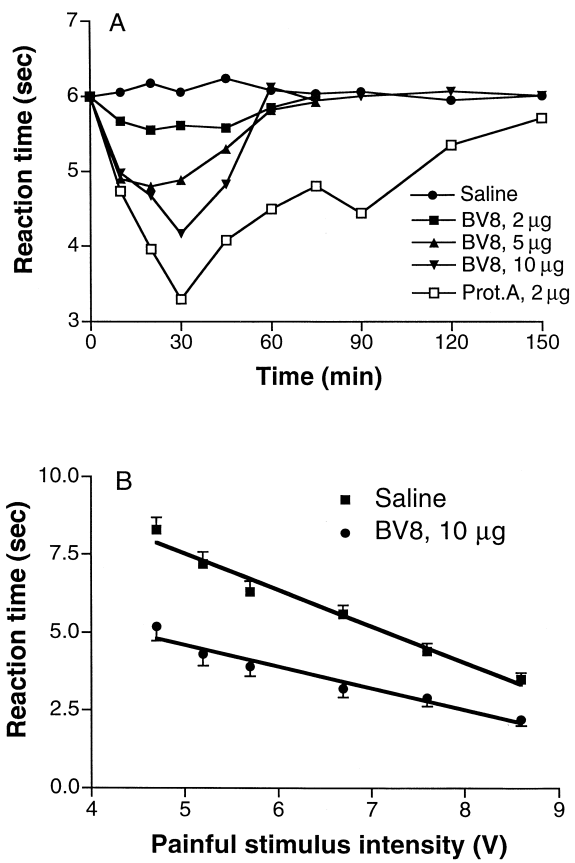

Fig. 5. I.c.v. injection of Bv8 or protein A into the brain of rats. (A) Time-course of hyperalgesia induced by i.c.v. administration of 2, 5 and 10 μg of Bv8 or 2 μg of protein A. Each point is the mean value obtained from four rats. (B) Reaction time to thermal stimulus (light beam) in rats injected with 10 μg Bv8 or saline and exposed to different levels of noxious stimulus intensity (expressed as voltage applied to the electric lamp). Each point corresponds to the mean value from four rats.

noxious stimulus (Fig. 5B). At the lowest intensity of the light beam (4.7 V), the reaction time was decreased by 3.1 s, while at the highest intensity (8.6 V), the reaction time was reduced by only 1.3 s.

Similar results were obtained with protein A which was, however, several times more potent in inducing hyperalgesia. Already at 2 μg protein per rat, a maximal effect was observed with a more than 50% reduction in baseline nociceptive reaction time (Fig. 5A). Moreover, the effects persisted for more than 1 h.

In the paw-withdrawal test, changes in the nociceptive thresholds of untreated and chronically inflamed rat hindpaw were measured after i.c.v. administration of 10 μg BV8 or 2 μg protein A to two groups of eight rats each. Saline-injected rats (eight animals) showed a lower pain threshold in the right inflamed than in left normal hindpaw (Fig. 6, time −30 to 0 min). As expected, the hyperalgesic response caused by Bv8 was more intense in the inflamed than in the untreated paw (Fig. 6).

Bv8 induced hyperalgesia in rats as assessed by the tail-flick test was not affected by pretreatment with naloxone 5 mg/kg s.c. We can thus exclude that this hyperalgesia somehow involves the opioid system. In addition, in experiments with isolated brain membranes, Bv8 at concentrations up to 10 μM did not displace the selective opioid ligands [^{3}H]DAGO, [^{3}H]naltrindole, [^{3}H]U-69596, and [^{3}H]nociceptin from their respective receptors (data not shown).

The i.c.v. injection of Bv8 or protein A also elicited major behavioural changes, which included scream on touch, chattering of the teeth, front-turned vibrissa, rhinorrhea, chewing, sniffing and urine loss (see Table 1). The time-courses of the hyperalgesia and of these behavioural changes were similar. The hyperalgesia was also accompanied by a moderate increase in mean arterial blood pressure (5–7 mm Hg).

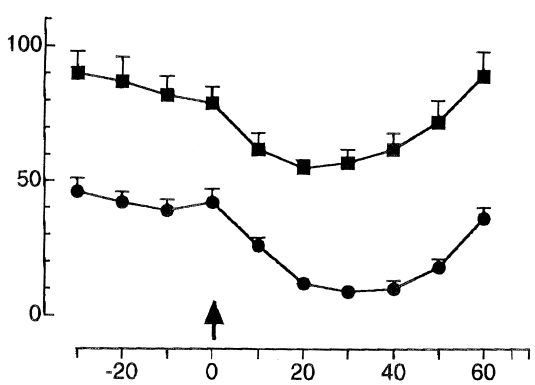

Fig. 6. Time-course of the pressure threshold of the healthy left (■) and inflamed right (●) hind paw of rats. Animals received 10 μg of Bv8 at time 0 (arrow). Each point represents the mean paw pressure threshold of eight rats ± S.E. ANOVA with repeated measures showed a significant interaction between time and dose (0, 10) for the time interval from −30 to 0 min (pre-treatment) and from 10–40 min (post-injection ($P < 0.01$)). Tukey's test revealed a significant difference between pressure thresholds of left and right paw before and after Bv8 injection ($P < 0.01$). Paw pressure (g) is plotted vs. time (min).

Table 1
Behavioural traits observed in rats after i.c.v. injection of Bv8 (10 μg) or protein A (2 μg)

Behaviour	Score	
	Bv8	Protein A
Front-turned vibrissae	+ + +	+ + +
Sniffing	+ + +	+ + +
Chewing	+ +	+
Teeth chattering	+	±
Rhinorrhea	+	–
Scream on touch	+	–
Urine loss	+ +	–

4. Discussion

From skin secretions of two *Bombina* species, we have isolated a small protein containing 77 amino acids, of which 10 are cysteines. The amino acid sequence has been established by automated Edman degradation and analysis of proteolytic fragments as well as independently by cDNA cloning. Possible homologues of Bv8 with the amino-terminal sequence AVITGA × ERDVQ... have also been detected in skin secretions of *Rana temporaria* and *Rana esculenta* (unpublished experiments). This frog skin product is similar to protein A, a constituent of the venom of the black mamba (Joubert and Strydom, 1980). As already mentioned, we could show that in the sample of protein A which we analyzed, residues 18 and 22 were found to be Cys and Ser, respectively. All the cysteines of Bv8 and protein A are thus located at homologous positions and their overall identity is 58%. In view of the structural and functional similarities, protein A could in fact be termed mamba Bv8 (we do not want to use the term protein A for the frog compound, since this name is used in other contexts). Besides the xenoxins (Kolbe et al., 1993), Bv8 is the second example of a product of amphibian skin secretions which is related to components of snake venoms.

The data bank search also revealed that a mouse exon had been found in exon trapping experiments which is potentially derived from the gene of a mammalian homologue of Bv8 (Nehls et al., 1994). Of the 42 amino acids encoded by this exon, 24 including six cysteines are identical to Bv8 and protein A. The cysteine pattern of Bv8 is also present in co-lipase from several mammalian species (Wicker and Puigserver, 1990). The three-dimensional structure of co-lipase has been elucidated (Van Tilbeurgh et al., 1992). In addition, during preparation of this manuscript, a study on the three-dimensional structure of protein A was published (Boisbouvier et al., 1998).

Using nuclear magnetic resonance techniques, these authors demonstrated that protein A has the same disulfide pattern as co-lipase. It seems likely that this is also true for Bv8. However, the sequence similarities between the co-lipases and Bv8 and protein A are so small that it seems unlikely that the latter interact with lipases. Indeed, we could not detect any stimulation of the activity of pancreatic lipase by Bv8 (unpublished experiments). Lastly, it is noteworthy that cysteine patterns similar to the one present in Bv8 have also been found in a domain of the product of the mouse and *Xenopus* dickkopf gene (Glinka et al., 1998) and in a protein identified in chicken lens fibers (Sawada et al., 1997).

Our results indicate that Bv8 and protein A interact with receptors present in mammalian tissues. In particular, it stimulates the contraction of the guinea-pig ileum in the nanomolar range. This stimulation is not inhibited by a variety of antagonists which we tested (see Section 3). Moreover, injection of a few micrograms of Bv8 or protein A into the brain of rats causes significant hyperalgesia as observed with the tail-flick test which lasts for 1 h or more. The magnitude of the hyperalgesia was inversely related to the intensity of the painful stimulus. Moreover, i.c.v. administration of BV8 produced a stronger hyperalgesic response to mechanical pressure in the rat inflammed hindpaw than in normal paw. These results allow us to exclude that possible changes in body or skin temperature after BV8 injection may be an artefactual source of hyperalgesia when radiant heat is used as noxious stimulus. To our knowledge, BV8 is a unique peptide in producing an hyperalgesic response to mechanical pressure after central administration. Animals injected with Bv8 or protein A also show behavioural changes and increase in mean blood pressure that suggest fear and pain.

When administered by i.c.v. injection, only few compounds are known to cause hyperalgesia in rats. One is nociceptin which, however, is considered to reverse the anti-nociception of morphine (Tian et al., 1997). It has in fact been suggested that the stress-induced analgesia associated with the i.c.v. injection, which is due to the activation of the endogenous opioid system, is reversed by nociceptin (Henderson and McKnight, 1997). In contrast, Bv8 antagonized neither opioid analgesia (unpublished experiments) nor the binding of opioids to brain receptors.

In rats, several naturally occurring peptides produce hyperalgesia when administered intrathecally (Woolf et al., 1997) or when their concentration in the spinal cord was increased in models of neuropathic pain (DeLeo et al., 1996; Dickinson and Fleetwood-Walker, 1998). It seems unlikely that the hyperalgesia caused by Bv8 or protein A proceeds via spinal release of any of these. Hyperalgesia occurs when Bv8 was injected i.c.v., but not after intrathecal injection (unpublished observation).

These results raise several questions. Foremost, it will be interesting to test whether mammalian homologues of Bv8 exist. The exon from mouse embryos (Nehls et al., 1994) potentially codes for a fragment of such a homologue. Frog Bv8 and snake protein A may in fact be members of a new family of biologically active peptides. In addition, we will search for Bv8 receptors which we expect to find in rat brain homogenates and possibly in different mammalian cells and tissues.

Acknowledgements

We thank Dr. Günter Lepperdinger for help with the fractionation of the mamba venom, Dr. Manfred Sippl (University of Salzburg) and Dr. Stefano Pascarella (University of Rome) for their findings on the similarity between Bv8 and co-lipases, Christa Tippelt for help with the collection of skin secretions and the fractionation procedures and Elisa Giannini for help in testing compounds on isolated organs. This work was supported in part by grants from the Italian Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST-CNR Biotechnology Program L 95/95), the Istituto Pasteur — Fondazione Cenci Bolognetti, Università La Sapienza, and the Austrian Fonds zur Förderung der wissenschaftlichen Forschung (grant #P13279).

References

Barra, D., Simmaco, M., 1995. Amphibian skin: a promising resource for antimicrobial peptides. Trends Biotechnol. 13, 205–209.

Bevins, C.L., Zasloff, M., 1990. Peptides from frog skin. Ann. Rev. Biochem. 59, 395–414.

Boisbouvier, J., Albrand, J.-P., Blackledge, M., Jaquinod, M., Schweitz, H., Lazdunski, M., Marion, D., 1998. A structural homologue of co-lipase in black mamba venom revealed by NMR floating disulphide bridge analysis. J. Mol. Biol. 283, 205–219.

D'Amour, F.E., Smith, D.L., 1941. A method for determining loss of pain sensation. J. Pharm. Exp. Ther. 72, 74–79.

DeLeo, J.A., Colburn, R.W., Nichols, M., Malhotra, A., 1996. Interleukin-6-mediated hyperalgesia/allodynia and increased spinal IL-6 expression in a rat mononeuropathy model. J. Interferon Cytokine Res. 16, 695–700.

Dickinson, T., Fleetwood-Walker, S., 1998. Neuropeptides and nociception: recent advances and therapeutic implications. Trends Pharmacol. Sci. 19, 346–348.

Erspamer, V., Falconieri-Erspamer, G., Inselvini, M., 1970. Some pharmacological actions of alytesin and bombesin. J. Pharm. Pharmacol. 22, 875–876.

Feinberg, A.P., Vogelstein, B., 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137, 266–267.

Frohman, M.A., Dush, M.K., Martin, D., 1988. Rapid production of full-length cDNAs from rare transcripts amplification using single gene specific oligo-nucleotide primers. Proc. Natl. Acad. Sci. U.S.A. 85.

Gibson, B.W., Tang, D., Mandrell, R., Kelly, M., Spindel, E.R., 1991. Bombinin-like peptides with antimicrobial activity from skin secretions of the Asian toad *Bombina orientalis*. J. Biol. Chem. 266, 23103–23111.

Glinka, A., Wu, W., Monaghan, A.P., Blumenstock, C., Niehrs, C., 1998. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391, 357–362.

Gubler, U., Hoffmann, B.J., 1983. A simple and very efficient method for generating cDNA libraries. Gene 25, 263–269.

Henderson, G., McKnight, A.T., 1997. The orphan opioid receptor and its endogenous ligand nociceptin/orphanin FQ. Trends Pharmacol. Sci. 18, 293–300.

Joubert, F.J., Strydom, D.J., 1980. The amino acid sequence of protein A from *Dendroaspis polylepis polylepis* (black mamba) venom. Hoppe-Seyler's Z. Physiol. Chem. 361, 1781–1794.

Kiss, G.M., Michl, H., 1962. Über das Gift der Gelbbauchunke, *Bombina variegata* L. Toxicon 1, 33–37.

Kolbe, H.J.V., Huber, A., Cordier, P., Rasmussen, U.B., Bouchon, B., Jaquinod, M., Vlasak, R., Delos, E.C., Kreil, G., 1993. Xenoxins, a family of peptides from dorsal gland secretion of *Xenopus laevis* related to snake venom cytotoxins and neurotoxins. J. Biol. Chem. 268, 16458–16464.

Lazarus, L.H., Attila, M.T., 1993. The toad, ugly and venomous, wears yet a precious jewel in his skin. Neurobiology 41, 473–507.

Mignogna, G., Simmaco, M., Kreil, G., Barra, D., 1993. Antibacterial and haemolytic peptides containing D-*allo*-isoleucine from the skin of *Bombina variegata*. EMBO J. 12, 4829–4832.

Mignogna, G., Pascarella, S., Wechselberger, C., Hinterleitner, C., Mollay, C., Amiconi, G., Barra, D., Kreil, G., 1996. BSTI, a trypsin inhibitor from skin secretion of *Bombina bombina* related to protease inhibitors of nematodes. Protein Sci. 5, 357–362.

Negri, L., Lattanzi, R., Melchiorri, P., 1995. Production of antinociception by peripheral administration of [Lys7]dermorphin, a naturally occurring peptide with high affinity for μ-opioid receptors. Br. J. Pharmacol. 114, 57–66.

Nehls, M., Pfeifer, D., Micklem, G., Schmoor, C., Boehm, T., 1994. The sequence complexity of exons trapped from the mouse genome. Curr. Biol. 4, 983–989.

Randall, L.O., Smith, J.J., 1957. A method for measurement of analgesic activity on inflamed tissue. Arch. Int. Pharmacodyn. Ther. 111, 409–419.

Sawada, K., Agata, K., Eguchi, G., 1997. Swiss Prot. Accession Number 2498517.

Schweitz, H., Bidard, J.N., Lazdunski, M., 1990. Purification and pharmcological characterization of peptide toxins from the black mamba (*Dendroaspis polylepis*) venom. Toxicon 28, 847–856.

Simmaco, M., Barra, D., Chiarini, F., Noviello, L., Melchiorri, P., Kreil, G., Richter, K., 1991. A family of bombinin-related peptides from the skin of *Bombina variegata*. Eur. J. Biochem. 199, 217–221.

Stein, C., Millan, M.J., Herz, A., 1968. Unilateral inflammation of the hindpaw in rats as a model of prolonged noxious stimulation: alteration in behavior and nociceptive threshold. Pharmacol. Biochem. Behav. 31, 445–451.

Taché, Y., Melchiorri, P., Negri, L., 1988. Bombesin-like peptides in health and disease. Ann. N.Y. Acad. Sci. 547, 1–541.

Tian, J.H., Xu, W., Fang, Y., Mogil, J.S., Grisel, J.E.G., D.K., Han, J., 1997. Bidirectional modulatory effect of orphanin FQ on morphine-induced analgesia: antagonism in brain and potentiation in spinal cord of the rat. Br. J. Pharmacol. 120, 676–680.

Van Tilbeurgh, H., Sarda, L., Verger, R., Cambillau, C., 1992. Structure of the pancreatic lipase–procolipase complex. Nature 359, 159–162.

Wechselberger, C., Kreil, G., Richter, K., 1992. Isolation and sequence of a cDNA encoding the precursor of a bombesin-like peptide from brain and early embryos of *Xenopus laevis*. Proc. Natl. Acad. Sci. U.S.A. 89, 9819–9822.

Wicker, C., Puigserver, A., 1990. Rat pancreatic colipase mRNA: nucleotide sequence of a cDNA clone and nutritional regulation by a lipidic diet. Biochem. Biophys. Res. Commun. 167, 130–136.

Woolf, C.J., Allchorne, A., Safieh-Garabedian, B., Poole, S., 1997. Cytokines, nerve growth factor and inflammatory hyperalgesia: the contribution of tumour necrosis factor alpha. Br. J. Pharmacol. 121, 417–424.