Biochimie 93 (2011) 1537–1542

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Research paper

Kasstasin: A novel potent vasoconstrictor peptide from the skin secretion of the African red-legged running frog, *Kassina maculata*

Xiaohe Li [a,b], Wanyu Feng [b], Mei Zhou [a], Chengbang Ma [a], Tianbao Chen [a], Martin Zeller [c,d], Martin Hornshaw [c,d], Lei Wang [a,e,**], Chris Shaw [a,*]

[a] Molecular Therapeutics Research, School of Pharmacy, Queen's University, 97 Lisburn Road, Belfast, BT9 7BL Northern Ireland, UK
[b] Department of Pharmacology, College of Pharmacy of China Medical University, Shenyang, Liaoning Province, PR China
[c] Thermo Fisher Scientific, Bremen, Germany
[d] Thermo Fisher Scientific, Hemel Hempstead, UK
[e] Clinical Medicine College of China Medical University, Shenyang, Liaoning Province, PR China

ARTICLE INFO

Article history:
Received 15 March 2011
Accepted 11 May 2011
Available online 23 May 2011

Keywords:
Amphibian
Mass spectrometry
Skin peptides
Molecular cloning
Vasoactive

ABSTRACT

Amphibian skin secretions are established sources of bioactive peptides. Here we describe the isolation, structural and pharmacological characterisation of a novel vasoconstrictor peptide from the skin secretion of the African hyperoliid frog, *Kassina maculata*, which exhibits no structural similarity to any known class of amphibian skin peptide. The peptide consists of 21 amino acid residues, FIKELLPHLSGIIDSVANAIK, and is C-terminally amidated. The provisional structure was obtained by MS/MS fragmentation using an Orbitrap mass spectrometer and L/I ambiguities were resolved following molecular cloning of biosynthetic precursor-encoding cDNA. A synthetic replicate of the peptide was found to possess weak antimicrobial and haemolytic activities but was exceptionally effective in constricting the smooth muscle of rat tail artery (EC_{50} of 25pM). In reflection of its exceptional potency in constricting rat arterial smooth muscle, the peptide was named kasstasin, a derivation of *Kassina* and "*stasis*" (stoppage of flow). These data illustrate the continuing potential of amphibian skin secretions to provide novel natural peptide templates for biological evaluation.

1. Introduction

The defensive skin secretions of amphibians are chemically-complex and are a particularly rich source of biologically-active peptides, many of which have proven to be new chemical entities [1–5]. As many established peptides from these sources have been found to act through specific molecular targets, such as ion channels and G-protein-linked receptors, many may ultimately find a role as lead compounds in drug development [2–5]. Other classes of peptides have been shown to have broad-spectrum antimicrobial actions and these have received increasing attention as potential therapeutic agents [6,7].

Antimicrobial peptides are often the most abundant group in amphibian skin secretions and are frequently produced in multiple, structurally-related isoforms with each displaying differential activity against a range of test microorganisms [8–10]. While the presence of antimicrobial peptides in the skin secretions of frogs and some toads appears to be widespread, only a few taxa have been subjected to in-depth systematic investigations and these include frogs of the families Pipidae, Ranidae and Hylidae, and toads of the family, Bombinatoridae [1,3,6,8]. The classical families of antimicrobial peptides currently described include the magainins (Pipidae), brevinins, ranatuerins, esculentins and temporins (Ranidae), dermaseptins and caerins (Hylidae) and bombinins (Bombinatoridae) [1,3,6,8]. Each of these peptide families have distinctive signature motifs but are highly variable in terms of primary structure [1,6,8]. This observation probably reflects the fact that their mode of biocidal action depends upon their usually cationic amphipathic characteristics that can be achieved, with some restrictions, by different amino acid residues with side-chains displaying similar physicochemical properties [9–12].

Pharmacologically-active peptides present in amphibian skin secretions are often identical to, or are analogues of, endogenous regulatory/neuropeptides. Examples include thyrotropin-releasing hormone (TRH), the bradykinin-related peptides (bradykinin),

* Corresponding author. Tel.: +44 0 2890 972129; fax: +44 0 2890 247794.
** Corresponding author. School of Pharmacy, Queen's University, 97 Lisburn Road, Belfast, BT9 7BL Northern Ireland, UK. Tel.: +44 0 2890 972129; fax: +44 0 2890 247794.
E-mail addresses: l.wang@qub.ac.uk (L. Wang), chris.shaw@qub.ac.uk (C. Shaw).

0300-9084/$ – see front matter © 2011 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2011.05.009

caeruleins (CCK/gastrin), bombesins (gastrin-releasing peptide) and various tachykinins [3]. Many of these peptides, particularly the bradykinins and caeruleins, are vasoactive, causing an intense but transient vasodilation [3].

Hyperoliidae is a family of some 209 species of frogs of 18 genera essentially of African origin, of which *Kassina* is one such genus, containing 16 species found in the sub-Saharan regions of the continent [13,14]. Kassinatuerin-1 and -2 were peptides originally reported from the skin secretion of *Kassina senegalensis* [15,16], of which only the first displayed antimicrobial activity. A series of kassinatuerin-2-related peptides have been described from the skin secretion of *Kassina maculata* [17], and more recently, from *K. maculata* and *K. senegalensis*, prototypes of a novel peptide family, named kassorins, have been structurally- and functionally-characterised [18]. The skin secretions of kassinid frogs have proven to be rich sources of pharmacologically-active peptides including tachykinins such as kassinin and hylambatin [19], FMRFamide-related peptides [20] and kassinakinin [16].

Here, we describe the identification, isolation/structural characterisation, molecular cloning of precursor cDNA and preliminary biological evaluation of a 21-mer peptide representing a novel structural class of amphibian skin peptide that we have named kasstasin, in reflection of its potent constricting effects on rat arterial smooth muscle.

2. Materials and methods

2.1. Specimen biodata and secretion harvesting

African red-legged running frogs, *K. maculata* ($n = 4$, 4–6 cm snout to vent length) of both sexes, were obtained from a local herpetological supplier and were from wild East African populations. All frogs were adults and skin secretion was obtained from the dorsal skin using gentle transdermal electrical stimulation as previously described [21]. These procedures were licensed under appropriate national legislation and all local and national ethical guidelines for such procedures were strictly adhered to. The stimulated secretions were washed from the skin using de-ionised water, snap frozen in liquid nitrogen, lyophilised and stored at −20 °C prior to analyses.

2.2. Reverse phase HPLC fractionation of skin secretion

Twenty micrograms (20 μg) of lyophilised skin secretion were fractionated by reverse phase nano-HPLC with the effluent directed into an LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). The spectrometer was used in a data-dependent acquisition mode in which all detected doubly- and triply-charged ions were subjected to MS/MS fragmentation using several different fragmentation technologies. Archiving of data permitted subsequent *de novo* sequencing analysis of selected peptides.

2.3. Cloning of novel peptide biosynthetic precursor-encoding cDNA

Five milligrams (5 mg) of lyophilised skin secretion were dissolved in 1 ml of cell lysis/mRNA protection buffer supplied by Dynal Biotec, UK. Polyadenylated mRNA was isolated by the use of magnetic oligo-dT beads as described by the manufacturer (Dynal Biotec, UK). The isolated mRNA was subjected to 5′ and 3′-rapid amplification of cDNA ends (RACE) procedures to obtain full-length peptide biosynthetic precursor nucleic acid sequence data using a SMART-RACE kit (Clontech UK) essentially as previously described. Briefly, the 3′-RACE reactions employed a nested universal (NUP) primer (supplied with the kit) and a degenerate sense primer (S: 5′-THAARGARYTIYTICCICAYYT-3′) that was complementary to the near N-terminal amino acid sequence, – L/IKEL/IL/IPH –, of the novel peptide established by *de novo* MS/MS sequencing. The 3′-RACE reactions were purified and cloned using a pGEM-T vector system (Promega Corporation) and sequenced using an ABI 3100 automated sequencer. The sequence data obtained from the 3′-RACE product was used to design a common and only partially degenerate antisense primer (AS: 5′-GACATCTGAATTATGAATATGTAWTCAG-3′) to a conserved site within the 3′-non-translated region of the novel peptide-encoding transcripts. 5′-RACE was carried out using these primers in conjunction with the NUP primer and resultant products were purified, cloned and sequenced.

2.4. Synthesis of the novel peptide

Following acquisition of two data sets, one from *de novo* sequencing by MS/MS and the other from biosynthetic precursor cloning, the unequivocal primary structure of the novel peptide was established. (*De novo* sequencing algorithms normally set a leucyl residue as default as such programs cannot differentiate the presence of the isobaric isoleucyl residue. The molecular cloning data established the identity of the isobaric amino acid residue, leucyl vs. isoleucyl, at each site.) A synthetic replicate of the novel peptide with corrected authentic primary structure was synthesised by solid-phase fmoc chemistry using an Applied Biosystems 433 peptide synthesiser. The synthetic mixture was purified and the primary structure of the major product (>90%) was subsequently confirmed by LC/MS/MS. For pharmacological experiments, standardisation of synthetic peptide was achieved by acid hydrolysis of a known gravimetric quantity of lyophilisate followed by amino acid analysis using an Applied Biosystems PTH-amino acid analyser.

2.5. Antimicrobial and haemolytic assays

Antimicrobial activity of the synthetic peptide was assessed by determination of minimal inhibitory concentrations (MICs) using a standard Gram-positive bacterium *Staphylococcus aureus* – NCTC 10788), a standard Gram-negative bacterium (*Escherichia coli* – NCTC 10418) and a standard pathogenic yeast (*Candida albicans* – NCPF 1467). The peptide was tested within the concentration range of 10–250 μM and solutions in this range were prepared by dilution of peptide in Mueller–Hinton broth (MHB). Peptide solutions were inoculated with microorganism cultures (10^5 colony forming units (CFU)/ml) and placed into 96-well microtitre cell culture plates. Plates were incubated for 18 h at 37 °C in a humidified atmosphere. Following this, the growth of bacteria/yeast was determined by means of measuring optical density (OD) at λ 550 nm by an ELISA plate reader (Biolise BioTek EL808). Minimal inhibitory concentrations (MICs) were defined as the lowest concentration at which no growth was detectable. Haemolytic assays were performed using defibrinated horse blood as described in detail previously [22].

2.6. Rat arterial smooth muscle pharmacology

Male adult Wistar rats, weighing 200–250 g, were killed by asphyxiation with CO_2 followed by cervical dislocation in accordance with institutional animal experimentation ethics and U.K. Department of Health guidelines. The animals were laid on their dorsal surfaces, followed by removal of the tail skin. The tail artery vascular bed was identified and moistened with Krebs' solution (NaCl-118 mM, KCl-4.7 mM, $NaHCO_3$-25 mM, NaH_2PO_4-1.15 mM, $CaCl_2$-2.5 mM, $MgCl_2$-1.1 mM, glucose-5.6 mM). The membrane and the connective tissue beneath the main artery were carefully