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Kassorins: Novel innate immune system peptides from skin secretions of the African hyperoliid frogs, *Kassina maculata* and *Kassina senegalensis*

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ABSTRACT

From defensive skin secretions acquired from two species of African hyperoliid frogs, *Kassina maculata* and *Kassina senegalensis*, we have isolated two structurally related, C-terminally amidated tridecapeptides of novel primary structure that exhibit a broad spectrum of biological activity. In reflection of their structural novelty and species of origin, we named the peptides kassorin M (FLEGLLNTVTGLLamide; 1387.8 Da) and kassorin S (FLGGILNTITGLLamide; 1329.8 Da), respectively. The primary structure and organisation of the biosynthetic precursors of kassorins M and S were deduced from cloned skin secretion-derived cDNA. Both open-reading frames encoded a single copy of kassorin M and S, respectively, located at the C-terminus. Kassorins display limited structural similarities to vespid chemotactic peptides (7/13 residues), temporin A (5/13 residues), the N-terminus of *Lv*-ranaspumin, a foam nest surfactant protein of the frog, *Leptodactylus vastus*, and an N-terminal domain of the equine sweat surfactant protein, latherin. Both peptides elicit histamine release from rat peritoneal mast cells. However, while kassorin S was found to possess antibacterial activity against *Staphylococcus aureus*, kassorin M was devoid of such activity. In contrast, kassorin M was found to contract the smooth muscle of guinea pig urinary bladder (EC_{50} = 4.66 nM) and kassorin S was devoid of this activity. Kassorins thus represent the prototypes of a novel family of peptides from the amphibian innate immune system as occurring in defensive skin secretions.

1. Introduction

Antimicrobial peptides are now well-established as vital components of innate immune system defence against microbial infection in a wide range of both vertebrates and invertebrates (Lazarus and Attila, 1993; Erspamer, 1994; Bulet et al., 2004; Saleem et al., 2010). Many anuran amphibians possess highly specialised dermal glands, the so-called poison or granular glands, which synthesise and store a complex chemical cocktail which is essentially peptide-based, and that is released onto the skin surface following stress or predator attack (Lazarus and Attila, 1993; Saleem et al., 2010). Major components of such secretions are antimicrobial peptides that are represented by multiple isomeric forms within several distinct structurally related families which are usually taxon-specific (Lazarus and Attila, 1993; Erspamer, 1994; Kreil, 1994; Simmaco et al., 1998; Saleem et al., 2010). In some species, potent mast cell-degranulating peptides are also found and these are thought to play a role, once ingested by the predator, in causing histamine release within exposed mucous membranes—a factor that causes discomfort in the form of irritation and possible bronchoconstriction, and that may facilitate, through enhanced vascular permeability, the uptake of other secretion components into tissues (Ziai et al., 1990). In many instances, both antimicrobial and mast-cell degranulation/histamine-releasing properties are possessed by the same peptides (Hider, 1988; Ziai et al., 1990; Fontana et al., 2004). These attributes of amphibian skin secretion-derived cationic amphipathic peptides are similar to those possessed by several peptides found in the venoms of hymenopteran insects—mellitin from honeybee (*Apis mellifera*), mast-cell degranulating peptides from honeybee, mastoparans from the venoms of *Polistes* wasps and vespid chemotactic peptides from the venoms of *Vespa* hornets (Hirai et al., 1980; Yasuhara et al., 1983; Hider, 1988; Fontana et al., 2004). Of particular interest in this respect, is the finding of structurally similar peptides in amphibian defensive skin secretions and hymenopteran venoms, for example, mellitin-related peptides in *Rana* frogs/mellitin in honeybee

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venom (Conlon et al., 2003), bradykinins in many frogs/kinins in wasp/hornet venoms (Bhoola et al., 1992) and temporins in *Rana* frogs/chemotactic peptides in wasp/hornet venoms (Simmaco et al., 1996; Habermann, 1972). These are excellent examples of convergent evolution and natural molecular design-for-purpose in respective taxa and would tend to suggest that their very similar structures reflect a near achievement of functional optimisation. It is thus becoming increasingly clear that one common strategy adopted by the molecular weapon design machinery found within the venom factories of widely different taxa, is directed to achieve the goal of stimulating endogenous pro-inflammatory mediators from immunocytes in unfortunate venom recipients. This action undoubtedly facilitates increased local vascular permeability leading to systemic dissemination of other venom components and these attributes could form the basis of their exploitation as therapeutics.

Here we describe the discovery of novel, structurally homologous peptides in the defensive skin secretions of two African hyperoliid frogs, *Kassina maculata* and *Kassina senegalensis*. Both species are known to produce a skin secretion with noxious, toxic and often lethal effects to other amphibians and higher vertebrates (Duellman and Trueb, 1994). These peptides, named kassorin M and kassorin S, from *K. maculata* and *K. senegalensis*, respectively, elicited mast cell histamine release but had differential antimicrobial and smooth muscle contracting effects.

2. Materials and methods

2.1. Acquisition of frog skin secretions

Adult *K. maculata* originated in Tanzania and were housed in a terrarium under a 12 h/12 h light/dark cycle. Skin secretions were obtained by transdermal electrical stimulation after the method of Tyler et al. (1992). Briefly, secretions were obtained from the dorsal skin surface by electrical stimulation (4 V DC, 4 ms pulse-width, 50 Hz) through platinum electrodes for two periods of 15 s duration. The obvious viscous white secretions were washed from the skin using deionised water, snap-frozen in liquid nitrogen and lyophilised. Lyophilisate was stored at $-20\,^{\circ}C$ prior to analysis. Adult *K. senegalensis* originated from Cameroon and were housed and sampled as above. Specimens of both species were devoid of any obvious signs of ill-health and secretions were sampled only after a 3-month period of acclimatisation. All procedures on living animals were approved through UK Department of Health licences and were carried out in a manner consistent with both national and local research ethics.

2.2. Fractionation of skin secretion by reverse-phase HPLC

Five milligram samples of lyophilised skin secretion from both *Kassina* species were reconstituted in 0.05/99.5 (v/v) trifluoroacetic acid (TFA)/water and clarified of particulates by centrifugation. Each sample was separately subjected to reverse-phase HPLC fractionation using a Thermoquest (San Jose, CA, USA) gradient HPLC system fitted with a Vydac semi-preparative C-18 column (30 cm × 1 cm). Bound peptides were eluted with a linear gradient formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 1 ml/min. Fractions (1 ml) were collected at minute intervals and the effluent absorbance was continuously monitored at λ 214 nm. Samples (100 μl) were removed from each fraction, lyophilised and stored at $-20\,^{\circ}C$ prior to biological testing. Additional purification of the peptides in the fractions displaying these bioactivities was achieved, where necessary, using a Phenomenex C-8 analytical column (250 mm × 4.6 mm) on the same gradient HPLC system. The column was equilibrated in 0.05/99.5 (v/v) TFA/water and eluted with a gradient rising to 0.05/19.5/80.0 (v/v/v) TFA/water/acetonitrile in 80 min at a flow rate of 1 ml/min.

2.3. Structural analyses and chemical synthesis of isolated peptides

Once the novel histamine-releasing peptides had been detected, an estimate of molecular mass was achieved by MALDI-TOF MS (Voyager DE, Perseptive Biosystems, Framingham, MA, USA) of peak active reverse phase HPLC fractions. Following the obtaining of these data, accurate molecular masses of the peptides, their amidation status and primary structures were determined using an LTQ Orbitrap XL ETD mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), following separate nanoHPLC runs of 20 μg of lyophilised skin secretion from both species of frog. After establishment of the unequivocal primary structure of both peptides, replicates were synthesised by solid-phase Fmoc chemistry using a PS3 automated peptide synthesiser (Protein Technologies, Tucson, AZ, USA). After completion of all amino acid coupling cycles, peptides were cleaved from the synthesis resin, deprotected and purified of contaminants by reverse phase HPLC. Both the degree of purity and authentication of structure of each synthetic peptide were determined using MALDI-TOF MS.

2.4. Molecular cloning of kassorin M and S biosynthetic precursor (preprokassorin M and S) -encoding cDNA

A 5 mg sample of each lyophilised skin secretion was 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 preprokassorin M nucleic acid sequence data using a SMART-RACE kit (Clontech, UK) essentially as described by the manufacturer. Briefly, the 3′-RACE reactions employed a nested universal (NUP) primer (supplied with the kit) and sense primers (SM: 5′-GARGGIYTIYTIAAYACIGT-3′; SS: 5′-GGIGGIATHYTIAAYACIAT-3′) that were complementary to the amino acid sequences, -EGLLNTV- of kassorin M and -GGILNTI- of kassorin S. The 3′-RACE reaction was 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 specific antisense primer (AS: 5′-GAATTGAATTCCACAGAGGTGGGAGT-3′) to a conserved site within the 3′-non-translated region of both kassorin M and S cDNAs. 5′-RACE was carried out using this specific primer in conjunction with the NUP RACE primer and resultant products were purified, cloned and sequenced.

2.5. Histamine release assay

Male hooded Lister rats (150–250 g body weight) were killed by cervical dislocation in accordance with local and national animal experimentation procedures. Mixed peritoneal cells were obtained as previously described in detail (Shore et al., 1959). The cells were washed twice in Tyrode's buffer (NaCl (137 nM), glucose (5.6 mM), HEPES (10 mM), KCl (2.7 mM), $MgCl_2{\cdot}6H_2O$ (1 mM), $CaCl_2{\cdot}2H_2O$ (1 mM) and $NaH_2PO_4{\cdot}2H_2O$ (0.4 mM), pH 7.4) and recovered by centrifugation (100 × g, 4 °C, 2 min). Samples of the isolated peritoneal cells were pre-warmed to 37 °C for 5 min. Lyophilised aliquots of chromatographic fractions of skin secretion from both *Kassina* species were reconstituted in Tyrode's buffer (100 μl) and added to cell suspensions. Following incubation (10 min, 37 °C), the reactions were quenched by addition of ice-cold Tyrode's buffer (2.8 ml). The cell suspensions were centrifuged and the supernatants removed for histamine assay as an indicator of mast cell