Toxicon 60 (2012) 967–981

Contents lists available at SciVerse ScienceDirect

Toxicon

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

Antimicrobial peptides and alytesin are co-secreted from the venom of the Midwife toad, *Alytes maurus* (Alytidae, Anura): Implications for the evolution of frog skin defensive secretions

Enrico König [a,*], Mei Zhou [b], Lei Wang [b], Tianbao Chen [b], Olaf R.P. Bininda-Emonds [a], Chris Shaw [b]

[a] AG Systematik und Evolutionsbiologie, IBU – Fakultät V, Carl von Ossietzky Universität Oldenburg, Carl von Ossietzky Strasse 9-11, 26129 Oldenburg, Germany
[b] Natural Drug Discovery Group, School of Pharmacy, Medical Biology Center, Queen's University, 97 Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK

ARTICLE INFO

Article history:
Received 23 March 2012
Received in revised form 20 June 2012
Accepted 28 June 2012
Available online 16 July 2012

Keywords:
Alyteserin
Host-defence peptides
Cytolytic peptides
Innate immune system
Bombesin-related peptides

ABSTRACT

The skin secretions of frogs and toads (Anura) have long been a known source of a vast abundance of bioactive substances. In the past decade, transcriptome data of the granular glands of anuran skin has given new impetus to investigations of the putative constituent peptides. *Alytes obstetricans* was recently investigated and novel peptides with antimicrobial activity were isolated and functionally characterised. However, genetic data for the evolutionarily ancient lineage to which *Alytes* belongs (midwife toads; Alytidae) remains unavailable.

Here we present the first such genetic data for Alytidae, derived via the granular gland transcriptome of a closely-related species of midwife toad, *Alytes maurus*. First, we present nucleotide sequences of the entire peptide precursors for four novel antimicrobial peptides (AMPs). The two precursors resemble those from Bombinatoridae in both their structural architecture and amino acid sequence. Each precursor comprises two AMPs as tandem repeats, with a member of the alyteserin-1 family (alyteserin-1Ma: GFKEVLKADLG SLVKGIAAHVAN-NH2 or alyteserin-1Mb: GFKEVLKAGLGSLVKGIPAHVAN-NH2) followed by its corresponding member from the alyteserin-2 family (alyteserin-2Ma: FIGKLIS AASGLLSHL-NH2 or alyteserin-2Mb: ILGAIIPLVSGLLSHL-NH2). Synthetic replicates of the four AMPs possessed minimal inhibitory concentrations (MICs) ranging from 9.5 to 300 μM, with the most potent being alyteserin-2Ma. Second, we also cloned the cDNA encoding an alytesin precursor, with the active alytesin exhibiting high sequence identity to bombesin-related peptides from other frogs. All putative mature peptide sequences were confirmed to be present in the skin secretion via LC/MS.

The close structural resemblance of the alyteserin genes that we isolated for *A. maurus* with those of *Bombina* provide independent molecular evidence for a close evolutionary relationship between these genera as well as more support for the convergent evolution of the AMP system within anurans. In contrast to the more evolutionarily conserved nature of neuropeptides (including alytesin, which we also isolated), the more variable nature of the AMP system together with the sporadic distribution of AMPs among anuran amphibians

Abbreviations: AMPs, antimicrobial peptides; BLPs, bombesin-like peptides; cDNA, complementary deoxyribonucleic acid; CFU, colony forming units; DMSO, dimethyl sulfoxide; GRP, gastrin-releasing peptide; HPLC, high performance liquid chromatography; LTQ, linear ion trap; MALDI-TOF, matrix-assisted laser desorption/ionisation, time-of-flight; MIC, minimal inhibitory concentration; MS, mass spectrometry; NCBI, National Center for Biotechnology Information; OD, optical density; TFA, trifluoroaceticacid.

* Corresponding author. Tel.: +49 441 798 3373; fax: +49 441 798 193965.
E-mail address: en.koenig@uni-oldenburg.de (E. König).

0041-0101/$ – see front matter
http://dx.doi.org/10.1016/j.toxicon.2012.06.015

fuels in part our hypothesis that the latter system was co-opted secondarily to fulfil a function in the innate immune system, having originally evolved for defence against potential macropredators.

1. Introduction

The skin secretion of anuran amphibians (frogs and toads) is known to be an extraordinarily rich source of diverse bioactive substances. More than half a century worth of research on several hundred species of frogs and toads, has unveiled a plethora of natural products including alkaloids, steroids (bufadienolids), amines and peptides (Daly et al., 2005; Erspamer, 1994; Pukala et al., 2006). Pharmaceutical studies undertaken over the past few decades have demonstrated the huge potential for drug discovery and medical therapeutics of frog skin compounds among which the biologically active peptides are by far the most promising candidates (Calderon et al., 2011; Clarke, 1997; Conlon et al., 2004; Lazarus and Attila, 1993). These peptides include neuropeptides that act as both neurotransmitters or neuromodulators (e.g., the gastrin-related bombesin, the cholecystokinin-like caeruleins, or the tachykinins, bradykinins and an array of other vasoactives) as well as opioids/dermorphins and antibiotic peptides (also known as antimicrobial peptides or AMPs) (Bevins and Zasloff, 1990).

In this study, we focus on two of these compound classes, the bombesin-like peptides (BLPs) and AMPs and their expression in the skin secretion of the Midwife toad *Alytes maurus*. Our research follows on from the early work of the group of Vittorio Erspamer, one of the pioneers in the field of frog skin peptides, who isolated alytesin and bombesin from the two closely-related species *Alytes obstetricans* (Alytidae) and *Bombina bombina* (Bombinatoridae), respectively (Anastasi, 1971). Since then, the skin secretions of several *Bombina* species have been investigated intensively and numerous substances have been characterised, including bradykinin-related peptides (Yasuhara et al., 1973), amines (Erspamer, 1994) and numerous AMPs (Simmaco et al., 2009). However, no further research was carried out on midwife toads until the recent identification of alyteserins from *A. obstetricans*, the first peptides with antimicrobial activity known from Alytidae (Conlon et al., 2009a).

Both BLPs and AMPs are widely distributed among anurans. The co-occurrence of AMPs whenever BLPs are present is an intriguing fact, and one that can apparently be generalised to neuroactive peptides, which appear to be always accompanied by AMPs in various frog species (Fig. 1). Otherwise, however, their potential defensive mode of action is quite different. The structural counterparts of the neuroactive BLPs in other amphibian tissues and in other vertebrates – neuromedin B and gastrin-releasing peptides – can stimulate or suppress gastrointestinal secretion, act directly on extravascular smooth muscles, and cause potent antidiuretic effects (Spindel, 2006). In the central nervous system, BLPs can modify thermoregulation, satiety and dipsogenia (Spindel, 2006). Such potent pharmacological properties make BLPs very efficient defensive agents against predator attack, especially given that the major predators of frogs are other vertebrates (e.g., snakes, birds, or mammals). In addition to *Bombina* and *Alytes*, BLPs also occur in skin secretions of other anurans including species of the genera *Litoria* (litorin), *Phyllomedusa* (phyllolitorin) and *Rana* (ranatensin) (Anastasi et al., 1975; Barra et al., 1985; Nakajima et al., 1970; Spindel, 2006; Yasuhara et al., 1983).

Because BLPs, together with other neuroactive peptides and their vertebrate structural counterparts, act on specific receptors in a stereo-specific manner, they tend to be under high selective pressure which is reflected in their more stringently-conserved amino acid sequences [unpublished observations: König, Clark, Shaw]. However, convergent evolution in the form of gene duplication also seems to have played an important role in this class of peptide toxins (Roelants et al., 2010; Wang et al., 2009). As indicated by studies on *Bombina orientalis*, the gastrin-releasing peptides (GRP) from intestine/brain and bombesin from skin secretion, although structurally similar to one another, are products of different genes. Both genes are expressed in the brain and stomach, but only that for bombesin is expressed in the skin (Nagalla et al., 1992; Spindel, 2006), suggesting that the latter tissue is the place of production for specialised neuroactive peptides in anurans that probably derive from gene duplication before the radiation of the group as a whole and were subsequently incorporated as a defensive weapon.

By contrast, despite their similar mode of action to one another, AMPs show a high degree of diversity among anurans. AMPs are united by their amphipathic and cationic nature, which enables them via electrostatic interaction to disrupt the integrity of the negatively charged lipids in the membrane of a target cell with their ability to adopt α-helical structures in membrane environments eventually causing pore formation and lysis (Matsuzaki, 1999). Because this mechanism is very similar to the actions of the complement system of other vertebrates, amphibian AMPs have been considered to be part of the amphibian innate immune system (Nicolas and Mor, 1995; Woodhams et al., 2007). According to this theory, AMPs evolved initially to counteract the permanent threat of potential infections posed by the moist, mucous amphibian skin, which provides an optimal breeding environment for microorganisms. Further support derives from the finding that the physiological active products of the AMP gene undergo positive selection (Tennessen, 2005) as would be expected for immune system components.

However, AMPs present a complex distribution across anurans and apparently do not occur in all frog species (Conlon, 2010; Conlon et al., 2009b) (Fig. 1). Closely related species do tend to share a common set of structurally-related AMPs that can be classified into distinct families (Amiche et al., 2008; Conlon, 2008a,b), leading to the hypothesis