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Pipa carvalhoi skin secretion profiling: Absence of peptides and identification of kynurenic acid as the major constitutive component

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ABSTRACT

The presence of peptides has been identified in all African pipid genera; nevertheless, little is known about skin secretion of South American frog genus *Pipa*. Skin secretion from captive and wild *Pipa carvalhoi* were obtained in the presence or absence of norepinephrine stimulation. The <10 kDa fraction was analyzed by liquid chromatography and mass spectrometry, searching for peptides. Chromatographic profiles show the presence of a major component in this secretion, regardless of the stimulation method (norepinephrine or mechanical stimulation) and the origin of the animal (captivity or wild), as well as in the absence of any stimulus. The general mass distribution profile in *P. carvalhoi* skin secretion shows numerous components below 800 Da. Moreover, no peptide could be identified, regardless of the chromatographic approach. The major component was purified and identified as kynurenic acid, an L-tryptophan derivative. *P. carvalhoi* does not secrete peptides as toxins in its skin. In addition, we here report that kynurenic acid is the main component of *P. carvalhoi* skin secretion. Although no biological activity was associated with kynurenic acid, we propose that this molecule is a pheromone that signals the presence of a co-specific in the shady environment in which this animal lives. In this study we demonstrate the absence of peptidic toxins in the skin secretion of *P. carvalhoi*, a break of paradigm in the pipid family.

1. Introduction

Amphibia were the first group of vertebrates to conquer the terrestrial environment and became dominant during the Carboniferous and Permian periods (Carroll et al., 1999; Schoch, 2009). The present amphibians lie in the subclass Lissamphibia that includes three orders: Anura, Caudata and Gymnophiona (Zardoya and Meyer, 2001; Frost, 2014). Anura is the most diverse group, containing circa 6200 species grouped in 54 families (Frost, 2014). Frogs of the family Pipidae comprise five genera, four present in Africa (*Hymenochirus*, *Pseudhymenochirus*, *Silurana* and *Xenopus*) and only the genus *Pipa* inhabiting South America (Frost, 2014). Pipids constitute the only exclusively aquatic anuran family and are characterized for being aglossa and having a dorsal-ventrally flattened body and hind limbs adapted to swimming (Pough et al., 2001; Fernandes et al., 2011).

The skin exerts important biological functions in all amphibians, such as gas-exchange, thermoregulation, and ionic and osmotic balance (Toledo and Jared, 1993, 1995; Amey and Grigg, 1995; Clarke, 1997; Larsen and Ramløv, 2013). In particular, *Pipa* possess a unique mode of parental care in which the fertilized eggs are placed by the male on the back of the female, where they are individually immersed inside the dorsal skin and develop. Depending on the species, the female releases either larvae, or fully metamorphosed froglets (Zippel, 2006; Greven and Richter, 2009; Fernandes et al., 2011).

The amphibian skin is rich in mucous and granular glands involved in physiological processes and protection (Toledo and Jared, 1993, 1995; Amey and Grigg, 1995; Clarke, 1997; Jared et al., 2009; Prates et al., 2012; Larsen and Ramløv, 2013; Mailho-Fontana et al., 2014). Granular glands are thought to accumulate several bioactive compounds, such as alkaloids, peptides, proteins and steroids that are used as an active (Mailho-Fontana et al., 2014) or passive chemical defense against predators or infections (Toledo and Jared, 1995; Jared et al., 2009; Conlon, 2011; Prates et al., 2012; Sciani et al., 2013a). Among these compounds, several biological activities have already been described, such as antimicrobial (Zasloff, 1987), antileishmanial and antitrypanosomal (Tempone et al., 2008), vasoactive (Conceição et al., 2009), cytotoxic (Sciani et al., 2013b) and neurotoxic effects (Toledo and Jared, 1995).

The first amphibian antimicrobial peptides (AMP) isolated and biochemically characterized were the magainins, molecules that present growth inhibitory activities against bacteria and fungi (Zasloff, 1987).

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These peptides were identified in the skin of the African clawed frog *Xenopus laevis* by Zasloff in 1987. Since the discovery of magainins, other AMPs, such as the peptides glycine–leucine-amide (PGLa), caerulein-precursor fragment (CPF) and xenopsin-precursor fragment have been described in the skin secretion of *Silurana* and *Xeponus* species after norepinephrine-stimulation (Conlon et al., 2010, 2012; Mechkarska et al., 2010). Mechkarska et al. (2012) and Conlon et al. (2013) also identified antimicrobial peptides in *Hymenochirus boettgeri* and *Pseudohymenochirus merlini*, respectively.

Besides the unusual parental care, very little is known about the biology of the different species of *Pipa* and about the biochemical and biological activity of their skin secretion. In this work, we have profiled the skin secretion collected from captivity and wildlife *P. carvalhoi* individuals, in the presence and absence of norepinephrine (NE) stimulation, aiming the biochemical characterization and, particularly, searching for peptides (magainin-like). With this study we intend to provide some insights about the evolutionary adaptations within *Pipa* genus that were isolated from other Pipidae from Africa during the continental drift.

2. Material and methods

2.1. Reagents

All the employed reagents were purchased from Sigma Co. (St. Louis, MO, USA), unless otherwise stated. Amicon Ultra-4 Centrifugal Filter Units were purchased from Millipore, USA.

2.2. Animals

Captive *P. carvalhoi* individuals were kept in 200 L water tanks at the animal facility of the Cellular Biology Laboratory (Zimmermann, 1995; Fernandes et al., 2011). Alternatively, wild *P. carvalhoi* were collected in Ilhéus (BA, Brazil) and introduced in the bioterium. All procedures were approved by the ethics committee under protocol number 892/12. Animal collecting was authorized by IBAMA (license # 15964-1-MMA) and ICMBio (license # 42500-1-CJ).

2.3. Skin secretion collection

Regardless of the method of collection, individual animal skin secretion solutions were pooled and lyophilized prior to subsequent analyses.

2.3.1. Norepinephrine-stimulated skin secretion collection

An adaptation of the protocol described by Ali et al. (2001) was employed. Briefly, *P. carvalhoi* were subcutaneously administrated with 1 nmol/g (body mass) norepinephrine hydrochloride and immersed in ammonium acetate (100 mL, 25 mM, pH 7.0) for 30 min, at room temperature. Alternatively, we tested the administration of a higher dose of norepinephrine hydrochloride (50 nmol/g) in *P. carvalhoi*, as described by Conlon et al. (2010) and Mechkarska et al. (2010), and follow the protocol described above.

2.3.2. Mechanically-stimulated skin secretion collection

An adaptation of the methodology employed by Sciani et al. (2013a) was used. Briefly, anurans were immersed in ammonium acetate (100 mL, 25 mM, pH 7.0), and gently compressed for 5 min at room temperature.

2.3.3. Absence of stimulation

P. carvalhoi were immersed in ammonium acetate (100 mL, 25 mM, pH 7.0) for 15 min, aiming to verify if anurans could release these skin secretions without the presence of any stimulation.

2.4. Sample preparation

Pooled lyophilized secretion was ressuspended in 5% acetonitrile (ACN) containing 0.1% trifluoroacetic acid (TFA), mechanically filtered on a 0.22 μm membrane (Millex-GV, Millipore) and then filtered in a 10 kDa cut-off centrifugal filter (Amicon Ultra-4, Millipore). Only the filtered material (<10 kDa) was further processed in this study.

2.5. Biochemical characterization

2.5.1. Reversed-phase liquid chromatography

Skin secretion solutions were analyzed by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) using a binary HPLC system (20A Prominence, Shimadzu Co., Japan). Aliquots of each material were loaded in a C18 column (ACE C18, 5 μm; 100 Å, 250 mm × 4.6 mm) in a two solvent system: (A_1) TFA/water (1/999, v/v) and (B_1) ACN/water/TFA (900/99/1, v/v/v). The column was eluted at a constant flow rate of 1 mL/min with a 0 to 100% gradient of solvent B_1 over 20 min. The eluates were monitored by a Shimadzu SPD-M20A detector at 214 nm. After skin secretion profiles, fractions were collected manually.

2.5.2. Mass spectrometry

Liquid chromatography–Mass Spectrometry (*LC–MS*). LC–MS analyses were performed using an Electrospray-Ion Trap-Time of Flight (ESI-IT-TOF) (Shimadzu Co., Japan) equipped with binary Ultra-Fast Liquid Chromatography system (UFLC) (20A Prominence, Shimadzu). Aliquots of both stimulations were dried, resuspended in water/formic acid (0.99/0.01, v/v) and loaded in a C18 column (Shimadzu-pack XR-ODS, 2.2 μm; 100 × 3 mm) in a binary solvent system: (A_2) water/formic acid (FA) (999/1, v/v) and (B_2) ACN/water/FA (900/99/1, v/v/v). The column was eluted at a constant flow rate of 0.2 mL.min^{-1} with a 0 to 100% gradient of solvent B_2 over 20 min. The eluates were monitored by a Shimadzu SPD-M20A PDA detector before introduction into the mass spectrometer, in which the spray voltage was kept at 4.5 KV, the capillary voltage at 1.76 KV, at 200 °C. MS spectra were acquired under positive mode and collected in the 80–2000 *m/z* range. Instrument control, data acquisition, and data processing were performed with LabSolutions (LCMSsolution 3.60.361 version, Shimadzu).

Direct infusion mass spectrometry. Mass spectrometric analyses were performed in an ESI-IT-TOF as described above. Skin secretion fractions (obtained in 2.5.1 subsection) were dried and ressuspended in 0.1% FA for positive mode electrospray ionization (ESI+) or in methanol for negative mode ionization (ESI−). The fractions were manually injected in a Rheodyne injector, at a flow rate 50 μL/min, in 50% B_2 (ESI+) or 50% methanol (ESI−). Instrument control, data acquisition, and data processing were performed with LabSolutions (LCMSsolution 3.60.361 version, Shimadzu).

2.5.3. Nuclear magnetic resonance (NMR)

The identification of the purified compound was carried out by ^{1}H and ^{13}C and 2D (HMBC) NMR analysis (Bruker DRX 500 MHz) in $CDCl_3$ with TMS as internal standard.

3. Results

3.1. RP-HPLC

In order to evaluate the <10 kDa skin secretion composition of *P. carvalhoi*, UV-monitored RP-HPLC profiles were obtained and compared under different conditions: NE stimulation (1 or 50 nmol/g; Fig. 1A and B) versus mechanical stimulation (Fig. 1D and E) versus no stimulation (Fig. 1F) and captivity versus wildlife animals (Fig. 1B and C). The complete scheme of collection is summarized on Table 1. Fig. 1A–E also shows that regardless of the stimulation method and origin of the anuran (captivity or wildlife), there is one clear major