Short communication

Major biological effects induced by the skin secretion of the tree frog *Phyllomedusa hypochondrialis*

Katia Conceição[a], Fernanda Miriane Bruni[a], Marta M. Antoniazzi[b], Carlos Jared[b], Antonio Carlos M. Camargo[a], Mônica Lopes-Ferreira[a], Daniel C. Pimenta[a],*

[a]*LETA (Laboratório Especial de Toxinologia Aplicada) Center for Applied Toxinology (CAT/CEPID), Instituto Butantan, 05503-900 São Paulo, SP, Brazil*
[b]*Department of Celullar Biology, Instituto Butantan, São Paulo, SP, Brazil*

Abstract

Amphibian skin secretions contain several bioactive compounds such as biogenic amines, alkaloids, steroids, proteins and peptides; being peptides a continuously growing field of interest. This work aims to describe the main physiopathological properties of the tree frog *Phyllomedusa hypochondrialis* skin secretion, obtained by manual stimulation of the dorsal skin surface. Intravenous skin secretion administration provoked lethal effect in mice after 5 min. Low doses induced significant systemic and local effects like edema and nociception in mice and topic administration induced myonecrosis in the endothelium of cremaster mice. The presence of phospholipase A_2 activity, proteolytic activity and creatine kinase activity (in the plasma of treated mice) are reported and are very likely to be related to the physiopathological (edematic and myotoxic) activities observed. These data provide in vivo evidence of the complex toxic effects of the *P. hypochondrialis* skin secretion as well as possible mechanisms of action for these effects.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: *Phyllomedusa hypochondrialis*; Amphibia; Anura; Skin secretion; Biological activities

1. Introduction

Amphibian skin secretions are one of the richest sources of natural compounds and bioactive molecules in the animal kingdom, in part because of some histological and physiological characteristics of this tissue. The amphibian skin contains two kinds of glands: (i) mucous glands, usually associated with breathing, reproduction and water balance, and (ii) granular glands, responsible for the secretion of toxins, generally related to the defense against microorganisms and predators (Stebbins and Cohen, 1995; Toledo and Jared, 1993).

So far, several molecules have been isolated from the anuran skin, and this number is still growing. Among them, there are biogenic amines, alkaloids, steroids and peptides with a plethora of biological effects (cytotoxic, bactericidal, fungicidal, lytic, neuromimetic, anesthetic and pheromonal) (Flier et al., 1980; Roseghini et al., 1989; Bevins and Zasloff, 1990; Barra and Simmaco, 1995; Daly,

---

*Corresponding author. Tel./fax: +55 11 3726 1024.
E-mail address: dcpimenta@butantan.gov.br (D.C. Pimenta).

1995; Clarke, 1997; Wegener et al., 2001; Kikuyama et al., 2002).

Amphibians possess specialized glands that take part into their defense systems, and also assist with the regulation of dermal physiological action. These glands are able to respond to a variety of stimuli by secreting several compounds onto the dorsal surface and even into the gut of the amphibian, acting, for instance, as antimicrobials and regulators of the number of nematodes in the gut, respectively (Apponyi et al., 2004). The granular glands are responsible for the production of noxious or toxic substances presenting several biological and pharmacological effects. Nevertheless, few studies on the *toxicity* of the skin secretions of frogs and toads glands are reported, and fairly recognized. Peptide isolation and characterization seems to be the preferred approach to studying these secretions, rather than crude skin toxicity assessment.

Among the tree frogs, the Phyllomedusinae comprises six genera, namely *Phyllomedusa* (being the genus having the majority of the known species), *Agalychnis*, *Pachymedusa*, *Phrynomedusa*, *Phasmahyla* and *Hylomantis* (Walls, 1996). The hylid genus *Phyllomedusa* contains 32 species distributed throughout southern Central America and much of South America (da Cruz, 1990; Frost, 2004). *Phyllomedusa hypochondrialis* (Daudin, 1802) is a typical South American genus, inhabiting from the east of the Andes from Colombia and the Guianas southward to Argentina, Paraguay, northeastern and southeastern Brazil (Frost, 2004).

The study and biochemical analysis of the skin secretions and/or skin extracts from different *Phyllomedusa* species have led to the identification and characterization of a large number of bioactive peptides (Erspamer et al., 1986; Simmaco et al., 1998; Leite et al., 2005; Chen et al., 2005, 2006; Brand et al., 2006). However, few studies have been conducted in attempt to demonstrate the toxic activities induced by skin secretions of Phyllomedusa in murine models. In view of these facts, the aim of the present study was the analysis of biological activities induced by the crude skin secretion of the northeastern Brazilian *P. hypochondrialis*. Our results will make possible a better understanding of the role of the proteic and enzymatic components present in the epidermal secretion of this Brazilian frog.

2. Materials and methods

2.1. Animals

Groups of six Swiss mice weighing 18–22 g were used throughout. The animals provided by Instituto Butantan animal house were kept in temperature and humidity-controlled rooms, and received food and water ad libitum. All the procedures involving mice were in accordance with the guidelines provided by the Brazilian College of Animal Experimentation.

2.2. Collection of skin secretion

Specimens ($n = 12$) of *P. hypochondrialis* were collected by Angicos in the state of Rio Grande do Norte, Brazil. The tree frogs were kept alive in the biotherium of the Department of Cellular Biology of Butantan Institute, São Paulo, Brazil. Glandular secretion of the frogs was obtained from adult specimens, which were submerged in a beaker containing deionized water and manually and gently compressed. These solutions were frozen and lyophilized prior to analysis, stored at −20 °C. Protein content was determined by the method of Bradford (1976) using bovine serum albumin (Sigma Chemical Co., St Louis, MO) as standard protein.

2.3. Nociceptive activity

Nociceptive activity of the skin secretion was assayed according to Hunskaar et al. (1985). Thirty-microliter aliquots of skin secretion (0.15, 0.30 and 0.60 μg of protein) were injected (i.pl.) in the right footpad of mice. Then, each mouse was kept in an adapted chamber mounted on a mirror for 10 min. The control-group was injected only with PBS. Each animal was then returned to the observation chamber and the amount of time spent licking or biting each hind paw was recorded. Each point represents mean ± SEM.

2.4. Vascular permeability

Evans Blue dye, 20 mg/kg in 200 μl of PBS was i.v. administered 20 min before the skin secretion (0.15, 0.30 and 0.60 μg of protein) or PBS i.p. After 2 h, mice were sacrificed, and their peritoneal cavity was washed with 2 ml of ice-cold PBS and 0.1% BSA. The cells were spun down

and the OD of the supernatant at 620 nm was measured as an indicator of Evans Blue leakage into the peritoneal cavity (Sirois et al., 1988). Five mice were used for each group per experiment, and the experiments were conducted three times. The results were expressed in μg of Evans Blue/ml and the concentration of Evans Blue was calculated from a standard curve of a known concentration.

2.5. Edema

Edematogenic activity of the skin secretion was assayed according to Lima et al. (2003). Thirty-microliter aliquots of skin secretion (0.15, 0.30 and 0.60 μg of protein) were injected (i.pl.) in the right footpad of mice. Local edema was quantified by measuring the thickness of the treated paws with a paquimeter (Mytutoyo Sul Americana, SP, Brazil) $\frac{1}{2}$, 1, 2, 4, 24 and 48 h after injection. PBS-treated mice were used as controls. The results are expressed by the difference between experimental and control footpad thickness. Each point represents mean $\pm$ SEM.

2.6. Intravital microscopy

Dynamics of the alterations in the microcirculation and muscle fibers can be evaluated by using transillumination intravital microscopy of mice cremaster muscle after topical application of skin secretions (0.6 μg of protein dissolved in 20 μl of sterile saline versus sterile saline as control). In two independent experiments ($n = 4$), mice were anesthetized with pentobarbital sodium (Hypnol® Cristália; 50 mg/kg, intraperitoneal route) and the cremaster muscle was exposed for microscopic examination in situ as described by Lomonte et al. (1994). The animals were maintained on a special board thermostatically controlled at 37 °C, which included a transparent platform on which the tissue to be transilluminated was placed. After stabilization of the microcirculatory network, alterations in muscle fibers for 30 min after topical application of skin secretion could be observed. The study of the microvascular system of the tissue transilluminated was accomplished with an optical microscope (Axiolab, Carl-Zeiss, Oberkochen, DE) coupled to a photographic camera (Coolpix 990-Nikon) using a ×10/025 longitudinal distance objective/numeric aperture and 1.6 optovar.

2.7. Proteolytic activity

N,N-dimethylated casein (Sigma) can be used as an unspecific proteic substrate for the assessment of global proteolytic activity of a complex mixture, as described by Menezes et al. (2006). 0.15, 0.30 and 0.60 μg skin secretion were incubated with 2% casein in 0.4 ml 0.1 M Tris–HCl, pH 8.8, containing 0.01 M $CaCl_2$ for 30 min, at 37 °C. The reaction was stopped by adding 1 ml 5% trichloroacetic acid. Then, the mixture was centrifuged at 14,000 rpm for 15 min and the absorbance at 280 nm was measured. One unit of activity was defined as the amount of skin secretion yielding an increase in OD of 1.0 per min at 280 nm. Specific activity was expressed as units/mg protein.

2.8. Phospholipase A_2 activity

Phospholipase A_2 activity was carried out according to the method of Holzer and Mackessy (1996). The protein of skin secretion was assayed using 4-nitro-3-(octanoyloxy) benzoic acid as substrate (Sigma Chemical Company). *Crotalus durissus terrificus* venom was used as control-group. Activity was expressed as nmol chromophore released/min/ mg protein.

2.9. Myotoxic activity

Myotoxic activity was assessed by means of evaluating creatine kinase (CK) activity as a revealer of myonecrosis. Groups of four mice were injected intramuscularly in the right gastrocnemius muscle with 0.6 μg *P. hypochondrialis* (dissolved in 100 μl PBS, pH 7.2). Control mice received 100 μl PBS. At two time intervals (3 and 24 h) a blood sample was collected by retroorbital bleeding into heparinized capillary tubes. The CK activity in serum was determined using the kit CK-NAC (Bioliquid Laborclin Ltda, Brazil). One unit corresponds to the amount of enzyme that hydrolyzes 1 μmol of creatine per min. Myotoxic activity was expressed as U/mg of skin secretion.

2.10. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out according to the method of Laemmli (1970). Twenty-microgram skin secretions were analyzed by 12% SDS-PAGE gels. Prior to electrophoresis, the samples were

mixed 1:1 (v/v) with sample buffer. The gel was stained with the Silver method.

2.11. Statistical analysis

All results were presented as means $\pm$ SEM of at least four animals in each group. Differences among data were determined by one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences between two means were determined using unpaired Student's t-test. Data were considered different at $p < 0.05$.

3. Results and discussion

Bioactive peptides, mainly cytotoxic, bactericidal, fungicidal, lytic, neuromimetic, anesthetic or pheromonal, have been the goal of the majority of the reports on the skin secretions of Phyllomedusinae (Lazarus et al., 1999; Basir et al., 2000; Séon et al., 2000; Salmon et al., 2001; Chen et al., 2002; Doyle et al., 2002; Severini et al., 2002). These peptides defend the naked frog skin from noxious microorganisms and assist during wound repair (Zasloff, 1987; Nicolas and Mor, 1995; Simmaco et al., 1998; Zasloff, 2002). However, in the present study, we describe for the first time that the crude skin secretion of the P. hypochondrialis contains bioactive proteins (and not only peptides) capable of inducing local or systemic physiopathological alterations in mice.

Once the protein content of the crude skin secretion from P. hypochondrialis tree frog was calculated (2.3 mg/ml), some biological activities were assessed using the crude skin secretions of P. hypochondrialis. This contrasts to the current peptide-driven approach, often performed with Phyllomedusinae specimens (Erspamer et al., 1986; Simmaco et al., 1998; Chen et al., 2005).

Intravenous skin secretion administration (0.6 µg protein) provoked lethal effect in Swiss mice after 5 min. Necropsy revealed hyperemia of the lungs (data not shown), while the remaining organs remained unaltered; this effect together with the observed convulsion in the mice probably are the death cause. Intradermal administration of this same dose in the footpad of the animals led to a motor compromising of their right hind leg within the first 2 min and also evident paw swelling. After 15 min systemic effects were observed as erection of the hair, tachycardia and slavering activity. In addition, it was observed a reduction in

spontaneous locomotor activity, and this prostrated state persisted for 30 min. Forty-eight hours after skin secretion administration, necrosis could be observed in the footpad of these mice, while PBS-control mice did not present any variation at all.

Skin secretions from other amphibians have already been described as cardiotoxic and hemolytic (Schwartz et al., 1998, 1999), effects that could be related to the lethality of some of these secretions. The observed reduction in the spontaneous locomotor activity elicited by the skin secretion administration may be caused by peptides interacting with adenosine receptors (Daly et al., 1992). Erspamer et al. (1993) have described some effects with dried secretions from Phyllomedusa bicolor that contains large amounts of toxins (i.e. peptides) potently affecting the cardiovascular system and visceral functions.

Subsequently, we investigated the role of the skin secretion in nociception, edema and vascular permeability. Mice were injected with P. hypochondrialis skin secretion at different doses (0.15, 0.30 and 0.60 µg) into the mouse hind paw for nociception and edema and intraperitonealy for permeability.

Fig. 1 shows that both 0.30 and 0.60 µg skin secretion doses caused similar increase on the paw licking duration for 30 min (40.4 $\pm$ 7.7 for dose 0.30 µg, 46.6 $\pm$ 8.4 for dose 0.60 µg and 1.2 $\pm$ 0.1 for PBS). The dose of 0.15 µg did not induce nociception activity in mice. Immediate and transient nociceptive response (characterized by licking,
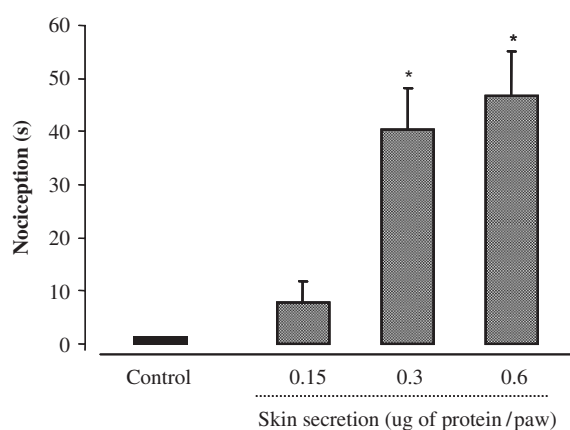

Fig. 1. Estimation of nociception-inducing activity. P.hypochondrialis skin secretion (0.15, 0.30 and 0.60 µg of protein) was injected (i.pl.) in the right footpad of mice. Amount of time spent licking or biting each hind paw was recorded for 30 min (A) or 0–5 and 15–40 min (B) and taken as the index of nociception. Each point represents mean $\pm$ SEM. *$p < 0.05$ compared with control-group.

biting and flinching of the injected paw) is a consequence of inflammatory mediators releasing. So, changes rapid occurring in primary afferent nociceptive fibers are likely to be the cause of the immediate nociceptive response induced by the secretion.

Fig. 2 shows that the peak of protein extravasation into peritoneal cavity is observed after 0.15 μg of protein injection (44.7 ± 12 vs. 4.7 ± 0.9). Moreover, larger doses (0.30 and 0.60 μg) induced a significant edematogenic activity when compared to the 0.15 μg dose and the control-group (Fig. 3). These higher doses maintained the edematogenic response up to 24 h, and only the dose of 0.6 μg of skin secretions induced a sustained edematogenic response until 48 h after injection when compared with the control-group (73.0 ± 8.7 and 5.0 ± 1.0, respectively). Edema formation is a common feature of the cutaneous inflammatory processes and is dependent on a synergism between mediators that increase vascular permeability and those that increase blood flow (Brain and Williams, 1985). The local edema induced by skin secretion of *P. hypochondrialis* was characterized by rapid onset, reaching a plateau at 30 min. After this early peak, the persistence of edema was related to higher doses. The rapid onset of the edematogenic response may also suggest a neurogenic driven response, i.e. an edema mainly produced by the vasodilator peptides released from nociceptors acutely activated by the noxious stimuli.

In order to assess the effects of the secretion in muscular fibers, intravital microscopy was
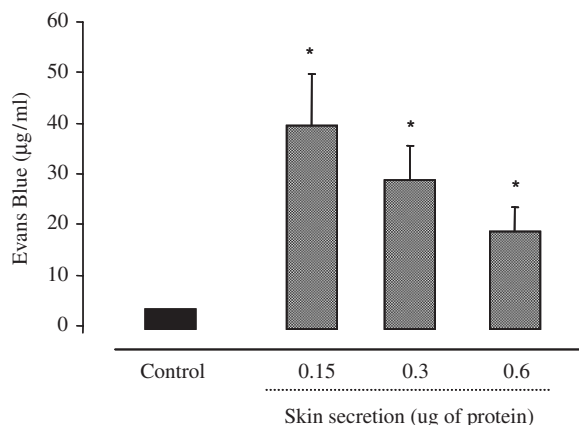
Fig. 3. *Estimation of edema-inducing activity. P.hypochondrialis* skin secretion (0.15, 0.30 and 0.60 μg of protein) was injected (i.pl.) in the right footpad of mice. Results were expressed as the difference between experimental and control footpad thickness. Data represent mean ± SEM. *$p < 0.05$ compared with control-group.

employed. This methodology allows the direct observation of changes in the muscular fibers, and is a plain and noninvasive approach to the pursue of biological effects (Lubbers, 1995). Topical application of 0.6 μg skin secretion induced myofibrilar hypercontraction of the muscle cells and leukocytes rolling 10 min after administration, being these effects more evident after 30 min (Fig. 4).

Also noteworthy is the detection of phospholipase A_2 activity in this secretion at 0.6 μg of protein (Table 1). The relative PLA_2 activity was considered relevant when compared to the well-known venom of *C. d. terrificus* (4.78 ± 0.21 vs. 24.9 ± 2.96 nmol chromophore released/min/mg venom). PLA_2s are frequent components found in many types of venom of vertebrates and invertebrates. On the other hand, mammalian-secreted PLA_2s are involved in inflammation, host defense and several inflammatory diseases, and are specifically distributed in several tissues, suggesting that they may play a role in a number of fundamental physiological processes (Valentin et al., 2000).

We also determined the proteolytic activity of *P. hypochondrialis* skin secretion on casein, a broadly used unspecific substrate that can be degraded by both serine proteinases and metalloproteinases. Our data show that the dose of 0.6 μg protein contains a significant caseinolytic activity (Table 1).

Plasmatic CK activity 2 and 24 h after injection of the skin secretion (0.60 μg) in the gastrocnemius

Fig. 2. *Evaluation of the vascular permeability*. 20 mg/kg Evans Blue dye was i.v. administered 20 min before the skin secretion (0.15, 0.30 and 0.60 μg of protein) and quantified in the peritoneal cavity wash. Results were expressed in μg of Evans Blue/ml.
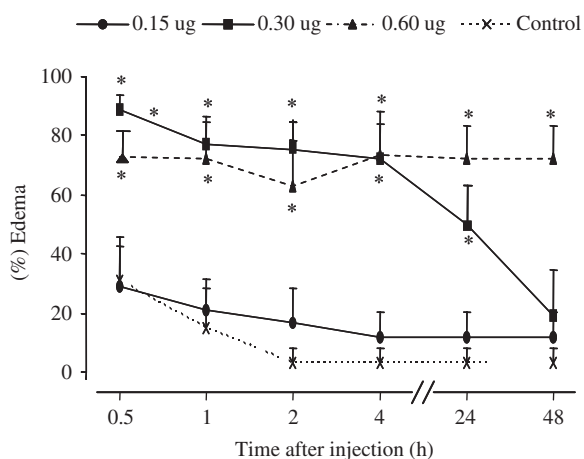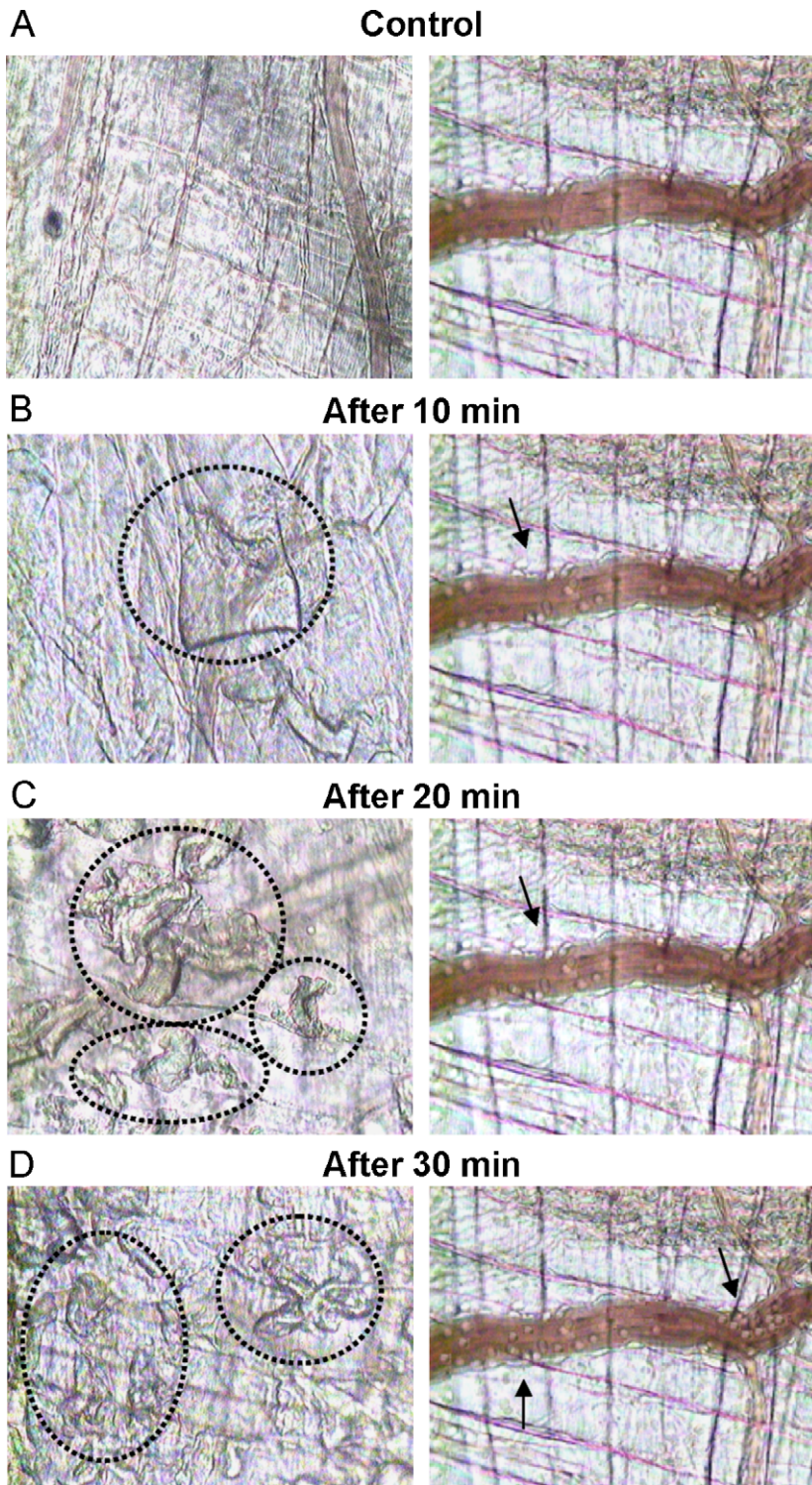
Fig. 4. Intravital micrograph of cremaster muscle after topical application of 0.6 µg of skin secretion of *P. hypochondrialis*. (A) Cremaster muscle examined before topical application of *P. hypochondrialis* skin secretion—Control. (B) Presence of myofibrillar hypercontraction in a affected muscle cell (circle) 10 min after skin secretion administration and (C) intense hypercontraction after 20 min and (D) 30 min (circle). The arrows indicate the rolling of leukocytes. Results were obtained in recorded images on optical microscope (Axiolab, Carl-Zeiss) coupled to a photographic camera (Coolpix 990-Nikon) using an ×10/025 longitudinal distance objective/numeric aperture and 1.6 optovar. Photographs were obtained from digitized images on the computer monitor. This data representative of observations in four mice, analyzed on different days.

muscle was assessed. No significant differences could be observed after 2 h, but increased plasmatic CK activity could be detected after 24 h (Table 1).

The electrophoretic profile of the skin secretion was also evaluated. A 12% SDS-PAGE was performed with 10 µg of protein/well and was able to reveal that *P. hypochondrialis* skin secretions present seven major bands, revealed after silver staining distributed as follows: above 68 kDa, around 25 kDa and below 14 kDa (Fig. 5).

Protein evaluation (chemically and/or kinetically) on the skin secretion of anurans is, in general, not very common, being peptide research the more

Table 1
Toxic activities induced by *P. hypochondrialis* skin secretion

Biological activity	Activity[a]
Proteolysis	1.64 ± 0.03 (U/mg)[b]
Phospholipase	4.78 ± 0.21(nmol chromophore released/min/mg)
Myotoxicity	184.3 ± 60.2 (U/ml)[c]

[a]Normalized data per 0.6 µg skin secretion of protein.
[b]Units are defined in Section 2.7.
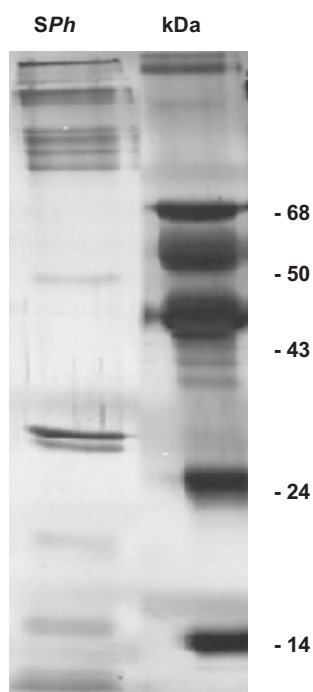[c]Units are defined in Section 2.9.

Fig. 5. *Phyllomedusa hypochondrialis* skin secretions (S Ph) SDS-PAGE using polyacrylamide gel 12%. And silver stained. Right lane, M_r markers and respective molecular weights.

usual form of describing biological effects. The 'toxins' clearly present in the *P. hypochondrialis* skin secretion are very likely to be bioactive, either participating in the skin homeostasis or acting like defense molecules as reported through the characterization of three enzymatic activities described in this report.

Further studies are necessary to clarify their biochemical role; but, some of the findings reported here are strictly related to enzymatic (therefore, proteic) functions, namely proteolytic activity and phospholipase activity. These enzymatic activities may be as important as the classically described peptidic activities, such as antimicrobial and/or kinin-related. Moreover, some enzymatic activities, such as proteolytic, may be responsible for the generation of the 'famous' bioactive peptides, and PLA_2 activity may be as important as the peptidic activities for the physiological antimicrobial effect, at the level of the amphibian skin.

Here, we propose that the biochemical and pharmacological events observed in mice caused by *P. hypochondrialis* crude skin secretion administration, even leading to the establishment of irreversible lesions, are due to the action of various proteic toxins (and, very likely, peptidic toxins as well). Our data present the first depiction of the biological activities induced by the Brazilian frog's skin secretion of the *P. hypochondrialis* in experimental animals. The isolation and characterization of the skin secretion proteins responsible for these effects is a relevant task and is currently ongoing in our laboratory.

Acknowledgments

References

Apponyi, M.A., Pukala, T.L., Brinkworth, C.S., Maselli, V.M., Bowie, J.H., Tyler, M.J., Booker, G.W., Wallace, J.C., Carver, J.A., Separovic, F., Doyle, J., Llewellyn, L.E., 2004. Host-defence peptides of Australian anurans: structure, mechanism of action and evolutionary significance. Peptides 25, 1035–1054.

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

Basir, Y.J., Knoop, F.C., Dulka, J., Conlon, M.J., 2000. Multiple antimicrobial peptides and peptides related to bradykinin and neuromedin N isolated from skin secretions of the pickerel frog, Rana palustris. Biochim. Biophys. Acta 1543, 95–105.

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

Bradford, M.M., 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72, 248–254.

Brain, S.D., Williams, T.J., 1985. Inflammatory mechanims of inflamed-tissue factor. Agents Actions 3, 348–356.

Brand, G.D., Krause, F.C., Silva, L.P., Leite, J.R., Melo, J.A., Prates, M.V., Pesquero, J.B., Santos, E.L., Nakaie, C.R., Costa-Neto, C.M., Bloch Jr., C., 2006. Bradykinin-related peptides from Phyllomedusa hypochondrialis, Peptides 27, 2137–2146.

Chen, T., Orr, D.F., Bjourson, A.J., McLean, S., O'Rourke, M., Hirst, D.G., Rao, P., Shaw, C., 2002. Novel bradykinins and their precursor cDNAs from European yellow-bellied toad (Bombina variegata) skin. Eur. J. Biochem. 269, 4693–4700.

Chen, T., Walker, B., Zhou, M., Shaw, C., 2005. Dermatoxin and phylloxin from the waxy monkey frog, Phyllomedusa sauvagei: cloning of precursor cDNAs and structural characterization from lyophilized skin secretion. Reg. Peptides 129, 103–108.

Chen, T., Zhou, M., Gagliardo, R., Walker, B., Shaw, C., 2006. Elements of the granular gland peptidome and transcriptome persist in air-dried skin of the South American orange-legged leaf frog, Phyllomedusa hypocondrialis. Peptides 27, 2129–2136.

Clarke, B.T., 1997. The natural history of amphibian skin secretions, their normal functioning and potential medical applications. Biol. Rev. Camb. Philos. Soc. 72, 365–379.

da Cruz, C.A.G., 1990. Sobre as relações intergenéricas de Phyllomedusinae da floresta atlantica (Amphibia, Anura, Hylidae). Rev. Bras. Biol. 50, 709–727.

Daly, J.W., 1995. The chemistry of poisons in amphibian skin. Proc. Natl. Acad. Sci. USA 92, 9–13.

Daly, J.W., Caceres, J., Moni, R.W., Gusovsky, F., Moos Jr., M., Seamon, K.B., Milton, K., Myers, C.W., 1992. Frog secretions and hunting magic in the upper Amazon: identification of a peptide that interacts with an adenosine receptor. Proc. Natl. Acad. Sci. USA 15, 10960–10963.

Daudin, F.M., 1802. Histoire Naturelle Des Rainettes, Des Grenouilles et Des Crapauds. Imprimerie de Bertrandet; libraire Leurault, Paris, 108pp (ex Easteal 1986).

Doyle, J., Llewellyn, L.E., Brinkworth, C.S., Bowie, J.H., Wegener, K.L., Rozek, T., Wabnitz, P.A., Wallace, J.C., Tyler, M.J., 2002. Amphibian peptides that inhibit neuronal nitric oxide synthase. Isolation of lesuerin from the skin secretion of the Australian Stony Creek frog Litoria lesueuri. Eur. J. Biochem. 269, 100–109.

Erspamer, V., Falconieri Erspamer, G., Cei, J.M., 1986. Active peptides in the skins of two hundred and thirty American amphibian species. Comp. Biochem. Physiol. C 85, 125–137.

Erspamer, V., Erspamer, G.F., Severini, C., Potenza, R.L., Barra, D., Mignogna, G., Bianchi, A., 1993. Pharmacological studies of 'sapo' from the frog Phyllomedusa bicolor skin: a drug used by the Peruvian Matses Indians in shamanic hunting practices. Toxicon 31, 1099–1111.

Flier, J., Edwards, M.W., Daly, J.W., Myers, C.W., 1980. Widespread occurrence in frogs and toads of skin compounds interacting with the ouabain site of Na+, K+-ATPase. Science 208, 503–505.

Frost, D.R., 2004. Amphibian species of the world, an online reference, v. 3.0 (22 August). American Museum of Natural History, New York. Electronic database accessible at ⟨http://research.amnh.org/herpetology/amphibian/index.html⟩.

Holzer, M., Mackessy, S.P., 1996. An aqueous endpoint assay of snake venom phospholipase A2. Toxicon 34, 1149–1155.

Hunskaar, S., Fasmer, O.B., Hole, K., 1985. Formalin test in mice, a useful technique for evaluating mild analgesics. J. Neurosci. Methods 14, 69–76.

Kikuyama, S., Yamamoto, K., Iwata, T., Toyoda, F., 2002. Peptide and protein pheromones in amphibians. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 132, 69–74.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lazarus, L.H., Bryant, S.D., Cooper, F.S., Salvadori, S., 1999. What peptides these deltorphins be. Prog. Neurobiol. 57, 377–420.

Leite, J.R., Silva, L.P., Rodrigues, M.I., Prates, M.V., Brand, G.D., Lacava, B.M., Azevedo, R.B., Bocca, A.L., Albuquerque, S., Bloch Jr., C., 2005. Phylloseptins: a novel class of anti-bacterial and anti-protozoan peptides from the Phyllomedusa genus. Peptides 26, 565–573.

Lima, C., Clissa, P.B., Piran-Soares, A.A., Tanjoni, I., Moura-da-Silva, A.A., Lopes-Ferreira, M., 2003. Characterization of local inflammatory response induced by Thalassophryne nattereri fish venom in a mouse model of tissue injury. Toxicon 42, 499–507.

Lomonte, B., Lungren, L., Johansson, B., Bagge, U., 1994. The dynamics of local tissue damage induced by Bothrops asper snake venom and myotoxin II on the mouse cremaster muscle: an intravital. Toxicon 32, 41–55.

Lubbers, D.W., 1995. Optical sensors for clinical monitoring. Acta. Anaesthesiol. Scand. Suppl. 104, 37–54.

Menezes, M.C., Furtado, M.F., Travaglia-Cardoso, S.R., Camargo, A.C., Serrano, S.M., 2006. Sex-based individual variation of snake venom proteome among eighteen Bothrops jararaca siblings. Toxicon 47, 304–312.

Nicolas, P., Mor, A., 1995. Peptides as weapons against microorganisms in the chemical defense system of vertebrates. Annu. Rev. Microbiol. 49, 277–304.

Roseghini, M., Falconieri Erspamer, G., Severini, C., Simmaco, M., 1989. Biogenic amines and active peptides in extracts of the skin of thirty-two European amphibian species. Comp. Biochem. Physiol. C 94, 455–460.

Salmon, A.L., Cross, L.J., Irvine, A.E., Lappin, T.R., Dathe, M., Krauser, G., Canning, P., Thim, L., Beyermann, M., Rothemund, S., Bienert, M., Shaw, C., 2001. Peptide leucine arginine, a potent immunomodulatory peptide isolated and structurally characterized from the skin of the Northern Leopard frog, Rana pipiens. J. Biol. Chem. 276, 10145–10152.

Schwartz, E.F., Schwartz, C.A., Sebben, A., 1998. Occurrence of hemolytic activity in the skin secretion of the caecilian Siphonops paulensis. Nat. Toxins 6, 179–182.

Schwartz, E.N., Schwartz, C.A., Sebben, A., Largura, S.W., Mendes, E.G., 1999. Indirect cardiotoxic activity of the

caecilian *Siphonops paulensis* (Gymnophiona, Amphibia) skin secretion. Toxicon 37, 47–54.

Séon, A.A., Pierre, T.N., Redeker, V., Lacombe, C., Delfour, A., Nicolas, P., Amiche, M., 2000. Isolation, structure, synthesis, and activity of a new member of the calcitonin gene-related peptide family from frog skin and molecular cloning of its precursor. J. Biol. Chem. 275, 5934–5940.

Severini, C., Improta, G., Falconieri-Erspamer, V., Salvadori, S., Erspamer, V., 2002. The tachykinin peptide family. Pharmacol. Rev. 54, 285–322.

Simmaco, M., Mignogna, G., Barra, D., 1998. Antimicrobial peptides from amphibian skin: what do they tell us? Biopolymers 47, 435–450.

Sirois, M.G., Jancar, S., Braquet, P., Plante, G.E., Sirois, P., 1988. PAF increases vascular permeability in selected tissues: effect of BN-52021 and L-655,240. Prostaglandins 36, 631–644.

Stebbins, R.C., Cohen, N.W., 1995. A Natural History of Amphibians. Princeton University Press, Princeton, NJ.

Toledo, R.C., Jared, C., 1993. Cutaneous adaptations to water balance in amphibians. Comp. Biochem. Physiol. 105, 593–608.

Valentin, E., Singer, A.G., Ghomashchi, F., Lazdunski, M., Gelb, M.H., Lambeau, G., 2000. Cloning and recombinant expression of human group IIF-secreted phospholipase A(2). Biochem. Biophys. Res. Commun. 279, 223–228.

Walls, J.G., 1996. Red-Eyes and Other Leaf-Frogs, Manufactured in the USA. T.F.H. Publications, Inc.

Wegener, K.L., Brinkworth, C.S., Bowie, J.H., Wallace, J.C., Tyler, M.J., 2001. Bioactive dahlein peptides from the skin secretions of the Australian aquatic frog *Litoria dahlii*: sequence determination by electrospray mass spectrometry. Rapid Commun. Mass Spectrom. 15, 1726–1734.

Zasloff, M., 1987. Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. 84, 5449–5453.

Zasloff, M., 2002. Antimicrobial peptides of multicellular organisms. Nature 415, 389–395.