Annual Research & Review in Biology
4(2): 378-391, 2014

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The Parotoid Gland Secretion of Bufonids Inhibits the Activity of the Multidrug Resistance Target Pdr5p from Yeast Plasma Membranes

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript

Original Research Article

Received 25th March 2013
Accepted 7th July 2013
Published 19th October 2013

ABSTRACT

Aims: Considering the well known biological effects of secretion of cutaneous glands in anurans and that Pdr5p belongs to the ATB-Binding Cassette (ABC) superfamily of membrane transporters, which efflux a large variety of xenobiotic compounds, and play an important role in the multidrug resistance (MDR), the parotoid gland of two bufonids were used in order to evaluate their secretion activity on Pdr5p of plasma membranes of the yeast *Saccharomyces cerevisiae* as a potential inhibitor of MDR.
Place and Duration of Study: Laboratory of Integrative Histology and Laboratory of Microbian Biochemistry, Federal University of Rio de Janeiro, between September 2009 and October 2011.
Methodology: Five male bufonids of *Rhinella icterica* and of *R. ornata* were used, and the parotoid gland secretion was obtained after manual gland comprehension and by dissection. ATPase assays as well as SDS-PAGE were performed, besides immunohistochemical analysis at light microscopy.
Results: Pdr5p ATPase activity was inhibited when the crude extract was obtained by

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manual compression from the parotoid gland in contrast to the isolated intake of the large granular alveoli, which showed no enzymatic activity. Moreover, the Pgp expression in acinar cells of both mixed and small granular glands as well as in a myoepithelial cells was visualized by immunohistochemistry, suggesting its participation in tissue defense against toxic compounds.

Conclusion: The inhibitory effect of crude extract obtained by manual comprehension was detected, while the isolated intake showed no effect on the Pdr5p ATPase activity. Both bufonid represent an important source of biomolecules, opening new perspectives of research on natural products.

Keywords: Parotoid gland; bufonid; multidrug resistance; Pdr5p.

1. INTRODUCTION

Multidrug resistance (MDR) refers to cell simultaneous resistance to several structurally and functionally different cytotoxic compounds owing to their increased efflux from the cells [1]. This is a kind of defense mechanism conserved in animal evolution from bacteria to humans that causes a decrease in effectiveness of antimicrobial therapy and tumor treatment [1,2]. Moreover, MDR is a crucial clinical problem for human cancer therapy and infection treatment caused by bacteria or fungi [3].

One of the best understood mechanisms behind the acquisition of MDR involves the overproduction of an ABC transporter protein designated MDR1, which acts as an ATP-dependent drug pump and prevents the accumulation of toxic levels of target compounds [4].

As a model for studying of the MDR mechanism, the yeast *Saccharomyces cerevisiae,* which has about 30 distinct genes encoding ABC proteins, is used since it shares homology with mammalian genes relative to MDR [3,5,6]. Among them, Pdr5p is one of the major sources of drug resistance to various compounds such as cycloheximide, cerulenin, rhodamine 6G, anticancer drugs, and steroids. Moreover, it is a plasma membrane protein associated to ATP-binding cassette (ABC) transporter as well as the major multidrug efflux of *S. cerevisiae* [7]. Therefore, Pdr5p obtained from *S. cerevisiae* has been used to study human MDR mechanism since it acts similarly to that of the human Pgp (P-glycoprotein) [6].

Bioactive substances have been isolated from different animal groups. Although amphibians are widely distributed in the world, there are a small number of reports on bioactive substances from bufonids [8,9]. They represent a source of chemical compounds, which are an integral part of their defense system and assisting the regulation of dermal physiology.

Anuran specialized cutaneous glands on the dorsal surface of the integument secrete several compounds [10]. Previous reports showed that the glandular crude secretion from *Bufo andrewsi* exhibits cytotoxic activities [11] and, recently, an anti-HIV-1 component was purified from this anuran [8]. Furthermore, peptides/proteins from amphibian integument have shown biological activities such as antimicrobial effect [8,12,13], hemolytic, platelet aggregation activating, bradykinin-like, insulin-releasing activities [14] and anticancer activity [12,15].

In bufonids, their integument exhibits different gland types, i.e., cutaneous glands scattered through body integument and the parotoid glands. Both of which are responsible for

producing bioactive substances. According to Clarke [16], cutaneous secretion may contribute to anuran protection against predators and parasites. Moreover, the parotoid gland of bufonids is not a simple gland, but it represent an aggregate of several glandular types (small glands, mucous glands, small granular glands and large granular glands) [17,18].

Based on these data, this work aimed to evaluate the bioactivity of the parotoid gland secretion of two bufonids (*Rhinella icterica* and *R. ornata*) on the ATPase activity of Pdr5p of the *S. cerevisiae* plasmatic membranes. Moreover, the Pgp expression in both bufonid integuments was accessed through Pgp immunolabeling since antibody against Pdr5p is not commercially available, and Pgp has similarities to Pdr5p.

2. MATERIALS AND METHODS

2.1 Animal and Secretion Obtainment

Five adult male bufonids of *R. icterica* (Spix, 1824) and of *R. ornata* (Spix, 1824) were collected according to the Brazilian laws (license no. 12164-1 – IBAMA/MMA). This study was performed in accordance with the guidelines for the care of laboratory animal used at the Federal University of Rio de Janeiro, and approved by its Ethics Committee. In addition, the toads were euthanized according to bioethics practices (resolution from the Brazilian Federal Committee of Veterinary Medicine).

R. icterica were captured in the Districts of Teresópolis and *R. ornata* in São Conrado (both areas of the Atlantic Forest in Rio de Janeiro State). The average weight of *R. icterica* was 120 grams and the average length (snout-vent) was 11 centimeters. For *R. ornata*, the average weight and average length were 12 grams and 6 cm, respectively.

2.2 Biochemical Analysis

For the biochemical study, the parotoid gland secretion was obtained by two procedures: (1) the parotoid glands were manually compressed with a constant pressure until extrusion of the secretion in order to obtain the crude extract; (2) after euthanasia, the parotoid glands were removed, and immediately frozen. Then, the frozen glandular intakes of the large alveoli were manually removed by dissection. After these procedures, the secretions were homogenized in 10mM Tris buffer adjusted for pH 7.6 with protease inhibitor and aliquoted.

2.3 Preparation of Plasma Membranes

The isolation of plasma membranes enriched in Pdr5p was carried out as described by Goffeau and Dufour (1998), following some modifications [3]. Cells from the AD1234567 strain of *S. cerevisiae* containing the *PDR5-6 HIS* allele at the *PDR5* locus were grown overnight in 2% peptone, 2% dextrose, 1% yeast extract, until the exponential phase of growth [3]. After washing with 10mM NaN_3, the cell walls were digested with zymolyase at 37ºC for 60 minutes. Unbroken cells and debris cells were removed by low-speed centrifugation (5.500 x g for 10 min) at 4ºC. The supernatant was centrifuged for 30 minutes at 9,000 x g in order to remove possible contaminants, as mitochondria, and the remaining supernatant was centrifuged at 20.000 x g for 45 minutes. The final pellet, highly enriched with plasma membranes, was stored at -70 ºC. In order to avoid possible contaminants, the

plasma membrane was preserved with a pool of protease inhibitors (Sigma-Aldrich®) and different inhibitors were added to ATPase assay medium as described below [3].
The protein intake of Pdr5p and of secretions was assayed according to Bradford [19], using bovine serum albumin as standard [20].

2.4 ATPase Assays

ATP hydrolysis was measured by incubating the Pdr5p-enriched membranes (5 µg of protein) at 37 ºC with the samples of the parotoid gland secretions, having a final volume of 1600 µL adjusted for pH 7.0 with HCl. The solution contained 100mM ATP (at pH 7.0), 10 mM $MgCl_2$, 2.5 mM EGTA, 2.5 mM NaN_3 (inhibitor of mitochondrial F_1F_0- ATPase), 1.25 mM ouabain (inhibitor of plasma membrane Na^+, K^+-ATPase), 125 mM KNO_3 (inhibitor of phosphatases) and 100 mM Tris.

The samples were previously incubated with Pdr5p for 15 minutes and the assays were carried out as described by Decottignies and coworkers [5]. After 1 hour, the reaction was stopped by the addition of 1% SDS (sodium dodecyl sulfate) as described by Dulley [21] and the inorganic phosphate was measured as described by Fiske and Subbarow [22].

For the control group, the samples of the parotoid gland secretions were not added, whereas for the experimental groups, the samples were tested in different concentrations (1µg/mL, 2µg/mL, 5µg/mL, 10µg/mL).

All the experiments were carried out at least three times and the results were obtained with the assistance of the computer program Sigma Plot version 8.0 (SPSS Science Marketing).

2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Ten percent (% w/w) of sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) was used to determine the apparent molecular weight of the protein intake of the parotoid gland secretion, according to the method of Laemmli [23]. The proteins (3 µg) were mixed in 0.5 M Tris buffer, containing 1% SDS in distilled water until a final volume of 40 µL. Molecular weight standards were “Page Ruler Protein Ladder” (Fermentas) and the gel was stained with silver nitrate.

2.6 Light Microscopy

For the light microscopic (LM) analysis, parotoid gland fragments were fixed with 10% buffered formaldehyde and/or Bouin’s liquid, and processed according to standard histological techniques for paraffin embedding. Five-micrometer thick serial slices were stained with haematoxylin-eosin (HE) [24].

Since the Pdr5p expression is related only to *S. cerevisae*, a similar protein, known as Pgp, was used. The Pgp immunolocalization was performed using standard immunoassaying procedures. Thus, slices were de-paraffinized, dehydrated, and washed in phosphate buffered saline (PBS). Endogenous peroxidase was blocked with 3% hydrogen peroxide solution for 30 minutes. The sections were incubated in a humidified chamber for 1 hour with 1% bovine serum albumin (BSA) and 0.1% Triton at room temperature. The sections were incubated with monoclonal antibody anti P-glycoprotein (Dako, Monoclonal Mouse Anti-Human, clone C494) diluted to 1:200 overnight. After being washed with PBS, the sections

were incubated with Envison (Dako, Envision + Dual link system peroxidase) for 30 minutes. Then, they were washed in PBS, and revealed using the 3´3-diaminobenzidine tetrahydrochloride (DAB) (Dako, Liquid DAB + Substrate chromogen system). The sections were counterstained with haematoxylin, and permanent preparations were finished according to the standard technique. As negative control procedure, the treatment with the primary antibody was omitted; as positive control, both human liver and toad kidney were used. After that, slices were observed under a Leica DMLS microscopy, and the images were captured using the Leica DFC 425 digital camera.

3. RESULTS

As previously described [17,18], the integument of the parotoid gland region of both anurans exhibits its basic morphology, i.e., it is constituted of an epidermis and a dermis formed by a spongious dermis, reticular dermis, and compact dermis, besides the Eberth-Katschenko layer scattered through the spongious dermis. In the parotoid gland region, mixed glands occur in the spongious dermis; however, the alveoli of the small and larger granular glands are located in the reticular dermis.

In this work, the histological analysis of the parotoid gland indicated that the glandular cells remained in the alveolus even after manual compression (Fig. 1), discharging only their secretion, i.e., the crude extract.

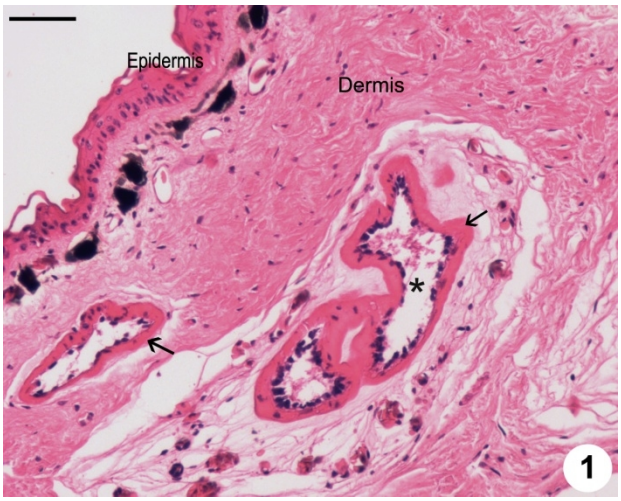

Fig. 1. Light micrograph of the parotoid gland after manual compression. Although the glandular alveoli exhibit reduction of its lumen (∗), the secretory cells remain and are surrounded by myoepithelial cells (arrows). Scale bar: 50 µm. HE-staining

The crude extract and the isolated glandular intake of the larger granular alveoli have shown different effects on the Pdr5p ATPase activity. These results revealed a reduction of the enzymatic activity when submitted to different concentrations of crude extract of both bufonids. In addition, the inhibitory effect of crude extracts was dose-dependent (Fig. 2A and 2B). The perceptual values of the Pdr5p ATPase activity on concentrations of 1µg/mL, 2µg/mL, 5µg/mL and 10µg/mL of the crude extract of *R. icterica* were 91.01(%), 55.68 (%), 37.42(%) and 31.13(%), respectively. The effect of the crude extract from the parotoid gland

of *R. ornata* on the same sequence of concentrations previously cited was 87.82 (%), 45.01 (%), 44.86 (%) and 24.63 (%). At dosages of 10 μg, the crude extracts inhibited more than 50% on the Pdr5p ATPase activity.

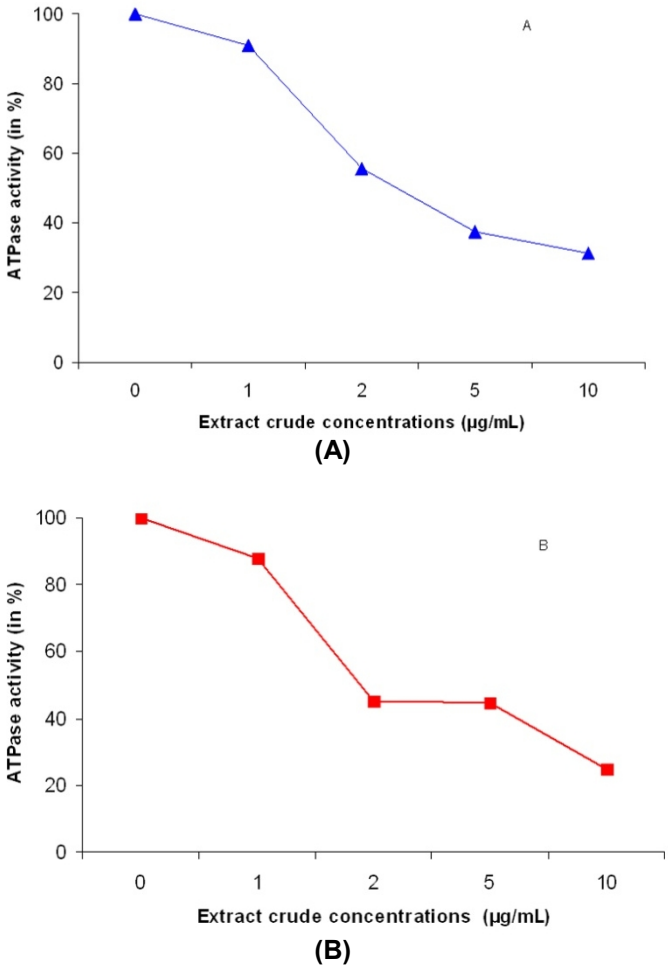

Fig. 2. Inhibition of the Pdr5p ATPase activity. The plasma membrane ATPase activity was evaluated in different concentrations of the crude extract. (A) R. icterica; (B) R. ornate

In addition, the isolated granular intakes of the parotoid glands have shown no significant effect on the Pdr5p ATP hydrolytic activity (Fig. 3). The values of the inhibition by isolated granular intake remained close to control, showing no interference in the action of the Pdr5p ATPase activity. For both species, the control activity corresponded to 1.20 μMol $Pi.mg^{-1}.min^{-1}$, and the results were shown in percentages, being the control activity considered as 100%. Thus, the effect of the isolated granular intake from the parotoid gland secretion of *R. icterica*, considering the concentrations of 1μg, 2μg, 5μg and 10μg, was 95.74(%), 102.16(%), 100.33(%) and 96.99(%), respectively. The values obtained from *R. ornata* were 97.74(%), 98.49(%), 103.66(%) and 95.49(%).

Due to different action mechanisms of the crude extract and the isolated granular intake from the larger alveoli on the Pdr5p ATP hydrolytic activity, the SDS-PAGE was used in order to separate proteins, revealing their patterns. Results indicated that the parotoid gland secretions of both bufonids had a wide molecular size range of approximately 25 – 120 kDa (Fig. 4).

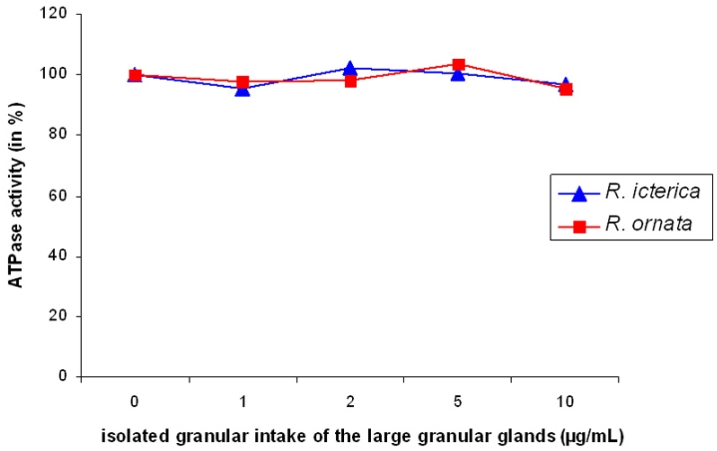

Fig. 3. Action of the isolated intake of the large alveoli on the ATPase activity. As control, ATPase activity was measured in the absence of the crude extract and corresponds to 0 concentration in the graph

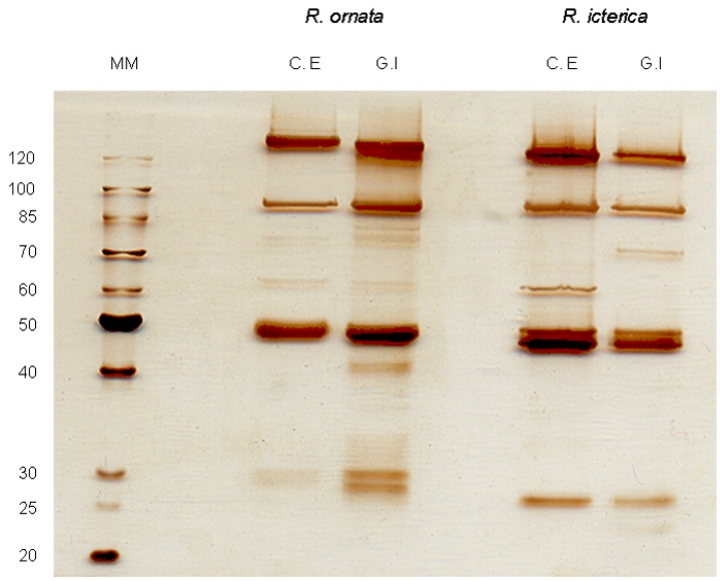

Fig. 4. SDS-PAGE profile of the parotoid gland secretions of both bufonids in concentration of 3 µg/mL. MM: Molecular mass (kDa); C.E: crude extract; G.I: granular intake of the large alveoli

Considering the SDS-PAGE, the crude extract and the isolated intakes from both species showed similarities regarding three protein bands: a protein band of approximately 50 kDa, a

protein band with relative molecular mass of 85 – 100 kDa and a protein band of approximately 120 kDa. However, the banding patterns exhibited differences depending on the species. In *R. icterica*, a protein band of 25 kDa was observed in the crude extract as well as in the isolated granular intake. Besides, in *R. ornata*, protein bands of approximately 28 kDa and 30 kDa were detected in both samples. Nevertheless, considering the secretion type, significant differences were observed. Although some protein bands were identified in the isolated granular intake of the larger gland alveoli, they were not observed in the crude extract. A protein band with molecular mass of approximately 70 kDa was detected in the granular intake of *R. icterica*, while a protein with molecular weight slightly above 40 kDa was visualized in *R. ornata*. Moreover, in *R. ornata*, a protein band of 60 kDa was found in the isolated secretion, while in *R. icterica* this protein band was only evident in the crude extract.

After immunohistochemistry, the Pgp expression was observed in the glandular cells of the mixed and small granular glands. Furthermore, the secretion of the small granular glands exhibited slightly positive reaction as well as in myoepithelial cells and in some cells scattered through the spongious dermis (Fig. 5). As positive reaction control, Pgp immunolabeling was observed in the human liver (Fig. 6A) and in both bufonid kidneys (Fig. 6B).

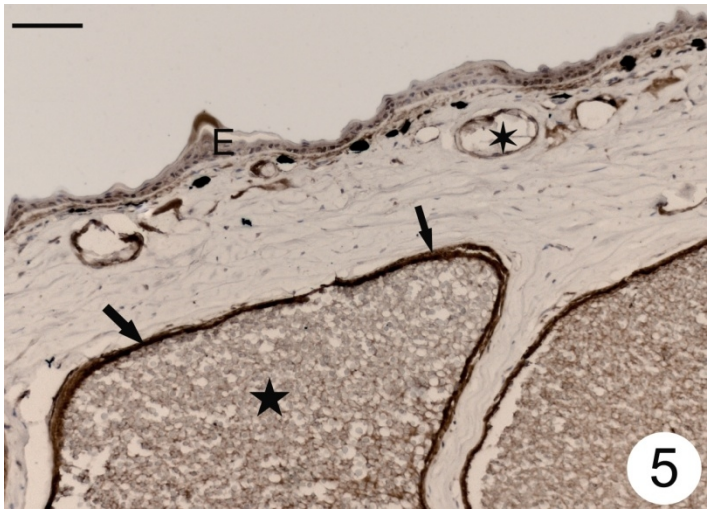

Fig. 5. Light micrograph of the parotoid gland of R. icterica. Note Pgp expression in secretory cells of the small granular (*) glands and in the glandular cells of large alveolus. Inset: negative control. Scale bar: 50µm; Pgp immunolabeling

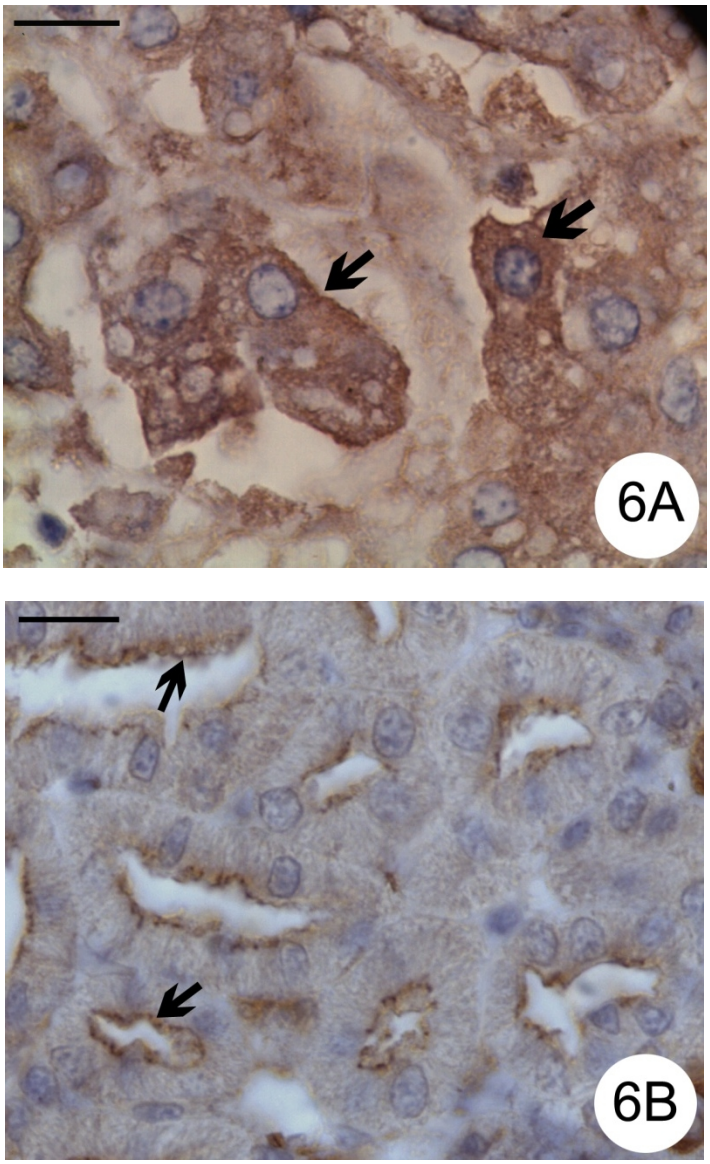

Fig. 6. Pgp Immunostaining: the human liver (A) (used as positive control) and the bufonid kidney (B) (used as molecular recognition in anuran tissue). Note Pgp positive reaction (→) in the hepatocytes and in the brush border of the renal tubules. Scale bar: 60µm

4. DISCUSSION

Due to the expansive employ of commercially available antibiotics, the ever growing number of resistant microorganism strains has stimulated researches on new alternatives for drug therapy [13]. Researches on MDR inhibitors required for cancer chemotherapy and for the elucidation of molecular mechanisms of drug efflux mediated by MDR proteins have been performed in order to find some strategies to reverse this drug resistance [6,24,25].

Therefore, studies about bioactivity of animal secretions could be useful. In amphibians, the major source of biochemical compound is cutaneous secretions. In addition, the scientific use of these compounds has contributed to the understanding of some physiological mechanisms [16,26]. Moreover, the extraordinary variety of bioactive substances (amines, alkaloids, steroids and proteins) found in anuran secretions and coupled with the high probability of their contents with a novel molecular structure and clinically useful function makes anurans an additional animal target group [16].

Although some molecules with remarkable biological function have been extracted from anuran integument such as dermaseptins, citropins, caerins [13,14,15], until now, there has been no information regarding the action of anuran secretions on the Pdr5p ATPase activity, which is responsible for multidrug resistance phenotype. In this work, different concentrations of crude extract of both bufonids blocked the enzymatic activity of Pdr5p in contrast to the isolated intake of the larger granular alveoli, which showed no biological effect.

Once the parotoid gland secretion represents a mixture of secretions produced by different kinds of cutaneous glands [17,18], the biochemical results indicated that the entire secretion, elaborated for these three glandular types, is crucial to biologically act on the Pdr5p ATPase activity. Pukala and coworkers [15] related that peptides are stored as a propeptide in inactive form inside the gland alveolus, being released from the gland on the integument surface as active peptides when anurans are submitted to some type of stimulus or stress. Since the isolated granular intake of the larger granular alveoli of both bufonids showed no effect, these data allow us to conclude that the protein intake is stored in an inactive form, being active when all secretions are discharged and mixed with each other onto the integument of the parotoid gland region. Thus, the inhibition of the Pdr5p ATPase activity occurs only when all compounds are associated, constituting the crude extract.

Pdr5p is an important model protein to study MDR function and search for inhibitors [27]; however, its ATPase activity has not been extensively characterized [28]. Rangel and coworkers [29] investigated different plant extracts and their fractions on the activity of Pdr5p. Crude extracts of *Bathysa australis*, *Mabea fistulifera* and *Virola oelifera* produced reasonable inhibition in the concentration of 200 μg/mL. The analysis of these fractions revealed flavonoids, considered as inhibitors and/or modulators of MDR activity. Some compounds, as FK506 (immunosuppressant), flavonoids, protein kinase C effectors, and phenotiazines, have been reported to act as inhibitors against Pdr5p and MDR proteins [6]. Ferreira and colleagues [30], testing ethanol extracts of sea sponges (*Petromica citrina* and *Agelas sp.*) in concentrations of 200 μg/mL on the Pdr5p enzymatic activity, observed a reduction of approximately 50% of the ATPase activity. Some Pdr5p inhibitors, as the enniatin obtained from fungi strain *Fusarium* sp. Y-53 and the isonitrile purified from *Trichoderma* sp. P24-3, were isolated from microorganisms. In Pdr5p over-expressing cells, the enniatin and the isontrile at non-toxic concentration exhibited inhibitory profiles on the Pdr5p-mediated efflux of cycloheximide or cerulenin [6]. For Yamamoto and coworkers [27] the carboxyl residue of the isonitrile appears to be essential for the inhibitory activity against Pdr5p.

Cutaneous secretions of bufonids contain much more organic molecules (steroids, alkaloids and biogenic amines) but fewer peptides or proteins [8]. In this study, SDS-PAGE revealed proteins with different molecular masses constituting the parotoid gland secretions of both *Rhinella* species. Some proteins were common in both bufonids, suggesting a protein pattern characteristic for *Rhinella* genus. However, differences on protein composition

depend on the bufonid as well as on the secretion type. These variations indicated that some proteins are species specific and their molecular mass varied according to the isolated granular intake in the same species. For Perry [31], the parotoid gland secretions from *Amietophrynus mauritanicus* (former *Bufo mauritanicus*) and *Epidalea calamita* (former *Bufo calamita*) are a mixture of proteins with the relative molecular mass range of approximately 12-200 kDa, and these proteins markedly exhibited different banding patterns according to the species.

Recent investigations on cutaneous molecules of *Bufo gargarizans* (former *Bufo andrewsi*) have shown some bioactive proteins, as protease inhibitor and lysozyme [8]. Moreover, Perry [31] suggested that the analysis of the protein profile could be used to taxonomic and evolutionary studies, helping species identification, particularly where anatomical criteria are ambiguous.

Immunohistochemical results showed the Pgp expression in the glandular portion cells of the mixed and small granular glands, in the myoepithelial cells, as well as in some cells in the spongious dermis. The Pgp immunolabeling in the bufonid integument suggests that this protein may be involved with the extrusion of cytotoxic compounds. Since the anuran integument is highly permeable, environmental xenobiotic compounds could penetrate into the integument, interfering with the quality of the glandular secretion. Thus, the Pgp expression may be related to bufonid protection strategy, which is important for the maintenance of its chemical defense.

Greenberg [32] commented that it is not clear how or why these pumps of efflux associated with MDR are related. However, he suggested that these pumps appeared to naturally eliminate environmental toxins.

Although Pgp has been identified in mouse, rat, bovine, dog, monkey, rodent and human [332], this work is the first relate in anuran integument. Pgp and other multidrug resistance associated proteins (MRP) are known to extrude xenobiotics from tissues and cells, such as liver, kidney, gut, trachea and tumor cells. In addition, in tadpoles of *Xenopus laevis*, Pgp was identified in the basolateral compartment of neurons of the olfactory epithelium. This neuronal class is directly exposed to environment and due to this exposure some odorants and other xenobiotic compounds constitute a potential cytotoxic element to these cells. Thus, the removal of xenobiotics from cells of the olfactory epithelium by Pgp is crucial [34]. According to Linardi and Natalini [33], Pgp plays an important role as a protective barrier against a wide variety of substrates, in absorption, disposition, metabolism and excretion of a number of drugs, having an enormous clinical significance. Thus, changes in the Pgp expression or function have been associated with several diseases in humans and animals.

The positive Pgp immunostaining in the glandular intake reflects their apocrine secretion mode, which has been described for other anurans [31,32]. Probably, this positive reaction is due to cytoplasmic endomembranes, which is discharged together with the secretion. For Baldini and coworkers [35], Pgp expression occurs not only in the plasma membrane, but also inside the cytoplasm and the nucleus.

5. CONCLUSION

This work revealed a new bioactivity to the cutaneous anuran secretions, since crude extract of the parotoid glands acted in the Pdr5p activity. This novel biological effect of the parotoid gland secretion represents a good source of new modulators for MDR transporters, and may

be used as a template for the design of new drugs. Thus, Pdr5p is important to contribute to the maintenance of both integrity and functionality of bufonid cutaneous secretion. Nevertheless, further investigations are essential to elucidate the mechanism of action of the crude extract of *R. icterica* and *R. ornata* on the Pdr5p activity, as well as to characterize the specific components of the parotoid gland secretion that block the Pdr5p-specific ATPase activity.

ACKNOWLEDGEMENTS

We wish to thank the Carlos Chagas Filho de Amparo à Pesquisa do Rio de Janeiro Foundation (FAPERJ) for the financial support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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