Identification and Characterization of New Protein Chemoattractants in the Frog Skin Secretome*

Baptiste Leroy‡§¶, Gerard Toubeau§, Paul Falmagne‡, and Ruddy Wattiez‡||

The vomeronasal organ is a chemosensory organ present in most vertebrates and involved in chemical communication. In the last decade, the deciphering of the signal transduction process of this organ has progressed. However, less is known about the vomeronasal organ ligands and their structure-function relationships. Snakes possess a highly developed vomeronasal system that is used in various behaviors such as mating, predator detection, or prey selection, making this group a suitable model for study of the vomeronasal chemoreception. In this work, we used a proteomics approach to identify and characterize proteins from frog cutaneous mucus proteome involved in prey recognition by snakes of the genus Thamnophis. Herein we report the purification and characterization of two proteins isolated from the frog skin secretome that elicit the vomeronasal organ-mediated predatory behavior of Thamnophis marcianus. These proteins are members of the parvalbumin family, which are calcium-binding proteins generally associated to muscular and nervous tissues. This is the first report that demonstrates parvalbumins are not strictly restricted to intracellular compartments and can also be isolated from exocrine secretions. Purified parvalbumins from frog muscle and mucus revealed identical chemoattractive properties for T. marcianus. Snake bioassay revealed the Ca$^{2+}$/Mg$^{2+}$ dependence of the bioactivity of parvalbumins. So parvalbumins appear to be new candidate ligands of the vomeronasal organ. Molecular & Cellular Proteomics 5:2114–2123, 2006.

Among vertebrates, chemical communication is involved in several species-specific complex behaviors including prey selection, territoriality, sexual behavior, or detection of predators (1). Functional studies have demonstrated that the vomeronasal organ (VNO) plays a key role in the detection of biologically active chemical cues (2–4). Significant progress has been made during the past 10 years in the study of vomeronasal receptors (5) and in the understanding of the molecular mechanisms involved in chemosensory transduction (6). In contrast, the available information on the chemical nature of the molecules recognized by the VNO remains particularly limited (7). These vomeronasal ligands appear to belong to a wide spectrum of compounds ranging from small volatile molecules to peptides or proteins. In rodent, the most investigated model, six small hydrophobic volatile molecules are known to induce vomeronasal organ-mediated behaviors (8–10), such as estrus synchronization or induction of puberty. Recent findings indicate that proteins/peptides could also play the role of chemosignals when coupled to VNO receptors. Major histocompatibility complex class I peptides are able to activate vomeronasal sensory neurons and are involved in the pregnancy block effect observed in mice (11). Moreover major urinary proteins, which are members of the lipocalin family, acting as pheromone carriers in mouse urine also seem to be endowed with pheromonal properties (12). Similarly another lipocalin called aphrodisin, isolated from female hamster vaginal secretions, could be involved in vomeronasal organ-mediated mating behavior (13–15).

Squamates, and principally snakes, possess a well developed VNO making this group a highly used model for the study of this particular chemosensory organ. Among snakes, many behaviors are mediated by the vomeronasal organ in the presence of specific chemical cues. It is now completely accepted that prey detection by snakes of the genus Thamnophis depends on the presence of a functional VNO (16, 17). Using Thamnophis as a model of predatory behavior, Halpern and co-workers (18) have already described the first protein ligand of the VNO. This protein, isolated from earthworm mucus and called ES20, binds to specific snake VNO receptors and possesses an intrinsic biological activity. Nevertheless ES20 has been harvested under mucus electrical stimulation and could not correspond to a physiological constituent of the earthworm mucus.

Crude protein extracts of mucus from frog skin were also able to elicit the predatory behavior of the Thamnophis snakes (19), indicating that this particular proteome contains ligands of the VNO. Although frog mucus has been widely investigated in search for antimicrobial activity, less is known about its protein content. In this context, we have undertaken an extensive study of the frog cutaneous mucus proteome in

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1 The abbreviations used are: VNO, vomeronasal organ; 2-DE, two-dimensional gel electrophoresis; aa, amino acids; DEAE, diethylaminoethyl; IB, immunoblot buffer.
search for protein chemoattractants. Here we report the first analysis of the frog mucus proteome leading to the identification, purification, and characterization of two new protein candidate ligands for the VNO. These proteins both belong to the parvalbumin family. Moreover, functional studies of these proteins revealed for the first time their presence in a physiological extracellular fluid as well as a \( \text{Ca}^{2+}/\text{Mg}^{2+} \) dependence of their chemotactic properties.

**EXPERIMENTAL PROCEDURES**

**Animals**—This study was approved by the ethics review committee. Maintenance and care of animals were in compliance with guidelines set by the institutional animal care committee. *Rana temporaria* subjects used for mucus collection were adult frogs collected under a Belgium state collecting permit in the Beloeil area (Hainaut, Belgium). *R. temporaria* experimental subjects used for muscular parvalbumin purification were purchased from Arbiotech society (Saint-Gilles, France). 13 adult or subadult *Thamnophis marcianus* snakes were used in this study and were obtained from the personal collection of Prof. G. Toubeau or from the Natural History Museum of Tournai (Belgium).

**Preparation of Mucus Extract from Frog Skin**—Mucus extracts were produced according to the procedure described by Wattiez et al. (19) with minor modifications. Briefly, frogs were immersed in distilled water or TBS (50 mM Tris-HCl, pH 7.2, 150 mM NaCl) (1 ml/g body weight) for 1 h at room temperature. The extracts contaminated by feces were discarded. DTT (50 mM) and PMSF (2 mM) were then added to the solution. After filtration through folded filters (75 µm; Schleicher & Schuell MicroScience), the wash solution was concentrated by lyophilization and dissolved in 50 mM DTT. The extract was then dialyzed three times against 50 mM DTT for 2 h at 4 °C and centrifuged for 20 min at 20,000 × g. The supernatant was collected, and protein concentration was measured using the Bio-Rad protein assay kit; 100-µl aliquots were stored at −80 °C.

**Snake Bioassay**—The chemoattractant activity of proteins was measured by a snake bioassay already validated by different laboratories (18–20). In our snake bioassay, an all-or-none procedure inspired by Wattiez et al. (19) was adopted. In this test, a lure (cooked macaroni, 5-mm diameter, 15-mm length) was set out in the test area, and a response was considered positive only if the lure was attacked by the snake within the 20 s following the first lure-directed tongue flick. To accelerate the testing procedure and avoid the denaturation of the test sample, a fish odor-charged air stream was blown into the cage. This device rapidly attracts snakes in the testing area and put them in a food searching context. Control tests were systematically realized using frog mucus crude extract as positive control and the sample buffer as negative control. Order of sample presentation was chosen randomly.

**Two-dimensional Polyacrylamide Gel Electrophoresis**—After lyophilization, the mucus crude extract (about 0.1 g) was suspended in 500 µl of denaturing buffer (7 M urea, 2 M thiourea, 4% (v/v) CHAPS, and 50 mM DTT) containing a complete set of protease inhibitors (Roche Applied Science) and centrifuged for 20 min at 20,000 × g to discard the insoluble components. The supernatant was collected, and protein concentration was measured using the Bradford method (21). IEF was performed using IEF strips (18 cm; Amersham Biosciences). The first dimension isoelectric focusing was carried out as described previously (22). The second dimension vertical slab SDS-PAGE (12%) was run for 4 h at 30 mA/gel using the Protean II Xi apparatus (Bio-Rad). The gels were stained with Coomassie Brilliant Blue R-250 (Amresco, Solon, OH).

**Isolation of Protein Chemoattractants from the Mucus Crude Extract**—Protein chemoattractants of the mucus crude extract were isolated by semipreparative SDS-PAGE as described in Jiang et al. (18). 15 µg of mucus crude extract proteins solubilized in Laemmli sample buffer were loaded onto an SDS-polyacrylamide gel slab gel (T: 14%; C: 3.3%; 7 × 8.5 cm). Electrophoresis was conducted at 20 mA/gel for 1 h. After running, the gel was vertically cut into two parts, one of which was stained with Coomassie Brilliant Blue R-250 and used as a guide strip for locating corresponding protein bands on the unstained part of the gel. Gel pieces containing the target proteins were excised from this unstained gel. Proteins were then recovered by soaking the pulverized gel pieces in 50 mM DTT for 24 h under strong agitation at 4 °C. The supernatants were collected, concentrated by lyophilization, and suspended in ultrapure water. The samples were then submitted to the snake bioassay or stored at −20 °C for further analysis.

**High Performance Liquid Chromatography of Bioactive Proteins**—The bioactive proteins isolated by semipreparative SDS-PAGE described above were submitted to HPLC on a C18 TSK-Gel column (30 cm × 7.8-mm inner diameter; TosohHaas) equilibrated with 0.1% (v/v) TFA. A flow rate of 0.4 ml/min was obtained with an L-6200 pump (Merck-Hitachi), and a two-step acetonitrile gradient in 0.1% (v/v) TFA (5–60% CH3CN in 5 min, 60–100% CH3CN in 30 min) was used for elution. An FC250 fraction collector (Gilson) was programmed to collect 0.4-ml samples. After solvent evaporation in a SpeedVac, fraction contents were analyzed by SDS-PAGE.

**Purification of Parvalbumins from Frog Muscle**—To confirm the intrinsic role of parvalbumin in the predatory behavior of the snake, parvalbumins from frog muscles were purified by semipreparative SDS-PAGE or according to the procedure described by Gosselin-rey and Gerday (23).

25 g of frog leg muscles were dissected, avoiding skin contamination. Muscle tissue was then homogenized in 100 ml of TBS containing a protease inhibitor mixture (Roche Applied Science) and incubated for 1 h at 4 °C under agitation. Insoluble material was washed by centrifugation at 20,000 × g for 30 min at 4 °C.

Supernatant was then submitted to semipreparative SDS-PAGE as described above or to the parvalbumin purification procedure in the latter case. Ammonium sulfate was added to the supernatant to produce 60% saturation and incubated overnight at 4 °C under slight agitation. The pellet was then precipitated by centrifugation, and proteins of the supernatant were then precipitated with ammonium sulfate at 100% saturation. The precipitate was dissolved and equilibrated in 50 mM NH4HCO3, 50 mM DTT and subsequently chromatographed on a G-75 Sephadex column (1.5 × 40 cm) equilibrated in 50 mM NH4HCO3, 50 mM DTT. The parvalbumin-containing fractions, identified by SDS-PAGE, were exhaustively dialyzed against a 15 mM piperazine HCl, pH 5.7, 50 mM DTT buffer and submitted to DEAE-cellulose chromatography (HiPrep DEAE FF, 1.6 × 10 cm; Amersham Biosciences) in a linear 0–100 mM NaCl gradient. The fraction contents were analyzed by SDS-PAGE. Parvalbumin-containing fractions were pooled and lyophilized. Samples were then submitted to the biological test or stored at −80 °C for further analysis.

**Edman Degradation**—Mucus extract proteins separated by SDS-PAGE (12%) or 2-DE were then electroblotted onto PVDF membranes (Sequí-Blot PVDF membrane, Bio-Rad) using a semidry blotting device (Trans-Blot SD dry transfer cell, Bio-Rad) for 40 min at 24 V and 500 mA. After the transfer, the membranes were washed three times with ultrapure water and then stained with 0.1% (v/v) Cooamassie Brilliant Blue R-250 in 50% (v/v) methanol for 5 min. The membrane was destained in 40% (v/v) methanol, 10% (v/v) acetic acid, air-dried, and stored at −20 °C. Selected protein bands or spots on the Cooamassie-stained PVDF membranes were excised, and the N-terminal amino acid sequences were determined by automated Edman degradation using a Beckman LF3400D protein-peptide microsequencer (22). Searches for protein
identity/similarity from sequence data were performed using the BLASTP algorithm on the Swiss-Prot or TrEMBL databases. The research was carried out in all species.

In-gel Protein Digestion and MALDI-TOF-MS—The Coomassie-stained protein spots of interest were cut out and in-gel digested as described previously (24). Peptides from digested proteins were solubilized in 2 μl of 0.5% TFA. Peptide mass fingerprints were obtained with a MaldiTOF mass spectrometer (Micromass, Manchester, UK) working in reflectron mode with 15 kV of source voltage, 2.5 kV of pulse voltage, and 2 kV of reflecting voltage. A saturated solution of α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 0.1% TFA was used for the matrix. A total of 1 μl of the matrix and sample solution were mixed in a 1:1 (v/v) ratio and applied onto the 96 target wells. Mass accuracy for peptide mass fingerprint analysis was 0.1 Da with external calibration, and internal calibration was carried out using enzyme autolysis peaks; resolution was 11,000. The resulting peptide masses were automatically searched for in a local copy of the Swiss-Prot and TrEMBL databases using the ProteinLynx global server and the Protein Probe (Micromass Ltd., Manchester, UK) and Mascot (Matrix Science) search engines. The research was carried out in all species. One missed cleavage per peptide was allowed, a mass tolerance of 50 ppm was used, and some variable modifications were taken into account such as carbamidomethylation of cysteines and oxidation of methionines. Protein identification results were manually evaluated. Only identification results with a confidence level above 95% were confirmed as positive hits.

Nanospray ESI-MS/MS—Peptides after in-gel protein digestion were recovered by sequential extractions with 25 mM NH₄HCO₃, 5% (v/v) formic acid, and 50% (v/v) CH₃CN. After lyophilization using a SpeedVac, extracts were reconstituted in 10 μl of 0.1% TFA and then desalted using ZipTip C₁₈ pipette tips (Millipore, Bedford, MA). ESI-MS/MS was carried out with a hybrid quadrupole orthogonal acceleration tandem mass spectrometer (Q-TOF2, Micromass Ltd.). The capillary voltage in MS and MS/MS experiments was set to an average of 900 V, and the sample cone voltage was 50 V. The collision gas was argon with a pressure of 0.1 megapascal by 3.5 10⁻⁷ mbar, and the sample cone voltage was 50 V. The collision energy was 25–50 eV. Glu-fibrinopeptide was used to calibrate the instrument in the MS/MS mode. MS/MS spectra were transformed using MaxEnt3 (MassLynx, Micromass Ltd.), and amino acid sequences were interpreted manually using PepSeq (BioLynx, Micromass Ltd.). Searches for protein identity from sequence data were performed using the BLASTP algorithm using the Swiss-Prot or TrEMBL databases. The research was carried out in all species. One missed cleavage per peptide was allowed, a mass tolerance of 50 ppm was used, and some variable modifications were taken into account such as carbamidomethylation of cysteines and oxidation of methionines. Protein identification results were manually evaluated. Only identification results with a confidence level above 95% were confirmed as positive hits.

In search for protein chemoattractants, all proteins from skin mucus were detected by a chemiluminescence detection kit (Lumiprobe). Western Blot Analysis—Proteins of the mucus crude extract (10 μg) were separated by SDS-PAGE and blotted at 4 °C on a nitrocellulose membrane (Hybond ECL; Amersham Biosciences) using a semidry blotting device (Trans-Blot SD dry transfer cell, Bio-Rad) for 40 min at 500 mA and 25 V. The membrane was then washed three times for 5 min in 50 mM PBS, 0.5% (v/v) Tween 20 (immunoblot buffer (IB)). IB was used for the matrix. A total of 1 μl of the matrix and sample solution were mixed in a 1:1 (v/v) ratio and applied onto the 96 target wells. Mass accuracy for peptide mass fingerprint analysis was 0.1 Da with external calibration, and internal calibration was carried out using enzyme autolysis peaks; resolution was 11,000. The resulting peptide masses were automatically searched for in a local copy of the Swiss-Prot and TrEMBL databases using the ProteinLynx global server and the Protein Probe (Micromass Ltd., Manchester, UK) and Mascot (Matrix Science) search engines. The research was carried out in all species. One missed cleavage per peptide was allowed, a mass tolerance of 50 ppm was used, and some variable modifications were taken into account such as carbamidomethylation of cysteines and oxidation of methionines. Protein identification results were manually evaluated. Only identification results with a confidence level above 95% were confirmed as positive hits.

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In search for protein chemoattractants, all proteins from skin mucus proteome of R. temporaria were screened. In this context, mucus proteins were separated by SDS-PAGE, eluted from the gel, and then submitted to the snake bioassay as described under “Experimental Procedures.” The response was considered positive only if the snake readily attacked the lure and tried to swallow it (Fig. 2). However, it is important to note that, in every positive test, the attack of the lure was always preceded by rapid tongue flicking of the sample suggesting the involvement of the VNO (16).

Under these experimental conditions, only four chemoattractive protein bands, Ph11, -14, -24, and -30, characterized by an apparent molecular mass of 11, 14, 24, and 30 kDa, respectively, were detected among all the mucus proteins revealed by SDS-PAGE (Fig. 1B). In contrast with the eluted proteins Ph24 and -30 (±50% positive tests), the protein bands Ph11 and -14 triggered snake attack in all
tests conducted (Table I). The bioactivity of these proteins was completely abolished after treatment with proteinase K. As negative controls, trypsin inhibitor and carbonic anhydrase were isolated following a similar procedure and tested.

Reverse phase HPLC analysis of the Ph14 band, isolated by semipreparative SDS-PAGE, revealed the presence, after chromatography, of two new protein bands at 24 and 30 kDa corresponding to different oligomeric states of Ph14 (Fig. 3A). These polymers were also observed in the crude protein fraction of the skin mucus by Western blot analysis using a specific anti-Ph14 antibody (Fig. 3B). These observations suggest that the chemoattractive activity sometimes associated with Ph24 and Ph30 bands could be due to the presence of Ph14 oligomers. We inferred that the variability observed in...

**Table I**

Snake bioassay of proteins purified from frog mucus and muscle

| Samples                  | Positive tests | Positive tests after proteolysis |
|--------------------------|----------------|----------------------------------|
| Ph30                     | 7/17           | 0/5                              |
| Ph24                     | 8/17           | 0/5                              |
| Ph14                     | 14/14          | 0/5                              |
| Ph11                     | 12/12          | 0/4                              |
| Muscular parvalbumin α<sup>a</sup> | 5/5         | 0/3                              |
| Muscular parvalbumin β<sup>a</sup> | 5/5         | 0/3                              |
| Trypsin inhibitor        | 0/7            | ND<sup>b</sup>                   |
| Carbonic anhydrase       | 0/7            | ND<sup>b</sup>                   |
| Buffer                   | 0/23           | ND<sup>b</sup>                   |

<sup>a</sup> Muscular parvalbumins were purified by semipreparative SDS-PAGE.

<sup>b</sup> ND, not determined.

**Fig. 1.** Isolation of chemoattractive proteins from the frog mucus proteome. A, 2-DE gel of the frog mucus crude extract (100 μg). B, proteins Ph11, -14, -24, and -30 were isolated by semipreparative SDS-PAGE (12%) from mucus crude extract as described under “Experimental Procedures” and analyzed by SDS-PAGE (12%). CE, mucus crude extract.

**Fig. 2.** Snake bioassay. A response was considered positive when the lure (cooked macaroni coated with 10 μl of protein sample) was attacked by the snake within the 20 s following the first lure-directed tongue flick.

**Fig. 3.** Polymerization of Ph14 protein in denaturing conditions. A, SDS-PAGE (12%) of the different fractions obtained after reverse phase HPLC of the Ph14 protein isolated by semipreparative SDS-PAGE. B, Western blot of the frog mucus crude extract using an anti-Ph14 polyclonal rabbit antibody (1:2000). This antibody was obtained from the Ph14 SDS-PAGE band. CE, crude extract. The quality (background and contrast) of the image blots was improved using Corel PhotoPaint<sup>™</sup> 8.0.
the biological tests with these samples could be due to a variable degree of polymerization as demonstrated by immunoblot analysis (data not shown).

**Identification of Phagostimulating Proteins**—To identify the phagostimulating protein localized in the Ph14 band, microsequence analysis was performed using automated Edman degradation and mass spectrometry. Interestingly only one N-terminal sequence (23 aa) was observed after Edman degradation of the eluted bioactive protein(s) (Fig. 4, upper panel). In parallel, the sequences of four tryptic peptides (41 aa) were obtained by ESI-MS/MS. Protein database searches revealed that these sequences belong to the \( \alpha \) parvalbumin family as they share 92% identity with the muscle \( \alpha \) parvalbumin of *Rana esculenta*. This identification was confirmed by immunoblot analysis using a commercial anti-rat parvalbumin antibody (Fig. 5). Automated Edman degradation of the Ph11 band of the mucus extract after CNBr treatment (12 aa) and tandem mass spectrometry of the tryptic peptides (33 aa) also revealed a unique protein sequence but this time with a high similarity (88%) to the \( \beta \) parvalbumin family (Fig. 4, lower panel). As for Ph14, this identification was confirmed by immunoblot analysis using a commercial anti-rat parvalbumin antibody (Fig. 5). The identified \( \alpha \) and \( \beta \) parvalbumins could therefore participate in the recorded bioactivity of the eluted material from the Ph14 and Ph11 SDS-PAGE bands, respectively, and have chemoattractive properties for *T. Marcianus*.

**Purification and Bioactivity Analysis of Parvalbumins from Frog Muscle**—To demonstrate the validity of our proteomics studies, we decided to confirm the chemoattracting properties of parvalbumins using their intracellular homologues. In this context, the \( \alpha \) and \( \beta \) parvalbumins from muscular tissue of *R. temporaria* were purified by semipreparative SDS-PAGE or chromatography method as described under “Experimental Procedures.” Their purity was both confirmed by SDS-PAGE and MALDI-TOF analyses (Fig. 6). The molecular mass of the muscle \( \alpha \) and \( \beta \) parvalbumins determined by MALDI-TOF was 12,122 ± 10.4 Da and 11,796 ± 0.2 Da, respectively.

Tryptic fingerprint comparison and ESI-MS/MS showed a great similarity between the muscular and mucus parvalbumins. Fingerprint comparison of muscle and mucus \( \alpha \) parval-
bumins revealed only a shift of 26 Da for two peptides of 1486.1 and 1460.1 m/z and 1614.2 and 1589.2 m/z, respectively (Fig. 7A). MS/MS analysis showed that these two peptides are identical and result from the cleavage of trypsin at two successive lysines. The 26-Da shift between muscular and mucous samples corresponds to a single amino acid substitution in position 26 of mucus parvalbumin where a Tyr residue of muscle parvalbumin is substituted for a His residue (Fig. 7B). No other difference was observed among sequences obtained by ESI-MS/MS or by Edman degradation.

On the other hand, fingerprints of the tryptic peptides obtained from mucus and muscle parvalbumins show no differences (data not shown). As observed with the mucus protein, the N terminus of muscle β parvalbumin was also blocked.

Finally the chemoattractive properties of the muscular parvalbumins purified by semipreparative SDS-PAGE were clearly demonstrated by the snake bioassay because they triggered the snake attack in all the tests (Table I). However, when the muscular parvalbumins were purified by chromatography, the snake bioassay showed a complete loss of bioactivity (Table II). Interestingly the chemoattractive activity of these purified muscular parvalbumins was recovered in the presence of calcium.

**Ion Binding and Chemoattractive Properties of Parvalbumins**—Muscular parvalbumins purified by chromatography require the presence of Ca^{2+}/Mg^{2+} to express their bioactivity. This was confirmed by the demonstration that the bioactivity of muscular parvalbumins was restored by dialysis against Tris-HCl buffer, pH 7.2, containing 1 mM Ca^{2+} (Table II). It was equally recovered in the presence of magnesium ions. In contrast, the bioactivity was abolished after dialysis against chelating agents such as EGTA (Table II). In this context, the predatory behavior of garter snakes was never triggered by calcium or magnesium ions alone, pointing to the pivotal role of parvalbumins for prey detection.

In this study, semipreparative SDS-PAGE was extensively used for isolation of fully chemoattractive parvalbumins. Therefore, the capacity of muscular α parvalbumin to bind calcium in SDS-PAGE conditions was analyzed. As shown in Fig. 8, the presence of CaCl₂ in electrophoresis gel and buffer resulted in the presence of three bands corresponding to calcium-free parvalbumin or to parvalbumin interacting with one or two calcium ions. In contrast, parvalbumin β appeared as a single band when gels contained EGTA. These observations suggest that the calcium-parvalbumin interaction persisted in SDS-PAGE conditions and explain why parvalbumins maintained their bioactivity even in such denaturing conditions.

**Fig. 6.** Purification of frog muscle parvalbumins. A. α and β parvalbumins purified by semipreparative SDS-PAGE (S) or chromatography methods (C) were analyzed by 15% SDS-PAGE. B. MALDI-TOF analysis of α and β parvalbumins purified from muscle.
Immunohistochemistry of Parvalbumin—Because parvalbumins are usually intracellular proteins, a rabbit polyclonal anti-Ph14 antibody was used to localize, by immunohistochemistry, the cellular source of parvalbumin in the skin of *R. temporaria*. Immunochemistry examination revealed positive cells located in the basal region of the glandular epithelium of the dermal mucous glands (Fig. 9). No labeling was observed without primary antibody or in the presence of preimmune serum. The proteins belonging to the parvalbumin family are known to be intracellular, and the present observation is the first evidence that parvalbumins could also be detected in extracellular fluids such as exocrine secretions. This result confirmed the natural presence of parvalbumin in the frog mucus proteome.

**DISCUSSION**

The identification of vomeronasal ligands is essential for the understanding of the molecular mechanisms involved in chemosensory transduction in general. Snakes possess a highly developed vomeronasal system that is used in various behaviors such as mating, predator detection, or prey selection, making this group a suitable model for study of the vomeronasal chemoreception. It is now well established that *Thamnophis* predatory behavior involves detection by VNO of chemical cues on the prey surface (16, 26, 27). For instance, a protein chemoattractant (ES20), mainly purified from earthworm mucus after electric stimulation, has been reported previously to bind to specific snake VNO receptors (18). Using a proteomics approach coupled to a bioassay, we attempted to find proteins from the cutaneous mucus of the frog *R. temporaria* able to trigger the predatory behavior of the snake *T. marcianus*.

During this study, crude mucus preparation was obtained by single immersion of the frogs in water or isotonic solution rather than generated by electric induction. Moreover the protein composition of mucus preparations studied by 2-DE was similar in water or isotonic solution extractions. These data suggest that the protein content of our mucus preparations represents the physiological protein composition of this fluid rather than gland content and/or proteins leaking from cellular lyses. Nevertheless we are confident that this mucus preparation only contains the proteins of the cutaneous mucus soluble in water or isotonic solution.

2-DE analysis revealed the complexity of the mucus proteome of the frog *R. temporaria* in which parvalbumins are the major proteins. No protein eluted from 2-DE maps showed any chemoattractive activity probably due to the excessive denaturation conditions of the 2-DE. This problem was circumvented by using semipreparative SDS-PAGE. A limitation of our purification protocol is the ability of the denaturation conditions to disturb the bioactivity of proteins. Unfortunately no separation was obtained in different non-denaturing conditions due to an excessive aggregation of the mucus proteins. Therefore, after elution, a renaturation step by extensive dialysis was used in all our experimental approaches.

Among all the protein bands isolated by semipreparative
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SDS-PAGE, four (Ph11, Ph14, Ph24, and Ph30) were able to trigger the snake predatory behavior. Treatment with a non-specific protease revealed the involvement of proteins in the chemoattractive activity of the eluted components from these four SDS-PAGE bands. Among these chemoattractive protein bands, only Ph11 and Ph14 displayed positive results in all the conducted snake bioassays and were selected for further analysis. As demonstrated by microsequence analysis (Edman degradation and ESI-MS/MS) and immunodetection studies, Ph11 and Ph14 bands only contain major protein corresponding to the β and α parvalbumins, respectively; they are the major acidic proteins observed in the mucus 2-DE map. No other proteins were detected by the mass spectrometry approach in the eluted bioactive proteins. Nevertheless, mucus crude extract 2-DE analysis revealed co-migration of some low abundance mucus proteins with parvalbumin. Evidence for the participation of parvalbumins in the chemoattractive activity of Ph11 and Ph14 bands was then gained by carrying out the purification of α and β parvalbumins from frog muscle. A clear phagostimulating activity was associated with muscular α and β parvalbumins. These findings confirm unequivocally the involvement of parvalbumins in the predatory behavior of T. marci anus.

By chromatography and Western blot analyses, α parvalbumin was shown to polymerize in a denaturing condition such as SDS-PAGE. It has been published previously that muscular parvalbumins are prone to form dimers and trimers (28). These data suggest that the biological activity sometimes associated with Ph24 and Ph30 could be due to the presence of polymers of α parvalbumin.

Parvalbumins are calcium-binding proteins present in muscular and nervous tissues (29). Our data suggest clearly the presence of high concentrations of parvalbumins in frog exocrine secretions, a surprising finding because parvalbumins have only been detected in intracellular localizations (29). In this context, the cellular source of the mucus α parvalbumin was identified by immunohistochemistry: this protein is secreted by the glandular epithelium of the dural mucous glands of the amphibian skin. α parvalbumin thus constitutes a physiological component of the frog skin secretome. To our knowledge, the presence of this protein in an extracellular fluid such as exocrine secretion from skin glands has not yet been reported, and the mechanism of its secretion as well as its functions is unknown. This protein could be involved in the transcutaneous calcium uptake known to occur in amphibians (30–33). In fish cutaneous mucus, a similar function was assigned to calmodulin, another calcium-binding protein (34). Alternatively parvalbumins could also be involved in the control of the calcium-dependent physical properties (35) of the mucus coating and play a role in the adaptation to variations in the environmental conditions.

The main characteristic of parvalbumins is their ability to bind calcium and magnesium ions. In this work, we demonstrate that the Ca$^{2+}$/Mg$^{2+}$ interaction with parvalbumins is necessary for the expression of the biological activity. No phagostimulating activity was detected with the apoparvalbumins or ions without parvalbumin. Parvalbumin purification by ion exchange chromatography or 2-DE caused the loss of the chemoattractive properties. This is not surprising because in these experimental conditions, especially in the presence of salts or ampholytes, the affinity of parvalbumin for calcium and magnesium is strongly reduced (36). In contrast, it is well known that SDS does not disturb the Ca$^{2+}$/Mg$^{2+}$ interaction with parvalbumin (37). The precise function of calcium ions remains unknown, but it is likely that the changes of parvalbumin conformation induced by calcium binding may be involved in the parvalbumin recognition by the receptors of VNO (38, 39).

In our frog-snake model, all positive tests in the bioassay of parvalbumin-containing samples were always preceded by tongue licking on the lure coated with these samples. It is well established that this tongue licking behavior necessarily implies the involvement of a functioning vomeronasal organ in the predatory behavior of snakes (16, 40).

To date, parvalbumins are the first effective chemoattractive compounds for snakes of the genus Thamnophis identified in the mucus proteome of frog. Another chemoattractive protein, ES20, has been identified in the mucus of earthworms (18). Interestingly the chemoattractive property of ES20 also depends on the presence of calcium or magnesium. However the ability of ES20 to interact with these ions has not been demonstrated. Nevertheless bioinformatics structural analysis of ES20 (data not shown) showed the presence of a potential helix-loop-helix domain similar to the calcium binding domain of the parvalbumins. Extracellular calcium-binding proteins could represent a protein family endowed with chemoattractive activity for the genus Thamnophis.

In conclusion, our proteomics approach allowed us to identify the first proteins, parvalbumins, from mucus of frog that play a central role in the vomeronasal organ-mediated predatory behavior of the garter snake T. marci anus. The precise role of calcium in the observed chemoattractive activity must now be investigated. The proteomics approach seems to be an effective tool for detecting protein chemoattractants in skin mucus from different frog species.

Acknowledgment—We thank Catherine S’Heeren for technical assistance.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by Fonds National pour la Recherche dans l’Industrie et l’Agriculture.

‡ A research associate of the National Funds for Scientific Research. To whom correspondence should be addressed. Tel.: 32-65373312; Fax: 32-65373320; E-mail: ruddy.wattiez@umhb.ac.be.
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