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# Frog secretions and hunting magic in the upper Amazon: Identification of a peptide that interacts with an adenosine receptor

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**ABSTRACT** **A frog used for "hunting magic" by several groups of Panoan-speaking Indians in the borderline between Brazil and Peru is identified as *Phyllomedusa bicolor*. This frog's skin secretion, which the Indians introduce into the body through fresh burns, is rich in peptides. These include vasoactive peptides, opioid peptides, and a peptide that we have named adenoregulin, with the sequence GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV as determined from mass spectrometry and Edman degradation. The natural peptide may contain a D amino acid residue, since it is not identical in chromatographic properties to the synthetic peptide. Adenoregulin enhances binding of agonists to $A_1$ adenosine receptors; it is accompanied in the skin secretion by peptides that inhibit binding. The vasoactive peptide sauvagine, the opioid peptides, and adenoregulin and related peptides affect behavior in mice and presumably contribute to the behavioral sequelae observed in humans.**

Carneiro (1, 2) called attention to the use of an unidentified Peruvian frog in the hunting magic of Indians in western Amazonia. This extraordinary custom is now known to be practiced by Brazilian Mayoruna (ref. 2, this paper, and K.M., unpublished data) and Marúbo (14) and by Peruvian Amahuaca (1) and Matses (ref. 3 and P. Gorman, personal communication) Indians—all culturally related speakers of the Panoan language family in the borderland between Brazil and Peru.

Skin secretion, previously scraped from a live frog (Fig. 1 *Upper*) and stored dry on a stick, is mixed with saliva and introduced into a line of fresh burns on the arms or chest (Fig. 1 *Lower*). This induces within minutes violent illness, including rapid pulse, incontinence, and vomiting, after which the recipient lapses into a state of listlessness and, finally, into a state perhaps to be described as euphoric; he later claims to be a better hunter, with improved stamina and keener senses.

The intensity of human reactions to frog secretion is doubtless dose-dependent. The period of intense illness (<1 hr) is followed by a state of listlessness and sleep lasting from one to several days. Carneiro (1, 2) reported that vivid hallucinations occur but this is not supported by other observations (ref. 3, K.M., and P. Gorman, personal communication). P. Gorman (1986, field notes) was administered a reduced dose and felt the urge for vomiting and incontinence, and an alarmingly rapid heartbeat, intense sweating, fearful incapacitation, and near delirium; after a day's rest, he noted in his journal that he had "not only recuperated but was beginning to feel quite godlike in my strength and the acuteness of my senses."

We here identify the frog used by the Peruvian Matses and Brazilian Mayoruna Indians as *Phyllomedusa bicolor* (Boddaert), a large green hylid that inhabits lowland rain forest throughout much of the Amazon basin and the Guayanan region (Fig. 1 *Upper*). Skin extracts from this species have been previously studied and are known to contain a variety of vasoactive peptides (4). These include high levels of phyllocaerulein, phyllokinin, and phyllomedusin and moderate levels of sauvagine. Small amounts of deltorphins, a class of opioid agonists selective for δ receptors, also are present (5). We initially felt it unlikely that either the vasoactive peptides or the small amounts of deltorphins would account for effects on humans attributed to secretions from *P. bicolor*. Therefore, an examination was initiated of the effects of extracts of dried secretion from a Mayoruna frog stick.

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## EXPERIMENTAL PROCEDURES AND RESULTS

**Extraction and Behavioral Effects.** A stick holding dried skin secretion from *P. bicolor* was acquired by K.M. at the Rio Lobo site in western Brazil. The Rio Lobo stick was scraped to provide 400 mg of dried frog secretion, which was triturated first with 6 ml of methanol to yield a pale yellow methanol extract and then with 6 ml of water to yield a pale yellow aqueous extract. Extracts were stored at −20°C. Subcutaneous injection into mice of methanol and aqueous extracts in doses equivalent to 0.1–6 mg of dried secretion resulted in a dose-dependent reduction in spontaneous locomotor activity. At higher doses mice were completely inactive for several hours, although when touched they would respond briefly and then reassume a lethargic state. The aqueous extract produced a less active state at a threshold dose equivalent to 0.1 mg of dried secretion, whereas the methanol extract required a dose of 0.3 mg to produce such a less active state. Increasing the threshold dose for either extract by a factor of 2 resulted in a profoundly inactive state that persisted for 1–2 hr. Similar results were later obtained from extracts of a half-stick donated by P. Gorman and acquired from Matses Indians on the Río Gálvez, Peru, roughly 60 km north of Rio Lobo. Injection of methanol extract from a skin of *Phyllomedusa lemur* that was equivalent to 100 mg of wet skin produced an inactive state similar to that caused by extracts of dry *P. bicolor* secretion, which persisted for about 30 min. The inactive state elicited by substance(s) in *Phyllomedusa* spp. skin or dried secretion is reminiscent of the inactive but responsive state caused by injection of adenosine analogs in mice (6).

**Binding Assays.** Extracts of frog-stick secretion were assayed for their ability to inhibit binding of the following: (*R*)-[$^3$H]-$N^6$-phenylisopropyladenosine to $A_1$ adenosine receptors in rat brain membranes (7); [$^3$H]*N*-ethylcarboxamidoadenosine to $A_2$ adenosine receptors in rat striatal membranes (8); [$^3$H]naloxone to opioid receptors in rat brain membranes (9); and [$^3$H]*N*-methylscopolamine to muscarinic receptors in rat brain membranes (10). A marked inhibition of binding to $A_1$ adenosine receptors occurred with half-

FIG. 1. (*Upper*) A green tree frog, *Phyllomedusa bicolor*, used as a source of emetic secretion at a Mayoruna settlement on the Rio Lobo (73°00′ W, 5°50′ S), Rio Javari drainage, Amazonas, Brazil. Identification is based on this and other photographs of individual frogs used by the Mayoruna (K.M.) and Matses (P. Gorman, field notes) groups and on examination of a single specimen collected by P. Gorman. The frog arrived at the American Museum of Natural History, New York, in poor condition but readily identifiable; it was retained as a skeletonized specimen (AMNH A-134148). *P. bicolor* also may be the frog used by the Marúbo Indians (see ref. 14). (*Lower*) Line of burns on chest of a Mayoruna at the Rio Lobo site; the burned skin has been scraped away prior to application of frog secretion to each open burn. (Photographs by K.M.)

maximal inhibition by the aqueous extract corresponding to 0.03 mg of dried frog secretion and complete inhibition corresponding to 0.06 mg. There was no effect on binding to $A_2$ receptors, whereas there was a modest inhibition of binding to opioid receptors, presumably due to deltorphins known to be present in skin of *P. bicolor* (5), and a modest

inhibition of binding to muscarinic receptors (data not shown). The principles active against $A_1$ adenosine receptor binding did not partition into an organic solvent (chloroform) and thus were polar compounds.

**Purification of Active Principles.** Fractionation of the extract was performed by reversed-phase high-pressure liquid chromatography (HPLC); a typical chromatogram is shown (Fig. 2*A*). The conditions for reversed-phase HPLC fractionation were as follows: $C_{18}$ bonded silica column (100 × 2.5 mm) with acetonitrile and 0.1% aqueous trifluoroacetic acid as the two solvents, going from 10% to 50% acetonitrile in 20 min. The ultraviolet detector was set at 220 nm with the flow rate at 1 ml/min. Multiple 200-μl injections were chromatographed. A total of 80 fractions were collected per injection at a rate of 2 fractions per minute. The fractions were lyophilized and redissolved in 500 μl of water. The fractions were assayed for inhibition of binding of the agonist (*R*)-[$^3$H]-$N^6$-phenyl isopropyladenosine to $A_1$ adenosine receptors (Fig. 2*B*). In each HPLC run, $A_1$ adenosine receptor binding was affected by fractions eluted from 12 to 18 min; based on the ultraviolet absorption, these fractions contained only a small portion of the total peptide constituents in the extract. The fractions from five HPLC runs (equivalent to 70 mg of secretion) were combined to yield five combined fractions, P-1 to P-5 (see Fig. 2*B*). These five fractions, each in a total volume of 0.5 ml, had different effects on agonist binding to $A_1$ adenosine receptors. The P-1 and P-3 fractions inhibited binding, whereas the P-2 fraction stimulated binding, and the P-4 and P-5 fractions had minimal inhibitory effects.

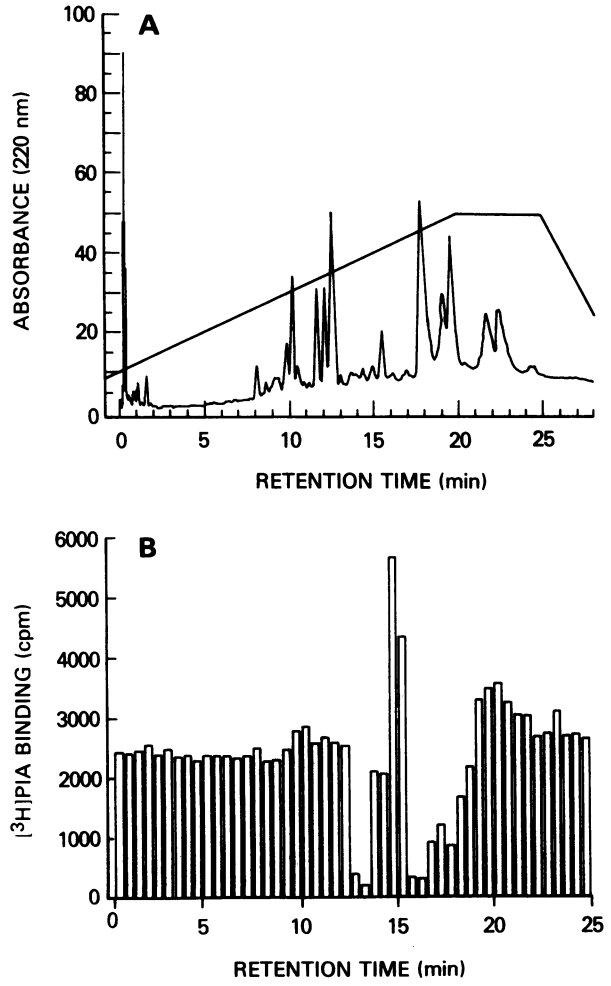


FIG. 2. Fractionation and analysis of an aqueous extract of dried skin secretion from *P. bicolor*. (*A*) Typical HPLC chromatogram of 200 μl of aqueous extract monitored at 220 nm. The ascending line represents the percentage of acetonitrile in a gradient of 0.1% aqueous trifluoroacetic acid and acetonitrile. (*B*) Effect of 10 μl of fractions (total volume of each, 500 μl) on binding of the agonist (*R*)-[$^3$H]-$N^6$-phenylisopropyladenosine (PIA) to rat brain membranes. Both inhibitory and stimulatory activities were detected. Aliquots of 0.1 to 30 μl of each fraction were assayed. A 10-μl aliquot would be equivalent to 1.4 mg of dried secretion. Dose-dependent inhibition of binding of agonist to $A_1$ adenosine receptors occurred with the P-1 and P-3 fractions, with 50% inhibition requiring about 7 μl and 20 μl, respectively. Dose-dependent stimulation of binding occurred with the P-2 fraction, with 3 μl causing a 1.2-fold stimulation and 30 μl causing a near-maximal 1.6-fold stimulation (see text for estimate of potencies). None of the fractions affected agonist binding to $A_2$ adenosine receptors. Retention times of fractions: P-1, 12.5–14.0 min; P-2, 14.0–15.5 min; P-3, 15.5–17.0 min; P-4, 17.0–18.0 min; P-5, 18.0–19.0 min.

Thin-layer chromatographic analysis with ninhydrin reagent for detection revealed the presence of peptides in the P-1 to P-5 fractions. The P-2 fraction had only one ninhydrin-positive spot and appeared homogeneous by amino acid analysis (data not shown). The amount of peptide present in the P-1, P-2, and P-3 fractions was estimated from ninhydrin reaction. The molecular weight of the major peptide of the P-2 fraction was 3186 (see below). The $EC_{50}$ value for stimulation of agonist binding to $A_1$ receptor by the P-2 peptide was estimated to be about 3 μM (data not shown). Based on the assumption that the inhibitory peptides also had molecular weights of about 3000, the P-1 and P-3 peptides had estimated $IC_{50}$ values versus binding of agonist to $A_1$ receptors of about 3 and 1.5 μM, respectively.

The sequence for the peptide in the P-2 fraction was determined by Edman degradation [Applied Biosystems model 477A sequencer equipped with a model 120A PTH analyzer (11, 12)] and corresponded to a 32-amino acid peptide. However, plasma desorption mass spectral analysis indicated a molecular weight of 3186. The analysis was performed on peptide delivered to a nitrocellulose-coated foil with spectra accumulated for $10^6$ fissions of $^{252}$Ca. This molecular weight is 99 mass units greater than that of the peptide sequence from Edman degradation. Amino acid analysis had indicated three valines (data not shown), only two of which were detected by Edman degradation. Thus, it appeared that a carboxyl-terminal valine (residue weight, 99) had not been detected in the Edman degradation. The sequence of the 33-amino acid residue peptide, for which the name adenoregulin is proposed, would therefore be as follows.

GLWSKIKEVGKEAAKAAAKAAGKAALGAVSEAV

Synthesis of this peptide was carried out using *t*-butoxycarbonyl chemistry on an Applied Biosystems 430A peptide synthesizer with chemical and program cycles provided by the manufacturer. The resulting peptide corresponded in mass spectrum (molecular weight 3186) and in sequence by Edman degradation, including lack of detection of the terminal valine, to the natural peptide (data not shown). However, the HPLC retention times of natural and synthetic peptide were slightly different (78 min and 77.5 min, respectively) and they partially separated on coinjection. Since the sequences and mass spectra of the natural and synthetic peptides were identical, the most likely difference would be the presence of D amino acid(s) in the natural peptide. The HPLC conditions for analysis and purification were as follows. Peptide samples in 8 M guanidinium chloride were passed through a 0.22-μm Millipore Ultrafree-MC filter and applied to a Vydac 214TP column (2.1 × 250 mm) equilibrated with 0.1% trifluoroacetic acid (solvent A) in a Hewlett–Packard model 1090 M with the column over at 60°C. The column was washed with solvent A for 10 min at 250 μl/min. The flow rate was reduced to 150 μl/min and peptides were

eluted with 0.85% trifluoroacetic acid in 80% acetonitrile (solvent B) by using a gradient in which the proportion of solvent B was brought to 35% over 60 min, to 75% over 30 min, and finally to 100% over 10 min (13). The column was washed for 5 min with 100% solvent B between runs to elute strongly retained peptides. Fractions were collected manually.

After HPLC purification of synthetic peptide and quantitation by ninhydrin reagent, an $EC_{50}$ of about 10 $\mu$M for stimulation of agonist binding and a maximal stimulation of 1.6-fold were determined (data not shown). There was insufficient natural adenoregulin after this HPLC purification to determine potency. From earlier data on the P-2 fraction (see above) it appears that natural adenoregulin is somewhat more potent than the synthetic material.

## DISCUSSION

Secretions from an Amazonian frog, now identified as a widespread large hylid, *P. bicolor*, elicit in humans profound malaise, followed by listlessness and then euphoria (see above). When injected into mice, extracts of the dried skin secretion cause a profound and long-lasting listless or lethargic state, reminiscent of the state caused by injection of adenosine analogs (6). Peptides that either enhance or inhibit binding of adenosine analogs to brain $A_1$ adenosine receptors proved to be present in extracts of the dried skin secretion. Several active fractions were isolated. The P-2 fraction contained a single 33-amino acid peptide, whose sequence was deduced by Edman degradation and mass spectral analysis. The trivial name adenoregulin, referring to its ability to modulate or "regulate" the $A_1$ adenosine receptor by enhancing binding of an agonist, is proposed. Adenoregulin contains many hydrophobic amino acid residues (11 alanines, 4 glycines, 3 valines, 2 leucines, and 1 isoleucine) along with 3 glutamate residues, 6 lysines, 2 serines, and 1 tryptophan. The sequence is quite different from those of other peptides known to be present in *P. bicolor*. Natural adenoregulin is not identical with synthetic material, although both stimulate binding of agonist to $A_1$ receptors. The natural peptide may contain one or more D amino acid residues, an occurrence that has precedent in other amphibian peptides (see ref. 5), including peptides from *P. bicolor*, but resolution of this question will require further supplies of natural adenoregulin.

Adenoregulin stimulates binding of an agonist to $A_1$ adenosine receptors, and the congeneric peptides in the P-1 and P-3 fractions inhibit binding of an agonist to $A_1$ adenosine receptors. The limited supply of natural adenoregulin prevented detailed investigation of its interaction with $A_1$ adenosine receptors. Adenoregulin had no effect on binding of an antagonist, [$^3$H]xanthine amine congener (XAC), to rat brain $A_1$ adenosine receptors, nor did it affect binding of an agonist, [$^3$H]*N*-ethylcarboxamidoadenosine, to rat striatal $A_2$ adenosine receptors (data not shown). It had no effect on binding of antagonists to rat brain opioid or muscarinic receptors (data not shown).

The significance of adenoregulin to the effects produced in humans by *P. bicolor* secretion is uncertain. When behavioral effects of HPLC fractions (see Fig. 2*A*) were assessed by injection of 50 $\mu$l into mice, the P-1, P-2, and P-3 fractions did cause modest behavioral depression (data not shown). These correspond to a dose of about 60 $\mu$g of peptide per mouse. The synthetic peptide appeared 2- to 3-fold less active than natural adenoregulin in causing behavioral depression in mice.

The skin secretion from *P. bicolor* also contains relatively large amounts of other peptides, especially various vasoactive peptides. The amounts estimated in skin of *P. bicolor* were reported as follows (4) (per 100 mg of skin) phyllocaerulein, 50–65 $\mu$g; phyllokinin, 30–50 $\mu$g; phyllomedusin, 50–140 $\mu$g; sauvagine, 5–10 $\mu$g; deltorphins, 0.5–0.8 $\mu$g. The levels of adenoregulin and congeneric peptides in skin of *P. bicolor* are unknown, but the HPLC absorbance profile (Fig. 2*B*) indicates that they represent minor constituents of the dried skin secretions.

The *in vivo* effects of peptides analogous in structure to three of the vasoactive peptides from *P. bicolor* were assessed. These were administered subcutaneously to mice at doses equivalent to the amount expected of such a peptide in 100 $\mu$g of frog skin. Caerulein (analogous to phyllocaerulein) at a dose of 40 $\mu$g per mouse, bradykinin (analogous to phyllokinin) at a dose of 40 $\mu$g, and physalaemin (analogous to phyllomedusin) at a dose of 40 $\mu$g had no effect except for a slight reduction in locomotor activity with physalaemin. Sauvagine at a dose of 10 $\mu$g caused marked inactivity in mice and should contribute to the behavioral depressant effects of dried extracts. [D-Ala$^2$]deltorphin I at a dose of 2.5 $\mu$g caused slight hyperactivity.

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