Widespread Occurrence in Frogs and Toads of Skin Compounds Interacting with the Ouabain Site of Na^+,K^+-ATPase

Abstract. *Amphibians of the family Bufonidae contain high levels of skin compounds that both inhibit Na^+- and K^+-dependent adenosinetriphosphatase and antagonize the binding of ouabain to the enzyme. In species of* Bufo *and* Atelopus, *these compounds are relatively nonpolar bufodienolides, whereas* Dendrophryniscus *and* Melanophryniscus *contain more polar compounds of unknown structure. Skin extracts from 30 of 48 species of frogs representing an additional eight families contained relatively low levels of compounds that inhibit binding of ouabain to Na^+,K^+-adenosinetriphosphatase. The widespread occurrence of low levels of inhibitory compounds is consonant with a role for these compounds as physiological regulators of Na^+,K^+-adenosinetriphosphatase in amphibian skin; high levels in the Bufonidae probably also serve as a defense against some predators.*

In amphibians, the skin is a major tissue for regulation of sodium and water homeostasis and contains large amounts of Na^+- and K^+-dependent adenosinetriphosphatase (ATPase), an enzyme crucial to this regulation (*1*). An endogenous regulator of Na^+,K^+-ATPase might be expected in such a tissue. Amphibian skin does contain a variety of substances affecting physiological transport of ions across membranes, but such substances have been presumed to be stored in granular secretory glands to serve mainly in defense against predators (*2*). One example is the bufodienolide toxins, which are potent inhibitors of Na^+,K^+-ATPase (*3*) and represent the animal equivalent of plant cardenolides such as ouabain. It is tempting to speculate that the bufodienolides evolved from a regulatory factor for Na^+,K^+-ATPase that was more widely distributed in amphibian secretory cells. Testing for antagonism of the binding of radioactively labeled ouabain to Na^+,K^+-ATPase is a sensitive assay for the presence of a regulatory factor for this enzyme in amphibian skin, and initial studies detected high levels of such compounds in certain bufonids (*4*, *5*). Extracts of skins from a wide range of anurans have now been tested for inhibition of ouabain binding (Table 1).

Skin extracts of eight species from four genera of Bufonidae contained high titers of compounds that inhibit ouabain binding. Parotid glands and skin of adult *Bufo* spp. are known to contain bufodienolides (*3*) that antagonize binding of ouabain. Skin from juvenile toads and even tadpoles of *B. marinus* also contained high titers of active compounds (*6*). Bufodienolides had not previously been reported from *Bufo* tadpoles or from *Atelopus*, *Dendrophryniscus*, or *Melanophryniscus*.

The major portion of the active compounds from skins of *Bufo*, *Atelopus*, *Dendrophryniscus*, and *Melanophryniscus* was extracted into 50 percent aqueous methanol, with 10 percent or less being recovered in a subsequent aqueous extract. For *Bufo*, *B. marinus* tadpoles, and *Atelopus* about 90, 70, and 70 percent of the active compounds, respectively, partitioned from aqueous methanol into three volumes of chloroform indicating that the active principles were relatively nonpolar—as would be expected if bufodienolides were the major active class in these species. In contrast, the active compounds from *Dendrophryniscus* and *Melanophryniscus* did not partition into chloroform (< 1 percent), indicating the presence of highly polar compounds rather than simple bufodienolides. The results of silica-gel chromatography support the presence of bufodienolides in extracts from skins of *B. regularis*, *B. marinus* (adult and tadpole), and *A. ignescens*. In all cases the majority of active substances was eluted at the point at which unconjugated bufodienolides would elute (Fig. 1, A to C). Small amounts of active substances found in later fractions may have been polar conjugates of such bufodienolides. The majority of active substances from *Dendrophryniscus* was eluted from the column much later than bufodienolides (Fig. 1D).

Bufodienolides contain an ultraviolet chromophore in the α-pyrone ring with an absorption maximum at 300 nm preceded by a minimum at about 254 nm (*3*). The active fractions obtained by silica-gel chromatography from the skins of *B. regularis*, *B. marinus* tadpoles, *A. ignescens*, and *Dendrophryniscus* were subjected to high-pressure liquid chromatog-

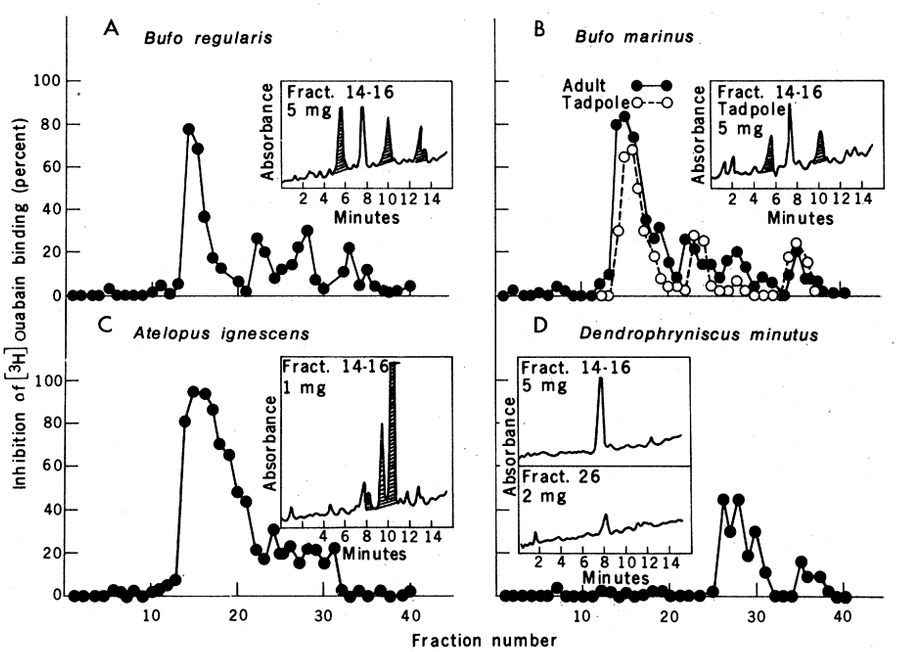

Fig. 1. Inhibition of binding of ouabain to Na^+,K^+-ATPase by chromatographic fractions from skin extracts. Methanol extracts corresponding to 0.5 g of skin were dissolved in 0.6 ml of methanol and chloroform (1:1), applied to a silica gel column (100 to 200 mesh, 20 by 1 cm), and eluted first with chloroform (60 ml); then with chloroform and methanol (10:1, 60 ml; then 2:1, 60 ml); and then with chloroform, methanol, and aqueous ammonia (1:4:0.05, 200 to 250 ml). Six-milliliter fractions were collected. (Fraction 40 represents a final 150 to 200 ml.) The fractions were evaporated to dryness and dissolved in 0.5 ml of methanol. Inhibition of the binding of radioactively labeled ouabain was assayed at similar dilutions (1:5000) for all the fractions. Insets show high-pressure liquid chromatograms of active fractions. Fractions that corresponded to 1 to 5 mg of skin were injected in 1 to 10 μl of methanol onto a 4.6 mm by 25 cm column (Altex Li-Chrosorb RP 18) with a flow of 2 ml/min and a linear gradient of 50 to 80 percent methanol in H_2O over 15 minutes. The absorbance at 300 nm is depicted. The cross-hatched peaks had a ratio of absorbances at 300 and 254 nm consonant with the presence of a bufodienolide chromophore. The empty peaks had incorrect absorbance ratios; they do not correlate with the presence or absence of compounds inhibiting the binding of ouabain. Marinobufagin emerges after about 10 minutes.

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Table 1. Inhibition of ouabain binding to Na^+-K^+-ATPase by methanol skin extracts from various anurans. After the animals were collected, their skins were removed and placed in methanol; extracts were prepared as described in (*13*). Limits of detection: 0.002 to 0.005 equivalent per milligram of skin; N.D., not detected.

Family, genus, and species	Country collected in	Ouabain equivalents per milligram of skin
Bufonidae		
Atelopus ignescens	Colombia	400*
Atelopus spurrelli	Colombia	400*
Atelopus varius zeteki	Panama	150*
Bufo granulosus	Venezuela	40*
Bufo marinus	Peru	80*
Bufo regularis	Malawi	80*
Dendrophryniscus minutus	Peru	80*
Melanophryniscus moreirae	Brazil	100*
Dendrobatidae		
Colostethus inguinalis	Panama	2.0*
Colostethus talamancae	Panama	0.05*
Dendrobates auratus	Panama	0.08*
Dendrobates histrionicus	Colombia	0.02
Dendrobates pumilio	Panama	0.01
Dendrobates steyermarki	Venezuela	N.D.
Dendrobates tricolor	Ecuador	N.D.
Phyllobates bicolor	Colombia	0.3*
Phyllobates vittatus	Costa Rica	N.D.
Hylidae		
Agalychnis callidryas	Panama	0.02
Hyla boans	Venezuela	N.D.
Phrynohyas venulosa	Panama	N.D.
Smilisca sila	Panama	0.02
Hyperoliidae		
Hyperolius marmoratus	Malawi	0.1*
Kassina senegalensis	Malawi	0.01
Leptopelis bocagei	Malawi	N.D.
Leptodactylidae		
Adenomera marmorata	Brazil	N.D.
Crossodactylus sp.	Brazil (Boracéia)	N.D.
Crossodactylus sp.	Brazil (Teresópolis)	0.1
Cycloramphus asper	Brazil	N.D.
Cycloramphus pinderi	Brazil	0.02
Edalorhina perezi	Peru	0.3*
Eleutherodactylus fitzingeri	Panama	N.D.
Eleutherodactylus guentheri	Brazil	N.D.
Eleutherodactylus parvus	Brazil	N.D.
Eleutherodactylus vilarsi	Venezuela	0.2*
Holoaden bradei	Brazil	0.02
Hylodes asper	Brazil	0.02
Hylodes glaber	Brazil	N.D.
Hylodes lateristrigatus	Brazil	0.8*
Hylodes pulcher	Brazil	0.6*
Leptodactylus pentadactylus	Panama	N.D.
Leptodactylus rugosus	Venezuela	0.1*
Megaelosia goeldi	Brazil	0.05
Paratelmatobius lutzi	Brazil	0.1
Physalaemus sp.	Brazil	0.01
Pleurodema brachyops	Panama	N.D.
Proceratophrys appendiculata	Brazil	0.01
Thoropa petropolitana	Brazil	0.4*
Microhylidae		
Breviceps poweri	Malawi	0.1*
Phrynomerus bifasciatus	Malawi	N.D.
Pipidae		
Xenopus muelleri	Malawi	0.1*
Ranidae		
Phrynobatrachus natalensis	Malawi	4.0*
Ptychadena mascareniensis	Malawi	0.8*
Pyxicephalus adspersus	Malawi	N.D.
Rana angolensis	Malawi	N.D.
Rana palustris	United States	0.05
Rhacophoridae		
Chiromantis xerampelina	Malawi	0.01

*Also analyzed after partition between chloroform and aqueous methanol (see text).

raphy. The active fractions from *Bufo* and *A. ignescens* contained ultraviolet-absorbing substances (insets in Fig. 1, A, B, and C), whereas the active fractions from *Dendrophryniscus* contained virtually none (inset in Fig. 1D). The relative absorbances at 300 and 254 nm were consonant with the presence of bufodienolides in active fractions from *Bufo* and *Atelopus* (*7*) and with their absence in active fractions from *Dendrophryniscus*. Thin-layer chromatographic analysis, in conjunction with detection of bufodienolides with $SbCl_3$, also supported these conclusions. The apparent presence of bufodienolides in the skin of *B. marinus* tadpoles is surprising since the granular glands, thought to represent the site of production and storage of most amphibian toxins, have been reported to appear only during or after metamorphosis (*8*).

Extracts from the skin of *B. regularis*, *A. ignescens*, *Dendrophryniscus*, and *Melanophryniscus* inhibited uptake of radioactive rubidium ions into red blood cells (*9*). Their potency in this regard was consonant with their potency in inhibiting ouabain binding to Na^+,K^+-ATPase. Thus the active principles in these extracts inhibit the activity of membrane Na^+,K^+-ATPase, as do ouabain and bufodienolides.

The Bufonidae species were the only anurans to contain high titers of substances that antagonize the binding of ouabain. However, there was a widespread occurrence of low titers of such compounds in extracts from many other amphibians (Table 1) (*10*). No obvious phylogenetic pattern was discernible, suggesting that such compounds (i) represent a primitive trait that is retained at detectable levels on a rather random basis or (ii) are physiological regulators of Na^+,K^+-ATPase, whose levels are correlated with unknown physiological or ecological factors (*11*). Extracts from 14 species were partitioned between aqueous methanol and chloroform (legend to Table 1). Only 10 percent or less of the active compounds from *Colostethus inguinalis* and *Hylodes pulcher* partitioned into chloroform, and only 20 to 30 percent of the active compounds from *Hylodes lateristrigatus* and *Thoropa petropoliana* did so. Thus the active compounds in these four species, like those of *Dendrophryniscus* and *Melanophryniscus*, are highly polar. In the other ten species, 60 to 80 percent of the active principles went into chloroform, as had the bufodienolides.

Among nonbufonids, the species containing the highest titers of ouabain equivalents (*12*) in skin were frogs that move back and forth from aquatic to ter-

restial environments: *H. pulcher, H. lateristrigatus, C. inguinalis, Ptychadena mascareniensis*, and *Phrynobatrachus natalensis*. Extracts from these five species and from the terrestrial *Phyllobates bicolor* all inhibited uptake of rubidium ions into red blood cells to an extent roughly consonant with their potency in inhibiting binding of ouabain to Na^+,K^+-ATPase. Thus, at least in these frogs, the active compounds not only compete for ouabain-binding sites but also inhibit Na^+,K^+-ATPase.

In summary, the skin of many amphibians contains inhibitory compounds that interact with the ouabain-binding site of Na^+,K^+-ATPase. The presence of such compounds appears highly variable except in the family Bufonidae, whose species contain high titers of compounds that inhibit ouabain binding and Na^+,K^+-ATPase activity. In skin secretions, these compounds probably serve in defense against predation. In *Bufo* and *Atelopus* (*7*), the active compounds are bufodienolides, whereas *Dendrophryniscus minutus* contains high titers of polar compounds that, based on the absence of a significant ultraviolet chromophore, do not appear to be bufodienolides. It would be inappropriate to draw taxonomic conclusions concerning intergeneric relations in the Bufonidae until the structures of the active polar compounds in *Dendrophryniscus* and *Melanophryniscus* are clarified. But it is of interest that extremely high levels of Na^+,K^+-ATPase inhibitors in the skin may be characteristic of the family and not limited to *Bufo*. The structures of inhibitory compounds present at low titers in species of other families are also unknown. They may represent physiological regulators for Na^+,K^+-ATPase in amphibian skin.

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References and Notes

1. M. G. Farquhar and G. E. Palade, *Proc. Natl. Acad. Sci. U.S.A.* **51**, 569 (1964).
2. M. Neuwirth, J. W. Daly, C. W. Myers, L. W. Tice, *Tissue Cell* **11**, 755 (1979).
3. K. Meyer and H. Linde, in *Venomous Animals and Their Venoms*, W. Bucherl and E. E. Buckley, Eds. (Academic Press, New York, 1971), vol. 2, p. 521.
4. J. S. Flier, *Nature (London)*, **274**, 285 (1978).
5. ———, E. Maratos-Flier, J. A. Pallotta, D. M. McIsaac, *ibid.* **279**, 341 (1979).
6. Skin from juvenile *B. marinus* (length, 2 to 3 cm) had levels of active substances only somewhat lower than those from adults. Skins from 80 tadpoles (Santa Barbara, upper Orinoco River, Venezuela) contained about 50 ouabain equivalents per milligram.
7. Methanol extracts from *A. ignescens* were analyzed further by high-pressure liquid chromatography (Zorbax ODS column with a linear gradient programmed from 50 to 80 percent methanol in H_2O over 30 minutes). The results suggested the presence of telocinobufagin and bufotalin as major constituents and marinobufagin, cinobufagin, bufalin, arenobufagin, and two unidentified bufodienolides as minor constituents. Standard compounds eluted in the following order: gamabufotalin, arenobufagin, hellebrigenin, bufotalin, telocinbufagin, bufalin, cinobufagin, resibufogenin, and marinobufagin. The two unidentified bufodienolides in the *Atelopus* extract eluted between hellebrigenin and bufotalin.
8. Y. L. Trong, *Bull. Soc. Zool. Fr.* **98**, 449 (1973).
9. Human red cells were prepared as described in (*4*) and resuspended at a hematocrit level of 15 percent in buffer containing 150 m*M* NaCl, 10 m*M* tris, 10 m*M* dextrose, and 2 m*M* RbCl at a *p*H of 7.4. Ouabain or skin extracts (25 μl) were added to 200 μl of cell suspensions and incubated at 37°C for 60 minutes. The cells were washed and reconstituted with 200 μl of buffer. After addition of 0.4 μCi of $^{86}Rb^+$ (3.1 mCi/mg), the cells were incubated for 70 minutes at 37°C and then washed three times in buffer containing 150 m*M* NaCl. The final pellet was treated with 100 μl of 10 percent perchloric acid. After sedimentation of the pellet, the tubes were inverted into scintillation vials containing 5 ml of 150 m*M* NaCl. Influx of $^{86}Rb^+$ was determined by subtraction of the values obtained at zero time.
10. Low titers of compounds that inhibit ouabain binding were detected in methanol extracts from the skins of the North American salamanders *Notophthalmus viridescens* and *Pseudotriton ruber*.
11. The possible significance of environmental factors is suggested from preliminary results with *Dendrobates auratus*: frogs obtained in the dry season on Isla Taboga, Panama, contained 0.08 ouabain equivalent per milligram of skin (Table 1), whereas skin from frogs collected in the wet season contained only 0.01 equivalent per milligram. Eight frogs maintained for 4 months in a terrarium had 0.5 equivalent per milligram of skin. After 5 hours in isotonic saline, levels of ouabain equivalents in two terrarium frogs had increased by >50 percent.
12. A ouabain equivalent is equal in activity to that amount of ouabain (4 ng) which inhibits binding of [^{3}H]ouabain to Na^+,K^+-ATPase by 50 percent.
13. Human red blood cells were prepared and incubated with $10^{-9}M$ [^{3}H]ouabain (8 Ci/mmole) for 60 minutes at 37°C, either alone or in the presence of increasing concentrations of nonradioactive ouabain ($10^{-8} \times 10^{-5}$ to $3 \times 10^{-5}M$) or portions of methanol or aqueous extracts equivalent to 0.001 to 20 mg of skin. Centrifugation, washing, and scintillation spectroscopy were carried out as described in (*4*), and ouabain equivalents were calculated through comparison with the ouabain displacement curve. (Maceration of skins twice each time with 5 ml of methanol per gram of skin had yielded the methanol extracts. In some cases, skins were then macerated twice with water to yield an aqueous extract.) When partition between aqueous methanol and chloroform was obtained, the chloroform phase was evaporated and redissolved in methanol.
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L-Ornithine Decarboxylase: An Essential Role in Early Mammalian Embryogenesis

Abstract. *The highly selective, enzyme-activated, irreversible inhibitor of* L-*ornithine decarboxylase,* DL-α-*difluoromethylornithine, suppresses the increase in uterine* L-*ornithine decarboxylase activity associated with early embryogenesis in the mouse and arrests embryonic development at that stage. Contragestational effects were confirmed in the rat and rabbit. An increase in* L-*ornithine decarboxylase activity that leads to a rapid increase in putrescine concentration appears to be essential during a critical period after implantation for continued mammalian embryonal growth.*

Decarboxylation of L-ornithine by L-ornithine decarboxylase (ODC) (E.C. 4.1.1.17) leads to the formation of putrescine and is the initial step in the biosynthesis of the polyamines spermidine and spermine (*1, 2*). Although basal activity of ODC is low in most tissues, marked increases are characteristically associated with rapid tissue growth (*3*) and particularly with mammalian and nonmammalian embryogenesis (*4*).

Although such observations imply a functional role for ODC and the polyamines in the regulation of growth processes, direct proof of this has been difficult to obtain, principally because of the lack of tools with which to manipulate selectively the polyamine biosynthetic pathway (*3*). A potent and selective inhibitor of ODC that works through the mechanism of substrate-induced, irreversible inhibition (*5*) was recently developed in our laboratories (*6*). This compound, DL-α-difluoromethylornithine (α-DFMO) (RMI 71782), proved an effective inhibitor of ODC in vivo (*7, 8*). We now report that α-DFMO arrests embryonic development in three mammalian species. The results provide direct evidence that the changes in ODC associated with the early stages of mammalian embryogenesis are essential to the process and not merely an incidental, noncausal event.

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