ATRAZINE INCREASES THE SODIUM ABSORPTION IN FROG (RANA ESCULENTA) SKIN

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Abstract—The presence of atrazine in agricultural sites has been linked to the decline in amphibian populations. The efforts of the scientific community generally are directed toward investigating the long-term effect of atrazine on complex functions (reproduction or respiration), but in the present study, we investigated the short-term effect on the short-circuit current (I_SC), a quantitative measure of the ion transport operated by frog (Rana esculenta) skin. Treatment with 5 μM atrazine (1.08 mg/L) does not affect the transepithelial outfluxes of [14C]mannitol or [14C]urea; therefore, atrazine does not damage the barrier properties of frog skin. Atrazine causes a dose-dependent increase in the short-circuit current, with a minimum of 4.64 ± 0.76 μA/cm² (11.05% ± 1.22%) and a maximum of 12.7 ± 0.7 μA/cm² (35% ± 2.4%) measured at 10 nM and 5 μM, respectively. An increase in I_SC also is caused by 5 μM ametryne, prometryn, simazine, terbuthylazine, or terbutryn (other atrazine derivatives). In particular, atrazine increases the transepithelial 28Na⁺ influx without affecting the outflux. Finally, stimulation of I_SC by atrazine is suppressed by SQ 22536, H89, U73122, 2-aminoethoxydiphenyl borate, and W7 (blockers of adenylate cyclase, protein kinase A, phospholipase C, intracellular kinase C, respectively), whereas indomethacin and calphostin C (inhibitors of cyclooxygenase and protein kinase C, respectively) have no effect.

Keywords—Atrazine Amphibian decline Anuran Aquatic toxicity

INTRODUCTION

Atrazine is a triazine herbicide widely used to grow crops such as maize, sorghum, and sugar cane. It is found, together with its dealkylated degradation products, in surface waters and groundwater [1]. Several countries in Europe simply ban any questionable pesticide found in drinking water at levels higher than 0.1 ppb. On the other hand, in the United States, where drinking water is allowed to have up to 3 ppb of atrazine, approximately 27,000 tons of atrazine are applied to fields each year. For these reasons, the scientific community is particularly interested in any investigation dealing with the possible effect of atrazine on living organisms [2].

The use of atrazine in agricultural sites has been linked to the decline in amphibian populations [3]. Such a decline has been observed especially in North America [4–6] and Australia, and it has several synergically interacting causes [7]. In particular, atrazine has been reported to negatively affect sexual development in African clawed frogs (Xenopus laevis) [8], although other authors have not been able to reproduce those results [9].

A direct link between pesticides and amphibian decline is difficult to prove, because the presence of contaminants at agricultural sites often is compounded with a less suitable habitat [10]. Therefore, such a link also is investigated during laboratory experiments that are a complement to field studies, because confounding variables can be eliminated.

The efforts of the scientific community generally are directed toward investigating the long-term effect of atrazine on complex biological functions, such as reproduction or respiration [8,9,11]. On the contrary, in the present study, we have investigated the short-term effect of atrazine on a simpler cellular process, the ion transport operated by the skin. Our choice is supported by the conviction that the myriad of biological functions of different cell types are all sustained by a common basic machinery. Frogs living in freshwater are hypertonic to the surrounding water; moreover, they have a permeable skin through which diffusion of many molecules, such as respiratory gases and water, takes place. Therefore, frogs produce large amounts of dilute urine and lose ions, which must be recovered. To this aim, the frog skin is the site of active ion absorption, which is necessary for survival and, consequently, is under continuous control. At the same time, this tissue is in direct contact with the aquatic environment and more readily available for experimental manipulation than, for example, fish gills. Therefore, isolated frog skin has been used as a model for studying the effects of pesticides on epithelial ion transport [12,13]. In previous studies, we have shown that the ion transport operated by the frog (Rana esculenta) skin is increased by 1 mM carbaryl [13] and 5 μM pyrethroids [14,15]. In the present study, we have extended our investigation to atrazine and five of its derivatives.

MATERIALS AND METHODS

The ventral skin, excised from an adult pithed R. esculenta (grown in the Naples region of Italy) was immediately mounted between two leucite chambers (exposed area, 2.09 cm²); each chamber contained 7 ml of perfusing solution. Fluids were gassed with atmospheric air at 22 ± 2°C. The composition of the perfusing solution was 112 mmol/L of NaCl, 5 mmol/L of KCl, 1 mmol/L of CaCl₂, and 2.5 mmol/L of NaHCO₃, with pH 8.1. Transepithelial fluxes and short-circuit current were measured with a specially constructed Ussing chamber [16].

In experiments with tracers (Fig. 1), any labeled compound
(final activity, 0.1 μCi/ml) was added to one compartment, and after 90 min, 1 ml was collected from the opposite side and replaced with 1 ml of Ringer solution. This step was repeated at least three times, every 30 min. The first period served as a control. Atrazine was then added, and the experiment was continued for two further half-hour experimental periods. Radioactivity was measured with a scintillation counter, and the transepithelial fluxes were calculated.

In Figure 2, after skin preparation and 90 min of incubation, a stable short-circuit current value was reached, and this value served as a control. Atrazine was then added, and values were recorded for 60 min. The highest value, representing the maximum increase, was used for calculation. Data, presented as the effect on short-circuit current (ΔI_SC) (Fig. 2, inset), were the mean value of the difference between the highest value of the short-circuit current after atrazine challenge and the control. When the effect of atrazine was measured under two different experimental conditions (see Figs. 3–6), the same skin was split into two symmetrical parts to be used for each treatment.

Ametryne (N^2-ethyl-N^4-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine), atrazine (6-chloro-N^2-ethyl-N^4-isopropyl-1,3,5-triazine-2,4-diamine), prometryn (N^2,N^4-di-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine), simazine (6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine), terbutylazine (N^2-tert-butyl-6-chloro-N^4-ethyl-1,3,5-triazine-2,4-diamine), and terbutryn (N^2-tert-butyl-N^4-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine) were obtained from Chem Service (West Chester, PA, USA). With the exception of amiloride, each drug was dissolved in ethanol to prepare a stock solution. Ten microliters of this stock solution were added to 7 ml of the medium, bathing the serosal side of the skin. Ethanol at the final concentration of 0.143% did not modify short-circuit current. Amiloride was dissolved in the perfusing medium, and 100 μl from a stock solution were added to the medium bathing the external side of the skin. Stock solutions were stored at −20°C.

The data reported in the present paper (mean ± standard error) were statistically analyzed with Student’s t test for paired values.

RESULTS

Effect on Na^+ absorption

A series of experiments was performed to clarify whether atrazine modifies the integrity of the frog skin barrier (Fig. 1). The transepithelial outflow (from the serosal to the mucosal compartment) of radioactively labeled mannitol, a model permeant and nonionic molecule, was measured in symmetrical halves of eight frog skins. Similar results were obtained in the absence and presence of atrazine (Fig. 1). Evidently, atrazine does not unspecifically damage the barrier properties of frog skin. This conclusion was strengthened by the observation that atrazine does not modify the transepithelial urea flux measured in a second series of eight experiments (Fig. 1).

Once we had established that atrazine does not damage the integrity of the skin barrier, we measured its effect on the ion-transport agencies present in frog skin (Fig. 2). The effect of atrazine on both the transepithelial electrical potential difference and the short-circuit current were monitored. The ele-
Atrazine increases the sodium absorption in frog skin

Table 1. Effect on short-circuit current (ΔIsc), transepithelial potential difference (ΔVte), or transepithelial resistance (ΔRte) induced by 5 μM atrazine or its derivatives in frog (Rana esculenta) skin

<table>
<thead>
<tr>
<th></th>
<th>ΔIsc (μA/cm²)</th>
<th>ΔVte (mV)</th>
<th>ΔRte (Ω cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine</td>
<td>12.7 ± 0.7</td>
<td>-1.1 ± 0.4</td>
<td>-398 ± 30</td>
</tr>
<tr>
<td>Ametryne</td>
<td>13.9 ± 1.2</td>
<td>0.3 ± 1.3</td>
<td>-258 ± 78</td>
</tr>
<tr>
<td>Prometryn</td>
<td>12.5 ± 1.9</td>
<td>-0.1 ± 0.8</td>
<td>-184 ± 54</td>
</tr>
<tr>
<td>Simazine</td>
<td>11.9 ± 2.0</td>
<td>2.3 ± 1.7</td>
<td>-231 ± 64</td>
</tr>
<tr>
<td>Terbuthylazine</td>
<td>14.7 ± 2.2</td>
<td>-2.4 ± 1.0</td>
<td>-348 ± 61</td>
</tr>
<tr>
<td>Terbutryn</td>
<td>12.8 ± 1.9</td>
<td>-1.5 ± 1.1</td>
<td>-299 ± 74</td>
</tr>
</tbody>
</table>

*Values are presented as the mean ± standard error of the difference between the highest value of the indicated parameter after challenge with atrazine or its derivative and the control from 102 (atrazine), 33 (ametryne), 13 (prometrym), or 14 (simazine, terbuthylazine, or terbutryn). Percentage values were calculated using the value measured under control conditions as 100%.

The following experiments were then performed to verify the role of Na⁺ and Cl⁻ pathways in response to atrazine challenge.

We measured the 22Na⁺ transepithelial influx and outflux across frog skin that was permanently short-circuited to avoid the effect of electrical potential on Na⁺ fluxes. Atrazine significantly (p < 0.01) increased the 22Na⁺ absorption without affecting the opposite flux (Fig. 3).

To confirm the presence of Cl⁻ secretion in the increase in short-circuit current observed after atrazine treatment, we took advantage of amiloride, a potent blocker of Na⁺ channels. In the presence of 0.1 mM amiloride in the medium bathing the external side of the skin, any increase in short-circuit current caused by adding a second drug is caused by the process of Cl⁻ secretion, which is amiloride insensitive [19]. On this basis, we investigated the effect of atrazine on short-circuit current in the absence and presence of amiloride. The first half-skin was used to measure the stimulation by atrazine, whereas the other symmetrical part was used to verify whether that stimulation was affected by amiloride. The addition of 5 μM atrazine increased the short-circuit current from 31 ± 3.42 to 47.8 ± 3.23 μA/cm² (p < 0.001) (Fig. 4). In the symmetrical part of each skin, the presence of amiloride induced a drastic drop in the short-circuit value from 31 ± 3.42 to 0.83 ± 0.39 μA/cm² (third bar). Finally, when atrazine was given in the presence of amiloride (fourth bar), the short-circuit current value was significantly increased to 2.33 ± 0.5 μA/cm² (p < 0.001). These data demonstrate that atrazine mainly increases the Na⁺ absorption and, to a lesser extent, the Cl⁻ secretion in frog skin, as reported previously for deltamethrin [14].

Fig. 3. Treatment with 5 μM atrazine increases the 22Na⁺ influx but does not affect the 22Na⁺ outflux across frog skin. Values are presented as the mean ± standard errors from eight experiments.

Fig. 4. The atrazine stimulation of short-circuit current (Isc) across frog skin in the absence or presence of amiloride. Values are presented as the mean ± standard errors from 12 experiments. In each experiment, the skin was split into two symmetrical parts, the first for control (C) and atrazine (AT) and the second for amiloride (AM) and AM with AT (AM+AT). *p < 0.001.
creased the short-circuit current ($I_{sc}$) across frog skin in the absence or presence of indomethacin (I). Values are presented as the mean ± standard errors from nine experiments. In each experiment, the skin was split into two symmetrical parts, the first for control (C) and AT and the second for I and I with AT (I+AT). *$p < 0.01$.

Interaction with intracellular signaling

We investigated some of the cellular mechanisms potentially involved in the stimulation of ion transport operated by atrazine with the same experimental approach as used for the amiloride treatment (Fig. 4). The first half-skin was used to measure the stimulation by atrazine, and the other symmetrical part was used to verify whether that stimulation was affected by the drug under investigation.

Under control conditions, 5 μM atrazine significantly increased the short-circuit current ($p < 0.01$). The difference between the value after atrazine (Fig. 5, second bar from left) and the basal short-circuit current (Fig. 5, first bar) is referred to as the atrazine-dependent increase in short-circuit current. In the symmetrical part of a skin, atrazine also produced a similar effect ($p < 0.01$) when 10 μM indomethacin was present. These data are summarized in Figure 6, in which the two bars on the left indicate the atrazine-dependent increase in short-circuit current and, therefore, the ion transport in $R. esculenta$ skin.

In frog skin, the lowest atrazine concentration stimulating the short-circuit current was 10 nM (2.2 μg/L). It has been reported that in regions where atrazine is used, the concentrations rarely exceeded 20 μg/L in rivers and streams but were approximately 5 μg/L in reservoirs [1]. Therefore, the atrazine concentrations found in the environment are comparable with those producing an increase in the short-circuit current in the present study. This phenomenon leads to a potential nonphysiological Na$^+$ disequilibrium that must be compensated for by other osmoregulating epithelia, with the only potential consequence being the dissipation of metabolic energy. Direct measurement of frog metabolic rate after cutaneous atrazine exposure has not yet been performed, but Allran and Karasov [11] have measured two parameters linked to metabolic rate, namely buccal and thoracic ventilation (breaths/min). Those authors found that the lowest-observed-adverse-effect levels were 4.32 and 12 mg/L, respectively. Although different frog species were used in the study by Allran and Karasov ($R. pipiens$) and in the present study ($R. esculenta$), it is noteworthy that the atrazine concentrations stimulating the short-circuit current (between 2.2 μg/L and 1.1 mg/L or 10 nM and 5 μM, respectively) do not increase buccal or thoracic ventilation [11]. Our conclusion is that atrazine, at concentrations similar to those found in the environment, increases the Na$^+$ absorption by frog skin. The consequent Na$^+$ disequilibrium has to be compensated for by other ion-transporting epithelia. Such a compensation occurs without any measurable dissipation of metabolic energy, however, because faster ventilation is not observed [11]. Therefore, the increased Na$^+$ absorption per se does not influence the frog life cycle.

On the other hand, the evidence that atrazine increases the short-circuit current clearly indicates interaction with a cellular target. Evidently, the physiological activators of ion transport trigger a cascade of intracellular events that is sensitive to atrazine or other xenobiotics. The fact that the most diverse biological activities operating in different cell types can be regulated by the same basic cellular machinery, however, pro-

Fig. 5. The atrazine (AT) stimulation of short-circuit current ($I_{sc}$) across frog skin in the absence or presence of indomethacin (I). Values are presented as the mean ± standard errors from nine experiments. In each experiment, the skin was split into two symmetrical parts, the first for control (C) and AT and the second for I and I with AT (I+AT). *$p < 0.01$.

Fig. 6. SQ 22536, H89, U73122, 2-aminoethoxydiphenyl borate (APB), W7, but not indomethacin and calphostin C, abolish the stimulation of short-circuit current ($\Delta I_{sc}$) by atrazine. Values are presented as the mean ± standard errors from 9, 16, 9, 8, 11, and 15 experiments. $A =$ atrazine; $A+I =$ atrazine with indomethacin; $A+S =$ atrazine with SQ 22536; $A+H =$ atrazine with H89; $A+U =$ atrazine with U73122; $A+P =$ atrazine with 2-APB; $A+W =$ atrazine with W7; $A+C =$ atrazine with calphostin C. *$p < 0.05$. 

DISCUSSION

The ion transport operated by frog ($R. esculenta$) skin can be stimulated physiologically by a variety of molecules, such as dopamine [21], norepinephrine [22], and substance P [23]. An increase in Na$^+$ absorption also has been observed after treatment with xenobiotics, such as 0.1 mM carbaryl, dichlorodiphenylchloroethane, dieldrin, or lindane in $Rana pipiens$ [24] or 1 mM carbaryl [13] and 5 μM pyrethroids in $R. esculenta$ [14,15]. In the present study, we show that 5 μM atrazine and its derivatives (ametryne, prometryn, simazine, terbutylazine, and terbutryn) increase the short-circuit current and, therefore, the ion transport in $R. esculenta$ skin.
vokes interest in studying of the cellular mechanism involved in the stimulation of the short-circuit current by atrazine.

In the present investigation, we performed experiments aimed at identifying the cellular steps between the initial atrazine interaction with the cell and the final increase in Na\(^+\) absorption. The increase in concentration of the second-messenger cAMP (inhibited by SQ 22536), protein kinase A (blocked by H89), and calmodulin (inhibited by W7) are involved in this intracellular cascade of events, whereas cyclooxygenase and protein kinase C (inhibited by indomethacin and calphostin C, respectively) are not. The evidence that the increase in Na\(^+\) absorption induced by atrazine can be inhibited supports the following linked conclusions. First, atrazine has an unknown cellular target that is regulated by cAMP, protein kinase A, and calmodulin and that, in turn, affects ion transport. Second, the atrazine effect should not involve direct interaction with any of the proteins operating the Na\(^+\) absorption.

The effects of atrazine on epithelial ion transport also have been described in other animals. In the Chinese mitten crab (Eriocheir sinensis), Silvestre et al. [25] observed that atrazine at 1 mg/L (4.64 \(\mu\)M) increased the transepithelial potential difference maintained by the isolated posterior gills. In that study, short-circuit current was not measured, and increases in Na\(^+\) and Cl\(^-\) influxes were observed but not significant.

Recently, Waring and Moore have investigated the effect of atrazine on Atlantic salmon (Salmo salar) smolts [26]. In that study, it was observed that atrazine in freshwater reduced the Na/K\(^+\)/K\(^-\)–adenosine triphosphatase (ATPase) activity located on the plasma membrane of gill cells. The same conclusion cannot be reached in our case, because in frog skin, the rate of active Na\(^+\) absorption is increased by the parallel increase, and not reduction, in the Na/K\(^+\)/K\(^-\)-ATPase activity [27].

In conclusion, at concentrations similar to those found in the environment, atrazine causes an increase in the short-circuit current and the Na\(^+\) absorption operated by frog skin. These phenomena should not influence the frog life cycle, but they indicate interaction between atrazine and the intracellular system regulating ion transport.

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