4-Aminopyridine Reverses Saxitoxin (STX)- and Tetrodotoxin (TTX)-Induced Cardiorespiratory Depression in Chronically Instrumented Guinea Pigs

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The extent to which cardiorespiratory infirmity and other sublethal effects of saxitoxin (STX) and tetrodotoxin (TTX) can be reversed by 4-aminopyridine (4-AP) was investigated in guinea pigs chronically instrumented for the concurrent electrophysiological recordings of electrocorticogram (ECoG), diaphragmatic electromyogram (DEMG), Lead II electrocardiogram, and neck skeletal muscle electromyogram. Animals were intoxicated with either STX or TTX (2 and 3 μg/kg, im) to produce a state of progressive cardiorespiratory depression (depicted by decreasing DEMG amplitude, bradypnea, and bradycardia). At the point where cardiorespiratory performance was most seriously compromised (≈30 min posttoxin), 4-AP (1 or 2 mg/kg, im) was administered. The therapeutic effect of 4-AP was striking in that, within minutes, the toxin-induced diaphragmatic blockade, bradypnea, bradycardia, and depressed cortical activity were all restored to a level either comparable to, or surpassing, that of control. The optimal 4-AP dose level was determined to be 2 mg/kg (im) based on analyses of cardiorespiratory activity profiles throughout the course of intoxication and 4-AP treatment. At the dose levels (either 1 or 2 mg/kg) used to restore ventilatory function and cardiovascular performance, 4-AP produced no sign of seizures and convulsions. Although less serious secondary effects such as cortical excitant/arousal effect (indicated by ECoG power spectral analysis) and transient periods of skeletal muscle fasciculation were observed, these events were of minor concern particularly in view of the remarkable therapeutic effects of 4-AP.

Saxitoxin (STX) and tetrodotoxin (TTX) are among the deadliest nonprotein neurotoxins known (lethal dose in guinea pigs ≈5 μg/kg, im; unpublished observations).

STX is elaborated by dinoflagellates of the genus *Gonyaulax* (Taylor and Seliger, 1979). Filter-feeding bivalves such as butterclam, cherrystone clam, sea scallop, mussel are among the known carriers of STX. TTX is believed to be expressed by several species of bacteria (Yasumoto et al., 1986). Transmitted through the food chain, TTX-producing bacteria can be found in the organs of a variety of marine and terrestrial animals such as fish, crab, octopus, frog, and newt (Mosher and Fuhrman, 1984; Schantz, 1986). Because of their presence in a variety of marine organisms that are routinely harvested for human consumption (for review, see Ragelis, 1984), STX and TTX pose a serious public health threat. The time to onset of symptoms in accidental poisoning depends on the type and the amount of toxin ingested and could vary from half an hour to several hours (Hughes, 1979; Lyn, 1985; Rodrigue et al., 1990; Sutherland, 1983; Valenti et al., 1979). Symptoms associated with STX and TTX poisoning are quite similar. These include dyspnea, dysphagia, paresthesia, dystonia, hot-cold reversal, nausea, gastrointestinal disturbance, and a state of generalized malaise. These symptoms often persist, albeit with abating severity, for many days. The most life-threatening component of STX and TTX poisoning is ventilatory failure attributable primarily to the blockade of diaphragmatic neurotransmission.

There is no specific antidote for STX and TTX poisoning. To this date, gastric lavage and prolonged periods of respiratory support are still the only available means to clinically manage accidentally poisoned victims. Findings from animal research indicated that immunopharmacological agents (i.e., STX- or TTX-specific antibodies and toxin-binding proteins) could be a promising alternative to this mode of therapy (Benton et al., 1994; Davio, 1985; Fukiya and Matsumura, 1993; Kaufman et al., 1991; Rivera et al., 1995; Smith et al., 1989). Notwithstanding, immunopharmacological agents are highly toxin-specific and are unable to recognize and neutralize other toxin analogues and toxic components found in the poison-tainted marine organisms. Moreover, consider...
lations such as availability, cost, and special storage condition (or shelf-life) of immunopharmacological agents may discourage their use, particularly in economically disadvantaged and geographically isolated locations. For these reasons, we started to search for other pharmacological compounds that can be used to antagonize STX and TTX toxicity. One of the compounds we have evaluated was 4-aminopyridine (4-AP). Using a urethane-anesthetized guinea pig electrophysiological model system developed in this laboratory (Chang et al., 1990; 1993), we have shown that 4-AP can antagonize lethal effects caused by either STX or TTX (Chang et al., 1996).

The purpose of this study is twofold. The first purpose was to validate the effectiveness of 4-AP in an unanesthetized, freely behaving electrophysiological animal model system. The use of an unanesthetized preparation should eliminate the confounding effects of anesthesia and concerns that the pharmacological attributes of the 4-AP may be modified by anesthetics. Second, clinical case reports indicated that many accidentally poisoned victims do not require emergency respiratory support despite manifestation of severe symptoms of intoxication (Sims and Ostman, 1986). For this reason, it is important to also examine 4-AP's ability to antagonize the sublethal effects of STX and TTX. Taken together, findings derived from this study would enable us to gain a more thorough understanding about the direction and the magnitude of cardiorespiratory changes in response to 4-AP treatment throughout the course of STX/TTX intoxication.

MATERIALS AND METHODS

Animal Use and Care

A total of 72 barrier-raised male Hartley albino guinea pigs (Cavia porcellus) weighing between 700 and 1100 g were used in this study. All animals were treated in accordance with AALAC guidelines. Upon arrival, the animals were quarantined and screened for evidence of disease in the vivarium. Prior to surgery or experimentation, the animals were housed individually in plastic cages with corn cob bedding. The bedding was changed twice per week. Commercial guinea pig ration and tap water were provided ad libitum. Animals were allowed to recover for at least 2 weeks before they were used for experimental purposes.

Surgery

1. Anesthesia

Surgical level of anesthesia was induced with sodium pentobarbital (38 mg/kg; ip). Corneal reflex and reactions to toe pinching were regularly examined to ensure adequate depth of anesthesia during surgery. Whenever necessary, a supplemental dose of pentobarbital (5-10 mg/kg, ip) was administered. The core temperature was maintained between 38 and 39°C with a servo-controlled thermal blanket throughout the course of surgery. All surgical procedures were performed under aseptic conditions. Each animal was surgically instrumented for chronic electrophysiological recordings of (i) electrocorticogram (ECOG), (ii) diaphragmatic electromyogram (DEMG), (iii) neck skeletal muscle electromyogram (NEMG), and (iv) Lead II electrocardiogram (ECG II). The ECG recordings were used to evaluate changes in CNS arousal state(s) throughout the course of intoxication and 4-AP treatment. DEMG activity was monitored to provide information concerning the animal's ventilatory status, as well as the extent of compromise and recovery in diaphragmatic function throughout the course of intoxication and 4-AP treatment. NEMG activities were documented to assess 4-AP-induced changes in skeletal muscle activity as a consequence of the enhanced acetylcholine release. Finally, ECG signals were used to reveal changes in general cardiovascular status of the animal during STX/TTX intoxication and 4-AP treatment. Surgical techniques and procedures used for electrophysiological instrumentation are described below.

2. Chronic Electrophysiological Instrumentation

a. Electrocorticogram (ECOG). Standard surgical instrumentation procedures were followed for chronic ECOG recording. A 5- to 6-cm-long mid-sagittal incision was made to expose the calvarium. The skull surface was cleared of periosteal tissues and allowed to dry completely prior to electrode implantation. Stainless steel skull screws with Teflon-insulated electrical leads (32 Ga) flux-soldered to the screw head were implanted bilaterally over the parietal cortices (2 mm caudal to the coronal suture, 1.5 mm medial to the temporal crest) for differentiation of ECOG signals. A third cortical screw serving as signal ground was placed in the frontal bone midway between the midline and the arched portion of the orbital crest.

b. Diaphragmatic electromyogram (DEMG, see Chang and Harper, 1989, for a more detailed technical description). DEMG electrodes were made from a pair of flexible, Teflon-coated, multistranded stainless steel wires (Type AS-634; Cooner Wire Co., Chatsworth, CA). Surgical cut-down involved a small incision on the right side of the body. The abdominal cavity was then exposed by teasing the muscle layers with blunt surgical tweezers. Access to the right costal diaphragm was accomplished by retracting the liver and other abdominal tissues with blunt surgical instruments. An optical fiber light source was used in visualizing the rhythmically contracting costal diaphragm at the time of electrode insertion. The DEMG electrodes were inserted through the diaphragm, into the pleural cavity, and retrieved after their exit through the intercostal space between the eighth and ninth ribs. Allowing adequate return tension, the wires were guided subcutaneously to the head. Abdominal muscles and dermal incision were closed with 4-O surgical silk. The final wiring and fashioning of an acrylic head mound were not made until the demarcation of the ECOG, NEMG, and ECG II recording electrodes had been completed.

c. Neck muscle electromyogram (NEMG). NEMG electrodes were made of Type AS-634, Teflon-coated, multistranded Cooner stainless steel wires. The dorsal surface of the neck muscles were exposed by extending the skull incision slightly. A pair of NEMG electrodes was inserted into the dorsal portion of the cervical trapezius muscle. Another multistranded wire was sewn into an adjacent muscle to serve as a ground wire.

d. Lead II electrocardiogram (ECG II). Two subdermal tunnels, starting at the site of incision for DEMG electrode implant and ending at (i) the upper right chest area and (ii) the left groin were created. Subcutaneous ECG electrodes (Type AS-634 Cooner wires) were inserted to the designated areas in a standard Lead II configuration for the recording of ECG. Heart rate was also derived from ECG II recording.

e. Construction of the head mound. The DEMG, NEMG, and ECG II recording electrodes and ground wires were all routed rostrally through a subcutaneous tunnel to a matrix of connector strips over the skull. Dental cement was applied to finally complete the fashioning of the head mound. Animals were allowed to recover for at least 2 weeks before they were used for experimental purposes.

Experimental Category and Group Design

Each animal served as its own control and a control period of at least 5 min was recorded prior to administration of 4-AP (groups 1 and 2) or toxin
4-AP ANTAGONIZES STX/TTX-INDUCED TOXICITY

TABLE 1
Summary of Experimental Category and Group Design

<table>
<thead>
<tr>
<th>Group</th>
<th>Toxin (dose, im)</th>
<th>4-AP dose (im)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (n = 4)</td>
<td>—</td>
<td>1 mg/kg</td>
</tr>
<tr>
<td>2 (n = 4)</td>
<td>—</td>
<td>2 mg/kg</td>
</tr>
<tr>
<td>3 (n = 4)</td>
<td>STX (2 μg/kg)</td>
<td>—</td>
</tr>
<tr>
<td>4 (n = 4)</td>
<td>STX (3 μg/kg)</td>
<td>—</td>
</tr>
<tr>
<td>5 (n = 4)</td>
<td>TTX (2 μg/kg)</td>
<td>—</td>
</tr>
<tr>
<td>6 (n = 4)</td>
<td>TTX (3 μg/kg)</td>
<td>—</td>
</tr>
<tr>
<td>7 (n = 6)</td>
<td>STX (2 μg/kg)</td>
<td>1 mg/kg*</td>
</tr>
<tr>
<td>8 (n = 6)</td>
<td>STX (3 μg/kg)</td>
<td>1 mg/kg*</td>
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</tr>
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<td>2 mg/kg*</td>
</tr>
<tr>
<td>14 (n = 6)</td>
<td>TTX (3 μg/kg)</td>
<td>2 mg/kg*</td>
</tr>
</tbody>
</table>

* For groups 1 and 2 (Category I) and 7–14 (Category III), electrophysiological monitoring were continuously made for at least 4 hr after 4-AP. Four groups 3–6 (Category II), electrophysiological monitoring were made for at least 6 hours after STX or TTX intoxication.

** 4-AP (1 or 2 mg/kg, im) was administered 30 min posttoxin (the point where cardiorespiratory performance was most seriously compromised).

1. Category I (4-Aminopyridine Control, Groups 1 and 2)

   Effects of two doses of 4-AP (without toxin) were evaluated in group 1 (1 mg/kg, im; n = 4) and group 2 (2 mg/kg, im; n = 4) animals.

2. Category II (STX and TTX Control, Groups 3–6)

   Animals in this category were used to document the sublethal effects of STX and TTX. Groups 3 and 4 received 2 and 3 μg/kg STX (im, n = 4 per group), respectively. Groups 5 and 6 received 2 and 3 μg/kg TTX (im, n = 4 per group), respectively.

3. Category III (STX/TTX Intoxication and Treatment with 4-AP, Groups 7–14)

   Animals in this category were exposed to sublethal doses of toxin (either STX or TTX; 2 or 3 μg/kg, im) and treated with 4-AP (either 1 or 2 mg/kg, im) 30 min postintoxication. A total of 8 groups (groups 7–14: 2 toxins × 2 toxin doses × 2 4-AP doses; n = 6 per group) of animals were used in this category.

   Table 1 provides a summary of experimental category and group design.

Electrophysiological Recording Environment and Equipment

Upon recovery from surgery, each guinea pig was placed in, and acclimated to, a noise-attenuated Faraday chamber (30 cm in width and length, 40 cm in height). Aside from the minor restriction imposed by the signal transmission tether, the animal was free to move within the perimeter of the Faraday cage. ECoG, DEMG, and NEMG signals were amplified and band-pass filtered (DEMG and NEMG, 50–7500 Hz; ECoG, 0.5–500 Hz). ECGs were recorded by an ECG biotach (Gould Electronics, Greenbelt, MD). All signals were displayed on an oscilloscope and recorded with a 14-channel FM tape recorder (TEAC XR510WB; TEAC, Montebello, California). The animals' behaviors were documented with an audio-visual recording system which consisted of a video camera (Panasonic WV-6000), a video cassette recorder (Panasonic AG-1800), and a video monitor (Sony CVM-1271).

Toxin and Drug Solutions

Working toxin/drug solutions were freshly prepared minutes before use. Saline was used as diluent. Working STX solutions were made from stock solutions of STX (1 μmol/ml in 0.01 M acetic acid; Calbiochem). TTX stock solutions were prepared by dissolving lyophilized tetradotoxin (Calbiochem) in physiological saline (1 mg/ml; pH adjusted to 4–5 with 0.01 N HCl). The toxin (STX or TTX) dose levels were either 2 or 3 μg/kg (im). The 4-AP doses were either 1 or 2 mg/kg (im). The dose volume of all working solutions (toxin and 4-AP) was 0.2 ml/kg.

Data Analysis

Heart rate and respiratory rate data were normalized and expressed graphically as percentage of control ± standard error of mean (SEM). Unpaired t tests were performed to assess (i) the effect of 4-AP on heart rate and respiratory rate and (ii) the extent of restoration in heart rate and respiratory rate resulting from toxin-induced cardiorespiratory depression.

Analog-to-digital conversions and electrophysiological analyses of ECoG and DEMG signals were accomplished with a Brainwave Neurophysiological Data Analysis System (Experimenter's WorkBench and Discovery, Boulder, CO). Power spectral analyses (Childers, 1978) were performed to evaluate the extent of changes in power spectral attributes of ECoG and DEMG activities. ECoG data were sampled at a rate of 625 Hz for 32.768 sec (20 epochs, 1024 points/epoch). DEMG data were sampled at a rate of 10 KHz for 32.768 sec (20 epochs, 8192 points/epoch). “Zero Mean” was applied to both ECoG and DEMG data. The running sums of DEMG data were “cosine-tapered” before fast Fourier transform computation. DEMG spectra were smoothed with a 15-point polynomial filter.
RESULTS

Electrophysiological Effects of 4-Aminopyridine (Category I)

Figure 1 (A and B) is an electrophysiographic depiction of cardiorespiratory and ECoG responses to 4-AP (Fig. 1A, 1 mg/kg; Fig. 1B, 2 mg/kg). A quantitative summary of 4-AP induced changes in cardiorespiratory activity profile over a period of 4 hr is shown in Fig. 2.

1. Cardiorespiratory Effects of 4-AP

Respiratory frequency and heart rate both increased in response to either 1 mg/kg (group 1, n = 4) or 2 mg/kg (group 2, n = 4) 4-AP. Changes in respiratory frequency

FIG. 1. (A and B) Electrophysiograms depicting changes in cardiorespiratory and electrocorticographic (ECoG) activities in response to intramuscular 4-AP (Fig. 1A, group 1 animal receiving 1 mg/kg; Fig. 1B, group 2 animals receiving 2 mg/kg) across control condition, 15, 30, and 240 min after administration. Signal trace description: ECoG, electrocorticogram; NEMG, neck (skeletal) muscle electromyogram; DEMG, diaphragmatic electromyogram; Int DEMG, integrated (λ = 20 msec) diaphragmatic electromyogram; ECG, Lead II electrocardiogram. Voltage calibrations: ECoG, 210 μV; DEMG, 2.60 mV. Time calibration, 5 sec.
Effects of 4-AP on Cardiorespiratory Activities

![Graph showing effects of 4-AP on heart rate and respiratory rate](image)

FIG. 2. Changes (percentage of control) in mean heart rate (filled circle/solid line) and mean respiratory rate (open triangle/dash line) in response to 1 mg/kg (left, group 1) and 2 mg/kg 4-AP (right, group 2) across control condition and various time points (5, 15, 30, 60, 120, 180, and 240 min) after the administration of each dose. The actual control values of heart rate and respiratory rate for group 1 animals (1 mg/kg) are 276.5 ± 14 and 110 ± 3.3, respectively. The actual control values of heart rate and respiratory rate for group 2 animals (2 mg/kg) are 287.5 ± 10 and 110.5 ± 6.4, respectively. The asterisks (*) denote significant differences between control and 4-AP data, *p* < 0.001. Error bar = standard error of the mean (SEM).

appeared to be dose-dependent. The magnitude of increase in respiratory frequency was particularly notable following 2 mg/kg 4-AP. The increase in respiratory rate in response to either dose of 4-AP was maintained for about 2 hr before returning to a level comparable to that of control.

The magnitude of increase in heart rate after either 1 or 2 mg/kg 4-AP was less striking compared to changes in respiratory rate and exhibited no obvious signs of dose dependency. After an initial increase during the first 5 min, the heart rate tended to return to a level only slightly above control for as long as 4 hr (Fig. 2).

2. Diaphragmatic Responses to 4-AP

Power spectral analysis of DEMG activities was performed to assess the effect of 4-AP on the diaphragm. Results derived from a representative animal are shown in Fig. 3. Spectrographs indicated a small decline in the overall (0-2000 Hz) magnitude of DEMG spectral power following 1 mg/kg 4-AP (group 1; see Fig. 3A). The decrease in the magnitude of diaphragmatic contraction was probably a compensatory response to the elevated respiratory frequency and heart rate (see Fig. 2). With 2 mg/kg 4-AP (see Fig. 3B, group 2); however, the diaphragmatic spectral power consistently showed an increase which typically lasted for at least 1.5 hr before returning gradually to the control level. At about 240 min post-4-AP, the magnitude of diaphragmatic contraction typically showed a slight decrease as result of a sustained period of hyperpnea.

3. Secondary Effects of 4-AP

In addition to an elevated cardiorespiratory performance, 4-AP also appeared to cause secondary effects that could be characterized on the basis of both electrophysiological and behavioral response attributes. These included (i) a heightened state of arousal, (ii) occasional fascicular twitches of the skeletal muscles, (iii) an increase in behavioral activity level, and (iv) irritability (increased sensitivity to sensory stimuli).

The heightened state of arousal, or elevated cortical excitability, following 1 or 2 mg/kg 4-AP was indicated by an increase in the power of a 10- to 40-Hz ECoG power spectral complex (typical alerting ECoG activity profile) and a concomitant reduction in the power of low-frequency (0.5-10 Hz) spectral varieties (see Fig. 4). The heightened level of arousal generally lasted for more than 4 hr. Despite such profound changes in ECoG activity pattern, we found no electrophysiological or behavioral indications of seizures or convulsions in animals that received either 1 or 2 mg/kg 4-AP.

Fascicular twitches, changes in behavioral patterns, and increased irritability were observed primarily in animals that received 2 mg/kg 4-AP (group 2 animals). Fascicular twitches could be seen approximately 10-15 min after 4-AP. These aberrant neuromuscular effects were sporadic and lasted only about an hour. The behavioral activity level, which included longer than usual periods of exploratory behavior (such as sniffing and walking) and grooming, was also increased after intramuscular 4-AP. The increase in behavioral activities began approximately 10-15 min post-4-AP and disappeared entirely after 45 min to an hour. Animals that received 2 mg/kg 4-AP appeared extremely irritable and
Effect of 4-AP on DEMG Spectral Power

A. 4-AP Control (1 mg/kg, im)

B. 4-AP Control (2 mg/kg, im)

FIG. 3. Power spectrographs showing changes in the amplitude of diaphragmatic activity across control conditions and 15, 30, and 240 min after intramuscular administration of either 1 mg/kg (Fig. 3A, group 1) or 2 mg/kg (Fig. 3B, group 2) 4-AP.

were easily startled and displayed augmented sensitivities to sensory stimuli (visual, auditory, tactile, and olfactory).

Sublethal Effects of Saxitoxin and Tetrodotoxin (Category II)

Sixteen guinea pigs were used to study the sublethal effects of STX (2 and 3 μg/kg, im) and TTX (2 and 3 μg/kg, im). The experimental group design is summarized in Table 1. Parenthetically, no animal died as a result of exposure to sublethal doses of toxin.

The first indication of intoxication was a state of progressive cardiorespiratory depression which began to emerge 3–6 (3.45 ± 0.47) min following either STX or TTX (2 or 3 μg/kg). Figure 5 is an electrophysiographic depiction of cardiorespiratory and ECoG responses to STX (3 μg/kg, im; group 4) over a period of 6 hr. The most notable sublethal effects occurred during the “Depression” stage (i.e., 30 min posttoxin; see the top right panels; also refer to Figs. 7 and 8). These were (i) bradypnea, (ii) bradycardia, (iii) a marked decrease in the amplitude of diaphragmatic activity, and (iv) visual indications of cyanosis, especially in animals challenged with 3 μg/kg toxin (either STX or TTX). Generally speaking, the sublethal cardiorespiratory effects of STX and TTX were qualitatively very similar. One notable exception was the bradycardiac condition which appeared to be slightly more profound in STX than TTX intoxication.

Somewhat unexpected was the long duration of cardiorespiratory depression following exposure to sublethal STX or TTX. As shown in Fig. 5 (bottom right), the cardiorespiratory activity profile remained depressed 6 hr after the toxin (3 μg/kg, im). Without therapeutic intervention, STX or TTX-induced sublethal effects could continue for as long as 8–12 hr. Throughout the course of intoxication, the animals typically remained prostrate and inactive.

Reversal of Sublethal Effects with 4-Aminopyridine

Forty-eight animals were used to evaluate the effectiveness of 4-AP in reversing STX- and TTX-induced cardiorespiratory depression. These animals were assigned to 8 experimental groups (groups 7–14; n = 6 per group; see Table 1 for group design). Four groups were challenged with a low sublethal dose (2 μg/kg) of either STX (groups 7 and 9) or TTX (groups 11 and 13) and treated 30 min later with either 1 mg/kg 4-AP (groups 7 and 11) or 2 mg/kg 4-AP (groups 9 and 13). Another four groups were intoxicated with a higher sublethal toxin dose (3 μg/kg) of either STX (groups 8 and 10) or TTX (groups 12 and 14). For antagonism of sublethal effects produced by the high toxin dose (i.e., 3 μg/kg), animals were treated with either 1 mg/kg 4-AP (groups 8 and 12) or 2 mg/kg 4-AP (groups 10 and 14).

Cardiorespiratory and ECoG responses to sublethal STX intoxication (3 μg/kg, im) and recovery after 4-AP treatment (1 mg/kg) are depicted in Fig. 6. Results of other toxin and 4-AP dose combinations are summarized in a quantitative format in Figs. 7 and 8 (vide infra).

For clarity of presentation, we have classified toxin-induced cardiorespiratory changes throughout the course of intoxication and 4-AP treatment into control stage and three experimental stages. The experimental stages were (i) the
4-AP ANTAGONIZES STX/TTX-INDUCED TOXICITY

**Effect of 4-AP on ECoG Spectral Power**

![Power spectrographs showing changes in the amplitude of electrocorticographic activity across control condition and 15, 30, and 240 min after intramuscular administration of either 1 mg/kg (Fig. 4A, group 1) or 2 mg/kg (Fig. 4B, group 2) 4-AP. Note that 4-AP, particularly at a dose level of 2 mg/kg, caused an increase in the power of a 10-40 Hz ECoG power spectral complex which is indicative of a heightened level of cortical excitability and arousal.](image)

"Depression" stage, (ii) the "Post-4-AP" stage, and (iii) the "Convalescence" stage.

1. The Depression Stage (30 min Posttoxin)

The cardiorespiratory performance was most seriously compromised about 30 min posttoxin. The temporal course along which the Depression stage emerged (30 ± 1.04 min; range, 24.1–32.6 min) varied only slightly across animals, the type of toxin (STX or TTX) used, or the toxin dose levels (2 or 3 µg/kg). Thus, the 30-min time point was designated as the beginning of the Depression stage. Events leading to a state of cardiorespiratory depression were similar to those seen in Category II animals (i.e., intoxication without 4-AP treatment; *vide supra*). In brief summary, the first indications of intoxication began to appear 3–6 (3.40 ± 0.51) min after administration of 2 or 3 µg/kg of toxin (STX or TTX). 4-AP treatment was given at the beginning of the Depression stage (30 min posttoxin) since respiratory frequency, heart rate, and amplitude of diaphragmatic oscillation were depressed the most at the 30-min time point.

2. The Post-4-AP Stage (10 min after 4-AP)

The effect of 4-AP was striking in that the dysfunctional cardiorespiratory status (bradycardia, bradypnea, and reduced amplitude of diaphragmatic activity) began to show signs of improvement within 2–4 min after 4-AP. Between 5 and 10 min post-4-AP, the animals were able to resume what appeared to be their "normal" behavioral repertoire (such as grooming, walking, sniffing, eating). Thus, despite some variability in the animals' response to 4-AP, there were clear signs of recovery by the end of first 10 min after 4-AP administration. The cardiorespiratory activity profile continued to show improvement during the next 20–30 min. By the end of 60 min post-4-AP, all signs of toxin-induced cardiorespiratory infirmity had disappeared. At the dose levels used to achieve the treatment responses described above, 4-AP (1 or 2 mg/kg) produced no sign of seizures and convulsions. Less serious side effects such as cortical excitant/arousal and rare occasions of mild fascicular twitches could be observed during the 30- to 90-min period following 4-AP. These events were of minor concern in consideration of 4-AP's remarkable therapeutic benefits.

3. The Convalescence Stage (2 hr Post-4-AP)

During the Convalescence stage, there was no sign of intoxication nor any indication of behavioral abnormality. The respiratory frequency was still slightly above control level which may be attributable to the residual respiratory stimulant effect of 4-AP. Somewhat unexpected was a slightly less than 100% recovery in heart rate. Otherwise, the animals' cardiorespiratory activity profile appeared to be comparable to that of control. The functional integrity of the cardiorespiratory function and, in particular, the animal's ability to maintain an eupneic breathing pattern were under continued observation for at least 4 hr following 4-AP therapy. The post-4-AP observation periods in 50% of randomly
Sub-Lethal Effects of STX (3 μg/kg)

Control

ECoG
NEMG
DEMG
Int DEMG
ECG

Depression

2 Hours Post STX

ECoG
NEMG
DEMG
Int DEMG
ECG

6 Hours Post STX

FIG. 5. Electrophysiograms depicting the extent of cardiorespiratory infirmity resulting from sublethal exposure of 3 μg/kg STX across control conditions. Depression stage (=30 min post toxin), 2 and 6 hr postintoxication. Note the severely depressed diaphragm in terms of magnitude and burst rate (respiratory rate) of DEMG activity profile. Without 4-AP treatment intervention, the toxin-induced cardiorespiratory depression could last as long as 8–12 hr. Signal trace description: ECoG, electrocorticogram; NEMG, neck (skeletal) muscle electromyogram; DEMG, diaphragmatic electromyogram; Int DEMG, integrated (λ = 20 msec) diaphragmatic electromyogram; ECG, Lead II electrocardiogram. Voltage calibrations: ECoG, 250 μV; DEMG, 2.88 mV. Time calibration: 5 sec.

selected animals were extended to 8–12 hr. During the extended observation period, there was no sign to indicate the return of either cardiorespiratory toxicity or secondary effects of 4-AP. It should be noted that, without 4-AP treatment (groups 3–6; vide supra), STX/TTX-induced sublethal effects could last as long as 8–12 hr.

Cardiorespiratory Responses to STX/TTX Intoxication and 4-AP Treatment

The extent of STX and TTX-induced cardiorespiratory infirmity and the effectiveness of 4-AP in restoring the cardiorespiratory activity profile are summarized in Figs. 7 and 8.

1. Cardiorespiratory Effects of STX and TTX

The respiratory frequency (or respiratory rate) consistently showed a dose-dependent decrease in response to either STX or TTX (see Depression stage, Figs. 7 and 8, dotted lines). The heart rate also declined after STX or TTX. Upon careful examination, however, it was found that the heart rate decreased in a dose-dependent fashion only in response to STX, but not to TTX (cf. Figs. 7 and 8, solid lines). These findings suggested that the heart rate appeared to be less sensitive to perturbation by TTX.

2. Effectiveness of 4-AP Treatment

The most notable change during the Post-4-AP stage was an increase in respiratory frequency, which remained elevated throughout the Convalescence stage and beyond (at least 4–6 hr). The recovery of heart rate, in terms of magnitude and duration, was less pronounced by comparison. Even during the Convalescence stage (i.e., 2 hr post-4-AP), the average heart rate was still in the range of 80–90% of control. The cause of this phenomenon is unknown. A ganglionic involvement is likely because the mild “bradycardia” condition could be easily eliminated with pressor agents (epinephrine or metaraminol; unpublished observation).

3. Treatment for Low Sublethal Toxin Exposure

Although exposure to a low sublethal toxin dose (i.e., 2 μg/kg, STX or TTX) could produce a severe and lasting state of cardiorespiratory depression, only 1 mg/kg 4-AP was needed to restore respiratory frequency and heart rate to a level comparable to that of control condition (see in Figs. 7D and 8D). Raising the 4-AP dose level to 2 mg/kg did not appear to offer any further benefit (see B in Figs. 7 and 8). That is, the higher 4-AP dose only caused an additional increase in respiratory frequency (therapeutically inconsequential) with no apparent changes in heart rate.
4-AP ANTAGONIZES STX/TTX-INDUCED TOXICITY

STX (3 μg/kg) and 4-AP (1 mg/kg)

Control

Depression

Post 4-AP

Convalescence

FIG. 6. STX electrophysiograms showing the cardiorespiratory activity profiles during (i) control condition, (ii) intoxication (Depression stage, 30 min post toxin), (iii) Post-4-AP stage (10 min after 4-AP), and (iv) Convalescence stage (recovery stage, 2 hr after 4-AP). The effectiveness of 4-AP (1 mg/kg) against the sublethal effects of STX (3 μg/kg) is demonstrated. It should be mentioned that without 4-AP treatment, the toxin-induced cardiorespiratory depression could last more than 8–12 hr. Signal trace description: ECoG, electrocorticogram; NEMG, neck (skeletal) muscle electromyogram; DEMG, diaphragmatic electromyogram; Int DEMG, integrated (λ = 20 msec) diaphragmatic electromyogram; ECG, Lead II electrocardiogram. Voltage calibrations: ECoG, 225 μV; DEMG, 2.14 mV. Time calibration: 5 sec.

4. Treatment for High Sublethal Toxin Exposure

Treatment of cardiorespiratory depression following 3 μg/kg toxin (STX or TTX) with 1 mg/kg 4-AP did not appear to be adequate. As shown in Figs. 7 and 8 (C), respiratory frequency and heart rate could be restored to only 80–90% of control throughout the Post-4-AP stage and the Convalescence stage. Raising the 4-AP dose level to 2 mg/kg produced a further increase in respiratory frequency, whereas heart rate remained fundamentally unaltered (see Figs. 7A and 8A). It should be noted that in STX-intoxicated animals, the additional increase in respiratory frequency occurred within 2–4 min after 4-AP (i.e., consistently observable during Post-4-AP stage; see panel 7A). In TTX-intoxicated animals, however, 2 mg/kg 4-AP did not cause an additional increase in respiratory frequency until about 30–45 min following 4-AP treatment (see Fig. 8A). Parenthetically, because of our scheme of designation of experimental stages, the additional increase in respiratory rate in TTX-intoxicated animals could only be shown during the Convalescence stage (see Fig. 8A).

Diaphragmatic Responses to Intoxication and 4-AP Treatment

One of the most notable sublethal effects of STX and TTX was a progressive reduction in DEMG activity amplitude. DEMG amplitude typically began to show a decrease 5–10 min after toxin and reached its lowest level during the Depression stage (30 min post toxin). Figure 9 (A–D) is a power spectrographic depiction of changes in DEMG activities in response to intoxication (STX or TTX, 3 μg/kg) and 4-AP (2 mg/kg) treatment. The DEMG power spectrographs revealed that the reduction in the amplitude of DEMG activity during the Depression stage appeared to span the entire frequency spectrum (0–2000 Hz) and was not limited to any particular spectral components or spectral range. Intramuscular administration of 2 mg/kg 4-AP produced prompt (within 5 min) and increasingly robust diaphragmatic oscillations with virtually no loss in power spectral complements (see Fig. 9).

Electrocorticographic (ECoG) Responses to Toxin and 4-AP Treatment

Figure 10 is a power spectrographic depiction of 4-AP-induced ECoG changes in an STX- (Fig. 10, top) and a TTX-intoxicated animal (Fig. 10, bottom).

1. Effects of STX and TTX

Between 5 and 10 min after either STX or TTX (2 or 3 μg/kg), the animals became increasingly restless and aroused...
Reversal of STX-Induced Cardiorespiratory Effects By 4-AP

FIG. 7. (A–D) Changes in heart rate (filled circle/solid line) and respiratory rate (open triangle/dash line) in response to STX (2 or 3 μg/kg) and 4-AP treatment (1 or 2 mg/kg 4-AP) across (i) control condition, (ii) Depression stage (30 min post-STX), (iii) Post-4-AP stage (10 min after 4-AP), and (iv) Convalescence stage (recovery stage, 2 hr after 4-AP). STX and 4-AP dose levels are indicated at the lower right hand corner of each panel. Changes in cardiorespiratory activities are indicated as a percentage of the control value. The actual control values of (heart rate)/(respiratory rate) are A (group 10), 290.5 ± 7.7/108 ± 8.6; B (group 9), 280 ± 8.3/111.8 ± 7.8; C (group 8), 288.5 ± 6.7/106 ± 6.6; D (group 7), 296.5 ± 8.2/111 ± 6.3. Asterisks (*) denote significant changes (p < 0.001) in heart rate and respiratory rate across Depression and Convalescence stages. Error bar = Standard Error of the Mean (SEM). Error bar, standard error of the mean (SEM).

Reversal of TTX-Induced Cardiorespiratory Effects By 4-AP

FIG. 8. Changes in heart rate (filled circle/solid line) and respiratory rate (open triangle/dash line) in response to TTX (2 or 3 μg/kg) and 4-AP treatment (1 or 2 mg/kg 4-AP) across (i) control condition, (ii) Depression stage (30 min post-TTX), (iii) Post-4-AP stage (10 min after 4-AP), and (iv) Convalescence stage (recovery stage, 2 hr after 4-AP). TTX and 4-AP dose combinations are indicated at the lower right hand corner of each panel. Changes in cardiorespiratory activities are indicated as a percentage of control value. The actual control values of (heart rate)/(respiratory rate) are A (group 14), 289.8 ± 5.7/114 ± 4.6; B (group 13), 288 ± 8/109.3 ± 7.5; C (group 12), 289 ± 11/104 ± 6.1; D (group 11), 290.5 ± 8.4/105 ± 6.9. Differences in heart rate and respiratory rate across Depression and Convalescence stages were indicated by two p values (*p < 0.001, *p < 0.05). Error bar, standard error of the mean (SEM). p < 0.001.
DEMGG Responses To 4-AP Treatment

A. Intoxication: STX, 3 μg/kg. Treatment: 4-AP, 2 mg/kg.

B. Intoxication: TTX, 3 μg/kg. Treatment: 4-AP, 2 mg/kg.

FIG. 9. Power spectrograms showing the extent of change in the amplitude of diaphragmatic electromyographic (DEMG) activity in response to 3 μg/kg toxin (STX, top; TTX, bottom) and 4-AP treatment (2 mg/kg) across control, Depression, Post-4-AP, and Convalescence stages.

by the cardiorespiratory effects of toxin which intensified over time. Concurrent with these events was the transformation of a wakefulness, resting ECoG pattern to an "arousal" profile characterized by a reduction in the amplitude of a low frequency (0.5–10 Hz) power spectral complex and an increase in the amplitude of a 5–30 Hz ECoG power spectral complex (see Depression stage, Fig. 10).

2. Effects of 4-AP Treatment

The only notable change in the ECoG power spectral profile which could be attributable to the pharmacological actions of 4-AP was a further increase in the amplitude of an asynchronous power spectral complex (≈5–30 and ≈5–40 Hz) indicative of a heightened state of cortical excitability (see Post-4-AP stage, Fig. 10). The amplitude of this spectral complex typically became smaller during the Convalescence stage with a concomitant reappearance of a low frequency (0.5–10 Hz) spectral waveform variety.

DISCUSSION

The sublethal toxicity of STX and TTX, as measured by the intensity and duration of cardiorespiratory infirmity, was considerably more severe than we first thought. As shown in Fig. 5, 3 μg/kg STX could produce a state of profound cardiorespiratory depression which lasted as long as 8–12 hr. Equally surprising was the remarkable effectiveness of 4-AP in reversing, within minutes, various toxin-induced cardiorespiratory dysfunctions such as bradycardia, bradypnea and reduced amplitude of diaphragmatic activity.

Precisely how and where 4-AP acts to restore STX- and TTX-induced cardiorespiratory infirmity is not completely understood. Nonetheless, much of 4-AP’s therapeutic actions may be attributed to its stimulant effects on the cardiorespiratory system.

Pharmacology of 4-Aminopyridine (4-AP)

4-AP, more commonly recognized as a potassium channel blocker, is best known for its ability to enhance the impulse-evoked acetylcholine release from presynaptic motor nerve terminals in phrenic-diaphragm preparations and other nerve–muscle model systems (Lundh, 1978; Lundh et al., 1990; Molgo et al., 1977). The cellular mechanism through which 4-AP facilitates neuromuscular transmission is generally believed to be a corollary of potassium channel blockade at an intracellular site (Kirsch et al., 1993; for review, see Glover, 1982). The blockade of potassium channels results in a profound alteration in the voltage-dependence of calcium channel activation/inactivation kinetics which allows a greater than normal calcium influx into the presynaptic terminals and ultimately causes an enhanced level of acetylcholine release from the motor nerve terminals. In addition to augmenting neuromuscular transmission, 4-AP is also known to have a respiratory stimulant effect (Folgering et al., 1979), a positive inotropic effect (Yanagisawa and Taira, 1979; Glover, 1981), and a CNS excitant effect (Rutecki et al., 1987).

Because of its cardiorespiratory stimulant effect, we began to investigate the possibility of using 4-AP in antagonizing STX- and TTX-induced cardiorespiratory depression and le-
FIG. 10. Power spectrographs showing changes in the amplitude of electrocorticographic (ECoG) activity in response to 3 μg/kg toxin (STX, top; TTX, bottom) and 4-AP treatment (2 mg/kg) across control, Depression, Post-4-AP, and Convalescence stages. Note that 4-AP (2 mg/kg) can produce an electrocorticographic activity profile indicative of enhanced cortical excitability even in the presence of toxin.

thality. As demonstrated in this study, and by findings from a series of earlier investigations (Benton et al., 1995; Chang et al., 1996) in this laboratory, we have shown that the pharmacological attributes of 4-AP, in particular, its cardiorespiratory stimulant action, could be exploited in clinical management of STX- and TTX-induced cardiorespiratory infirmity.

Effective Dose Level

The effectiveness of 2 mg/kg 4-AP was unequivocal. As illustrated in Figs. 7 and 8, cardiorespiratory infirmity resulting from STX or TTX intoxication (either 2 or 3 μg/kg) could be restored to a level either comparable to, or surpassing, that of control condition in less than 10 min (i.e., before the end of the Post-4-AP stage). The effectiveness of 1 mg/kg 4-AP was somewhat ambivalent. That is, while 1 mg/kg 4-AP appeared adequate for managing the toxic effects resulting from low-level intoxication (i.e., 2 μg/kg STX or TTX), it was uncertain whether this dose level was adequate for treating animals intoxicated with 3 μg/kg toxin. More specifically, as Figs. 7C and 8C showed that, in animals intoxicated with 3 μg/kg toxin (STX or TTX), respiratory frequency and heart rate were restored to approximately 80% of control during the Post-4-AP stage (10 min post-4-AP) and increased only slightly (80–90% of control) during the Convalescence stage (2 hr post-4-AP). Since side effects resulting from 2 mg/kg 4-AP were minor (see Figs. 1–4), and in view of the fact that it would be difficult, if not impossible, to assess the severity of sublethal intoxication in actual cases of poisoning in humans, a higher 4-AP dose (human equivalent of 2 mg/kg) would probably be a more prudent pharmacological means of treating the sublethal effects of STX or TTX.

Secondary Effects of 4-AP

We were unable to see any undesirable effects in animals treated with 1 mg/kg 4-AP. In animals treated with 2 mg/kg 4-AP, the side effects were limited to only occasional fascicular twitches and electrocorticographic changes indicative of a heightened state of cortical excitability. Finally, no serious complications such as seizures and convulsions were observed in response to either 1 or 2 mg/kg 4-AP.

The Potential Therapeutic Role of 4-Aminopyridine

More investigations are needed before the precise role of 4-AP in clinical management of paralytic shellfish poisoning can be defined. 4-AP is a potent potassium channel blocker (Pelhate and Pichon, 1974; Yeh et al., 1976). Because of the ubiquitous presence of potassium channels in excitable tissues, it would be difficult, if not impossible, to selectively target 4-AP's pharmacological actions on any particular site or mechanism of interest. Another important consideration is 4-AP's seizurogenic and convulsant potential due to the ease with which it can penetrate the "blood–brain barrier" (see Glover, 1982). While still under investigation, we have sufficient data to conclude that, at higher dose levels (viz., >4 mg/kg), not only can 4-AP cause severe seizures and
convulsions, but it also becomes quite lethal. At first glance, these considerations seem to make 4-AP less appealing as a potential therapeutic compound for STX/TTX poisoning. Mindful of its untoward side effects, we believe, for the reasons to follow, that 4-AP could still be of considerable therapeutic value in the treatment of paralytic shellfish poisoning.

First, there is little doubt that site- and mechanism-specific antibodies or toxin-binding proteins would be ideally suited for treating paralytic shellfish poisoning. However, it is known that STX and TTX, as well as many analogs of STX and TTX, do coexist in many marine species (Yasumoto et al., 1986). Thus, in actual paralytic shellfish poisoning, the immunopharmacological agents could not possibly recognize all the toxic components (or toxin analogs) within the poison. Other considerations, such as availability, shelf-life, and cost of these agents, could also significantly discourage their use, particularly in economically disadvantaged locations. Second, the life-threatening aspects of paralytic shellfish poisoning could very well demand aggressive therapeutic measures even at the expense of serious untoward side effects such as seizures and convulsions. However, side effects such as muscle fasciculation, seizures, and convulsions induced by 4-AP are a dose-dependent phenomenon. Thus, as long as 4-AP and its dose levels are administered with prudence, the severity of these side effects could be greatly reduced. Finally, adjuncts to 4-AP therapy that will either optimize its therapeutic effectiveness or reduce the severity of side effects should also be taken into consideration in the process of defining the therapeutic role of 4-AP.

**Conclusion**

In this report, we have described the extent of cardiorespiratory dysfunctions resulting from sublethal exposure to STX or TTX and the degree to which each dysfunctional variable could be reversed by a potassium channel blocker, 4-AP. The optimal dose level necessary to achieve the desired therapeutic responses, in our opinion, is 2 mg/kg (im). At this dose level, 4-AP produced no sign of seizures or convulsions. Although side effects such as electrocortico-graphic indications of a heightened state of arousal and short periods of fascicular twitches could be observed, these events, however disquieting, seemed to be trivial vis-à-vis the remarkable symptomolytic effects of 4-AP.

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**REFERENCES**


