Studies on curare-like action of 2,2',2''-tripyridine in the mouse phrenic nerve-diaphragm

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1 The curare-like action of 2,2',2''-tripyridine (a synthetic by-product of the herbicide, paraquat) was studied in mouse phrenic nerve-diaphragm preparation. The inhibition by 2,2',2''-tripyridine of nerve-evoked twitches was dependent on the concentration, ranging from 1 to 100 μM , which had no significant effect on the twitch amplitudes evoked by direct muscle stimulation.

2 The twitch inhibition by 2,2',2''-tripyridine was reversible and could be antagonized by anticholinesterase agents such as neostigmine, physostigmine as well as ecothiophate.

3 Pretreatment with either 0.7 μM (+)-tubocurarine or 2.2 μM succinylcholine shifted the concentration-inhibition curve of 2,2',2''-tripyridine to the left.

4 2,2',2''-Tripyridine inhibited not only acetylcholine-induced contracture of the denervated mouse diaphragm but also that of the chick biventer cervicis muscle. Like (+)-tubocurarine, 2,2',2''-tripyridine protected the twitches from the inhibition by α -bungarotoxin and also specifically inhibited the binding of [¹²⁵I]- α -bungarotoxin to the mouse diaphragm. All of these findings indicate that 2,2',2''-tripyridine possesses curare-like action and inhibits the muscle contractions through binding to postsynaptic acetylcholine receptors.

5 The postsynaptic inhibition exhibited by 2,2',2''-tripyridine was also implicated in the tetanic fade, a decrease in the amplitude of miniature endplate potential (m.e.p.p.) and endplate potential (e.p.p.).

6 The clinical implication of these findings is that 2,2',2''-tripyridine may be involved in the cause of respiratory failure in paraquat-intoxicated workers since 2,2',2''-tripyridine is a by-product of paraquat synthesis.

Keywords: 2,2',2''-Tripyridine; curare-like action; α -bungarotoxin binding; nicotinic acetylcholine receptor

Introduction

2,2',2''-Tripyridine is a pyridine derivative (Figure 1) which has been found to be a synthetic by-product of the herbicide paraquat. Epidemiological studies showed that the incidence of skin cancer of paraquat manufacturers was much higher than that of the general population in Taiwan (Wang *et al.*, 1987). We have identified 2,2',2''-tripyridine in the crude products of paraquat and found it to be a strong mutagen (Kuo *et al.*, 1986; Lin *et al.*, 1988). In addition, 2,2',2''-tripyridine was more potent than paraquat in producing carcinogenic action and DNA damage (Jennette *et al.*, 1974; Lin *et al.*, 1988). Although 2,2',2''-tripyridine has long been known to be a metal chelating agent derived from 1,10-phenanthroline (Howe-Grant *et al.*, 1976), the pharmacological actions of 2,2',2''-tripyridine have not been well characterized.

In this paper, we have investigated the effects of 2,2',2''-tripyridine on muscle contraction and transmitter release in the mouse phrenic nerve-diaphragm preparation. The results show that 2,2',2''-tripyridine is a specific antagonist of nicotinic acetylcholine receptors comparable to (+)-tubocurarine. The findings suggest the possible involvement of 2,2',2''-tripyridine in the respiratory failure of paraquat-intoxicated workers.

Methods

Mouse phrenic nerve-diaphragm preparation

Phrenic nerve-hemidiaphragm preparations were isolated from ICR strain mice (20–30 g) according to the method of Bül-

bring (1946). The muscle preparation was suspended in 10 ml of modified Krebs solution (composition in mM: NaCl 130.6, KCl 4.8, MgSO₄ 1.2, NaHCO₃ 12.5, CaCl₂ 2.5 and glucose 11.1) maintained at $37.0 \pm 0.5^\circ\text{C}$ and oxygenated with 95% O₂ and 5% CO₂. The phrenic nerve was stimulated with 0.05 ms rectangular pulses at the frequencies indicated. Muscle contractions were recorded via an isometric transducer (Grass FT.03) on a Grass Model 7 polygraph.

Chick biventer cervicis nerve-muscle preparations

Chicks aged 3–5 days were used in all experiments. The biventer cervicis nerve-muscle preparation was isolated according to the method of Ginsborg & Warriner (1960). The muscle was suspended in 10 ml of modified Krebs solution which was constantly gassed with 95% O₂ + 5% CO₂ at $37.0 \pm 0.5^\circ\text{C}$. The nerve was stimulated through the tendon with supramaximal rectangular pulses of 0.05 ms at a rate of 0.2 Hz. The contraction of the muscle was recorded isometrically with a force-displacement transducer (Grass FT.03) on a Grass Model 7 polygraph.

Intracellular recordings

Conventional microelectrode recording techniques (Fatt & Katz, 1951) were used. Glass microelectrodes filled with 3 M

Figure 1 Chemical structure of 2,2',2''-tripyridine.

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KCl had resistances in the range of 3–10 M Ω . The mouse diaphragm was placed in modified Krebs solution at $37.0 \pm 0.5^\circ\text{C}$ and oxygenated with 95% O₂ + 5% CO₂. A high impedance amplifier (WPI) and Hitachi V-352 oscilloscope were used for recordings. The amplitude and frequency of miniature endplate potentials (m.e.p.ps) were measured by conventional techniques and recorded with a Data 6100 waveform analyzer. The amplitude of endplate potentials (e.p.ps), elicited at 1 Hz was determined in a modified Krebs solution containing 0.3 mM Ca²⁺ and 1.4–2.2 mM Mg²⁺.

[¹²⁵I]- α -bungarotoxin binding studies

The binding of [¹²⁵I]- α -bungarotoxin was studied in the mouse diaphragm preparations. The preparations were incubated with [¹²⁵I]- α -bungarotoxin at $37.0 \pm 0.5^\circ\text{C}$ for 3.5 h in the presence or absence of various concentrations of 2,2',2''-tripyridine. It was then washed repeatedly with 5 ml modified Krebs solutions for 5 min duration, and with three changes of bathing solution. The treated diaphragms were trimmed, blotted with filter paper and weighed. The level of radioactivity was measured on a gamma counter (LKB 1282).

Drugs

α -Bungarotoxin was isolated from the venom of *Bungarus multicinctus* by the method described by Lee *et al.* (1972). The homogeneity of the purified toxins was verified by disc gel electrophoresis (David, 1964).

All of the chemical compounds listed above for the test experiments were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A. [¹²⁵I]- α -bungarotoxin was obtained from Amersham, Buckinghamshire, England.

Statistics analysis

Results are given as mean $\pm$ s.e.means. Numbers of experiments are indicated by *n*. The significance of difference was evaluated by Student's *t* test. When more than one group was compared with one control, significance was evaluated according to ANOVA. Probability values (*P*) of less than 0.05 were considered to be significant.

Results

Twitch inhibition of mouse diaphragm induced by 2,2',2''-tripyridine

2,2',2''-Tripyridine inhibited the nerve evoked twitches of the mouse diaphragm but had no effect on the twitches evoked by direct muscle stimulations (Figure 2). Anticholinesterase agents such as 0.3 μM neostigmine, 0.4 μM physostigmine and 0.5 μM ecothiophate all completely reversed the twitch inhibition by 2,2',2''-tripyridine (Figure 2b). As shown in Figure 3, the concentration-inhibition curve of 2,2',2''-tripyridine was parallel to those of (+)-tubocurarine and succinylcholine respectively. From these curves, the concentrations of (+)-tubocurarine, succinylcholine and 2,2',2''-tripyridine required for 50% inhibition were estimated to be 0.7, 13 and 48 μM respectively. Pretreatment with a low concentration of either 0.7 μM (+)-tubocurarine or 2.2 μM succinylcholine shifted the concentration-inhibition curve of 2,2',2''-tripyridine to the left in a parallel manner (Figure 4).

Antagonistic action of 2,2',2''-tripyridine on nicotinic acetylcholine receptors

As shown on Figures 5a and b, 2,2',2''-tripyridine inhibited acetylcholine contracture of denervated mouse diaphragm and of chick biventer cervicis muscle in a manner similar to (+)-tubocurarine. The concentration-contracture curves for acetylcholine were shifted in a parallel fashion to the right by

Figure 2 Effects of 2,2',2''-tripyridine and (+)-tubocurarine on twitch responses of mouse diaphragm. (a) Twitch responses to the nerve stimulation were partially inhibited by treatment with 2,2',2''-tripyridine (T, 43 μM), while the twitches to direct muscle stimulation remained unchanged after prolonged incubation of 120 min. The twitches blocked by 2,2',2''-tripyridine could be completely recovered either by washout (W) with modified Krebs solution (a) or (b) by neostigmine (Neo, b). (+)-Tubocurarine ((+)-TC, 4.2 μM) reversibly inhibited the twitches in response to the nerve stimulations (c).

Figure 3 Concentration-dependent inhibition of nerve evoked twitches of the mouse diaphragm induced by 2,2',2''-tripyridine ($\circ$, $n = 6-8$), (+)-tubocurarine (Δ , $n = 6-8$) and succinylcholine ($\bullet$, $n = 4-6$). The nerve-muscle preparation was suspended in 10 ml of modified Krebs solution and stimulated indirectly. The twitch amplitude was measured 60 min after the addition of various concentrations of inhibitors and calculated as a percentage of the control twitches.

2,2',2''-tripyridine and (+)-tubocurarine (Figure 5b). Furthermore, both 2,2',2''-tripyridine and (+)-tubocurarine protected the muscle contractions of mouse diaphragm from the inhibitory action of α -bungarotoxin (Figure 6). As shown in Table 1, α -bungarotoxin alone inhibited the twitch amplitude by $5.2 \pm 2.8\%$, $23.6 \pm 3.7\%$, $42.8 \pm 2.3\%$ and 100% at about 16, 30, 40 and 74 min respectively after the application of the toxin. However, pretreatment with either 22 μM and 43 μM 2,2',2''-tripyridine or 1 μM (+)-tubocurarine enhanced twitch blockade induced by α -bungarotoxin; the time required for complete blockade was found to be 41.7 ± 6.5 min, 33.2 ± 3.7 min and 16.3 ± 3.7 min respectively after addition of the toxin. Although the twitches recovered almost completely by washout immediately after the complete twitch blockade (% twitch restored in Table 1) at the above indicated time, it was considered to be due to an incomplete blockade by α -bungarotoxin. Therefore, some mouse diaphragm preparations

Figure 4 Enhancement by (+)-tubocurarine and succinylcholine of the twitch-inhibitory action of 2,2',2''-tripyridine in the mouse diaphragm. The nerve-muscle preparation was suspended in 10 ml of modified Krebs solution and stimulated indirectly at 0.2 Hz. The preparations were pretreated for 30 min in order to reach a steady twitch amplitude with either 0.7 μM (+)-tubocurarine (Δ , $n = 4-6$) or 2.2 μM succinylcholine ($\bullet$, $n = 4-6$) prior to the addition of 2,2',2''-tripyridine. Twitch-inhibition by 2,2',2''-tripyridine was estimated as a percentage of the respective control prior to 2,2',2''-tripyridine. 2,2',2''-Tripyridine alone ($\circ$, $n = 6-8$).

Figure 5 Inhibition by 2,2',2''-tripyridine and (+)-tubocurarine on the dose-response curve of acetylcholine-contraction of denervated mouse diaphragm (a) and the chick biventer cervicis muscle (b). 2,2',2''-Tripyridine ($\circ$, $n = 4-6$) and (+)-tubocurarine (Δ , $n = 4-6$) at various concentrations were applied to denervated mouse diaphragm for 30 min. The acetylcholine (0.1 mM)-induced contracture was then recorded and calculated as a percentage inhibition of the contracture prior to the addition of 43 μM 2,2',2''-tripyridine or 7 μM (+)-tubocurarine.

undergoing similar treatments were left in contact with the toxin plus either 2,2',2''-tripyridine or (+)-tubocurarine for 75 min after the application of the toxin (delay twitch recovery as indicated on Table 1). It was clearly shown that the blockade by α -bungarotoxin alone could not be reversed

Figure 6 Protection by (+)-tubocurarine and 2,2',2''-tripyridine from the irreversible inhibition on twitches of mouse diaphragm induced by α -bungarotoxin. The phrenic nerve of mouse diaphragm was electrically stimulated and the contraction of the diaphragm was recorded isometrically. The irreversible twitch inhibition was produced by α -bungarotoxin (α -BuTX, 0.125 μM), (a). Pretreatment with either (+)-tubocurarine (b) or 2,2',2''-tripyridine (c) completely restored the twitches inhibited by α -BuTX even when washout is carried out 30 min after complete twitch-inhibition, (d). The reversal of twitch-inhibition is also exhibited by the application of 2,2',2''-tripyridine 20 min after α -bungarotoxin, (e). W denotes washout with modified Krebs solution.

by washout but pretreatment with either 2,2',2''-tripyridine or (+)-tubocurarine allowed delayed twitch recovery by about 40% and 62% respectively (Table 1). The most prominent effect of 2,2',2''-tripyridine was to inhibit the binding of [^{125}I]- α -bungarotoxin to the mouse diaphragm. The concentration of 2,2',2''-tripyridine required for 50% inhibition on the binding of [^{125}I]- α -bungarotoxin was 48 μM which was comparable to that for 50% inhibition of nerve-evoked twitches (Figure 7).

Effects of 2,2',2''-tripyridine on tetanic contraction, m.e.p.ps and e.p.ps

As shown on Figure 8a, 2,2',2''-tripyridine progressively inhibited the tetanic contractions. By contrast, the control tetanic contractions remained sustained without fading. (+)-Tubocurarine produced a similar tetanic fade (Figure 8b). On the other hand, 2,2',2''-tripyridine inhibited the single twitches dependent on the frequency of electrical stimulations; the higher the stimulation frequency was, the greater the inhibition that was produced (Figure 9). Moreover, 2,2',2''-tripyridine reduced the frequency and amplitude of miniature endplate potentials (m.e.p.ps) and endplate potentials (e.p.ps), but did not change the resting membrane potential (Table 2).

Discussion

In this paper, we have shown that 2,2',2''-tripyridine preferentially inhibited the nerve-evoked twitches of the mouse diaphragm and left the muscle-evoked twitches unaffected, indicating that 2,2',2''-tripyridine might either inhibit acetylcholine release from motor nerve terminals or antagonize the postsynaptic acetylcholine receptors. Evidence obtained from this study suggests that 2,2',2''-tripyridine pos-

Table 1 Twitch inhibition by α -bungarotoxin in mouse diaphragm treated with either 2,2',2''-tripyridine or (+)-tubocurarine

Treatment	Conc. (μ M)	Time to twitch block (min)	Twitch restored (%)	Delayed twitch recovery (%)
α -Bungarotoxin	0.125	74.1 $\pm$ 3.6 (48)	—	0
α -Bungarotoxin + 2,2',2''-Tripyridine	0.125			
	22	41.7 $\pm$ 6.5* (21)	94.2 $\pm$ 2.3* (10)	38.8 $\pm$ 4.7* (11)
	43	33.2 $\pm$ 3.7* (38)	97.2 $\pm$ 6.5* (28)	39.2 $\pm$ 4.1* (10)
α -Bungarotoxin + (+)-tubocurarine	0.125			
	1	16.3 $\pm$ 3.7* (8)	98.2 $\pm$ 3.4* (4)	62.3 $\pm$ 6.7* (4)

Data are presented as means $\pm$ s.e.means.

The twitches of the mouse diaphragm in the modified Krebs solution were evoked by electrical stimulations of the phrenic nerve. 2,2',2''-Tripyridine (43 μ M, n = 34) and (+)-tubocurarine (1 μ M, n = 6) alone did not produce neuromuscular blockade after 3 h of treatment and the partial twitch inhibition is reversible after washout. 'Twitch restored' and 'Delayed twitch recovery' are defined as the recovery of twitches by washout of the toxins immediately or 30–60 min after the neuromuscular blockade produced by α -bungarotoxin in the presence or absence of either 2,2',2''-tripyridine or (+)-tubocurarine.

The numbers in parentheses are the number of experiments.

* P < 0.05 as compared with control.

Figure 7 Concentration-inhibition curve of 2,2',2''-tripyridine on [125 I]- α -bungarotoxin binding to the mouse diaphragm. Data are presented as mean $\pm$ s.e.means (vertical bars, n = 6–8). The mouse diaphragm was incubated with [125 I]- α -bungarotoxin plus various concentrations of 2,2',2''-tripyridine at $37 \pm 0.5^\circ\text{C}$ for 3.5 h. The inhibition was calculated as a percentage of the specific binding of [125 I]- α -bungarotoxin in the absence of 2,2',2''-tripyridine.

sesses a curare-like action: 2,2',2''-tripyridine directly inhibited acetylcholine-contraction of the denervated mouse diaphragm and chick biventer cervicis muscle. Like (+)-tubocurarine, 2,2',2''-tripyridine shifted the concentration-contraction curve of acetylcholine to the right in a parallel manner. Pretreatment with a low concentration of either 0.7 μ M (+)-tubocurarine or 2.2 μ M succinylcholine potentiated the inhibitory action of 2,2',2''-tripyridine on the nerve-evoked twitches and also shifted its concentration-inhibition to the left in a parallel manner. Based on these findings together with the fact that both (+)-tubocurarine and succinylcholine are known to be an antagonist and an agonist respectively of the nicotinic acetylcholine receptors (Paton & Zaimis, 1952; Liley & North, 1953), it is proposed that 2,2',2''-tripyridine is a competitive inhibitor of nicotinic acetylcholine receptors.

α -Bungarotoxin which was isolated from the venom of *Bungarus multicinctus* is known to be a specific irreversible antagonist of nicotinic acetylcholine receptors (Lee & Chang, 1966). Pretreatment with (+)-tubocurarine protected the nicotinic acetylcholine receptors from the binding of α -bungarotoxin (Miledi *et al.*, 1971). Similarly, we have demonstrated that pretreatment of the mouse diaphragm with 2,2',2''-tripyridine and then subsequent application of α -

Figure 8 Inhibition by 2,2',2''-tripyridine and (+)-tubocurarine of the tetanic contraction of the mouse diaphragm. The phrenic nerve of the mouse diaphragm was stimulated (50 Hz, 1 s pulse duration) every 30 s in modified Krebs solution. The five successive panels show the tetanic responses of control (C) and those treated with either 43 μ M 2,2',2''-tripyridine (a) or 1 μ M, (+)-tubocurarine (b) for various time intervals.

bungarotoxin reversibly blocks the twitches which were resumed after washout, while the paired control treated with α -bungarotoxin alone irreversibly blocked twitches. This finding indicates that the occupation of 2,2',2''-tripyridine on nicotinic acetylcholine receptors excluded the binding of α -bungarotoxin and the subsequent washout of the reversible binding of 2,2',2''-tripyridine allowed muscle contractions to recover. The reversal effect of 2,2',2''-tripyridine could also be shown even when 2,2',2''-tripyridine was applied 20 min after α -bungarotoxin. More direct evidence was obtained by the

Figure 9 Frequency-dependent inhibition by 2,2',2''-tripyridine of twitch amplitude of mouse diaphragm. The mouse phrenic nerve-diaphragm preparation was suspended in 10 ml of modified Krebs solution and stimulated indirectly on the nerve. Note that the higher the stimulation frequency, the greater was the inhibition by 2,2',2''-tripyridine (43 μ M) ($n = 4-6$).

competitive inhibitory action of 2,2',2''-tripyridine on specific binding of radioactive [125 I]- α -bungarotoxin. The concentration (48 μ M) of 2,2',2''-tripyridine required for 50% inhibition of the binding of [125 I]- α -bungarotoxin was coincidentally similar to that for 50% inhibition of nerve-evoked twitches as well as that of acetylcholine contractures respectively. All of these results support the theory that the neuromuscular blocking action of 2,2',2''-tripyridine was mainly due to the antagonistic action on nicotinic acetylcholine receptors.

The chemical structures of an antagonist required for binding to nicotinic acetylcholine receptors were claimed to be a mono-quaternary or bistertiary amines (Kitz *et al.*, 1969; Kharkevich & Skoldinov, 1980). The mono-quaternary compounds were claimed to have less stable bonds with acetylcholine receptors of skeletal muscle than the bistertiary compounds. 2,2',2''-Tripyridine possesses three tertiary nitrogen atoms in the pyridine rings so that the bonds between nicotinic acetylcholine receptors and 2,2',2''-tripyridine may

be less stable than compounds (e.g. (+)-tubocurarine, succinylcholine) with one or two quaternary nitrogen atoms. The fact that IC_{50} values (concentrations for 50% inhibition on the twitch amplitude) of (+)-tubocurarine, succinylcholine and 2,2',2''-tripyridine were 0.7, 13 and 48 μ M respectively supports the contention of a weaker but specific competitive inhibitor of 2,2',2''-tripyridine on nicotinic acetylcholine receptors.

It has been reported that nicotinic acetylcholine receptors were also present on the motor nerve terminals (Miyamoto, 1978; Standaert, 1982). The presynaptic acetylcholine receptors were claimed to function as modulators of acetylcholine release from the motor nerve terminals (Bowman, 1980; Bowman *et al.*, 1986; Chang & Hong, 1986). Binding of (+)-tubocurarine to the presynaptic receptors decreased the release of acetylcholine (Wilson, 1982; Chang & Hong, 1986). In this paper, we have found that 2,2',2''-tripyridine decreased not only the frequency and amplitude of m.e.p.ps but also the amplitude of e.p.ps. It was considered that 2,2',2''-tripyridine probably inhibited the presynaptic nicotinic acetylcholine receptors which resulted in the reduction of acetylcholine release. The tetanic fade phenomenon as well as stimulation-frequency dependent inhibition on twitches also implied that 2,2',2''-tripyridine might exert effects on the presynaptic sites. A similar conclusion was reached by Bowman (1980) and Bowman *et al.* (1986) for the presynaptic action of (+)-tubocurarine. This possibility of the presynaptic action of 2,2',2''-tripyridine merits further study.

In conclusion, 2,2',2''-tripyridine inhibited the nerve-evoked twitches of the mouse diaphragm through a curare-like action on postsynaptic nicotinic acetylcholine receptors. This inhibitory action of 2,2',2''-tripyridine on the diaphragm contraction may contribute to the respiratory failure of paraquat intoxicated workers since 2,2',2''-tripyridine was proved to be a synthetic by-product of paraquat from the condensation of a pyridine moiety.

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Table 2 Decrease in amplitude of miniature endplate potentials (m.e.p.ps) and endplate potentials (e.p.ps) of the mouse diaphragm induced by 2,2',2''-tripyridine or (+)-tubocurarine

Treatment	Conc. (μ M)	M.e.p.ps		E.p.ps	
		Frequency (event/s $^{-1}$)	Amplitude (mV)	Amplitude (mV)	RMP -mV)
Control		1.21 $\pm$ 0.7	1.21 $\pm$ 0.5	4.28 $\pm$ 0.41	77.8 $\pm$ 4.9
2,2',2''-Tripyridine	4.3	0.99 $\pm$ 0.08*	0.75 $\pm$ 0.04*	2.78 $\pm$ 0.19*	76.5 $\pm$ 3.7
	22	0.74 $\pm$ 0.06*	0.59 $\pm$ 0.03*	1.42 $\pm$ 0.37*	74.8 $\pm$ 3.7
	43	0.49 $\pm$ 0.08*	0.47 $\pm$ 0.03*	0.73 $\pm$ 0.28*	76.8 $\pm$ 4.2
(+)-Tubocurarine	0.28	1.20 $\pm$ 0.03	0.84 $\pm$ 0.07*	3.21 $\pm$ 0.13*	75.2 $\pm$ 3.7

Data are presented as mean $\pm$ s.e. means obtained from 4 preparations, for each preparation: 6-8 endplates were studied.

2,2',2''-Tripyridine and (+)-tubocurarine were added cumulatively and the incubation time was more than 20 min for each concentration added.

The endplate potentials (e.p.ps) were evoked by 1 Hz nerve stimulation on the mouse diaphragm in 0.3 mM Ca^{2+} and 1.4-2.2 mM Mg^{2+} modified Krebs solution.

* $P < 0.05$ as compared with control.

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