Br. J. Pharmacol. (1993), **110**, 163–168

Blockade by 2,2′,2″-tripyridine of the nicotinic acetylcholine receptor channels in embryonic *Xenopus* muscle cells

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1 The effects of 2,2′,2″-tripyridine on the nicotinic acetylcholine (ACh) receptor channels were studied in the cultured myocytes of 1-day-old *Xenopus* embryos.

2 2,2′,2″-Tripyridine depressed the amplitude of iontophoretic ACh-induced current at a low frequency of 0.7 Hz stimulation and it not only decreased the initial responses but also enhanced the run-down of ACh-induced current at higher frequency stimulation of 7 Hz and 30 Hz.

3 Single ACh channel recordings showed that 2,2′,2″-tripyridine decreased the channel conductance, the opening frequency and mean open time of both types of low- and high-conductance channels.

4 These results suggest that the blocking actions of 2,2′,2″-tripyridine on ACh receptor channels in the skeletal muscle may contribute to the depression of the nerve-evoked contraction of the mouse diaphragm as reported previously.

Keywords: 2,2′,2″-Tripyridine; nicotinic ACh receptor channel; *Xenopus* muscle cell

Introduction

2,2′,2″-Tripyridine is a pyridine derivative that has been found to be a synthetic by-product of the herbicide, paraquat and to be a strong mutagen (Kuo *et al.*, 1986; Lin *et al.*, 1988). Epidemiological studies showed that the incidence of skin cancer of paraquat manufacturers was much higher than that of the general population of Taiwan (Wang *et al.*, 1986). In addition 2,2′,2″-tripyridine was more potent than paraquat in producing carcinogenic actions and DNA damage (Lin *et al.*, 1988).

In our previous study, we have shown a curare-like action of 2,2′,2″-tripyridine in the mouse phrenic nerve-diaphragm preparation (Lin-Shiau *et al.*, 1992). At the mouse neuromuscular junction, 2,2′,2″-tripyridine not only depressed the amplitudes but also shortened the decay time constant of both endplate potentials (e.p.ps) and miniature endplate potentials (m.e.p.ps). In addition, it produced a fade of tetanic muscle tension and a run down of successive endplate potentials (e.p.ps) during repetitive stimulation in cut muscle preparations of mouse phrenic nerve-diaphragm (Lin-Shiau *et al.*, 1992). The present study was aimed at the exploration of the action of 2,2′,2″-tripyridine on the acetylcholine receptor channel of *Xenopus* muscle cells in culture, and attempted to distinguish the actions of the neuromuscular blocking agent, 2,2′,2″-tripyridine on the ACh receptor and its associated ionic channel.

Methods

Preparation of Xenopus *nerve-muscle cultures*

The *Xenopus* nerve-muscle cultures were prepared as previously reported (Spitzer & Lamborghini, 1976; Sanes & Poo, 1989). Briefly, the neural tube and the associated myotomal tissues of 1-day-old *Xenopus* embryos (stage 20–22) were dissociated in the Ca^{2+}- and Mg^{2+}-free saline. The cells were plated on clean glass coverslips and were used for experiments after 24 h at room temperature (20–22°C). The culture medium consisted of 50% (vol/vol) Ringer solution (composition mM: NaCl 115, $CaCl_2$ 2, KCl 2.5, HEPES 10 (pH 7.6), 49% L-15 Leibovitz's medium (Sigma), and 1% foetal bovine serum (Gibco).

Electrophysiological recording and analysis

Gigaohm-seal whole-cell and cell-attached patch clamp recording methods followed those described previously (Hamill *et al.*, 1981; Young & Poo, 1983). Recordings were made at room temperature in culture medium. The solution inside the whole-cell recording pipettes contained (mM): KCl 150, NaCl 1, $MgCl_2$ 1 and HEPES 10, pH 7.2. Iontophoresis of ACh was applied to the surface of the myocytes by conventional glass microelectrodes filled with 3 M ACh (resistance, 100–200 MΩ). The iontophoretic ACh pulses of 2 ms duration were supplied by a Grass stimulator (SD9) through a microelectrode amplifier (WPI S-7061A), which provides a breaking current between 2 to 10 nA. For cell-attached recording, the pipette was filled with Ringer solution containing low concentrations of ACh (3–5 nM) to avoid the simultaneous opening of the multiple channels. In all recordings, the membrane currents were monitored by a patch-clamp amplifier (EPC-7), and the single channel current signal was filtered at 3 kHz. The data were digitalized by a digitizing unit (Neurocorder DR-384) and stored on a videotape recorder. For single channel analysis, the current signals were digitized at 100 μs intervals and analysed with a PClamp programme (Axon Instruments). Events corresponding to opening of more than one channel were excluded from the open time analysis. Events with open time shorter than 500 μs were not analysed because of possible attenuation and distortion. Open duration histograms were fitted with a single exponential and the amplitude histograms were fitted with Gaussian distribution curves, using least-squares methods in both cases. Effects of drugs were compared from different patches of the same myocyte.

Drugs

All of the chemical compounds listed above for the test experiments were purchased from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

Results

Effects of 2,2′,2″-tripyridine on the ACh-induced whole-cell currents

ACh channels were found on the surface of isolated myocytes of *Xenopus* at relatively high density and uniform

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distribution (Poo, 1982; Young & Poo, 1983). Repetitive iontophoretic applications of identical ACh pulses at one spot on the myocyte surface resulted in transient inward membrane currents. Figure 1 shows an example of whole-cell voltage-clamp recording of membrane currents induced by iontophoretic ACh pulses. The ACh-induced currents remained relatively constant at 0.7 Hz and 7 Hz application, but showed gradual decline at 30 Hz. Bath application of 43 μM 2,2',2''-tripyridine inhibited the ACh-induced currents when the iontophoretic ACh pulses were applied at 0.7 Hz (Figure 1b). In addition, 2,2',2''-tripyridine not only reduced the amplitude of initial responses but also enhanced the rate of decline at either 7 Hz or 30 Hz stimulation within 10 min (Figures 1c and d). The decay time constant of ACh-induced current recorded at a holding potential of − 60 mV was significantly shorter in myocytes treated with 43 μM 2,2',2''-tripyridine ($\tau = 2.7 \pm 0.4$ ms as compared with control; $\tau = 4.1 \pm 0.3$ ms, $n = 12$) (Figure 1a). 2,2',2''-Tripyridine decreased whole-cell ACh-induced currents in a concentration-dependent manner when ACh pulses were applied at 0.7 Hz (Figure 2). As shown in Figure 3, the amplitude and decay time constant of ACh-induced currents were linearly related to the membrane potential. 2,2',2''-Tripyridine inhibited the ACh-induced currents more effectively at hyperpolarized membrane potential but did not significantly affect the reversal potential of ACh-induced whole-cell currents.

Effects of 2,2',2''-tripyridine on ACh single channel currents

Cell-attached patch clamp recordings were made on isolated myocytes in 1-day-old *Xenopus* cultures. The cultured myocytes possess two populations of ACh channels, i.e. embryonic-type channels (low-conductance channels) which have a prolonged mean open time and lower current amplitude and adult-type channels (high-conductance channels) which have 50% greater unitary conductance than embryonic-type channels and shorter mean open time. A representative example of the single channel recordings is shown in Figure 4. The average channel opening frequency, the amplitude of single channel events, and mean channel

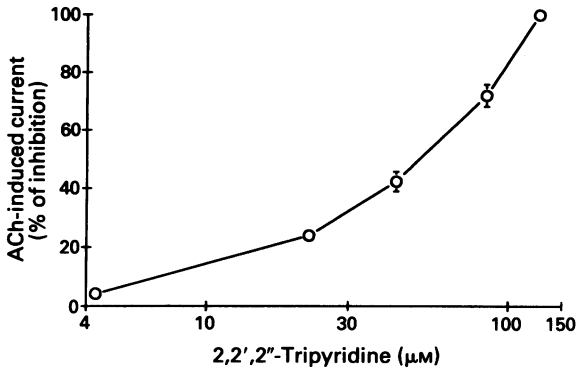

Figure 2 Concentration-dependent inhibition of acetylcholine (ACh)-induced current of the *Xenopus* muscle cells by 2,2',2''-tripyridine. The ACh-induced membrane currents were recorded by whole-cell recording from the myocytes before and 20 min after bath application of various concentrations ($n = 3-6$) of 2,2',2''-tripyridine. The ACh-induced current was recorded by repetitive iontophoretic ACh pulses on the surface of the myocyte at 0.7 Hz.

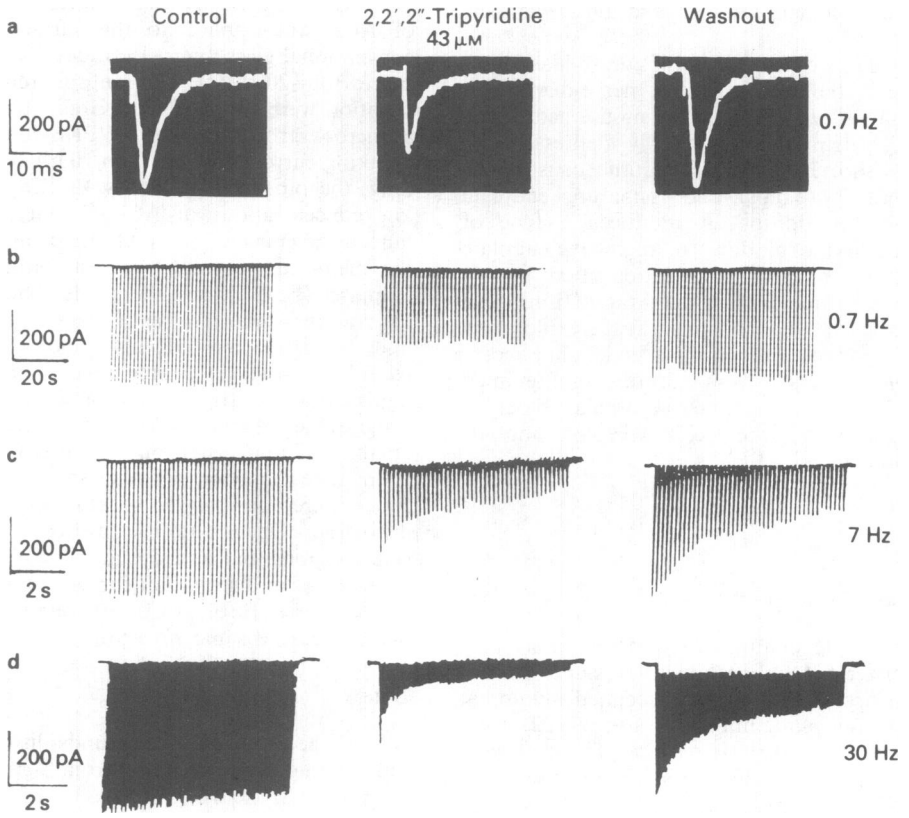

Figure 1 Effects of 2,2',2''-tripyridine on the acetylcholine (ACh)-induced whole-cell currents in *Xenopus* cultured myocytes. The myocytes were voltage-clamped at a holding potential of − 60 mV. Continuous traces showed the membrane currents recorded by whole-cell recording from the same myocyte before (left), 20 min after bath application of 43 μM 2,2',2''-tripyridine (middle) and 20 min after washout (right). The identical iontophoretic ACh pulses were applied on the surfae of the myoctye at a frequency of 0.7 (b), 7 (c) and 30 Hz (d), respectively (filtered at 150 Hz). Samples of current are shown on the upper trace at higher time resolution (filtered at 10 kHz). Note that 2,2',2''-tripyridine inhibited the initial responses of ACh-induced currents and enhanced the run-down of the currents at higher frequency of stimulation.

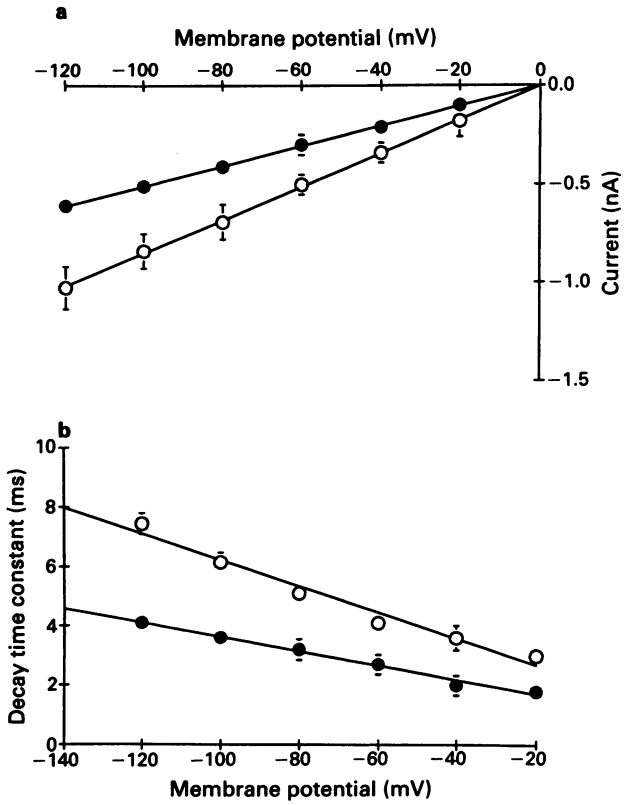

Figure 3 Inhibitory action of 2,2',2''-tripyridine on the acetylcholine (ACh)-induced current as a function of membrane potential. The ACh-induced membrane currents were recorded by whole-cell recording from the myocytes in the absence (○) or 20 min after bath application of 43 μM 2,2',2''-tripyridine (●). The ACh-induced currents were recorded by repetitive iontophoretic ACh pulses on the surface of the myocyte at 0.7 Hz. Note that more pronounced inhibitory effects of 2,2',2''-tripyridine on both the amplitude (a) and decay time constant (b) of ACh-induced currents at hyperpolarizing potentials. The symbols show mean ± s.e.means for the six experiments. The reversal potentials were estimated at 0 mV (control) and − 2 mV (43 μM 2,2',2''-tripyridine) respectively. Resting membrane potentials in these cells were from − 75 mV to − 80 mV.

Table 1 Comparison of the kinetics of acetylcholine single channel between control and 2,2',2''-tripyridine-treated *Xenopus* embryonic myocytes in culture

Channel property	*Control*	*2,2',2''-Tripyridine (43 μM)*
Low-conductance ACh channel		
Amplitude (pA)	5.21 ± 0.06 (12)	3.64 ± 0.09 (12)*
Mean open time (ms)	4.92 ± 0.17 (12)	3.68 ± 0.14 (12)*
Opening frequency (s^{-1})	7.4 ± 0.4 (12)	2.7 ± 0.3 (12)*
High-conductance ACh channel		
Amplitude (pA)	7.71 ± 0.11 (12)	5.59 ± 0.10 (12)*
Mean open time (ms)	1.96 ± 0.05 (12)	1.60 ± 0.04 (12)*
Opening frequency (s^{-1})	4.0 ± 0.3 (12)	1.8 ± 0.2 (12)*

Single ACh channel currents were obtained from cell-attached patch recording on the surface of isolated *Xenopus* muscle cells and the patch was hyperpolarized to 60 mV from rest.
Pipettes were filled with Ringer solution containing acetylcholine (3–5 nM).
*$P < 0.05$ as compared with control.
Data are presented as mean ± s.e.mean (n).
n represents the number of myocytes examined.

open time before and after 2,2',2''-tripyridine treatment were analysed from different patches of the same myocyte. As shown in Figure 4b, 2,2',2''-tripyridine reduced the current amplitude, mean open time as well as average opening frequency of both types of low- and high-conductance ACh channels. Results obtained from 12 cells are summarized in Table 1. Similar to the reversible blockade of 2,2',2''-tripyridine on the ACh-induced whole-cell current, the inhibitory effects of 2,2',2''-tripyridine (43 μM) on the single ACh channel were restored by washout (Figure 4c). As shown in Figure 5, the amplitude of single channel currents, activated by ACh, was linearly related to the membrane potential and the linear regression yielded one high-conductance channel of 61.8 ± 0.9 pS ($n = 12$) and another low-conductance channel of 42.6 ± 0.8 pS ($n = 12$). After exposure to 2,2',2''-tripyridine (43 μM), the conductance decreased to 42.8 ± 1.2 pS and 31.4 ± 0.7 pS for high- and low-conductance channel, respectively. The reversal potentials of single ACh channels were correlated with the data obtained from ACh-induced whole-cell currents and 2,2',2''-tripyridine did not significantly affect the reversal potential of ACh channels. This reduction of channel activity may indicate that 2,2',2''-tripyridine blocks the open conformation of the nicotinic ACh receptor channels.

Discussion

The action of the nondepolarizing neuromuscular blocking agents, (+)-tubocurarine and lobeline on both the ACh receptor and ACh receptor-activated ionic channel at the neuromuscular junction have been studied in some detail (Katz & Miledi, 1978; Lambert *et al.*, 1980; 1981; Gibb & Marshall, 1984). Initial work indicated that (+)-tubocurarine competitively blocked ACh receptors with no effect on the channel lifetime (Katz & Miledi, 1978), despite some earlier evidence that the drug shortened endplate current (e.p.c) decay (Beranek & Vyskocil, 1968) and blocked ACh-activated channels in *Aplysia* neurone (Marty *et al.*, 1976). Manalis (1977) demonstrated that the e.p.c. produced by iontophoretic application of ACh at frog endplates was shortened by (+)-tubocurarine, which produced a voltage-dependent decrease of peak e.p.c. amplitude. This effect became more pronounced at hyperpolarizing potentials. These observations led to a 'reexamination' of the action of (+)-tubocurarine by Katz & Miledi (1978), who confirmed the voltage-dependent channel-blocking action of the drug; in contrast, this effect was not seen with α-bungarotoxin.

The present results clearly showed that 2,2',2''-tripyridine not only depressed the amplitude but also shortened the decay time constant of ACh-induced whole-cell current in *Xenopus* myocyte. The reduction in peak amplitude of the ACh-induced current could be due to the inhibition of either or both the ACh receptor and its associated ionic channel. The sudden rise of the ACh concentration at the *Xenopus* myocyte causes a number of channels to open. Each channel will stay opened for a random, experimentally distributed period of time. The rising phase of the current reflects the number of channels opening as a function of time and the falling phase reflects the distribution of channel closing. The exponential time constant of the current decay is a measure of mean channel open time, a finding that has been confirmed by independent techniques such as noise analysis and patch clamp (Anderson & Stevens, 1973; Neher & Steinbach, 1978). In our experiments, the decay time constant of ACh-induced current recorded at a holding potential of − 60 mV was 4.1 ± 0.3 ms in control compared with 2.7 ± 0.4 ms in the presence of 43 μM 2,2',2''-tripyridine. From the result of single channel recording, 2,2',2''-tripyridine reduced the mean open time of both types of low- and high-conductance ACh channels which could explain the effects of 2,2',2''-tripyridine on the shortening of ACh-induced current decay time constant. Furthermore, the total

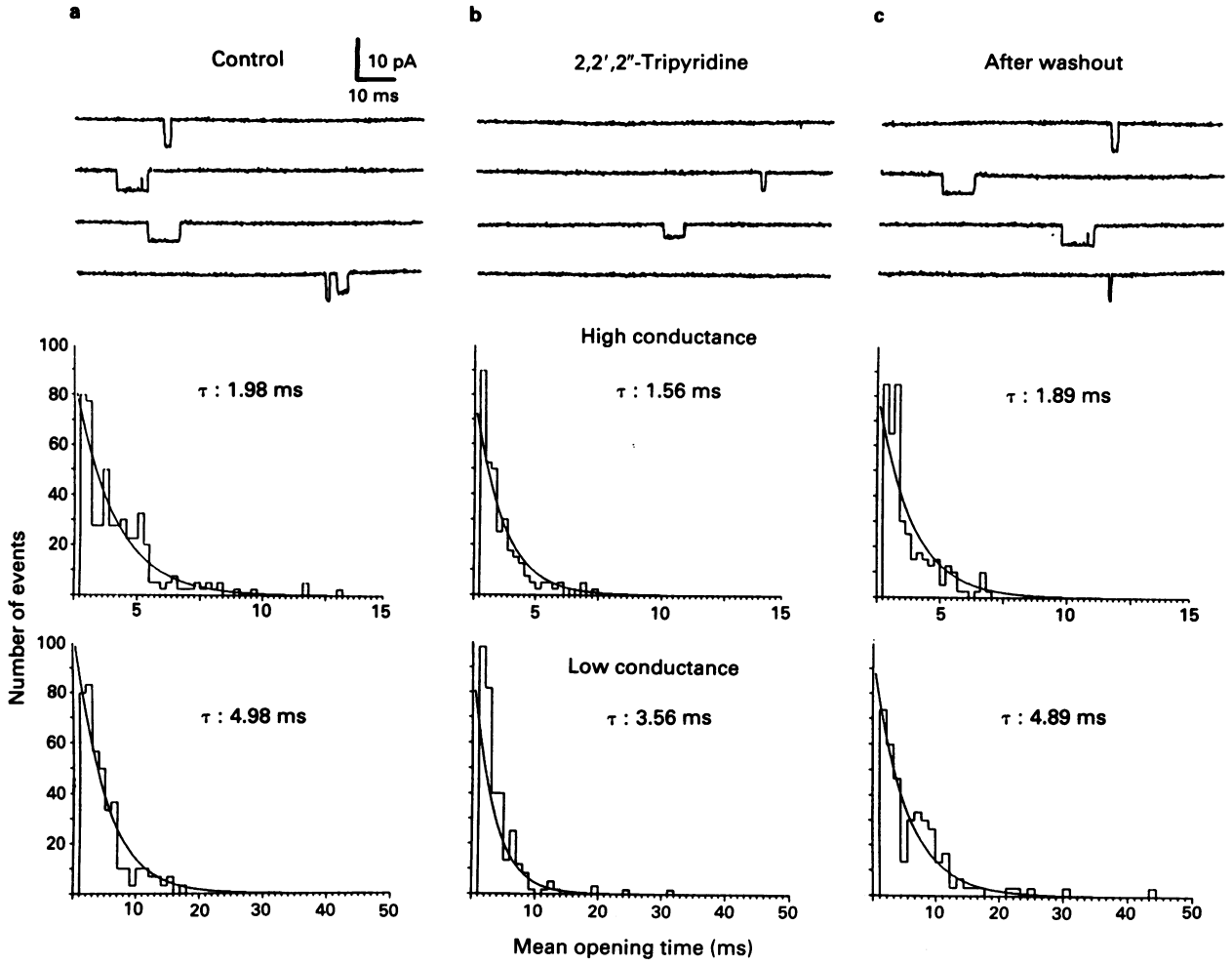

Figure 4 Effects of 2,2',2''-tripyridine on single acetylcholine (ACh) channel currents. Samples of recordings of single ACh channel currents and histograms of channel open time before (a) and after (b) treatment with 2,2',2''-tripyridine (43 μM) and 20 min after washout (c) were obtained from different cell-attached patches of the same *Xenopus* myocyte. Pipettes were filled with Ringer containing 3 nM ACh. The records were low-pass-filtered at 3 kHz and the patch was hyperpolarized to 60 mV from rest. Note that 2,2',2''-tripyridine decreased the open time and opening frequency of both classes of ACh channels.

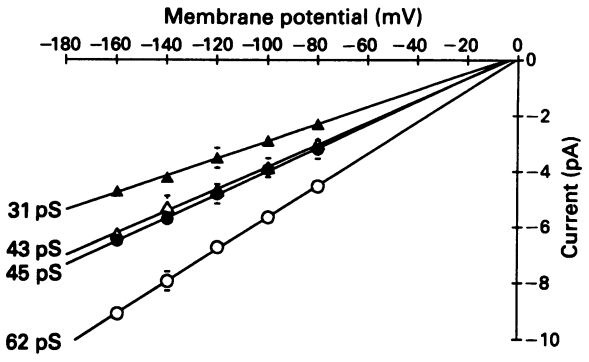

Figure 5 Effects of 2,2',2''-tripyridine on the conductance of acetylcholine (ACh) channels. This figure showed the current-voltage relations for high- (○,●) and low- (△,▲) conductance of ACh-activated channels in *Xenopus* muscle cells in culture. The open symbols (control) and filled symbols (20 min after bath application of 43 μM 2,2',2''-tripyridine) were recorded from the five cells. The indicated conductances were computed by the inverse slope of the line fitted by least squares of the five data points. Note that 2,2',2''-tripyridine reduced the conductance of both classes of ACh channels to the same degree. The symbols show mean ± s.e.means for the five experiments. The reversal potentials of the currents were estimated at − 3 mV (high conductance) and − 6 mV (low conductance) for the control condition and at − 4 mV (high conductance) and − 7 mV (low conductance) for the application of 43 μM 2,2',2''-tripyridine. Resting membrane potentials in these cells were − 75 mV to − 80 mV.

conductance of the membrane induced by ACh is supposed to be the sum of the conductance of individual channels and that each channel can show multiple open states (Sakmann *et al.*, 1980). The peak amplitude of ACh-induced currents in the whole cell recording was reduced by 2,2',2''-tripyridine in a concentration-dependent manner which corresponded to the depression of both types of low- and high-conductance ACh channels in the single channel recordings. Similar effects have been reported for (+)-tubocurarine (Katz & Miledi, 1978; Lambert *et al.*, 1980; 1981; Gibb & Marshall, 1984) and other neuromusclar blocking agents (Colquhoun & Sheridan, 1981).

2,2',2''-Tripyridine increased the run-down of ACh-induced currents at higher frequency stimulations of 7 Hz and 30 Hz, which could be accounted for either by enhancement of the rate of agonist-induced desensitization or by a direct action on the receptor-activated ionic channel. From the data of single channel recording, it seems unlikely that the enhancement of run-down of trains of ACh-induced current by 2,2',2''-tripyridine can be accounted for by postulating enhancement of desensitization of the receptor-channel complex (Bowman *et al.*, 1986; Wilson & Thomsen, 1991) since the conductance of ACh-activated single channels appeared to be independent of ACh receptor desensitization (Sakmann *et al.*, 1980); however, 2,2',2''-tripyridine could decrease the conductance of both types of low- and high-conductance ACh-activated channels. Furthermore, if 2,2',2''-tripyridine possessed an ability to enhance desensitization of the receptor-channel complex, it could potentiate the occurrence

of grouping or clustering of ACh channels opening in the presence of higher concentrations of ACh (> 1 μM). While, in our experiments with 2,2',2''-tripyridine on the patch pipettes with 1 μM ACh on cell attached patches, we saw less evidence for grouping or clustering of openings than in the absence of 2,2',2''-tripyridine. Thus, it was considered that 2,2',2''-tripyridine probably inhibited the nicotinic acetylcholine channels which resulted in the reduction of conductance of both types of ACh channel in the *Xenopus* muscle cells. Furthermore, 2,2',2''-tripyridine decreased the opening frequency and the mean open time of both types of low- and high-conductance ACh channel in *Xenopus* muscle cells. These decreasing effects on ACh channel activity were compared with those of typical blockers of nicotinic ACh receptor channels: bupivacaine as an open channel blocker (Aracava *et al.*, 1984), chlorpromazine as a closed or nonconducting channel blocker (Carp *et al.*, 1983) and phencyclidine as an open and closed channel blocker (Aguayo *et al.*, 1986). These three drugs manifested different blocking actions on single channel currents (Kimura *et al.*, 1991). 2,2',2''-Tripyridine was shown to be a mixed type of channel blocker of open and closed conformation of the ionic channel (like phencylidine) because of the simultaneous decrease of both the open time and open frequency.

2,2',2''-Tripyridine exerted two effects on the endplate potentials (e.p.ps) on the mouse phrenic nerve-diaphragm preparations: a depression in the peak amplitude and a decrease in decay time constant. Both effects seemed to be affected independently for the following reasons: (a) the onset of the depression of decay time constant was much faster than the depression of the peak amplitude; (b) the peak amplitude but not the decay time constant was progressively reduced by the repetitive stimulations. It is not known whether this dichotomy in the actions of the toxin reflects two different binding sites or a single topographical site possessing two conformations, one of which alters the decay time constant and the other immobilizes the ACh receptor in its closed form (Hsu *et al.*, 1992). A similar result was found in the present study. ACh-induced whole-cell current shows as a function of membrane potential under control and in the presence of 2,2',2''-tripyridine. 2,2',2''-Tripyridine reduces the peak amplitudes and decay time constant of ACh-induced whole-cell current in a voltage-dependent manner. Like (+)-tubocurarine, 2,2',2''-tripyridine is more effective in reducing ACh-induced whole-cell currents at hyperpolarized potentials. Furthermore, the reversal potential for both ACh-induced whole-cell currents and single ACh channel currents were not altered by 2,2',2''-tripyridine. In addition, 2,2',2''-tripyridine has no effect on the membrane potential of myocytes. These observed effects of 2,2',2''-tripyridine on the *Xenopus* muscle cells were correlated with findings on the mouse phrenic nerve-diaphragm preparations.

In conclusion, these results together with our previous report (Lin-Shiau *et al.*, 1992) which showed that 2,2',2''-tripyridine inhibited the binding of α-bungarotoxin in the mouse diaphragm, suggest that 2,2',2''-tripyridine, like (+)-tubocurarine, has at least two distinct postjunctional actions. 2,2',2''-Tripyridine appears to interact with both the ACh receptor and its ionic channel. It would be of interest to compare the binding characteristics of these two sites and to determine if 2,2',2''-tripyridine, (+)-tubocurarine and local anaesthetics share common channel binding sites.

This work was supported by a research grant from the National Science Council, Republic of China (NSC81-0412-B002-06z).

References

ANDERSON, C.R. & STEVENS, C.F. (1973). Voltage clamp analysis acetylcholine produced end-plate current fluctuations at frog neuromuscular junction. *J. Physiol.*, **235**, 665–691.

AGUAYO, L.G., VITKOP, B. & ALBUQUERQUE, E.X. (1986). Voltage- and time dependent effects of phencyclidines on the endplate current arise from open and closed channel blockade. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 3523–3527.

ARACAVA, Y. & ALBUQUERQUE, E.X. (1984). Meproadifen enhances activation and desensitization of the acetylcholine receptor-ionic channel complex (AChR), single channel studies. *FEBS Lett.*, **174**, 267–274.

BERANEK, R. & VYSKOCIL, F. (1968). The effect of atropine on the frog sartorius neuromuscular junction. *J. Physiol.*, **195**, 493–503.

BOWMAN, W.C., GIBB, A.J., HARVEY, A.L. & MARSHALL, I.G. (1986). Prejunctional actions of cholinoceptor agonists and antagonists, and of anticholinesterase drugs. In *New Neuromuscular Blocking Agents. Handb. Exp. Pharmacol.*, Vol. 79, ed. Kharkevich, D.A. pp. 141–170. Berlin: Springer-Verlag.

CARP, J.S., ARONSTAM, R.S., WITKOP, B. & AIBUOUEROUE, E.X. (1983). Electrophysiological and biochemical studies on enhancement of desensitization by phenothiazine neuroleptics. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 310–314.

COLQUHOUN, D. & SHERIDAN, R.E. (1981). The modes of action of gallamine. *Proc. R. Soc. B*, **211**, 181–203.

GIBB, A.J. & MARSHALL, I.G. (1984). Pre- and post-junctional effects of d-tubocurarine and other nicotinic antagonists during repetitive stimulation in the rat. *J. Physiol.*, **351**, 275–297.

HAMILL, O.P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cell and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HSU, K.S., FU, W.M. & LIN-SHIAU, S.Y. (1992). Further studies on the mode of twitch depression induced by 2,2',2''-tripyridine in mouse phrenic nerve-diaphragm. *Joint Annual Conference Neurosci.* Oct. 30–31, Taipei, Taiwan. Abs.p. 8.

KATZ, B. & MILEDI, R. (1973). The binding of acetylcholine to receptors and its removal from the synaptic cleft. *J. Physiol.*, **231**, 549–574.

KATZ, B. & MILEDI, R. (1978). A re-examination of curare action at the motor endplate. *Proc. R. Soc. B*, **203**, 119–133.

KIMURA, M., NOJIMA, H., MUROI, M. & KIMURA, I. (1991). Mechanisms of the blocking action of β-eudesmol on the nicotinic acetylcholine receptor channel in mouse skeletal muscles. *Neuropharmacol.*, **30**, 835–841.

KUO, M.L., WANG, L.D. & LIN, J.K. (1986). Mutagenicity and cytotoxicity of dipyridyl derivatives and waste water discharged from paraquat manufactory. *J. Chin. Oncol. Soc.*, **2**, 1–8.

LAMBERT, J.J., VOLLE, R.L. & HENDERSON, E.G. (1980). An attempt to distinguish between the actions of neuromuscular blocking drugs on the acetylcholine receptor and on its associated ionic channel. *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 5003–5007.

LAMBERT, J.J., DURANT, N.N., REYNOLDS, L.S., VOLLE, R.L. & HENDERSON, E.G. (1981). Characterization of endplate conductance in transected frog muscle: modification by drugs. *J. Pharmacol. Exp. Ther.*, **216**, 62–69.

LIN, J.K., KUO, M.L., LEE, K.W. & LIN-SHIAU, S.Y. (1988). DNA cleavage effect and cytotoxicity of the waste water discharged from paraquat manufactory. *J. Chin. Oncol. Soc.*, **4**, 1–12.

LIN-SHIAU, S.Y., HSU, K.S. & FU, W.M. (1992). Studies on the curare-like action of 2,2',2''-tripyridine in the mouse phrenic nerve-diaphragm. *Br. J. Pharmacol.*, **106**, 55–60.

MANALIS, R.S. (1977). Voltage-dependent effect of curare at the frog neuromuscular junction. *Nature*, **267**, 366–368.

MARTY, A., NEILD, T. & ASCHER, P. (1976). Voltage sensitivity of acetylcholine currents in *Aplysia* neurons in the presence of curare. *Nature*, **261**, 501–503.

NEHER, E. & STEINBACH, J.H. (1982). Local anaesthetics transiently block currents through single acetylcholine-receptor channels. *J. Physiol.*, **227**, 153–176.

POO, M.M. (1982). Rapid lateral diffusion of functional ACh receptors in embryonic muscle cell membrane. *Nature*, **299**, 332–334.

SAKMANN, B., PATLAK, J. & NEHER, E. (1980). Single acetylcholine-activated channels show burst-kinetics in presence of desensitizing concentrations of agonist. *Nature*, **286**, 71–73.

SANES, D.H. & POO, M.M. (1989). In vitro analysis of position- and lineage-dependent selectivity in the formation of neuromuscular synapse. *Neuron*, **2**, 1237–1244.

SPITZER, N.C. & LAMBORGHINI, J.C. (1976). The development of the action potential mechanisms of amphibian neurons isolated in culture. *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 1641–1645.

WANG, J.D., LI, W.M., HU, F.C. & HU, K.H. (1987). The occupational risk and the development of premalignant skin lesions among paraquat manufacturers. *Br. J. Indust. Med.*, **44**, 196–200.

WILSON, D.F. & THOMSEN, R.H. (1991). Nicotinic receptors on the phrenic nerve: evidence for negative feedback. *Neurosci. Lett.*, **132**, 163–166.

YOUNG, S.H. & POO, M.M. (1983). Topographical rearrangement of ACh receptor alters channel kinetics. *Nature*, **304**, 161–163.

YOUNG, S.H. & POO, M.M. (1983a). Spontaneous release of transmitter from growth cones of embryonic neurons. *Nature*, **305**, 634–637.

(Received April 16, 1993
Accepted April 29, 1993)