THE ACTION OF PHENOL ON NEUROMUSCULAR TRANSMISSION IN THE RED MUSCLE OF FISH

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INTRODUCTION

Since the beginning of this century, it has been known that phenolic substances antagonize the paralytic action of curare on the skeletal muscle (ROTHBERGER, 1902).

TOIDA (1940) observed that the amplitudes of twitch tension and extra-cellularly recorded action potentials of the frog sartorius muscle evoked by nerve stimulation were increased by phenol. Moreover, phenol had no direct effects either on the nerve or on the muscle. From the above results he deduced that phenol might facilitate the neuromuscular transmission due to the enhanced excitability of the nerve ending.

Many other investigators also reported the facilitatory action of phenol on the neuromuscular junction of the skeletal muscle (MOGEY and YOUNG, 1949; HOBBERGER, 1952; TAKAGI and TAKAYANAGI, 1965).

OTSUKA and NONOMURA (1963) studied the site of action of phenol on the sciatic nerve-sartorius muscle preparation of the frog by means of intracellular microelectrode technique. Phenolic substances greatly increased the size of the endplate potential in the curarized preparation without altering the sensitivity of the endplate to iontophoretically applied acetylcholine. In addition, phenol increased the degree of neuromuscular depression on double stimuli. OTSUKA and NONOMURA concluded from their observations that phenolic substances acted on the motor nerve endings and increased the amount of the chemical transmitter released by single nerve impulses.

NISHIHARA (1967) showed in his electronmicroscopic studies on the silver carp red muscle (fin muscle) that the muscle had multifocal diffuse innervation. These electronmicroscopic findings were confirmed by electrophysiological observations by HIDAKA and TOIDA (1969a). They also reported that the membrane resistance and capacitance were generally similar to those of the frog slow fibre. Action potentials could be barely initiated in this muscle in response to both direct and indirect stimuli (HIDAKA and TOIDA, 1969a and b).

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The purpose of this study was to determine whether phenol acts on the neuromuscular junction of the fin red muscle of the silver carp in the same way as on the frog fast muscle.

Furthermore, an attempt has been made to investigate the phenolic action on the transmitter release in the resting stage, namely, on miniature excitatory junction potentials and also on the postjunctional membrane properties.

METHOD

The pectoral fin muscles (*M. levator pinnae pectoralis*) with their nerves (Th. 1 and Th. 2) were dissected from the silver carp, *Carassius auratus* Linne. The pectoral fin muscle consists of the red muscle fibres. A 2-3 cm length of nerve and a 1-2 cm length of muscle were used. The preparation was placed in a lucite dish (capacity: 20 cc) at room temperature (17-22°C) and perfused with physiological saline.

The physiological solution contained; NaCl; 129.6 mM, KCl; 2.7 mM, CaCl_2 ; 1.8 mM, NaHCO_3 ; 2.5 mM (HIDAKA & TOIDA 1969a & b).

The intracellular recording electrodes were glass micropipettes filled with 3 M KCl (LING & GERARD type, 1949). Their resistances ranged from 10 to 30 M Ω . The recording system had a conventional cathode follower input stage. The grid current was less than 10^{-12} A (Nihon Kohden Ltd's MZ-3A). After DC-amplification, responses were displayed on a dual beam oscilloscope (Tektronix type 502).

The motor nerve was stimulated in paraffin oil by two 0.2 mm platinum wires. Phenol was prepared with distilled water to 10^{-3} g/ml, and diluted with physiological saline to final concentrations. In order to investigate the membrane properties two microelectrodes were inserted into single muscle fibers; one for passing current and the other for recording potential.

The action current of the nerve ending and the excitatory junction current were recorded simultaneously with single extracellular glass electrodes. The extracellular glass electrodes were about 2-10 μ in diameter and filled with isotonic saline as described by KATZ and MILEDI (1965).

Iontophoretic application of acetylcholine was used to measure the sensitivity of the postjunctional membrane to acetylcholine (Ach.) (KRNJJEVIĆ & MILEDI, 1958a). The current pulse was $0.5-1.25 \times 10^{-7}$ A in intensity and 0.1-100 msec in duration. Spontaneous diffusion of Ach. from a pipette was prevented by an adjustable inward current.

Throughout the experimental procedures, a normal response was compared with the response after phenol application on the same junction.

RESULTS

Resting membrane potential of the muscle fibres

The mean resting membrane potential of the fish red muscle fibres was 79.5 mV (S.E. = ± 0.94 , $n=320$). TABLE I shows the effects of phenol on the membrane potentials. They were measured 5-10 min after application of phenol, whose concentration ranged from 5×10^{-5} g/ml to 1×10^{-7} g/ml. At such concentrations, phenol had no marked effect on the resting membrane potential.

TABLE 1.
Effect of phenol on resting membrane potentials.

			Resting potential
Control		(n=320)	79.5 ± 0.9 (mV ± S. E)
Phenol	1 × 10 ⁻⁵ g/ml	(n=120)	78.8 ± 0.8
	1 × 10 ⁻⁶ g/ml	(n=120)	78.5 ± 0.9
	1 × 10 ⁻⁷ g/ml	(n=120)	78.4 ± 0.9
	5 × 10 ⁻⁵ g/ml	(n=120)	79.3 ± 0.9
Control (after washing)		(n=280)	80.1 ± 1.1

Excitatory junction potential (e.j.p.)

In twelve out of fourteen observations the amplitude of e.j.p. was enhanced to 1.5 or 2 times the control after the preparation was perfused for 2-3 min with the solution containing phenol (5×10^{-5} g/ml). However, there was no prolongation in the duration of e.j.p. when measured at 50% of the peak amplitude.

FIG. 1 shows the typical effects of phenol (5×10^{-5} g/ml) on the fin red muscle. Phenol increased the amplitude of e.j.p., but did not change the time course. In three fibres, abortive spikes were generated from augmented e.j.p. by phenol as shown in FIG. 2. The two fibres exhibited repetitive spike discharges as illustrated in the record shown at right hand side of FIG. 2.

FIG. 1. Effect of phenol 5×10^{-5} g/ml on the e.j.p.s which were evoked by supramaximal nerve stimuli of 0.02 msec at 0.25/sec.

FIG. 2. Spike discharges recorded from the single muscle fibre by electrical stimulation of the nerve in the presence of phenol (5×10^{-5} g/ml). Resting membrane potential in this fibre was -70 mV. Note no overshoot of the spikes.

Miniature excitatory junction potential (m.e.j.p.)

The effects of phenol on the amplitude and frequency of the m.e.j.p. were investigated. The frequency of m.e.j.p. was calculated by counting the number of m.e.j.p.

Phenol (1×10^{-6} g/ml) increased the frequency of the miniature excitatory junction potential two to three times over the control. These effects fully developed within 2-5 minutes of application.

The amplitude of m.e.j.p. was unaffected by phenol in the ranges between 10^{-5} and 10^{-6} g/ml while the frequency was moderately increased two to three times. In some fibres, however, phenol ($1-5 \times 10^{-5}$ g/ml) enormously increased the frequency and enhanced the amplitude of m.e.j.p. (FIG. 5). This increase in m.e.j.p. amplitude seemed to be due partly to the simultaneous discharges of more than one quantum of the transmitter from the single nerve terminal and partly to temporal and spatial summation of m.e.j.p. The above results might indicate that phenol increases the transmitter liberation, namely acetylcholine release, from the nerve ending (HIDAKA and TOIDA, 1969a).

FIG. 3 shows sample records of m.e.j.p. with and without the presence of phenol. The frequency of m.e.j.p. was increased after treatment with phenol (1×10^{-6} g/ml), but their amplitude remained unchanged.

FIGS 4 and 5 show with histograms the occurrence of m.e.j.p. before and after treatment with phenol (10^{-6} g/ml and 10^{-5} g/ml). The numbers of m.e.j.p. were taken as ordinates, the amplitudes of m.e.j.p. as abscissae. At 10^{-6} g/ml, phenol increased the frequency from 15/sec to 50/sec, while the mean amplitude stayed almost unchanged (FIG. 4). In the experiment of FIG. 5 an enormous increase in m.e.j.p. frequency was accompanied by the augmentation of m.e.j.p. In this case, the mean amplitude of m.e.j.p. was increased from 0.42 mV to 0.62 mV by phenol (1×10^{-5} g/ml).

FIG. 3. Effect of phenol (1×10^{-6} g/ml) on the appearances of m.e.j.p.s. Records were taken successively 2 min, 10 min and 20 min after treatment with phenol.

FIG. 4. Histograms showing effect of phenol (1×10^{-6} g/ml) on the occurrence of m.e.j.p.s. in a period of 140 sec. Calculations were made 10 min after perfusion with the test solution.

FIG. 5. Histograms showing effect of phenol (1×10^{-5} g/ml) on the occurrence of m.e.j.p.s. in a period of 140 sec. Calculations were made 2 min after application of phenol.

Postjunctional membrane properties

The passive electrical properties of the postjunctional membrane were obtained by measuring the electrotonic potentials produced by intracellularly applied weak hyperpolarizing currents. The current electrode was inserted into the muscle fibre at a point within 200μ from the recording electrode.

FIG. 6. Left side: Effect of phenol at 5×10^{-5} g/ml on the electrotonic potentials evoked by intracellularly applied weak hyperpolarizing currents. Right side: current voltage relations obtained from the experiment shown in the left.

FIG. 6 shows the effect of phenol (5×10^{-5} g/ml) on the current-voltage relationships of the muscle fibre membrane. The electrotonic potentials were taken as ordinates and the applied currents as abscissae. The input resistance of this fibre was 1.31 M Ω and 1.29 M Ω before and after treatment with phenol respectively.

The time constant of the muscle membrane was measured at 63% of the steady potential difference produced by weak hyperpolarizing current. The membrane time constant and the effective resistance in ten fibres were listed in TABLE II. The mean time constants were 18.6 ± 1.6 msec and 19.5 ± 2.0 msec before and after treatment with phenol. The mean effective resistance in the normal solution was 1.04 ± 0.21 M Ω ($n=10$) and it was 1.17 ± 0.29 M Ω ($n=10$) in the solution containing phenol. It is evidently safe to conclude that phenol does not change the passive membrane properties.

TABLE 2.
Characteristic constants of the membrane calculated from analyzing electrotonic potentials evoked by intracellularly applied weak hyperpolarizing current with or without presence of phenol (5×10^{-5} — 1×10^{-7} g/ml).

Exp. No.	Control		Phenol		
	(msec)	R _{eff} (M Ω)	Concentration	(msec)	R _{eff} (M Ω)
1	27.3	2.30	5×10^{-5} g/ml	28.2	2.50
2	24.1	1.12	"	24.3	1.15
3	16.4	0.67	"	12.7	0.66
4	15.0	0.76	1×10^{-5} g/ml	14.1	0.94
5	27.3	2.30	"	32.1	2.60
6	16.4	0.67	"	14.8	0.69
7	18.2	0.51	1×10^{-6} g/ml	19.1	0.41
8	15.0	1.35	"	14.1	1.20
9	16.4	0.67	"	21.8	0.84
10	14.1	0.67	1×10^{-7} g/ml	13.4	0.67
Mean	18.6 ± 1.6 (S.E.)	1.04 ± 0.21		19.5 ± 2.0	1.17 ± 0.29

Acetylcholin potential

The transmitter at the neuromuscular junction of the silver carp red muscle had already been discovered to be acetylcholine by HIDAKA and TOIDA (1969a). The sensitivity of the postjunctional membrane to acetylcholine was estimated by iontophoretic application of Ach. before and after treatment with phenol.

As shown in FIG. 7, the amplitude and the time course of Ach-potential were unaffected by phenol (5×10^{-5} g/ml). The above results were confirmed in six different fibres. This fact might indicate that phenol acts neither on

FIG. 7. Effect of phenol 5×10^{-5} g/ml on the acetylcholine potentials evoked by iontophoretic application of acetylcholine (2M). Applied current was 1×10^{-7} A, with a duration of 20 msec.

the sensitivity of postjunctional membrane nor on the cholinesterase activity.

Action current of nerve terminal and excitatory junction current (e.j.c.)

FIG. 8 shows the effects of phenol (1×10^{-5} g/ml) on the action currents of the nerve terminal and excitatory junction currents recorded with an extracellular electrode. The recording electrode was placed in close vicinity to the neuromuscular junction. The upper traces showed single sweeps, while lower traces superimposed recordings of 20 sweeps. Phenol (1×10^{-5} g/ml) increased the size of e.j.c. by 1.5 times, but did not increase its duration. The increase in e.j.c. size is consistent with the enhancement of e.j.p. by phenol. The same results were also obtained in other 33 fibres. In FIG. 9,

FIG. 8. Effects of phenol (1×10^{-5} g/ml) on the action currents of the nerve ending and excitatory junction currents simultaneously recorded with an extracellular electrode. The upper traces were recording of single sweep, and the lower traces were superimposed recordings of 20 sweeps.

FIG. 9. The mean height and the mean duration of the e.j.c.s of 33 fibres in the presence of phenol (5×10^{-5} g/ml), which were shown as multiples of the control values in the ordinate ($\% \pm$ S.E.).

the height and the duration of e.j.c. obtained from 33 fibres were plotted as multiples of the control against the time after treatment with phenol (1×10^{-5} g/ml).

Phenol (1×10^{-5} g/ml) had no effect on either the height or the duration of the action current of the nerve ending in four fibres.

The mean synaptic delay as measured from the peak of action current to the onset of the e.j.c. was 0.37 ± 0.05 (S.E.) msec., and the value after treatment with phenol (1×10^{-5} – 10^{-6} g/ml) was 0.35 ± 0.02 msec. No significant difference could be observed in the synaptic delay before and after application of phenol.

DISCUSSION

Various explanations may be possible for the action of phenol on neuromuscular transmission. Firstly, phenol might increase the quantity of the transmitter (acetylcholine) released from the nerve endings. This explanation was substantiated by the facts that phenol enhanced the heights of both e.j.p. and e.j.c. and increased the frequency of spontaneous m.e.j.p.s as shown in FIG. 1, 2, 3, 4, 5, 8 and 9. Secondly, phenol might inhibit cholinesterase activity. Thirdly, phenol might increase the sensitivity of the post-junctional membrane to acetylcholine. But the latter two possibilities were ruled out, because the configuration of Ach-potential was not affected by phenol (FIG. 7). Furthermore, the durations of e.j.c. and of e.j.p. were not influenced by phenol (FIG. 1, 8 and 9). Fourthly, phenol might increase the

resting membrane potential or the input resistance of the postjunctional membrane (FATT, P. and KATZ, B., 1951; KATZ and THESLEFF, 1957). Apparently these possibilities were also eliminated by the experimental results. Namely, the resting membrane potential, the input resistance of the postjunctional membrane and the amplitude of m.e.j.p. were unaffected by phenol (See TABLE I, TABLE II and FIG. 4 and 6). The fact that the duration of e.j.p. remained constant also supports this conclusion.

It was concluded from these results that phenol facilitates the neuromuscular transmission of the silver carp red muscle by increasing the amount of the transmitter liberated from the nerve endings. This conclusion is in agreement with the earlier suggestion on the phenolic action on motor nerve endings reported by TOIDA (1940) and with the recent electrophysiological observations of phenolic action on the frog fast muscle (OTSUKA and NONOMURA, 1963). The action of phenol is similar to that of noradrenaline (HIDAKA and KURIYAMA, 1969), adrenaline (KRNEVIC and MILEDI, 1958b) and guanidine (OTSUKA and ENDO, 1960) in that all of them act on the nerve ending and cause an increase in the transmitter release. Furthermore, it was shown that phenol also increases the transmitter liberation in the resting state and has no action on the passive membrane properties of the silver carp red muscle.

Though it is not known exactly how phenol increases the quantity of the transmitter released from the nerve endings, the effect of phenol can not be attributed to its action on the resting membrane potential of the nerve endings. Because, in such a reasoning, the increase in the size of e.j.p. should be related to a hyperpolarization of the membrane leading to an increased amplitude of the action current (TAKEUCHI and TAKEUCHI, 1962, HUBBARD and WILLIS, 1962), whereas the increase in m.e.j.p. frequency which was also observed should be related to a decrease in the resting potential and therefore to a decrease of the action current of the nerve ending (LILEY 1956). Actually the amplitudes of action currents of the terminal regions of nerve fibre were unchanged by phenol. Both the e.j.p. amplitude and the m.e.j.p. frequency were increased. These findings contradict the idea that the change in the resting membrane potential of the nerve ending might play a role in increasing the transmitter release by phenol. A more likely explanation would be that phenol may increase the amount of available transmitters which are located in close proximity to the inner surface of the membrane of the nerve terminal, ready for release by depolarization (ECCLES, 1963). There is, however no direct evidence for this.

Synaptic delay in the silver carp red muscle is 0.34 msec in Ringer solution and 0.35 msec after application of phenol. In 1965, KATZ concluded that the chief contributory factor to the synaptic interval was a delay in the quantal release of the transmitter after the arrival of the nerve impulse. He also reported that lengthening the current pulse, which depolarized the

nerve ending, caused a larger amount of the transmitter to be released after a longer delay. Increasing the intensity of current pulse, however, shortened the synaptic delays (KATZ, 1967b). According to KATZ's experimental results, no action of phenol on synaptic delay can be expected from unchanged action current of the nerve endings.

In normal condition, silver carp red muscle fibres never gave rise to action potential in response either to direct intracellular stimulation or to nerve stimulation. When a preparation was perfused with a solution containing phenol abortive spike activities occurred in a few fibres. Some of these spikes were apparently originated from enhanced e. j. p. Also, nerve stimulation set up spikes in this muscle when it was perfused with calcium rich solutions or with chloride deficient solutions in which Cl^- was substituted by NO_3^- , Br^- or glutamate (HIDAKA and TODA, 1969b).

TAKEUCHI (1959) reported that in the red muscle of the snake fish (*Ophichthys argus*), small spike potential appeared at the break of a strong inward current. He believed that in the red muscle of the snake fish, the mechanism for spike generation exists, but is not manifest in normal conditions since only a strong inward current activated the mechanism.

In the extraocular slow muscle fibres of the cat, a small spike activity occurred during long tetanic nerve stimulation (PILAR, 1967). Pilar described that it was not feasible to deny the possibility that some slow fibres might give rise to an action potential. It might well be that under the experimental conditions used, the slow fibres were already depolarized and the spike mechanism for that reason was inactivated (PILAR, 1967).

However, it is unknown whether phenol has a restorative activity on the silver carp red muscle such as shown by anodal polarization in the red muscle of the snake fish.

SUMMARY

The effect of phenol on neuromuscular transmission was investigated in the fin red muscle fibres of the silver carp, *Carassius auratus* Linne. Results are as follows:

1. Phenol (5×10^{-5} to 1×10^{-7} g/ml) had no effect on resting membrane potentials.
2. Phenol greatly increased the amplitude of excitatory junction potentials, but did not change the duration.
3. No spike generation from the muscle membrane in normal solution in response to nerve stimulation was observed. However, in some muscle fibres, spikes were generated by phenol from augmented excitatory junction potential.
4. Phenol enormously increased the frequency but did not change the amplitude of miniature excitatory junction potentials.

5. Phenol enhanced the size of extracellularly recorded excitatory junction currents with no change in the duration. There was no effect on simultaneously recorded action currents of the nerve ending. No effect was also observed on synaptic delay.
6. Phenol had no effect on acetylcholine potential evoked by iontophoretic application of acetylcholine.
7. Phenol had no effect on the input resistance and time constant of the muscle membrane.

From these, it was concluded that phenol facilitates the neuromuscular transmission of the silver carp fin red muscles by increasing the quantity of Ach. released from the nerve ending. Phenol had no residual transmitter action. The increase in Ach. release by phenol is not due to changes in the resting membrane potential of the nerve ending.

The possible mechanisms involved in the action of phenol for the increased release of chemical transmitter from the nerve terminal were discussed.

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