Br. J. Pharmac. (1979), **66**, 525–530

THE RELATIONSHIP BETWEEN THE PHARMACOKINETICS, CHOLINESTERASE INHIBITION AND FACILITATION OF TWITCH TENSION OF THE QUATERNARY AMMONIUM ANTICHOLINESTERASE DRUGS, NEOSTIGMINE, PYRIDOSTIGMINE, EDROPHONIUM AND 3-HYDROXYPHENYLTRIMETHYLAMMONIUM

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1 The relationship between the concentration of drug in plasma, the inhibition of erythrocyte acetylcholinesterase and the facilitation of neuromuscular transmission has been studied in the rat after the administration of neostigmine, pyridostigmine, edrophonium and 3-hydroxyphenyltrimethylammonium (3-OH PTMA).

2 After the administration of neostigmine or pyridostigmine, acetylcholinesterase activity recovered only slowly due to the covalent nature of the inhibition. In contrast, recovery from the reversible inhibition caused by edrophonium or 3-OH PTMA was rapid and showed a direct relationship to the plasma concentration of these drugs.

3 There was a statistically significant linear correlation between the logarithm of the plasma concentration of the drugs and the increase in the tibialis twitch tension.

4 The relationship between the inhibition of acetylcholinesterase and the facilitation of neuromuscular transmission was complex. When the enzyme was less than 85% inhibited no facilitation occurred. Between 85% and 98% inhibition, facilitation was linearly related to enzyme inhibition. Above 98% inhibition, facilitation was unrelated to inhibition of the enzyme.

Introduction

Previous pharmacokinetic studies on neostigmine, pyridostigmine and edrophonium have shown that the principle metabolite of neostigmine is 3-hydroxyphenyltrimethylammonium (3-OH PTMA) which is further metabolized to a glucuronide conjugate (Hussain, Roberts, Thomas & Wilson, 1969). It has been reported that 3-OH PTMA has a facilitatory action at the neuromuscular junction (Randall, 1950). Pyridostigmine is metabolized to 3-hydroxy-*N*-methylpyridinium (3-OH NMP) (Somani, Roberts & Wilson, 1972) to which no previous reference of a biological action has been made. Edrophonium is metabolized to a glucuronide conjugate (Back & Calvey, 1974) which has been reported to have little or no biological activity (Barber, Calvey, Muir & Taylor, 1976).

In this paper, the relationship between the concentration of drug in plasma, the inhibition of erythrocyte acetylcholinesterase from the same blood samples and the simultaneous facilitation of neuromuscular transmission has been studied in the rat after the administration of neostigmine, pyridostigmine, edrophonium or 3-OH PTMA. Similar work has been carried out in Riker's laboratory (Kuperman, Gill & Riker, 1961).

Methods

Experimental procedure

Male Wistar rats (250 to 350 g) were anaesthetized with ethyl carbamate (1.4 g/kg, i.p.) and a polyethylene cannula was inserted into a jugular vein. Respiration was assisted when necessary by means of an endotracheal tube and a miniature respiration pump. The contraction of the tibialis anterior muscle in response to continuous supramaximal stimulation throughout the experiments of the sciatic nerve (0.5 ms, 0.33 Hz) was measured from both hind limbs by means of a strain gauge. Arterial blood pressure was recorded from a common carotid artery by

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0007-1188/79/080525-06 $01.00

means of a polyethylene cannula filled with heparin sodium (100 u/ml) which was attached to a pressure transducer. The contralateral common carotid artery was also cannulated. Heparin sodium (200 u in 0.2 ml) was injected intravenously at the beginning of each experiment.

[^{14}C]-neostigmine bromide (sp. act. 5.2 mCi/nmol; dose: 1.0 μmol/kg), [^{14}C]-pyridostigmine bromide (sp. act. 14.3 mCi/nmol; dose: 1.0 and 4.0 μmol/kg), [^{14}C]-edrophonium chloride (sp. act. 13.7 mCi/mmol; doses: 1.0, 4.0 and 10.0 μmol/kg) or [^{14}C]-3-OH PTMA iodide (sp. act. 10.2 mCi/mmol; doses: 2.5, 4.0 and 10.0 μmol/kg) were injected into the jugular vein. All of these drugs were obtained from the Radiochemical Centre, Amersham. Samples of arterial blood (approximately 250 μl) were taken at 1, 3, 5, 10, 15, 30, 45, 60, 90, 120 and 180 min. Plasma was obtained from part of each sample by centrifugation and the plasma concentration of each drug was determined. The remainder of the blood sample was used for the determination of acetylcholinesterase activity in red cells as described by Barber *et al.* (1976). Thus the design allowed the simultaneous monitoring of plasma concentration, acetylcholinesterase inhibition and muscle twitch tension throughout the experiments.

Measurement of drugs in plasma

Plasma samples were assayed for total radioactivity by liquid scintillation spectrometry in 9 ml NE 260 (Nuclear Enterprises, Ltd.) liquid scintillation fluid and 1 ml toluene/iso-amyl alcohol (5:1 v/v). Samples were counted in a Nuclear Chicago Unilux II or Intertechnique SL 33 liquid scintillation spectrometer. Quench correction was effected by the channels ratio or external standard channels ratio method.

Neostigmine Samples of plasma were extracted for [^{14}C]-neostigmine by a liquid cation exchange procedure (Barber, Bourne & Buckley, 1972). NaOH (10 μl) and ethyl-butyl ketone (100 μl) containing sodium tetraphenyl boron (20 mg/ml) was added to plasma and extractions effected by shaking in a vortex mixer. After centrifugation, the upper organic layer (50 μl) was removed and the radioactivity due to [^{14}C]-neostigmine determined by liquid scintillation spectrometry.

3-OH PTMA This compound was separated from its metabolite by the liquid cation exchange procedure without the addition of NaOH (Barber & Bourne, 1974).

Pyridostigmine Plasma samples were assayed for total radioactivity prior to separation of pyridostigmine and its metabolite by paper electrophoresis in borate buffer (0.1 mol/l, pH = 9.2) for 2 h at 300 V (Somani *et al.*, 1972). Radioactivity on the paper electrophoretograms was identified on a Tracerlab 4 π radiochromatogram scanner. The concentration of unchanged [^{14}C]-pyridostigmine and its metabolite was then determined from the total radioactivity in plasma and the results of paper electrophoresis.

Edrophonium Total radioactivity in plasma was determined as described above. Unchanged [^{14}C]-edrophonium was separated from its glucuronide metabolite by descending paper chromatography (Back & Calvey, 1972). The plasma concentration of unchanged drug was then determined from the total radioactivity in plasma and the results of paper chromatography.

Plasma concentration of the drugs was related to inhibition of erythrocyte acetylcholinesterase and the facilitation of neuromuscular transmission which was assessed by the increase in tibialis twitch tension above control values.

The effect of unlabelled 3-OH NMP (Roche Products Ltd.) on the *in vitro* inhibition of erythrocyte acetylcholinesterase at concentrations between 10^{-8} mol/l and 10^{-5} mol/l and on the *in vivo* facilitation of neuromuscular transmission (doses: 4.0 and 10.0 μmol/kg) was also studied.

Results

Clearance of the drugs from plasma

After intravenous injection, the plasma concentration of the unchanged drugs decreased rapidly for the first 30 min. After 30 min, the plasma concentration decreased more slowly. A semi-logarithmic plot relating plasma concentrations of unchanged drug to time was resolved into both two and three exponential components by the method of residuals (Riggs, 1963) utilizing a digital computer programme. The residual sums of squares between the experimental data and the computed curve was calculated and in all instances there was a significant reduction in the sums of squares when the data were analysed in terms of a tri-exponential function. In consequence, the plasma concentration-time curves were invariably expressed as the tri-exponential equation:

$$Cp = Pe^{-\pi t} + Ae^{-\alpha t} + Be^{-\beta t}$$

where P, A and B represent intercepts on the ordinate at zero time and $\pi/2.3$, $\alpha/2.3$ and $\beta/2.3$ are the slopes of the respective exponential components. Individual half lives and volumes of distribution of the drugs are shown in Table 1. A full pharmacokinetic analysis on edrophonium at 4.0 μmol/kg was not undertaken as the duration of these experiments was only 45 min.

Time course of the inhibition of acetylcholinesterase

The decline in enzyme inhibition after the administration of edrophonium or 3-OH PTMA was rapid and showed a direct relationship to the decline in plasma concentration of the drug. On the other hand, after neostigmine or pyridostigmine there was a very slow recovery of acetylcholinesterase with time. Examples of these results are shown in Figure 1.

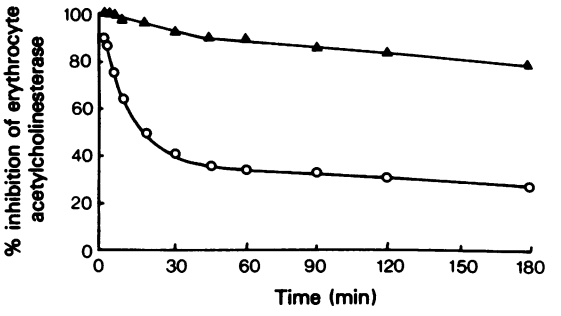

Figure 1 Time course of the inhibition of erythrocyte acetylcholinesterase after intravenous injection of either neostigmine (▲, 1 μmol/kg) or edrophonium (○, 1 μmol/kg).

Relationship between the concentration of the drugs in plasma and the inhibition of acetylcholinesterase

Measured concentrations of the drugs in plasma were related to the inhibition of erythrocyte acetylcholinesterase *in vivo* (Figure 2). When the activity of 3-OH NMP was studied it was found that this compound had no effect on erythrocyte acetylcholinesterase activity.

Relationship between the concentration of the drugs in plasma and the facilitation of neuromuscular transmission

After intravenous injection of edrophonium (1.0 μmol/kg), 3-OH PTMA (2.5 μmol/kg) and pyridostigmine (1.0 and 4.0 μmol/kg) neuromuscular transmission was enhanced in a monophasic manner. Different results were obtained after the administration of higher doses of edrophonium (4.0 or 10.0 μmol/kg), 3-OH PTMA (4.0 or 10.0 μmol/kg) and with neostigmine (1.0 μmol/kg). In these conditions, neuromuscular transmission was enhanced biphasically. An immediate evanescent increase in twitch tension rapidly declined and was succeeded by a secondary facilitatory phase which decreased to control levels more slowly.

The data preceding the peak of the secondary facilitatory phase were not included in the statistical analysis as this primary facilitation and subsequent decline has been shown to be quite unrelated to the plasma concentration of drug (Barber *et al.*, 1976).

The relationships between concentration of drug in plasma and the facilitation of neuromuscular transmission is shown in Figure 3. There was a statistically significant linear correlation between the logarithm of the plasma concentration and the increase in the tibialis twitch tension when the drugs were considered individually. These results are shown in Table 2. When 3-OH NMP was administered intravenously (4.0 or 10.0 μmol/kg) no effect on neuromuscular transmission was seen.

Relationship between inhibition of erythrocyte acetylcholinesterase and facilitation of neuromuscular transmission

The relationship between the inhibition of erythrocyte acetylcholinesterase and the facilitation of neuro-

Table 1 Half-lives and volumes of distribution of edrophonium, 3-hydroxyphenyltrimethylammonium (3-OH PTMA), neostigmine and pyridostigmine after intravenous administration in the rat

Drug	Dose (μmol/kg)	n	Half-lives (min) 1st Exponential	2nd Exponential	3rd Exponential	Volumes of distribution (ml/kg) V(central)	V(extrap)
Edrophonium	1.0	4	1.11 ± 0.16	10.5 ± 4.5	112 ± 22	201 ± 28	4910 ± 650
Edrophonium	10.0	3	0.98 ± 0.19	8.2 ± 1.8	156 ± 17	211 ± 36	5132 ± 699
3-OH PTMA	2.5	4	1.08 ± 0.11	7.8 ± 1.5	272 ± 51	241 ± 17	5120 ± 790
3-OH PTMA	4.0	4	1.23 ± 0.10	9.9 ± 2.1	281 ± 60	184 ± 11	5031 ± 522
3-OH PTMA	10.0	4	1.08 ± 0.07	11.5 ± 1.2	156 ± 86	181 ± 35	4891 ± 717
Neostigmine	1.0	6	2.07 ± 0.34	10.6 ± 0.7	232 ± 31	228 ± 37	4710 ± 450
Pyridostigmine	1.0	4	1.99 ± 0.23	9.7 ± 1.6	254 ± 34	181 ± 22	6540 ± 1170
Pyridostigmine	4.0	4	1.40 ± 0.33	7.5 ± 2.0	251 ± 74	157 ± 15	5840 ± 936

Data are mean ± s.e. mean.

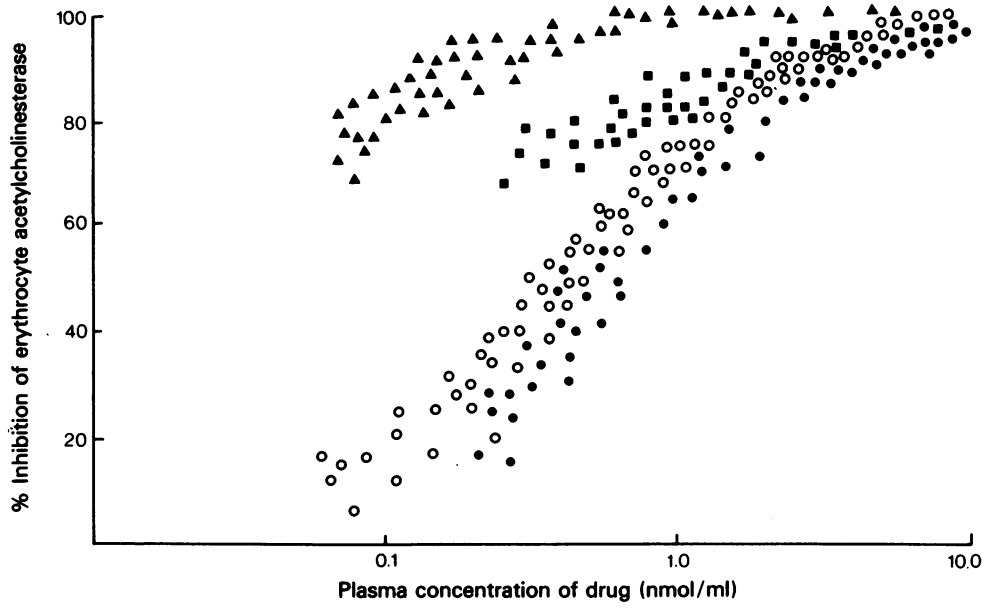

Figure 2 The relationship between the % inhibition of erythrocyte acetylcholinesterase and the concentration of drug in plasma: (▲) neostigmine; (■) pyridostigmine; (○) edrophonium; (●) 3-hydroxyphenyltrimethylammonium.

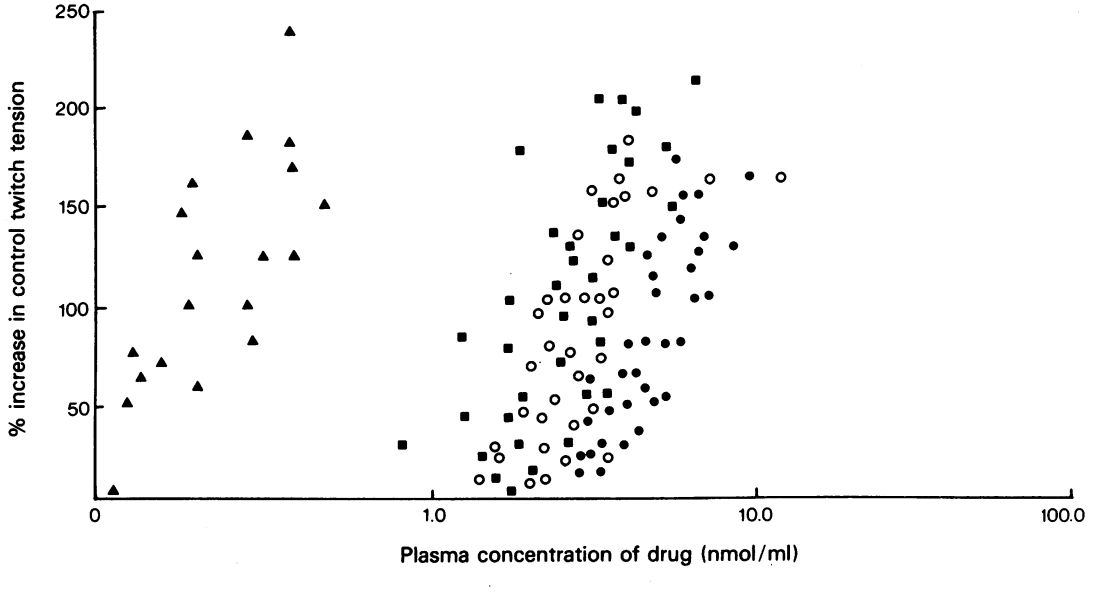

Figure 3 The relationship between the facilitation of neuromuscular transmission and the concentration of drug in plasma: (▲) neostigmine; (■) pyridostigmine; (○) edrophonium; (●) 3-hydroxyphenyltrimethylammonium

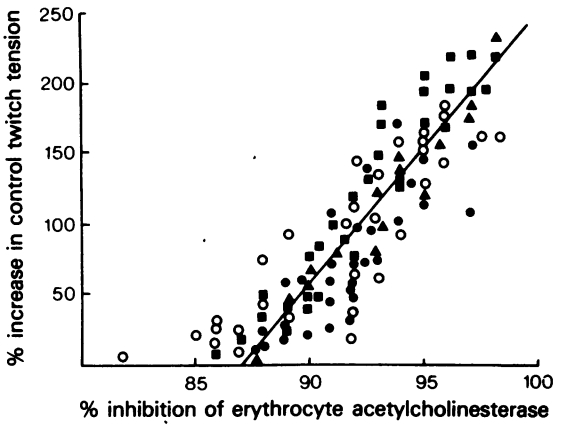

Figure 4 Correlation betweeen the facilitation of neuromuscular transmission and the inhibition of erythrocyte acetylcholinesterase; (▲) neostigmine; (■) pyridostigmine; (○) edrophonium,; (●) 3-hydroxyphenyltrimethylammonium.

muscular transmission in response to all four drugs is shown in Figure 4. When the enzyme was less than 85% inhibited no facilitation occurred. Between 85% and 98% inhibition the facilitation of neuromuscular transmission was linearly related to inhibition of erythrocyte acetylcholinesterase ($r = 0.81$, $P < 0.001$). However, because of the two mechanisms of enzyme inhibition, either reversible or covalent, which are responsible for the different time course of inhibition of these drugs (Figure 1), the facilitation of neuromuscular transmission lasted longer with pyridostigmine and neostigmine (up to 2 h) than for 3-OH PTMA and edrophonium (approx. 5 min).

Above 98% inhibition, facilitation of neuromuscular transmission was unrelated to inhibition of the enzyme as this corresponded to data preceding the peak of the second facilitatory phase.

Discussion

The qualitatively similar decline in plasma concentration of neostigmine, pyridostigmine, edrophonium and 3-OH PTMA with time indicates similar pharmacokinetic properties for all the drugs. The initial volume of distribution ($V_{(central)}$ 200 ml/kg or 20% of body weight) is not inconsistent with the drugs rapidly distributing into a central compartment consisting of the plasma and perhaps also other extracellular fluid. The immediate onset of action of these drugs and the correlation between the plasma concentration of the drug and the facilitation of neuromuscular transmission during the distributive decline in plasma concentration with time is indicative of a site of action within a central compartment (Levy, 1972) as the motor end plate is located within the extracellular space. The subsequent passage of these drugs into a total volume $V_{(extrap)}$ of approximately 5 l/kg indicates considerable tissue distribution despite their quaternary ammonium structure.

In contrast to the qualitatively similar decline in drug concentration with time, the decline in the *in vivo* inhibition of erythrocyte acetylcholinesterase in response to the four drugs was not similar (Figure 1). The very slow recovery of enzyme activity after the administration of neostigmine or pyridostigmine is probably due to the progressive carbamylation of the enzyme and is in contrast to the rapid recovery from the reversible ionic interaction caused by edrophonium or 3-OH PTMA. Previous studies have shown that edrophonium and 3-OH PTMA had little anticholinesterase activity at concentrations that facilitated neuromuscular transmission and cast serious doubts upon the classical views that these drugs acted via the inhibition of acetylcholinesterase at the motor end plate (Macfarlane, Pelikan & Unna, 1950; Randall & Lehmann, 1950). However, these results were obtained using assays of enzyme activity which employed relatively high substrate concentrations and dilution factors. Such methods are therefore rather inapplicable to *in vivo* experiments with reversible inhibitors of acetylcholinesterase as they would cause significant dissociation of the inhibited complex and hence reduce the measured inhibition below that actually occurring *in vivo* (O'Brien, 1967). The advantage of the radiometric assay used in these experiments has been discussed previously (Smith, 1974; Barber *et al.* 1976) and the results obtained indicate both edrophonium and 3-OH PTMA to be far more effective in inhibiting this enzyme than was originally suggested.

There was a statistically significant linear correlation between the logarithm of the plasma concentration and the increase in the tibialis twitch tension when the drugs were considered individually. However, when the drugs were considered collectively this relationship appears to be divided into two separate groups, one for neostigmine and one for pyridostigmine, edrophonium and 3-OH PTMA. Neostigmine

Table 2 Correlation between concentration of drug in plasma and % increase in control twitch tension

Drug	r	*d.f.*	P
Neostigmine	0.73	17	<0.01
Pyridostigmine	0.78	38	<0.01
Edrophonium	0.69	38	<0.01
3-OH PTMA	0.86	46	<0.01

increases the twitch tension at concentrations which are much lower than for the other three drugs (Figure 2). It is unlikely that the active metabolite of neostigmine, 3-OH PTMA, is responsible for this observation since its concentration during the period of observable pharmacological activity (0.15 to 0.20 nmol/ml) was incapable of inhibiting significantly erythrocyte acetylcholinesterase activity and insufficient to elicit a twitch response. Smith, Mead & Unna (1957) reported that neostigmine was about 25 times more potent than edrophonium and pyridostigmine in the *in vitro* facilitation of the frog rectus muscle in response to exogenously applied acetylcholine. Their results are consistent with the findings of this study. It is possible that the action of these drugs on the neuromuscular junction is in consequence of a direct pre- or postsynaptic membrane depolarization. However, Hobbiger (1952) concluded from his work with the frog rectus abdominus muscle that the direct action of these drugs in facilitating neuromuscular transmission was only evident at very high concentrations and that the primary facilitatory action was not due to a direct depolarization of the membrane receptor sites.

An alternative explanation may be evident from the relationship between the facilitation of neuromuscular transmission and the inhibition of erythrocyte acetylcholinesterase. While it is possible that both the facilitation of neuromuscular transmission and the inhibition of erythrocyte acetylcholinesterase are independently related to the plasma concentration of the drugs, the results shown in Figure 4 are not inconsistent with the facilitation of neuromuscular transmission being in consequence of the inhibition of acetylcholinesterase at the motor endplate whose inhibitory time course would parallel erythrocyte acetylcholinesterase inhibition. Further evidence for this is the observation that 3-OH NMP is devoid of anticholinesterase activity and did not facilitate neuromuscular transmission at the doses administered.

We should like to thank Dr R. F. Long, of Roche Products Limited, Welwyn Garden City, Herts., for the gift of 3-OH NMP. We should also like to thank Professor A. Breckenridge for his advice in the writing of this manuscript.

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(*Received August* 16, 1978.
Revised December 14, 1978.)