STUDIES ON THE ANTICHOLINESTERASE AND TWITCH POTENTIATION ACTIVITIES OF GALANTHAMINE*

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Following the isolation of galanthamine by Proskurina (1) and Uyeo (2), Mashkovskiy (3, 4) reported that cholinesterase activity was depressed and sensitivity of striated muscles to acetylcholine was increased by the administration of galanthamine. Sedova (5) also recognized that galanthamine administration caused myosis, salivation, and the activation of smooth muscle movement in experimental animals. Hence the application of galanthamine for the therapy of myopathy, myasthenia gravis, radiculitis, and polyneuritis was suggested. Anticholinesterase activities of galanthamine were further examined by Irwin *et al.* (6, 7). After examining both the anticholinesterase activity and twitch potentiation of several galanthamine derivatives, they concluded that galanthamine methiodide was the most active.

It seems, however, that something still remains to be clarified in the correlation between the twitch potentiation and anticholinesterase activity of galanthamine derivatives. The present experiments were chiefly carried out to clarify this point. Subsequently, comparisons of galanthamine were made with other anticholinesterases *i.e.* neostigmine, physostigmine, and DFP, in regards to muscle twitch potentiation, anticholinesterase activity, and in the activation of the motor nerve terminals.

METHODS

For the comparison of the twitch potentiation of anticholinesterases the sciatic gastrocnemius preparation and the phrenic nerve diaphragm preparation of Bülbring (8, 9) were used. Contractions of M. gastrocnemius and isolated diaphragm were obtained either by direct or indirect electric stimulation (0.2 c/s, 1 msec). Also the M. gastrocnemius paralyzed with 0.25~0.30 mg/kg of *d*-tubocurarine was employed for comparison of the anticholinesterase activity. This activity was determined 3 minutes after the administration of anticholinesterases. In addition, after 15 minutes of incubation with anticholinesterases, the contraction of M. rectus abdominis of frogs by acetylcholine was compared in order to evaluate the potency of anticholinesterases. The effects of anticholinesterases on the movements of rabbit intestine *in vitro* and *in vivo* were observed using the Magnus and Trenderburg method respectively. Anticholinesterase activity was also tested bioche-

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mically using the Warburg apparatus following Ammon's (10) method. The liberation of CO_2 in 30 minutes was determined under the condition of 95% N_2 , with 5% CO_2 . Antidromic discharges from the ventral nerve fibres in sciatic gastrocnemius preparation of cats were traced oscillographically with the technique described by Riker *et al.* (11, 12). The electro-myograph of M. gastrocnemius was also observed in this preparation. Cats were slightly anaesthetized with 30 mg/kg of methylhexital intraperitoneally.

RESULTS

I. Effects on the Striated Muscle

Twitch potentiation: Following the intravenous administration of galanthamine, neostigmine, and physostigmine in cats, the contractions of M. gastrocnemius by indirect

FIG. 1. Potentiation of anticholinesterases on the twitch reflexes of the M. gastrocnemius of cats.

TABLE 1. The comparison of effects between galanthamine and neostigmine on the twitch reflexes of M. gastrocnemius of rats.

Compound	Dose mg/kg	No. of Rats Responded	% Increase of Twitch	Duration min
Galanthamine	0.1	3/3	111.8 $\pm$ 10.2	93.0
	0.05	3/3	48.4 $\pm$ 8.3	16.0
	0.025	2/3	10.0 $\pm$ 4.0	7.0
Neostigmine	0.005	3/3	107.5 $\pm$ 7.3	120.0
	0.0025	3/3	29.6 $\pm$ 5.1	13.3
	0.00125	3/3	11.9 $\pm$ 4.8	11.7

Electric stimulation (0.2 c/s, 1 msec) was applied to the sciatic nerve.

TABLE 2. The comparison of effects between galanthamine and neostigmine in the phrenic nerve diaphragm preparation of rats.

Compound	Dose	No. of Preparation Responded	% Increase of Twitch	Time for Max. Twitch
Galanthamine	10^{-7}	2/4	27.9	3.7 min
	5×10^{-7}	4/4	61.8	5.6 "
	10^{-6}	3/3	91.0	2.7 "
Neostigmine	10^{-8}	2/5	29.7	4.5 "
	2.5×10^{-8}	2/2	32.6	6.9 "
	5×10^{-8}	5/5	84.0	7.3 "

Electric stimulation (0.2 c/s, 1 msec) was applied to phrenic nerve.

FIG. 2. The effect of anticholinesterases on the contraction of the M. gastrocnemius of cats.

electric stimulation were markedly activated (Fig. 1). The potency of galanthamine is supposed to be about 1/10.5 of neostigmine and 1/3.5 of physostigmine respectively. The duration of twitch potentiation was most lasting with physostigmine followed by neostigmine or galanthamine. These twitch potentiations by neostigmine and galanthamine were also observed in the sciatic gastrocnemius preparation or phrenic nerve diaphragm preparation of rats (Table 1, 2). In these experiments the potency of galanthamine is supposed to be about 1/10~1/20 of that of neostigmine. Quarternary galanthamine, *i.e.* galanthamine methiodide, however, showed a marked increase of drug action as Irwin had reported (Fig. 2). These anticholinesterases also had twitch potentiating effects in the direct electric stimulation of M. gastrocnemius in cats. So it is assumed that these compounds activate the muscle fibres as well.

Anti-curars activity: The contraction of M. gastrocnemius of cats, completely inhibited by the intravenous administration of *d*-tubocurarine in a dose of 0.25~0.3 mg/kg, showed

TABLE 3. Antagonism between anticholinesterases and *d*-tubocurarine in the contraction of the M. gastrocnemius of cats.

Anticholinesterase	Dose mg/kg	No. of Exp.	% Recovery of the Contraction
Galanthamine	0.8	3	72.9
	0.4	4	56.4
	0.2	4	24.2
	0.1	3	1.3
Neostigmine	0.2	2	89.3
	0.1	3	84.8
	0.05	4	60.0
	0.025	4	14.9
Physostigmine	0.4	4	30.7
	0.2	4	11.0
	0.1	3	5.5
	0.05	2	0

Each figure shows the mean value of the percentage recovery of the contraction of M. gastrocnemius 3 minutes after administration of anticholinesterase.

FIG. 3. The effects of anticholinesterases on the contraction of the M. rectus abdominis of frogs. Dots show when 5×10^{-7} of ACh were applied.

a prompt recovery after the administration of anticholinesterases. Table 3 shows the recovery of twitch reflexes 3 minutes after the injection of anticholinesterases. Anticuraric activities of these drugs decreased in the following order: neostigmine, galanthamine, physostigmine.

Effects on the M. rectus abdominis of frogs: galanthamine, neostigmine markedly sensitized the M. rectus abdominis to acetylcholine (Fig. 3). ED_{50} concentrations of neostigmine, galanthamine, and physostigmine are 2.1×10^{-7} ($6.0 \times 10^{-7} \sim 7.1 \times 10^{-8}$), 4.8×10^{-7} ($2.7 \times 10^{-6} \sim 8.4 \times 10^{-8}$), and 4.1×10^{-6} ($2.0 \times 10^{-5} \sim 8.4 \times 10^{-7}$) respectively.

II. Effects on the Smooth Muscles

Effects on the isolated rabbit intestine: The tonus of the isolated intestines was markedly elevated by the administration of galanthamine. In concentrations above 10^{-6} , tetanic contractions of the intestines were observed. The effects of both neostigmine and physostigmine are supposed to be 10 times stronger than that of galanthamine. But the effects of DFP, however, were rather mild and both mild contraction and irregular peristalsis of isolated intestines were observed even by concentrations of 10^{-5} .

Effects on the rabbit intestine "in situ": Following intravenous administration of galanthamine in rabbits, both the tonus and peristaltic amplitude of the rabbit intestine *in situ* were increased as observed in the isolated intestines. In this preparation the effects of other anticholinesterases were almost the same as that of galanthamine. But the effects of both galanthamine and DFP are about 1/10 of those of neostigmine or physostigmine.

III. Effects on the Cholinesterase Activity

Anticholinesterase activity: Anticholinesterase activities of galanthamine, neostigmine, and physostigmine are summarized as Table 4. Although the anticholinesterase activity of galanthamine is far inferior to those of neostigmine and physostigmine, the twitch potentiation by galanthamine administration is only 1/10 of that of neostigmine or physostigmine. So it is presumed that factors other than anticholinesterase activity are concerned with the twitch potentiating effects of galanthamine.

In comparing the anticholinesterase activities of galanthamine and its methiodide, no increase of anticholinesterase activity was found by this quaternization. In the homogenate of M. gastrocnemius of rats, there was also no difference in the anticholinesterase

TABLE 4. Comparison of I_{50} between anticholinesterases *in vitro*.

Compound	In the Brain Homogenate of Rats	In the Hemolized Erythrocytes of Rats	In the Homogenate of M. Gastrocnemius of Rats
Galanthamine	2.3×10^{-6} M	1.2×10^{-6} M	3.5×10^{-6} M
Neostigmine	1.1×10^{-8} "	1.9×10^{-8} "	1.5×10^{-8} "
Physostigmine	7.0×10^{-8} "	4.2×10^{-8} "	3.6×10^{-8} "

I_{50} shows the concentration of anticholinesterases by which the 50% inhibition of cholinesterase is recognized.

activity between galanthamine and its methiodide (Fig. 4). Therefore the increase of twitch potentiation by the quaternization of galanthamine can not be explained by the increase of anticholinesterase activity.

Mode of anticholinesterase activity: As shown in Table 5, even the 90% inhibition of erythrocyte cholinesterases by the incubation with galanthamine, disappeared after washing three times with bisulphate buffer. The anticholinesterase activity of neostigmine resembles that of galanthamine. The marked inhibitions of galanthamine and neostigmine on erythrocyte cholinesterase recovered following overnight dialysis with bisulphate buffer, but not that of DFP.

FIG. 4. Anticholinesterase activities of galanthamine, galanthamine methiodide, and neostigmine in the homogenate of *M. gastrocnemius* of cats.

Substrate; ACh 5×10^{-3} M.

ChE; Homogenate of *M. gastrocnemius* $\times 5$.

TABLE 5. The effects of washing on the anticholinesterase activity of galanthamine, neostigmine, and DFP.

Compound	Dose	No. of Exp.	% Inhibition before Washing	% Inhibition after Washing
Galanthamine	10^{-6} M	3	17.3 ± 0.04	0
	10^{-5} M	3	62.3 ± 2.8	1.6 ± 1.6
	10^{-4} M	3	95.2 ± 2.8	7.0 ± 3.5
Neostigmine	10^{-8} M	3	33.4 ± 5.3	1.5 ± 1.3
	10^{-7} M	3	60.7 ± 5.5	8.3 ± 0.6
	10^{-6} M	3	88.8 ± 6.1	27.2 ± 6.3
DFP	2.5×10^{-7} M	3	21.1 ± 9.3	21.8 ± 2.8
	5×10^{-7} M	3	45.1 ± 10.1	47.0 ± 5.3
	10^{-6} M	3	80.5 ± 3.6	74.4 ± 4.8

Erythrocytes suspension of rats diluted 10 times was employed as crude cholinesterase.

IV. Effects on the Antidromic Discharges

As Figs. 5 and 6 show, repetitive antidromic discharges were observed within one minute after the intravenous administration of galanthamine. Repetitive antidromic discharges caused by galanthamine, however, disappeared in a few minutes after the intravenous administration of 10 mg/kg of either pentobarbital or procaine. Galanthamine methiodide and neostigmine also elicited repetitive antidromic discharges. These discharges caused with 0.8 mg/kg of galanthamine are comparable to those caused by 0.1 mg/kg of its methiodide or 0.05 mg/kg of neostigmine.

FIG. 5. Effects of galanthamine both on the antidromic discharge of ventral root fibres and EMG of *M. gastrocnemius* of cats.

Between (1) and (2) galanthamine, 0.8 mg/kg *i.v.* inj. (1) Control (2) instantly after inj. (3) 1 min after inj. (4) 3 min after inj. (5) 5 min after inj. (6) 10 min after inj. (7) 20 min after inj. (8) 30 min after inj. (9) 40 min after inj. (10) 50 min after inj.

FIG. 6. Effects of procaine on the repetitive antidromic discharge of ventral root fibres induced by galanthamine.

(1) Control (2) 1 min after galanthamine 0.8 mg/kg (3) 5 min after galanthamine 0.8 mg/kg (4) 1 min after procaine 10 mg/kg (5) 5 min after procaine 10 mg/kg (6) 10 min after procaine 10 mg/kg.

TABLE 6. Acute toxicity of anticholinesterases in mice.

Compound	Route of Administration	LD ₅₀	Fiducial Limit
Neostigmine	Intravenous	0.1181 γ /g	0.1089 γ /g ~ 0.1281 γ /g
Physostigmine	Intravenous	0.255 γ /g	0.245 γ /g ~ 0.270 γ /g
Galanthamine	Intravenous	3.117 γ /g	2.934 γ /g ~ 3.320 γ /g
	Subcutaneous	6.600 γ /g	6.240 γ /g ~ 6.980 γ /g
	Oral	14.15 γ /g	13.38 γ /g ~ 14.69 γ /g

V. Toxicity of Galanthamine

In mice slight tremor in the upper parts of the body with salivation was observed in 3~10 minutes after the subcutaneous injection of galanthamine in a dose of 0.01~0.05 mg/kg. Then the animals died of respiratory arrest following marked tremor. The LD₅₀s of the anticholinesterases are shown in Table 6.

DISCUSSION

Although the anticholinesterase activity of galanthamine is about 1/100 of that of neostigmine or physostigmine, the twitch potentiating effects of galanthamine in the nerve muscle preparation is about 1/10 of the latter. In addition marked twitch potentiation of galanthamine methiodide is not followed with the rise of anticholinesterase activity as shown in the experiments. These contradictions between twitch potentiation and anticholinesterase activity are also not solved by the direct effects of galanthamine on the muscle fibres or by the difference of species in the experimental animals.

Following the studies of Brown (13), Eccles (14, 15) attributed the twitch potentiation of anticholinesterases to the accumulation of acetylcholine at the endplate of nerve muscle junction. Riker and Wercøe (16-19) in agreement with Masland and Wigton (20), considered that the twitch potentiation by neostigmine is mainly concerned with its activation at the motor nerve terminals. They criticized the classical view of Eccles stressing the following points; (1) Potent depolarizers do not necessarily show the marked twitch potentiation. (2) Twitch potentiations caused by anticholinesterase like neostigmine appeared soon after the administration of the compound. We also observed the marked repetitive antidromic discharges soon after the intravenous administration of galanthamine. The repetitive antidromic discharges caused with 0.8 mg/kg of galanthamine are almost comparable to those caused with 0.2 mg/kg of galanthamine methiodide or with 0.05 mg/kg of neostigmine. The relative potencies between anticholinesterases in the antidromic discharges are in good agreement with those observed in the nerve muscle preparation of cats and rats. So it is presumed that the twitch potentiation caused by these compounds are more concerned with the activation of motor nerve terminals than anticholinesterase activity. It is worthy of notice that studies on the effects of galanthamine on the spinal reflexes are in progress (21, 22).

SUMMARY

The effects of galanthamine on the nerve muscle preparation, smooth muscles, cholinesterases, and on the motor nerve terminals were compared with its methiodide, neostigmine, physostigmine, and DFP. Although galanthamine activated the nerve muscle preparation as well as smooth muscles like other anticholinesterases, these effects were not explained by the anticholinesterase activity only. It was suggested that the twitch potentiations caused by galanthamine, galanthamine methiodide, and neostigmine were more concerned with the activation of motor nerve terminals.

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