Br. J. Pharmac. (1979), **65**, 565–571

EFFECTS OF CERTAIN MUSCARINIC ANTAGONISTS ON THE ACTIONS OF ANTICHOLINESTERASES ON CAT SKELETAL MUSCLE

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1 The effects of some muscarinic antagonists, namely, N-ethyl-2-pyrrolidylmethyl-cyclopentylphenyl glycollate (PMCG), N-methyl-4-piperidyl-phenylcyclohexyl glycollate (PPCG, racemate and R and S enantiomers) and 4'-N-methyl-piperidyl-1-phenyl-cyclopentane carboxylate (G3063) on organophosphate (sarin, soman)- and carbamate (neostigmine)-induced twitch augmentation have been studied in cat soleus muscle.

2 The results of a preliminary study comparing the potency of sarin and soman in inhibiting the acetylcholinesterase activity of muscle in relation to the effect on the maximal twitch response indicated that there is not a simple relationship between degree of enzyme inhibition by these drugs and alteration of muscle function.

3 The muscarinic antagonists studied were capable of preventing or reversing sarin-, soman- or neostigmine-induced twitch augmentation. Doses sufficient to give complete protection from the effects of the anticholinesterase agents had little or no effect on the twitch response of normal muscle.

4 The protective action of these muscarinic antagonists is dose-dependent but independent of known antagonist actions at muscarinic receptors.

5 The effects of some local anaesthetics (lignocaine, prilocaine, cinchocaine, procaine) and other membrane stabilizers (quinine, ketamine, chlorpromazine, trifluprazine) were compared with those of the muscarinic antagonists in an attempt to elucidate the mode of action of these acetylcholine antagonists. The evidence is insufficient to exclude the involvement of a membrane stabilizing action.

Introduction

It was shown by Brimblecombe & Everett (1969a; 1970b) that some muscarinic antagonists, notably N-ethyl-2-pyrrolidylmethyl-cyclopentyl-phenyl glycollate (PMCG), cause augmentation of the indirectly or directly stimulated twitch of both slow-twitch and fast-twitch muscle of the cat hind limb. The same antagonists prevent or reverse sarin-induced twitch augmentation of these muscles (Brimblecombe & Everett, 1969b; 1970a).

The work described in the present paper was designed to explore the latter effects in more detail. In particular, the aims of the experiments described here were to ascertain whether these effects of the drugs on muscle were independent of their muscarinic antagonist activities and to study their interactions with both organophosphate and carbamate type anticholinesterase agents. In addition, in some experiments acetylcholinesterase activity in muscle was measured to investigate the relationship between enzyme inhibition and the physiological state of the muscle.

Some of the results have been presented to the British Pharmacological Society (Brimblecombe & Webb, 1973).

Methods

Experimental animals

The experiments were performed on cats of either sex, weighing between 2.0 and 2.75 kg, anaesthetized with pentobarbitone sodium (40 mg/kg) injected intraperitoneally.

All drugs (except pentobarbitone sodium) were injected intra-arterially (i.a.) to the soleus or flexor digi-

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0007-1188/79/040565-07 $01.00

torum longus (FDL) muscle through a fine polythene cannula inserted retrogradely into the sural artery so that its tip reached the junction with the popliteal artery. Intra-arterial injections were made in a volume of 0.2 ml and washed in with 0.2 ml of 0.9% w/v NaCl solution (saline) containing heparin (250 units/ml).

Blood samples of 0.4 ml were taken from the tibial artery and in some cases from a jugular vein.

Recording of muscle contractions

The method used for recording contractions of the soleus muscle, and in some cases of the flexor digitorum longus (FDL) was essentially that described by Buller & Lewis (1965a, b). The muscles were stimulated indirectly via the cut proximal end of the motor nerves every 20 s with pulses of 0.1 ms duration and supramaximal strength (4 × maximal).

Acetylcholinesterase (AChE) assay

AChE activity was measured at 30°C by the spectrophotometric method of Ellman, Courtney, Andres & Featherstone (1961). The reaction was measured at 412 nm with a Pye Unicam SP 1800 coupled to a Weyfringe ADCP-2 digital printer. The reaction rate was linear for 6 min.

The muscles were washed free of blood, blotted and weighed and then homogenized for 1 min in 2 vol of 0.1 M, pH 8.0 phosphate buffer with a Silverson homogenizer and sonicated for 30 s at 1.5 A with an MSE ultrasonicator. The homogenates were stored at −15°C until assayed. An optimal substrate concentration of 1.0 mM acetylthiocholine was used; with higher concentrations substrate inhibition occurred. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

No specific substrate for AChE could be used since acetyl-β-methylcholine is hydrolysed by both AChE (EC 3.1.1.8) and ChE (EC 3.1.1.7) in contrast to its oxygen analogue (Heilbronn 1959). Measurement of the contribution of ChE was therefore necessary. The ChE activity of 6 control muscles was 3.50 ± 0.47 μmol min^{-1} g^{-1} protein with butyrylthiocholine as substrate. It was calculated that this enzyme concentration would increase the hydrolysis of 1 mM acetylthiocholine by $<5\%$ using a calibration curve previously described (French, Sellers & Wilkinson, 1977). The term AChE strictly refers to acetylchiocholine esterase activity of the tissue.

Experiments with sarin and soman

Effects on twitch response and inhibition of muscle acetylcholinesterase. Twenty male cats were used to assess the effect of a single dose of either sarin or soman on the maximal twitch response and on muscle AChE activity. In these experiments each animal served as its own control for muscle AChE determinations. The right soleus muscle was stimulated indirectly via its motor nerve once every 20 s for 30 min and then removed for AChE assay. The left soleus muscle was then stimulated indirectly every 20 s for 15 min before a single dose of either sarin (dose range 2.5 to 15.0 μg) or soman (dose range 0.625 to 5.0 μg) was injected intra-arterially. The resultant twitch augmentation was recorded for a further 15 min before the muscle was removed for AChE assay.

Comparison of effects on right and left leg. Since the effects of organophosphate anticholinesterase agents persist over many hours it is virtually impossible to use any muscle as its own control. In the protection studies to be described, the contralateral muscle acted as control so it was necessary to check for similarity in sensitivity of the soleus muscles from both hind limbs. To do this, cumulative dose-response curves were determined, the same incremental dose of anticholinesterase agent being injected intra-arterially into both legs simultaneously and the resultant effect on maximal twitch tension noted.

Protection studies

Similar muscles were prepared in both hind limbs and stimulated alternately every 10 s. To assess the protective activity of a compound, it was injected into one leg prior to injection of the anticholinesterase agent to both legs. The muscarinic antagonists were given 5 min before the organophosphates or neostigmine. All the other drugs tested were injected 1 min before the anticholinesterase agents.

The doses of sarin (5 μg) and soman (2.5 μg) used in these protective studies were chosen to give approximately 45 to 50% inhibition of muscle AChE as this corresponded to near maximal twitch augmentation. A 10 μg dose of neostigmine was used because this dose consistently produced marked twitch augmentation. Since the effects on skeletal muscle following a single intra-arterial dose of either sarin or soman are so long lasting, it was not possible to assess whether the protective action of the muscarinic antagonists or the other drugs used was dose-dependent. Therefore, the short acting carbamate, neostigmine, was used for dose-response studies. In these experiments both legs received a 10 μg control dose of neostigmine (i.a.) before injection of a low dose (e.g. 0.25 mg) of the chosen antagonist into one leg only. Further 10 μg doses of neostigmine were then given and the dose of antagonist progressively increased by a factor of 2 until complete protection was afforded. The dose of neostigmine (10 μg i.a.) was then repeated at hourly intervals until the response in the experimental leg had returned to the control level.

Reversal experiments with sarin and soman

An attempt to quantify the protective action of the muscarinic antagonists was made by determining the dose required to produce a 50% reduction in either sarin or soman-induced twitch augmentation. The protective effect of a 2 mg dose of muscarinic antagonist was first determined, as already described, and when the augmented maximal twitch on the unprotected side had reached a steady state, doses of the same antagonist beginning at 0.0625 mg and increasing by a factor of 2 were given until the control tension was again reached.

Drugs

The following drugs were used:- pentobarbitone sodium (Nembutal, Abbot), heparin (Evans Medical), neostigmine methylsulphate (Roche; mol. wt. 334), isopropylmethylphosphonofluoridate (sarin, synthesized at CDE, Porton; mol. wt. 140), 1,2,2,-trimethylpropylmethylfluoridate (soman, synthesized at CDE, Porton; mol. wt. 182), atropine sulphate (BDH; mol. wt. 695), caffeine citrate (Evans Medical; mol. wt. 386), chlorpromazine hydrochloride (May & Baker; mol. wt. 355), cinchocaine hydrochloride (Parke Davis; mol. wt. 380), ketamine hydrochloride (Parke Davis; mol. wt. 274), lignocaine hydrochloride (Astra; mol. wt. 289), N-ethyl-2-pyrrolidylmethylcyclo-pentylphenyl glycollate (PMCG, synthesized at CDE, Porton; mol. wt. 368), N-methyl-4-piperidyl-phenylcyclohexyl glycollate (PPCG racemate and R and S enantiomers, synthesized at CDE, Porton; mol. wt. 348), 4′-N-methyl-piperidyl-1-phenyl-cyclopentane carboxylate (G3063, synthesized at CDE, Porton; mol. wt. 362), prilocaine hydrochloride (Astra; mol. wt. 257), procaine hydrochloride (Hopkin & Williams; mol. wt. 273), quinine dihydrochloride (BDH; mol. wt. 397), triflupromazine hydrochloride (synthesized at CDE, Porton; mol. wt. 394). Sarin and soman were dissolved in isopropyl alcohol and diluted with saline immediately before use.

Doses quoted in the text refer to the salts.

Results

Experiments with sarin and soman

Effects on twitch and inhibition of muscle acetylcholinesterase The effect of incremental doses of sarin and soman on twitch response and muscle AChE activity are presented graphically in Figure 1. The control value for muscle AChE activity, expressed as mean ± s.e. mean for 20 determinations from cats used in experiments described later, was 11.52 ± 0.75 μmol min^{-1} g^{-1} protein. The results are expressed as a percentage of the control values obtained for each animal.

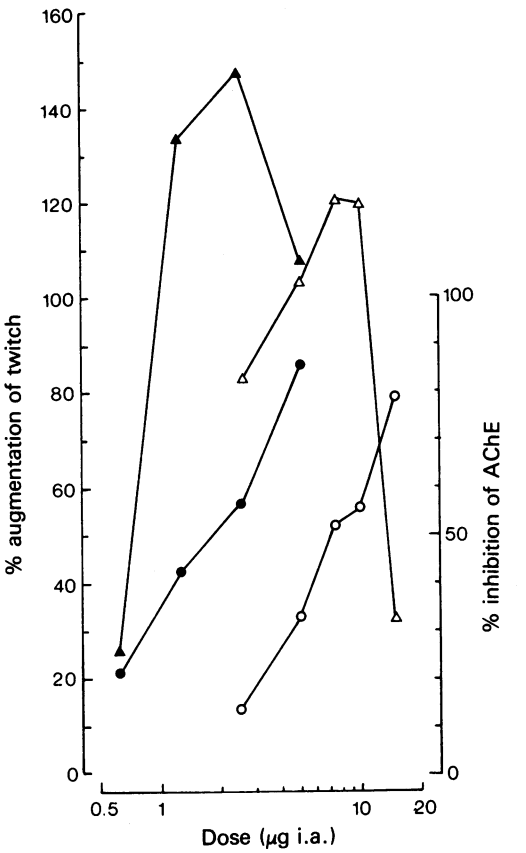

Figure 1 Effects of intra-arterial sarin (open symbols) and soman (filled symbols) on maximal twitch response of soleus muscle (△, ▲), and muscle acetylcholinesterase (AChE) activity (○, ●). Each point is the mean of a single dose in 2 to 3 animals.

Maximal twitch augmentation was obtained with doses of 1.25 to 2.5 μg for soman and 5.0 to 7.5 μg for sarin. This corresponded to an inhibition of muscle AChE activity of 43 to 57% for soman and 33 to 52% for sarin. When inhibition of muscle AChE was greater than 55% the twitch augmentation produced by either soman or sarin was reduced. The relative potency of soman to sarin was 4.2:1 for inhibition of muscle AChE.

Comparison of effects on left and right leg Both sarin and soman produced similar responses in soleus muscles from the right and left leg of the same animal. A dose-related twitch augmentation was followed by twitch depression at higher doses. With these higher doses (sarin, 64 μg i.a., soman, 32 μg i.a.) cardiovascular and respiratory effects were marked. The dose pro-

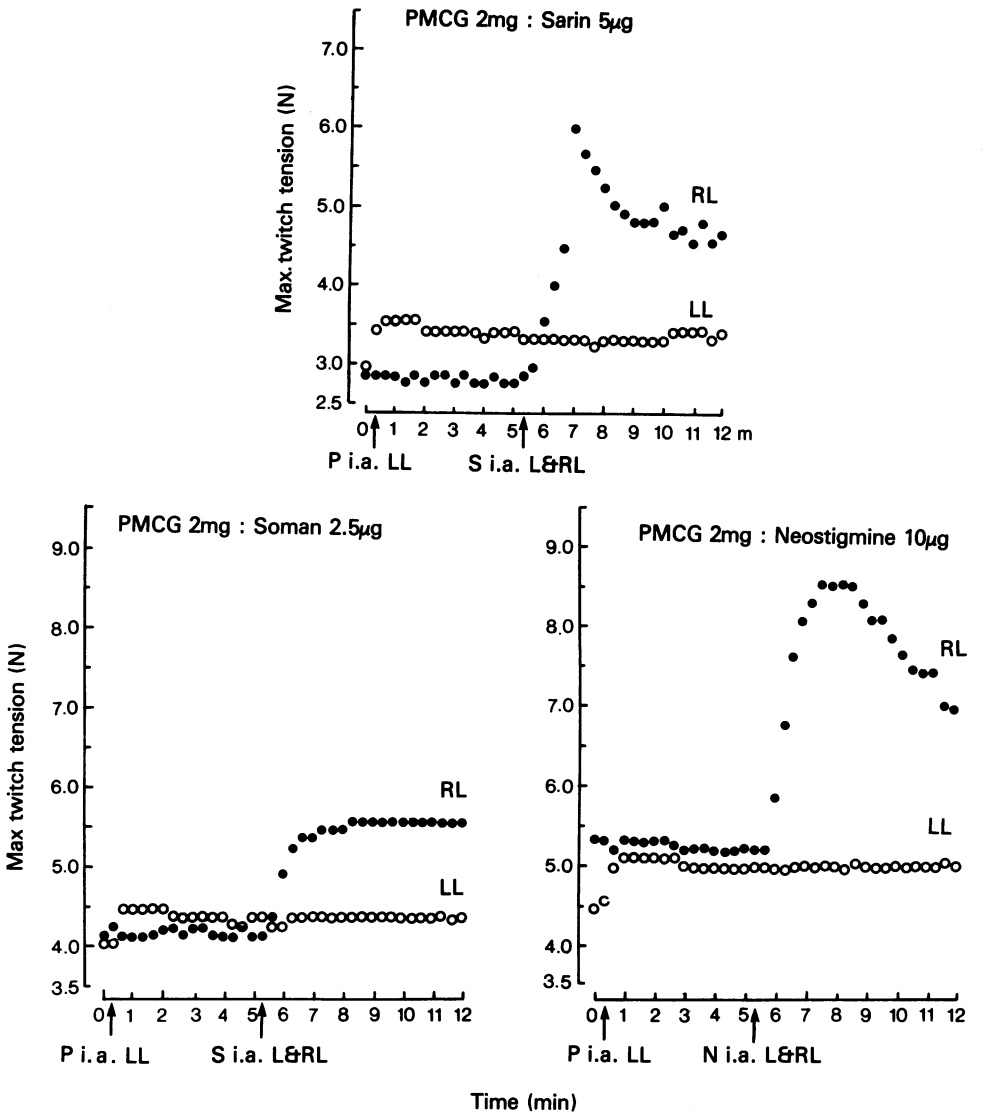

Figure 2 Soleus muscle. Results obtained from three different experiments in which N-ethyl-2-pyrrolidylmethyl-cyclopentylphenyl glycollate (PMCG) was administered to one leg only 5 min before giving an organophosphate (sarin, soman) or carbamate (neostigmine) anticholinesterase agent to both legs. The pretreated muscles failed to respond in the characteristic manner to these agents. Open symbols, left leg (LL); filled symbols, right leg (RL). Ordinate scale: maximal twitch tension in Newtons (N); abscissa scale: time in minutes.

ducing peak twitch augmentation ranged from 4 to 8 μg for sarin and 2 to 4 μg for soman.

Protection studies with sarin and soman

Muscarinic antagonists Figure 2 shows the effects of a 2 mg dose of PMCG injected intra-arterially into one leg 5 min before administration to both legs of either sarin (5 μg) or soman (2.5 μg); the effects of both agents were prevented completely. This protection occurred in the presence of the same degree of muscle AChE inhibition in both legs, i.e. 45 to 50%. Slight twitch augmentation (8 to 24%) was often, but not always, evident after the 2 mg dose of PMCG. Similar results were obtained with a 2 mg dose of either G3063 or PPCG (Rac). The enantiomers of PPCG were equally effective in preventing sarin or soman-induced twitch augmentation. The effects of

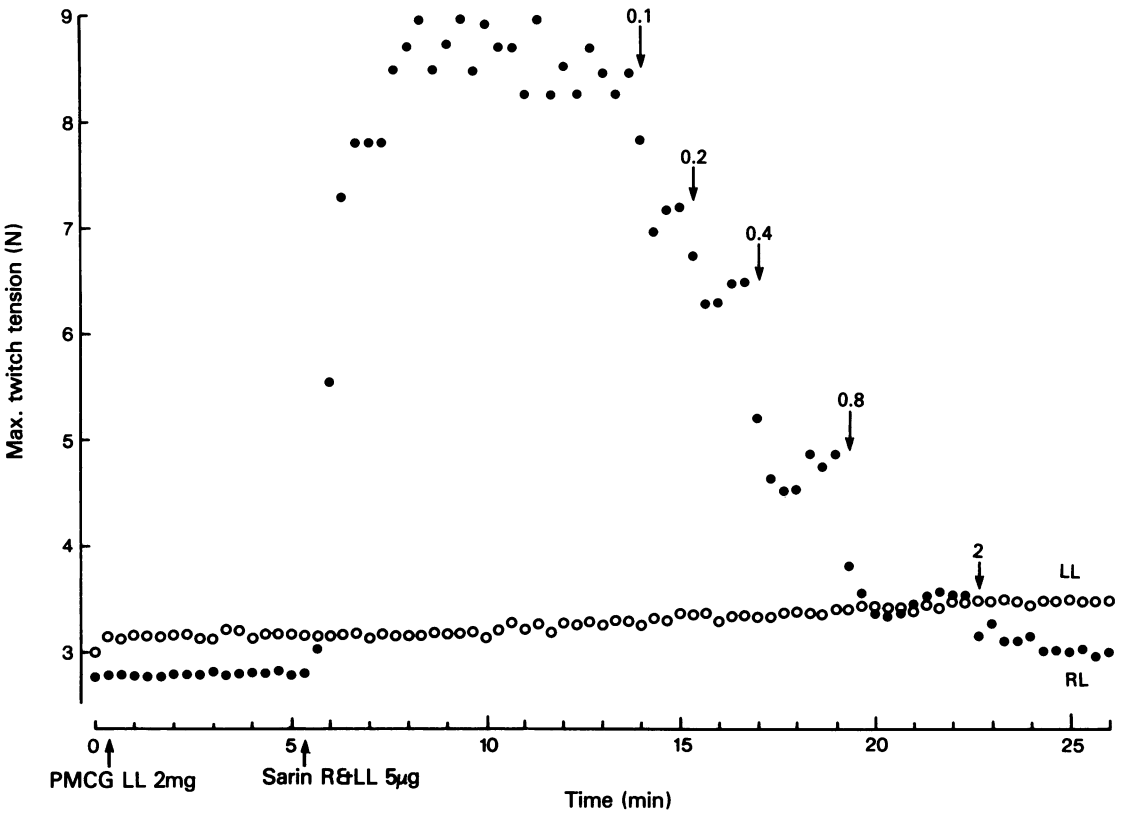

Figure 3 Soleus muscle. Dose-related effect of N-ethyl-2-pyrrolidylmethyl-cyclopentylphenyl glycollate (PMCG) on sarin-induced twitch augmentation. The doses (in mg) at the arrows are cumulative values. This figure also illustrates the protective effect of a 2 mg dose of PMCG given 5 min before administering sarin. Open symbols, left leg (LL); filled symbols, right leg (RL). Ordinate scale: maximal twitch tension in Newtons (N); abscissa scale: time in minutes.

sarin or soman were prevented by all these muscarinic antagonists in doses which had little or no effect on the twitch response of the soleus muscle.

In contrast, a 2 mg dose of atropine failed to protect against the effects of sarin or soman and complete protection was not achieved even with a dose of 8 mg. The protective action of PMCG, PPCG and G3063 lasted for 15 to 30 min and sometimes up to 1 h, after which it slowly declined.

Other drugs Both lignocaine and quinine completely protected the soleus muscle from the effects of sarin (5 μg); the dose of each required was 4 mg. Little or no effect on twitch response was seen with this dose. The duration of the protection afforded was short-lived lasting only 10 to 15 min. Caffeine in a dose of 8 mg was ineffective.

Reversal experiments with sarin and soman

The degree of twitch augmentation produced by 5 μg sarin or 2.5 μg soman varied considerably from experiment to experiment, i.e. sarin 59 to 145%, soman 85 to 180%. PMCG, PPCG, G3063, atropine, lignocaine and quinine all reversed the twitch augmentation (Figure 3) but due to the variability in the results it was not possible reliably to rank drugs in order of potency in producing this reversal. With all compounds the dose required to reverse the effect of sarin appeared to be lower than that necessary to reverse the effects of soman.

Protection studies with neostigmine

Muscarinic antagonists The dose-related nature of the protective effect of the muscarinic antagonists against neostigmine on the soleus muscle is summarized in Table 1. The doses affording complete protection against neostigmine (10 μg) had little or no effect on the twitch response of this muscle. These doses were also capable of preventing the effects of larger

doses of neostigmine, at least up to 100 μg. The protective action persisted for up to 1 to 1.5 h.

The protective action of PMCG, PPCG, G3063 and atropine was also investigated in 'fast-twitch' skeletal muscle. The results obtained with FDL were similar to those already described for the 'slow-twitch' soleus muscle i.e. the protection afforded was dose-related. The doses affording complete protection were the same for the two different muscles but the effect of these compounds on the twitch response of the FDL muscle was more pronounced, with up to 30% twitch augmentation being recorded.

Other drugs The local anaesthetics lignocaine, prilocaine, cinchocaine and procaine, the tranquillizers chlorpromazine and trifluprom azine, the intravenous anaesthetic ketamine and the anti-arrhythmic agent quinine were all capable of preventing the effects of neostigmine and they did so in a dose-related manner (see Table 1). The dose of both procaine and cinchocaine required to protect the soleus muscle from the effects of neostigmine caused twitch depression (10% for procaine, 30 to 40% for cinchocaine) whereas lignocaine, prilocaine, ketamine, quinine, chlorpromazine and trifluprom azine produced slight (5%) twitch augmentation or no effect on the twitch response.

Neither caffeine (up to 20 mg) nor aminophylline (up to 40 mg) modified the effects of neostigmine on soleus muscle.

Discussion

These results confirm previous findings (Brimblecombe & Everett, 1969b; 1970a) that certain muscarinic antagonists are capable of preventing or reversing sarin-induced twitch augmentation of cat hind-limb muscles. It has also been shown that these antagonists are effective against another organophosphate anticholinesterase agent, soman, and against the carbamate neostigmine. At higher dose levels the antagonists themselves produce augmentation of the twitch of directly or indirectly stimulated muscles but doses sufficient to give complete protection from the effects of the anticholinesterase agents have little or no effect on twitch of the normal muscle. The experiments with neostigmine demonstrated the dose-dependent nature of the protective action.

With the exception of atropine, all the muscarinic antagonists studied were approximately equiactive in protecting skeletal muscle from the effects of sarin, soman or neostigmine. However, they differ quite markedly, in their muscarinic antagonist activities. Most significantly the R and S enantiomers of PPCG, which would be expected to be absorbed, distributed and metabolized in a similar manner, differ by a factor of at least 20 in their potency as muscarinic antagonists (see Table 1). This indicates that the protective actions of the drugs are independent of their antagonist actions at muscarinic receptors.

Table 1 Some pharmacological properties of the muscarinic antagonists and other drugs used in these studies

Compound	*Effect on twitch response of cat soleus muscle to neostigmine (10 μg i.a.) Dose (mg i.a.)* 0.25	0.5	1	2	4	8	*Potency relative to atropine*[1] *Isolated guinea-pig ileum*	*Production of mydriasis in mice*	*Local anaesthetic activity relative to procaine Frog sciatic nerve*
PMCG	0	±	+	+	ND	ND	2	1	0.4
PPCG (Rac)	0	0	±	+	ND	ND	43	1.2	ND
PPCG (R)	0	0	±	+	ND	ND	100	2.4	ND
PPCG (S)	0	0	±	+	ND	ND	0.4	0.12	ND
G3063	0	±	±	+	ND	ND	0.6	0.3	ND
Atropine	ND	0	0	±	±	+	1	1	ND
Cinchocaine	0	±	+	ND	ND	ND			ND
Lignocaine	ND	0	±	±	+	ND			3
Prilocaine	ND	0	±	±	±	+			ND
Procaine	ND	ND	0	±	±	+			1
Ketamine	ND	0	±	±	+	ND			ND
Chlorpromazine	ND	0	±	+	ND	ND			ND
Trifluprom azine	ND	0	±	+	ND	ND			ND
Quinine	ND	ND	0	0	±	+			0.3

[1]From Brimblecombe, Green, Inch & Thompson (1971).
PMCG = N-ethyl-2-pyrrolidylmethyl-cyclopentylphenyl glycollate; PPCG = N-methyl-4-piperidyl-phenylcyclohexyl glycollate; G3063 = 4'-N-methyl-piperidyl-1-phenyl-cyclopentane carboxylate; 0 = no protection; ± = incomplete protection; + = complete protection; ND = not determined.

The effects of these acetylcholine antagonists on skeletal muscle contactility are also unrelated to their antagonist activity at muscarinic receptors (Brimblecombe & Everett, 1970b; Brimblecombe & Webb, unpublished observations) but on present evidence it is not possible to conclude whether the two effects, augmentation of twitch and antagonism of the effects of anticholinesterase agents, are related or not. Similarly the mechanism of action of the acetylcholine antagonists in preventing or reversing the effects of anticholinesterase agents is still not clear.

Curare-like compounds antagonize or prevent the effects of anticholinesterase agents on skeletal muscle (Rump & Kaliszan, 1968). Since weak curare-like properties have been described for PMCG (Brimblecombe & Everett, 1970b), procaine (Harvey, 1939b) and quinine (Harvey, 1939a) the possibility exists that this action plays a role in their effects described in this paper. Local anaesthetics have also been shown to antagonize the effects of anticholinesterases (Harvey, 1939b). Our study has shown that in addition to conventional local anaesthetics, other membrane stabilizers including chlorpromazine, trifluprom azine and ketamine antagonize the effects of neostigmine on the twitch response of cat soleus muscle. PMCG also possesses local anaesthetic activity (see Table 1) so that this action cannot be ruled out as being responsible for the effects on muscle. As a general point it should be recognized that in the experiments reported here, the drugs were given by close intra-arterial injection whereas their potencies in producing other pharmacological effects have been measured *in vitro* or *in vivo* by other routes of administration.

One interesting feature of the results relating to inhibition of AChE was that there was a significant regression of response on log dose for the inhibition of muscle AChE but not for twitch tension. This indicates that there is not a simple relationship between anticholinesterase action and alteration of muscle function. Barber, Calvey, Muir & Taylor (1975) also reported no correlation between the potentiation of the tibialis twitch tension and the plasma concentration of edrophonium or the inhibition of erythrocyte AChE. In order to study this aspect further, the relationship between inhibition of endplate AChE and alteration of muscle function should be studied on an isolated preparation rather than a muscle homogenate.

References

BARBER, H.E., CALVEY, T.N., MUIR, K.T. & TAYLOR, K. (1975). The relation between the plasma concentration of edrophonium, inhibition of erythrocyte acetylcholinesterase, and the facilitation of neuromuscular function in the rat. *Br. J. Pharmac.*, **54**, 263–264P.

BRIMBLECOMBE, R.W. & EVERETT, S.D. (1969a). Actions of a cholinergic antagonist of mammalian skeletal muscle. *Br. J. Pharmac.*, **36**, 172–173P.

BRIMBLECOMBE, R.W. & EVERETT, S.D. (1969b). Interactions between sarin and the cholinergic antagonist PMCG in fast and slow skeletal muscle of the cat. *J. Physiol.*, **203**, 19–20P.

BRIMBLECOMBE, R.W. & EVERETT, S.D. (1970a). Actions of sarin on fast-twitch and slow-twitch skeletal muscles of the cat and the protective action by anticholinergic drugs. *Br. J. Pharmac.*, **40**, 57–67.

BRIMBLECOMBE, R.W. & EVERETT, S.D. (1970b). Actions of some cholinergic antagonists on fast-twitch and slow-twitch skeletal muscle of the cat. *Br. J. Pharmac.*, **40**, 45–56.

BRIMBLECOMBE, R.W., GREEN, D.M., INCH, T.D. & THOMPSON, P.B.J. (1971). The significance of differences in the potency of enantiomers of antiacetylcholine drugs. *J. Pharm. Pharmac.*, **23**, 745–757.

BRIMBLECOMBE, R.W. & WEBB, S.N. (1973). Further studies on the interaction between acetylcholine antagonists and anticholinesterases on cat soleus muscle. *Br. J. Pharmac.*, **48**, 361–362P.

BULLER, A.J. & LEWIS, D.M. (1965a). The rate of tension development in isometric tetanic contractions of mammalian fast and slow skeletal muscle. *J. Physiol.*, **176**, 337–354.

BULLER, A.J. & LEWIS, D.M. (1965b). Further observations on the differentiation of skeletal muscles in the kitten hind limb. *J. Physiol.*, **176**, 355–370.

ELLMAN, G.L., COURTNEY, K.D., ANDRES, V. & FEATHERSTONE, R.M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmac.*, **7**, 88–95.

FRENCH, M.C., SELLERS, D.J. & WILKINSON, R.G. (1977). A comparison of methods for measuring acetylcholinesterase activity in blood samples inhibited by carbamates. *Biochem. Pharmac.*, **26**, 1263–1266.

HARVEY, A.M. (1939a). The actions of quinine on skeletal muscle. *J. Physiol.*, **95**, 45–67.

HARVEY, A.M. (1939b). The actions of procaine on neuromuscular transmission. *John Hopkins Hosp. Bull.*, **65**, 223–238.

HEILBRONN, E. (1959). Hydrolysis of carboxylic acid esters of thiocholine and its analogues. *Acta chem. scand.*, **13**, 1547–1560.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

RUMP, S. & KALISZAN, A. (1968). Effects of some local anaesthetics on abnormalities of neuromuscular transmission produced by neostigmine in rats. *Archs int. Pharmacodyn. Thér.*, **175**, 401–406.

(Received December 15, 1977, Revised October 31, 1978.)