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Interactions between oxotremorine and inhibitors of cholinesterase enzymes

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Interactions between oxotremorine and physostigmine or dyflos have been investigated in mice using doses giving approximately 50% inhibition of mouse brain cholinesterase. Oxotremorine LD50 was unaffected by pretreatment of mice with the anticholinesterases. Its ED50 (tremor) was decreased by physostigmine and neostigmine but not by dyflos. Dyflos antagonized oxotremorine hypothermia, physostigmine was without effect. Oxotremorine did not alter the inhibitory activity of the anticholinesterases. The lack of potentiation of oxotremorine by cholinesterase inhibitors suggests it acts directly in these tests by a mechanism independent of its ability to increase tissue acetylcholine concentration.

An increase in brain acetylcholine concentration after injection of oxotremorine into rats and mice has been reported (Holmstedt, Lundgren & Sundwall, 1963; Holmstedt & Lundgren, 1966; Everett, 1967; Holmstedt, 1967; Lévy & Michel-Ber, 1967; Cox & Potkonjak, 1969). However, the mechanism by which this increase occurs is still in some doubt (Holmstedt, 1967; Jenden, 1968). It is also not certain if the central pharmacological actions of oxotremorine are mediated by acetylcholine. The rise in brain acetylcholine concentration could be associated with either increased or decreased release of acetylcholine from cholinergic neurons. Studies on peripheral isolated tissues have produced conflicting evidence. Thus György, Pfeifer & Kenyeres (1970) found that oxotremorine released acetylcholine into the bath fluid of the rat isolated intestine preparation, whereas Cox & Hecker (1971) could find no evidence for oxotremorine-induced release of acetylcholine from guinea-pig isolated ileum.

Cox & Tha (1972), using an antinociceptive test, have shown that though physostigmine did not potentiate a low dose of oxotremorine, there was some evidence for potentiation of higher doses. Therefore high doses of oxotremorine could act, at least in part, by a mechanism involving acetylcholine release, while this seems much less likely with a low dose.

To further elucidate the mechanism of action of oxotremorine, its interactions with inhibitors of cholinesterase have been examined by measuring their effect on the acute toxicity (a high dose effect), and the tremor (an intermediate dose effect) and fall in oesophageal temperature (a low dose effect) it produces. Also, the interactions between oxotremorine and cholinesterase inhibitors on mouse brain cholinesterase activity have been examined.

METHODS

Albino mice (ICI Swiss strain), of either sex, 27 to 42 g, were used at 22 $\pm$ 1°. All injections were made subcutaneously and drug combinations were injected simultaneously.

Acute toxicity. Groups of 10 mice were housed in Perspex cages and allowed free access to food and water. Those surviving at 24 h after injection were counted. The median lethal dose, or LD50, was then calculated (see Statistics).

Tremor. Groups of 5 mice were housed in Perspex cages and allowed access to food and water. Those displaying tremor were noted at 10 min intervals for 60 min after drug injection. The median effective dose, or ED50 (tremor), was then calculated (see Statistics).

Oesophageal temperature. Oesophageal temperature was measured by a heat sensitive thermistor probe inserted to a depth of 2 cm, and retained *in situ* until a constant temperature was obtained. Time course experiments were made by injecting mice with either 0·9% saline or drug and recording temperature at 10 min intervals for 90 min.

Brain homogenate. Brain homogenates from two mice were prepared as described previously (Cox & Tha, 1972) from either 0·9% saline-pretreated or drug pretreated mice. The pretreatment time was 30 min. In the *in vitro* experiments non-pretreated mice were used, and the drugs were added to the reaction vessel.

The ability of mouse brain homogenate to hydrolyse acetylcholine was determined according to Alles & Hawes (1940). The activity of the homogenate was expressed as μmol acetylcholine hydrolysed min^{-1} mg $protein^{-1}$ (estimated by the Biuret method, Gornall, Bardawill & David, 1949).

Statistics. In the acute toxicity and tremor tests the LD50 and ED50 (tremor) respectively were calculated by the method of Litchfield & Wilcoxon (1949). Student's *t*-test was used to compare means in the oesophageal temperature experiments. Mann-Whitney *U* test (Siegel, 1956) was applied to assess the difference between groups in the brain homogenate experiments. Unless otherwise stated a significant difference between means or groups was taken as $P < 0{\cdot}05$.

Drugs. Acetylcholine perchlorate and physostigmine sulphate (BDH); dyflos (Koch-Light); neostigmine methylsulphate (Roche); physostigmine salicylate (T. & H. Smith); atropine methonitrate (kindly supplied by Winthrop Laboratories); oxotremorine (obtained as a gift from the Chemical Defence Establishment, Porton Down). All doses refer to the free base unless otherwise stated.

RESULTS

Acute toxicity. The LD50 of oxotremorine alone was not significantly different from that in the presence of physostigmine or dyflos (Table 1). Atropine methonitrate significantly increased ($P < 0{\cdot}05$) the LD50 (Table 1). There was no significant

Table 1. *Acute toxicity of oxotremorine in mice (after Litchfield & Wilcoxon, 1949).*

Drug	Treatment Dose (mg kg^{-1})	Number of mice	Oxotremorine lethal dose (mg kg^{-1}) LD50	95% confidence limits
Normal saline		150	7·7	7·1–8·4
Physostigmine	0·0625	30	7·5	6·7–8·4
Dyflos	2·0	30	7·2	5·5–9·4
Atropine methonitrate ..	0·1	40	15·6*	13·2–18·5

* $P < 0{\cdot}05$ when compared to saline control.

difference between the slopes of any of the dose-response lines. Mice that had not died within 40 min of injection survived longer than 24 h. A typical sequence of signs and symptoms of oxotremorine poisoning was found; cardiac arrest always followed respiratory failure.

Tremor. The ED50 (tremor) of oxotremorine alone was not significantly different from that obtained when the drug was used with dyflos (Table 2). Physostigmine and

Table 2. *Oxotremorine tremor in mice (after Litchfield & Wilcoxon, 1949).*

Treatment			Oxotremorine tremor dose (mg kg^{-1})		
Drug	Dose (mg kg^{-1})	Number of mice	ED50 (tremor)	95% confidence limits
Normal saline		288	0·188	0·165–0·214
Physostigmine	0·0625	40	0·081*	0·049–0·134
Neostigmine	0·0625	85	0·064*	0·043–0·095
Dyflos	2·0	40	0·152	0·106–0·214

* $P < 0{\cdot}05$ when compared to saline control.

neostigmine significantly decreased ($P < 0{\cdot}05$) the ED50 (tremor) of oxotremorine (Table 2), but there was no significant difference between the physostigmine and neostigmine-treated mice. No significant difference occurred in the slopes of any of the dose-response lines.

Oesophageal temperature. The time course for the effect of subcutaneous injection of oxotremorine, physostigmine or dyflos on oesophageal temperature in mice is shown in Fig. 1. Oxotremorine and physostigmine produced dose-related decreases in temperature which were maximal within 40 min but were not significantly different

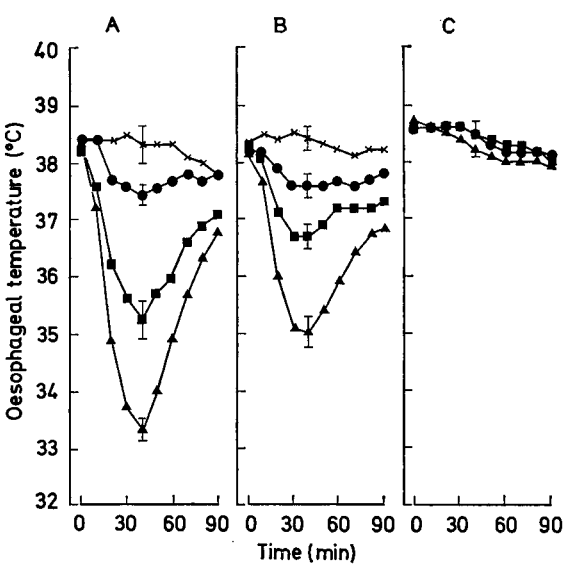

FIG. 1. The effect of subcutaneous injection of oxotremorine, physostigmine and dyflos on oesophageal temperature in mice. A, Oxotremorine (●, 0·025 mg kg^{-1}; ■, 0·05 mg kg^{-1}; ▲, 0·1 mg kg^{-1}). B, Physostigmine (●, 0·0625 mg kg^{-1}; ■, 0·125 mg kg^{-1}; ▲, 0·25 mg kg^{-1}). C, Dyflos (●, 0·5 mg kg^{-1}; ■, 1 mg kg^{-1}; ▲, 2 mg kg^{-1}). Saline controls (×). Each point represents mean of 10 observations. Vertical bars indicate ±s.e.

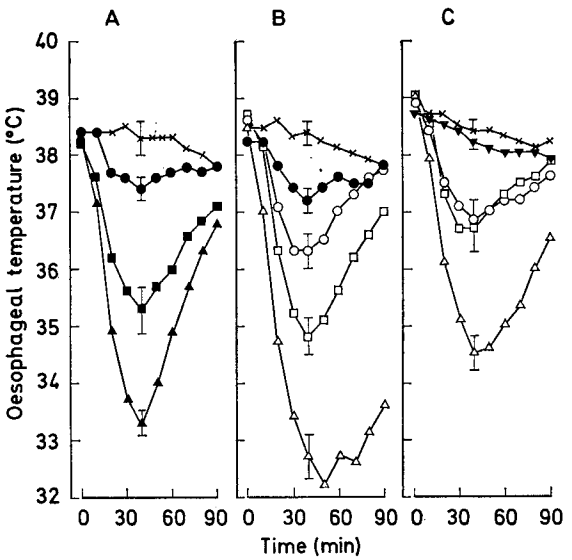

FIG. 2. The effect of oxotremorine on oesophageal temperature of mice injected subcutaneously with or without cholinesterase inhibitor. A, Saline plus oxotremorine (●, 0·025 mg kg^{-1}; ■, 0·05 mg kg^{-1}; ▲, 0·1 mg kg^{-1}). B, Physostigmine 0·0625 mg kg^{-1} plus oxotremorine (●, 0; ○, 0·025 mg kg^{-1}; □, 0·05 mg kg^{-1}; △, 0·1 mg kg^{-1}). C, Dyflos 2 mg kg^{-1} plus oxotremorine (▼, 0; ○, 0·025 mg kg^{-1}; □, 0·05 mg kg^{-1}; △, 0·1 mg kg^{-1}). Saline controls (×). Each point represents mean of 10 observations. Vertical bars indicate ±s.e.

from saline controls at 90 min. Dyflos produced no significant changes when compared with concurrently tested saline controls.

The time course for the effect of oxotremorine on oesophageal temperature when injected alone or in combination with cholinesterase inhibitors is shown in Fig. 2. The effects were maximal at 40–50 min and had returned to normal at 90 min except for the highest dose of oxotremorine used. Physostigmine 0·0625 mg kg^{-1} did not significantly alter the decrease produced by oxotremorine irrespective of the dose of oxotremorine used, but the effect of oxotremorine was significantly reduced ($P < 0{\cdot}05$) by dyflos pretreatment. Tremor was not observed during these experiments.

Brain homogenate. There was no significant difference between the ability of homogenates prepared from oxotremorine-pretreated (30 min; 0·025–0·1 mg kg^{-1}) and saline-pretreated mice to hydrolyse acetylcholine. Physostigmine 0·0625 mg kg^{-1}, injected 30 min previously into mice, inhibited the ability of homogenates to hydrolyse acetylcholine by 44%. Injection of oxotremorine together with physostigmine produced no change in its inhibitory action. When dyflos 2 mg kg^{-1} was substituted for physostigmine 62% inhibition resulted, and with oxotremorine there was no effect on the inhibitory action of dyflos.

DISCUSSION

If the central pharmacological actions of oxotremorine were mediated by acetylcholine release (see Introduction), then it might be expected that the inhibitors would potentiate oxotremorine. Oxotremorine itself is not an ester, and therefore not susceptible to hydrolysis by cholinesterase. Thus any pharmacological actions due to a direct effect should not be potentiated by inhibition of cholinesterase.

Of the three pharmacological tests selected to study the interactions between oxotremorine and cholinesterase inhibitors, acute toxicity was chosen because it allowed the use of the highest possible dose of oxotremorine while the dose of cholinesterase inhibitor was that giving approximately 50% inhibition of brain cholinesterase (measured as the ability of mouse brain homogenate to hydrolyse acetylcholine). As the doses of inhibitors did not cause any deaths in this test, the possibility of interpreting synergism as potentiation is not as great as in tests where the inhibitors themselves give a positive response. In the present experiments both physostigmine and dyflos produced no change in the LD50 of oxotremorine. Therefore, its toxicity was not increased by cholinesterase inhibition. Some protection against its toxic effect was, however, afforded by pretreatment of mice with atropine methonitrate. These results confirm the findings of Gerald & Maickel (1970) and indicate that oxotremorine lethality is, at least partly, peripheral in origin. A widespread increase in acetylcholine content of both central and peripheral tissues after oxotremorine has been reported (Pepeu, 1964; Holmstedt & Lundgren, 1966; Cox & Potkonjak, 1969; Ganguly & Saha, 1969). Our present findings suggest that these increases are not a causative factor in oxotremorine toxicity.

Tremor was chosen for two reasons. Firstly, the tremor-producing doses of oxotremorine were intermediate between the doses used by Cox & Tha (1972) in the hot plate test and those required for acute toxicity. Secondly, the tremor has been reported to be purely central in origin as its effects are blocked by atropine but not by quaternary atropine derivatives which do not pass the blood-brain barrier (George, Haslett & Jenden, 1962). Physostigmine caused a significant reduction in the ED50 (tremor) of oxotremorine but did not itself cause tremor, yet it produced approximately 50% inhibition of brain cholinesterase activity. In contrast, dyflos in a dose which caused a 62% inhibition had no significant effect on the ED50 (tremor) of oxotremorine. These observations appear contradictory and suggest that inhibition of brain cholinesterase is not likely to be an important factor for this potentiation of oxotremorine by physostigmine. However, whilst both physostigmine and dyflos pass the blood-brain barrier (Frawley, Hagan & Fitzhugh, 1952; Paulet, Marsol & Coq, 1957; Keranen, Zaratzian & Coleman, 1961; Cox & Potkonjak, 1969), physostigmine inhibits cholinesterase and acetylcholinesterase equally whereas dyflos preferentially inhibits cholinesterase (Augustinsson, 1948; Cox, Hecker & Weston, 1970). Thus one explanation for the potentiating action of physostigmine could be protection of centrally released acetylcholine from acetylcholinesterase. An alternative possibility is that the increased tremor seen in the presence of physostigmine is due to an effect at the neuromuscular junction and not an interaction between the two drugs in the central nervous system. The tremor experiments were repeated using the quaternary reversible cholinesterase inhibitor neostigmine. This drug, like physostigmine reduced the ED50 (tremor) of oxotremorine thereby indicating a peripheral site for potentiation.

The oesophageal temperature response was chosen because the dose range was the same as that employed in the antinociceptive test by Cox & Tha (1972) when apparent potentiation of the higher doses of oxotremorine occurred. In contrast to the findings in that test, there was no evidence of potentiation by physostigmine of oxotremorine's effect on temperature. Physostigmine itself caused only a small (significant) fall in temperature. Therefore, as in the acute toxicity test, the danger of interpreting synergism as potentiation is unlikely. The results would support the concept that

oxotremorine is a directly acting acetylcholine-like drug in the central nervous system and that the increase in brain acetylcholine has no relevance to its effects.

The interaction between oxotremorine and dyflos also proved interesting; not only was there a lack of potentiation of oxotremorine in the oesophageal temperature experiments, but an antagonism occurred. A similar response was seen in rats by Cox & Potkonjak (1969) with respect to the increase in brain acetylcholine. They suggested that oxotremorine might be interfering with the anticholinesterase activity of dyflos in the rat brain. Oxotremorine has been reported to be only weakly active against the acetylcholinesterase of a mouse brain homogenate (Holmstedt, Lundgren & others, 1965), but there is no evidence concerning its effects on the inhibitory potency of the anticholinesterase agents. From the brain homogenate studies there was no evidence that oxotremorine interfered with the inhibitory actions of the anticholinesterases. Therefore, this unexpected interaction between oxotremorine and dyflos still awaits elucidation.

In conclusion, therefore, the lack of potentiation of oxotremorine by effective inhibitory concentrations of anticholinesterase drugs in a series of pharmacological tests involving a 300-fold increase in dose (0·025 to 7·7 mg kg^{-1}), argues in favour of a direct action of this drug in both its central and peripheral cholinergic effects.

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