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The antinociceptive activities of oxotremorine, physostigmine and dyflos

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Oxotremorine and physostigmine caused dose-related increases in the hot plate reaction time (HPRT) of mice, but dyflos did not. Physostigmine did not potentiate a low dose of oxotremorine (0·025 mg/kg) in the HPRT test, but some potentiation of higher doses of oxotremorine (0·05 and 0·1 mg/kg) may have occurred. Dyflos did not potentiate any dose of oxotremorine. Physostigmine and dyflos inhibited mouse brain cholinesterase but oxotremorine was without effect. The relation between change of HPRT and cholinesterase inhibition was different for physostigmine and dyflos.

Oxotremorine and other acetylcholine-like drugs, that pass the blood-brain barrier, have given positive results in tests designed to demonstrate antinociceptive activity (Chen, 1958; Herz, 1962; Jacob & Barthelemy, 1965; Leslie, 1969). Oxotremorine also increases the brain concentration of acetylcholine (Holmstedt & Lundgren, 1967). Howes, Harris & others (1969) have reported a good correlation of inhibition of the tail-flick reflex and elevation of brain acetylcholine in mice after oxotremorine injection. These authors noted that the anticholinesterase physostigmine also had antinociceptive activity in this test. It is uncertain whether the increase in brain acetylcholine produced by the acetylcholine-like drugs has any relevance to their antinociceptive activity and whether the antinociceptive activity of physostigmine and other anticholinesterase agents is related to their ability to inhibit brain cholinesterase enzymes. We have investigated the antinociceptive activity of oxotremorine and the anticholinesterase drugs dyflos and physostigmine. Interactions between oxotremorine and either physostigmine or dyflos have also been examined.

METHODS

Albino mice (ICI Swiss strain), of either sex, 27 to 42 g, were used. Hot plate reaction time (HPRT) was measured by a method similar to that of Woolfe & Macdonald (1944). The plate was maintained at $56 \pm 1°$. Lifting and shaking of the hind limb was taken as a positive response to the noxious stimulus. A maximum cut off time of 90 s was used; the observer was unaware of the nature of the injection. Time course experiments were made by injecting mice with either 0·9% saline or drug and testing at 15 min intervals for 60 min. Investigations of drug interactions were made at the time of maximal drug effect as predicted by the time course experiments. Mice were tested immediately before the injection of drug or drug combinations so that the increase in reaction time could be measured; each mouse acted as its own control.

The ability of mouse brain homogenate to hydrolyse acetylcholine was measured by a titrimetric method similar to that of Alles & Hawes (1940). Two mice were killed by cervical stretch. The brains were removed and homogenized together in a cold solution containing $MgCl_2$ 0·04N and NaCl 0·05N to give a concentration of

100 mg brain per ml final solution. Homogenates were prepared from 0·9% saline pretreated or drug pretreated mice. The rate of hydrolysis of acetylcholine was determined at pH 7·4 and 37° by titrating the liberated acetic acid with 0·02N NaOH. The average velocity of the reaction was measured during the second and third minutes after addition of substrate, when the rate of hydrolysis was stable. The activity of the homogenate was expressed as μmol acetylcholine hydrolysed min^{-1} mg $protein^{-1}$. Protein content of the homogenate was estimated by the Biuret method (Gornall, Bardawill & David, 1949).

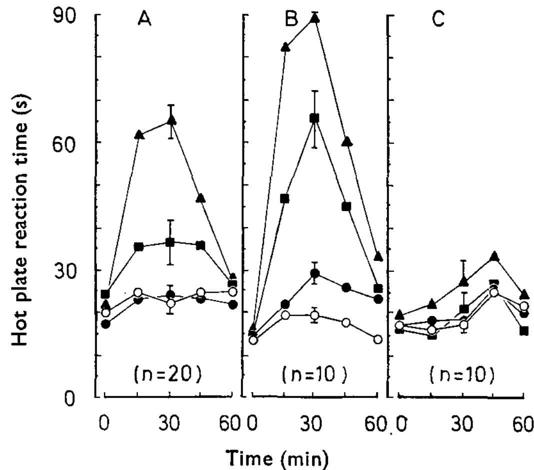

FIG. 1. The effect of oxotremorine, physostigmine and dyflos on hot plate reaction time in mice Each point represents mean of (n) number of observations. Vertical bars indicate standard error of the mean. A, Oxotremorine; 0 (○), 0·025 (●), 0·05 (■), 0·1 (▲) mg/kg, B, Physostigmine; 0 (○), 0·0625 (●), 0·125 (■), 0·25 (▲) mg/kg. C, Dyflos, 0 (○), 0·5 (●). 1·0 (■), 2·0 (▲) mg/kg.

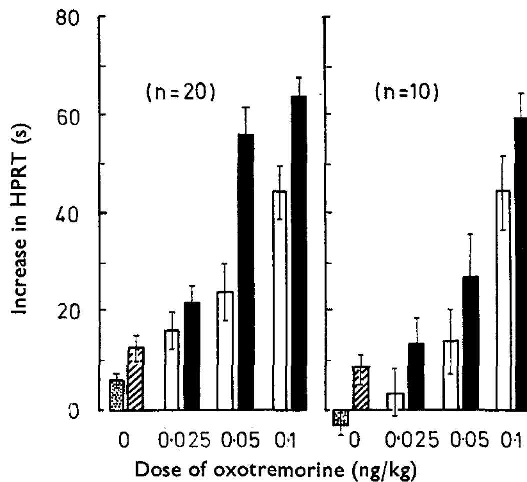

FIG. 2. The increase in hot plate reaction time (HPRT) measured 30 min after subcutaneous injection of oxotremorine alone compared with the increase after oxotremorine and cholinesterase inhibitor. Each column represents mean of (n) number of observations. Vertical bars indicate standard error of the mean. Stippled column = saline; hatched column = inhibitor alone; open column = oxotremorine alone; closed column = oxotremorine plus inhibitor. Left: oxotremorine-physostigmine 0·0625 mg/kg. Right: oxotremorine-dyflos 2·0 mg/kg.

Drugs. Acetylcholine perchlorate (BDH); dyflos (Koch Light); physostigmine salicylate (T. & H. Smith). Oxotremorine was obtained as gift from Dr. R. W. Brimblecombe, Ministry of Defence, Porton Down. Drug concentrations are expressed in terms of the free base.

RESULTS

The time course for the effect of subcutaneous injection of either oxotremorine, physostigmine or dyflos on HPRT in mice is shown in Fig. 1.

Oxotremorine (0·025, 0·05 and 0·1 mg/kg) and physostigmine (0·0625, 0·125 and 0·25 mg/kg) produced dose-related increases in HPRT which were maximal within 30 min but were not significantly different from saline controls at 60 min. Dyflos (0·5, 1 and 2 mg/kg) produced no significant changes in the HPRT of mice when compared with concurrently tested saline controls.

The effect of oxotremorine and cholinesterase inhibitors alone and in combination on HPRT in mice is shown in Fig. 2. Each mouse was tested immediately before and 30 min after drug injection. Thus the results show the mean change in time for the various drug combinations. Physostigmine (0·0625 mg/kg) caused a small non-significant increase in the time when compared with saline controls. Oxotremorine, 0·025 mg/kg, and physostigmine injected together did not increase the HPRT significantly from the result obtained with oxotremorine alone. After injection of oxotremorine, 0·05 or 0·1 mg/kg, into mice that were also injected with

Table 1. *Effect of oxotremorine pretreatment on the ability of mouse brain homogenate to hydrolyse acetylcholine.* Drug administred subcutaneously to mice 30 min before killing and preparing the brain homogenate.

Oxotremorine mg/kg	Number of experiments	Velocity of hydrolysis in μmol min^{-1} mg^{-1} (mean $\pm$ s.e.)
0	5	0·317 $\pm$ 0·014
0·1	5	0·346 $\pm$ 0·023

physostigmine, the increase in HPRT was significantly greater than that obtained with oxotremorine alone ($P < 0{\cdot}05$; Student's t-test). Dyflos (2 mg/kg) caused a small non-significant increase in HPRT when compared with saline controls. The increase in time produced by simultaneous injection of oxotremorine and dyflos was not significantly different from that after injection of oxotremorine alone.

The ability of mouse brain homogenate to hydrolyse acetylcholine was not significantly affected by an injection of oxotremorine (0·1 mg/kg) 30 min before killing and preparing the homogenate (Table 1). This dose produced a marked increase in HPRT (Fig. 1).

Pretreatment with either physostigmine or dyflos in mice produced dose-related inhibition of mouse brain cholinesterase (measured as the ability of mouse brain homogenate to hydrolyse acetylcholine). The relations between inhibition of mouse brain cholinesterase and increase in HPRT measured after 30 min drug pretreatment is shown in Fig. 3. After physostigmine injection, as the percentage inhibition of brain cholinesterase increased, there was also a marked increase in HPRT. When

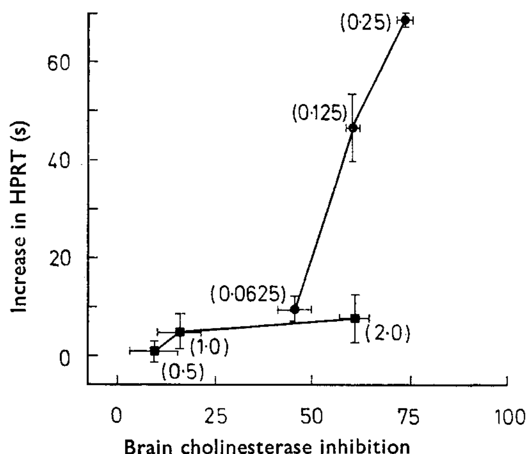

FIG. 3. Correlation of percentage inhibition of mouse brain cholinesterase and increase in hot plate reaction time (HPRT) produced by pretreatment of mice with either physostigmine or dyflos, from data in Fig. 1B and C. Dose of drug used for each point is shown in parentheses. Number of mice for each point = 10. Vertical and horizontal bars indicate standard errors of the means of the increase in HPRT and the percentage brain cholinesterase inhibition respectively. (●) = physostigmine; (■) = dyflos.

dyflos was used there was only a small increase in HPRT compared with a large change in brain cholinesterase inhibition.

DISCUSSION

As reported by Hendershot & Forsaith (1959), Herz (1962), Jacob & Barthelemy (1965), Leslie (1969) and Metys, Wagner & others (1969) the results with oxotremorine showed a dose-related increase in HPRT which was maximal at 30 min. Therefore in the drug interaction studies 30 min was chosen as the appropriate pretreatment time for oxotremorine. Similarly, a pretreatment time of 30 min was chosen for physostigmine which also gave a positive result on the hot plate test. The dose of physostigmine chosen for the interaction with oxotremorine (0·0625 mg/kg) gave approximately 50% brain cholinesterase inhibition and yet produced only a relatively small change in HPRT. This dose did not significantly change the increase in HPRT produced by the low dose of oxotremorine, but there was a significant increase when it was combined with the two higher doses. There are two possible explanations for this significant increase in HPRT. The cholinesterase inhibitor may be potentiating oxotremorine thereby indicating acetylcholine involvement. Alternatively, the result obtained is simply due to the summation of oxotremorine and physostigmine effects. If there was true potentiation of oxotremorine, then values derived by the addition of the results for oxotremorine and physostigmine individually should be lower than the experimentally determined values for simultaneous injection of oxotremorine and physostigmine (Fig. 2). Using this criterion there is not good evidence for potentiation of oxotremorine by the cholinesterase inhibitor with the low dose of oxotremorine, but there may be potentiation of the higher doses. Ireson (1970) reported that physostigmine potentiated oxotremorine in a writhing test but antagonized it in an electroshock test. In the test which showed potentiation, physostigmine itself had antinociceptive activity. Therefore summation rather than potentiation could be occurring. Ireson explains his results by suggesting that physostigmine is a partial agonist. He does not consider the

results with physostigmine in terms of cholinesterase inhibition. The dose of physostigmine (0·1 mg/kg) that he used in the electroshock test would, according to the present work, give 60% inhibition of the brain cholinesterase. The dose of physostigmine (0·03 mg/kg) which he used in the writhing experiment would give an inhibition of approximately only 30%. Thus the test showing potentiation was the one in which there was the smaller inhibition of brain cholinesterase. Therefore it would appear that inhibition of cholinesterase by physostigmine is not important for the reported interaction between physostigmine and oxotremorine.

Dyflos, in the doses used, did not have antinociceptive activity, neither did it significantly increase the effect of oxotremorine on HPRT. The dose used (2 mg/kg) gave 62% inhibition of whole brain cholinesterase which was similar to the inhibition given by physostigmine, 0·0625 mg/kg. These findings support the concept that the inhibition of cholinesterase would not potentiate oxotremorine. The results in Fig. 3 show an apparent correlation of increase in HPRT and inhibition of brain cholinesterase with physostigmine but not with dyflos. There are several possible explanations for this finding. There may not be a causal relation. Increase in HPRT and inhibition of brain cholinesterase may be separate phenomena occurring with dose as the common denominator. Alternatively, the lack of correlation found with dyflos may be because this inhibitor predominantly affects cholinesterase rather than acetylcholinesterase (Aldridge, 1953), and is less likely to potentiate neuronally released acetylcholine.

The lack of potentiation of low doses of oxotremorine by physostigmine suggests that the release of acetylcholine from cholinergic neurons is not an important mechanism for oxotremorine antinociceptive activity. It is not yet clear whether the possible potentiating effect on high doses of oxotremorine is related to the anticholinesterase action of physostigmine or to a different phenomenon.

REFERENCES

Alles, G. A. & Hawes, R. C. (1940). *J. biol. Chem.*, **133**, 375–390.

Aldridge, W. N. (1953). *Biochem. J.*, **53**, 62–67.

Chen, G. (1958). *J Pharmac. exp. Ther.*, **124**, 73–76.

Gornall, A. G., Bardawill, C. S. & David, M. M. (1949). *J. biol. Chem.*, **177**, 751–766.

Hendershot, L. C. & Forsaith, J. (1959). *J. Pharmac. exp. Ther.*, **125**, 237–240.

Herz, A. (1962). *Arch. exp. Path. Pharmak.*, **242**, 414–429.

Holmstedt, B. & Lundgren, G. (1967). *Ann. N.Y. Acad. Sci.*, **142**, 126–142.

Howes, J. F., Harris, L. S., Dewey, W. L. & Voyda, C. A. (1969). *J. Pharmac. exp. Ther.*, **169**, 23–28.

Ireson, J. D. (1970). *Br. J. Pharmac.*, **40**, 92–101

Jacob, J. & Barthelemy, C. (1965). In *Methods in Drug Evaluation.* Editors: Mantegazza, P. & Piccinni, F. Amsterdam: North-Holland.

Leslie, G. B. (1969). *J. Pharm. Pharmac.*, **21**, 248–250.

Metys, J., Wagner, N., Metysova, J. & Herz, A. (1969). *Int. J. Neuropharmacology.* **8**, 413–425

Woolfe, G &. Macdonald, A. D. (1944). *J. Pharmac. exp. Ther.*, **80**, 300–307.