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EFFECT OF OXOTREMORINE AND PHYSOSTIGMINE ON CHOLINE LEVELS IN MOUSE WHOLE BRAIN, SPLEEN AND CEREBELLUM*

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Abstract—The effect of oxotremorine and physostigmine on acetylcholine and choline levels in mouse whole brain, spleen, cerebellum and plasma was determined by a radiochemical method. Oxotremorine, 0·7 mg/kg, and physostigmine, 0·5 mg/kg, increased choline levels in mouse whole brain and cerebellum. Their activity differed in spleen, oxotremorine being highly active in increasing choline while physostigmine was ineffective. The increased cerebral choline produced by physostigmine is not related to its anticholinesterase activity since diisopropylfluorophosphate (DFP) at 10–20 higher molar doses had no effect on whole brain choline although almost doubling the acetylcholine level. Atropine sulfate, 5 mg/kg, completely antagonized oxotremorine and physostigmine induced increase in cerebellar choline and this effect of oxotremorine on the spleen. It is therefore concluded that both oxotremorine and physostigmine act directly through muscarinic receptors to increase choline levels in cerebellum. The possibility of the existence of muscarinic receptors in the mouse spleen, a tissue probably lacking cholinergic nerves is considered. It is postulated that oxotremorine first increases tissue choline which then results in increased acetylcholine synthesis.

OXOTREMORINE is a potent experimental drug which produces, besides tremor, several parasympathomimetic symptoms[1,2] probably related to a stimulation of muscarinic receptors since these symptoms are blocked by low doses of atropine. Oxotremorine also has several biochemical activities[3,4] including the increase of brain acetylcholine levels in pigeons and rats[5–8] which is apparently not due to an inhibition of brain cholinesterase.[1,9] It was recently found that oxotremorine increased mouse whole brain choline, the precursor of acetylcholine, as well as acetylcholine,[10] activity which was confirmed by Trabucchi and Salmoiraghi.[11]

Physostigmine, a potent cholinesterase inhibitor, has many pharmacological properties similar to oxotremorine and was found to also increase both choline and acetylcholine in mouse whole brain.[10]

This paper reports further observations concerning the mechanism of action of oxotremorine and physostigmine by utilizing tissues such as spleen and cerebellum which contain, respectively, no acetylcholine (unpublished results) or very low levels (0·3 μg/g wet wt,[12]) although they both contain relatively high levels of choline.

MATERIALS AND METHODS

Tissue preparation and extraction. Female Albino Swiss mice (20 g body wt) were killed by 3 sec immersion in liquid nitrogen. Whole brain, spleen and cerebellum

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were removed within 30 sec and immediately frozen in liquid nitrogen. After weighing in the frozen state, the tissues were pulverized in a specially designed mortar and pestle which permitted rapid, quantitative transfer of the frozen powder to test tubes for extraction.[13]

Plasma was obtained by low speed centrifugation of the mouse whole blood collected over 0·1 ml of 2% heparin.

Acetylcholine and choline were extracted from the tissues or plasma with 15% 1N aqueous formic acid–85% acetone.[14]

The radiochemical method of Saelens *et al.*[15] was then used for the measurement of acetylcholine and choline with slight modifications.[12] The method consists in separation of the acetylcholine from choline by low voltage paper electrophoresis, elution of the two bands and hydrolysis of the acetylcholine to choline. The choline is then acetylated with acetyl-[^{14}C]coenzyme A and previously prepared choline acetyltransferase. The acetyl-[^{14}C]choline formed is separated by paper electrophoresis and quantitated against appropriate standards. The results are expressed as μg free base of acetylcholine or choline per g wet wt of tissue or per ml of plasma.

Choline acetyltransferase activity was measured by the radiochemical method of McCaman and Hunt[16] modified by using paper electrophoresis rather than reinecke salt to separate the labelled acetylcholine formed.[17]

Drugs and reagents. Oxotremorine oxalate was purchased from Fluka A. G., isopropylfluorophosphate (DFP) from Schuchardt, Milan, physostigmine sulfate from Merck, Germany. Atropine sulfate was a gift from Hoffmann–La Roche, Basel. Acetyl-[^{14}C]coenzyme A, sp. act. 50–60 mC/mM was purchased from New England Nuclear.

The drugs were freshly prepared just before use and administered intraperitoneally at the doses and times shown in the figures and tables. None of the agents interfered with the analysis of acetylcholine and choline as determined with appropriate blanks.

The drugs were given as their salts and no corrections were made for their free base concentrations.

Statistics. Statistical significance was determined by the use of Student's *t*-test and Duncan's New Multiple Range test for 2 × 2 factorial analysis of variance.

Differences were considered significant if $P < 0.05$.

RESULTS

Table 1 compares oxotremorine and physostigmine at two concentrations on whole brain acetylcholine and choline 20 min after administration. Physostigmine, 0·5 mg/kg, is equivalent to oxotremorine, 0·7 mg/kg, on a molar basis. Both agents increased acetylcholine and choline. Physostigmine was more potent in increasing acetylcholine levels while they were equipotent in raising choline levels. That the increase in brain choline caused by physostigmine is not due to its anticholinesterase activity is shown in Table 1. DFP, at doses of 0·8–3·2 mg/kg, significantly increased mouse whole brain acetylcholine levels without affecting choline. The highest concentration of DFP almost doubled acetylcholine and it should be pointed out that this dose, on a molar basis, is 10–20 times higher than the doses of physostigmine used in these experiments.

Table 2 compares the effect of these two drugs on cerebellar acetylcholine. Physostigmine, 0·5 mg/kg, had no effect on this level up to 120 min after administration

TABLE 1. EFFECT OF OXOTREMORINE, PHYSOSTIGMINE AND DFP ON MOUSE WHOLE BRAIN ACETYLCHOLINE AND CHOLINE LEVELS

Drug	Dose (mg/kg i.p.)	Time (min)	Acetylcholine (μg/g wet wt)	Choline (μg/g wet wt)
Saline	—	20	2·31 ± 0·05 (8)	6·04 ± 0·19 (11)
Oxotremorine	0·7	20	4·05 ± 0·19† (5)	8·32 ± 0·73† (6)
	3·0	20	3·68 ± 0·10† (8)	9·53 ± 0·36† (8)
Saline		20	2·25 ± 0·07 (4)	5·88 ± 0·20 (10)
Physostigmine	0·25	20	3·74 ± 0·14† (7)	6·90 ± 0·25* (7)
	0·50	20	5·35 ± 0·12† (8)	8·29 ± 0·43† (9)
Ethanol 20%	—	20	2·07 ± 0·09 (4)	5·55 ± 0·38 (5)
DFP	0·8	20	2·54 ± 0·13† (6)	6·39 ± 0·43 (6)
	1·6	20	3·33 ± 0·14† (6)	6·21 ± 0·47 (6)
	3·2	20	3·87 ± 0·20† (6)	6·11 ± 0·62 (6)

Oxotremorine oxalate and physostigmine sulfate were dissolved in saline while DFP was dissolved in 20% ethanol for administration.

Figures are mean values ± S.E. of mean.

Figures in parenthesis represent number of animals used.

Group comparison Student *t*-tests were performed with saline (0·9% NaCl) or 20% ethanol treated animals to each drug treatment as noted.

* $P < 0·05$.

† $P < 0·01$.

while oxotremorine, 0·7 mg/kg, produced a small but significant increase at 30 min.

Figure 1 presents the time course of the effect of oxotremorine, 0·7 mg/kg, on choline levels in the spleen, cerebellum and plasma. Peak increases in choline occurred at around 30 min in spleen and plasma while in the cerebellum the peak was reached at 60 min. Spleen and plasma choline returned to normal by 120 min while cerebellar choline still remained elevated after 120 min followed by a return to normal by 180 min.

It is seen in Table 3 that increasing the dose of oxotremorine up to 3 mg/kg resulted in further increases in splenic choline 20 min after administration. No acetylcholine was detectable in the mouse spleen either in controls or after oxotremorine (Table 3) or physostigmine 0·5 mg/kg (data not shown). Since the lower sensitivity

TABLE 2. EFFECT OF OXOTREMORINE AND PHYSOSTIGMINE ON ACETYLCHOLINE LEVELS IN MOUSE CEREBELLUM WITH TIME

Time (min)	Cerebellar acetylcholine (μg/g wet wt) Oxotremorine	Physostigmine
0	0·34 ± 0·01 (16)	0·35 ± 0·02 (10)
30	0·44 ± 0·03* (7)	0·37 ± 0·02 (9)
60	0·42 ± 0·02 (16)	0·40 ± 0·03 (7)
120	0·38 ± 0·02 (9)	0·42 ± 0·04 (7)

Values represent mean ± S.E. of mean.

Figures in parenthesis represent number of animals used.

Oxotremorine oxalate was administered at the dose of 0·7 mg/kg, i.p.; physostigmine sulfate 0·5 mg/kg, i.p.

Group comparison Student *t*-tests were performed with saline (0 min) to each time period as noted.

* $P < 0·01$.

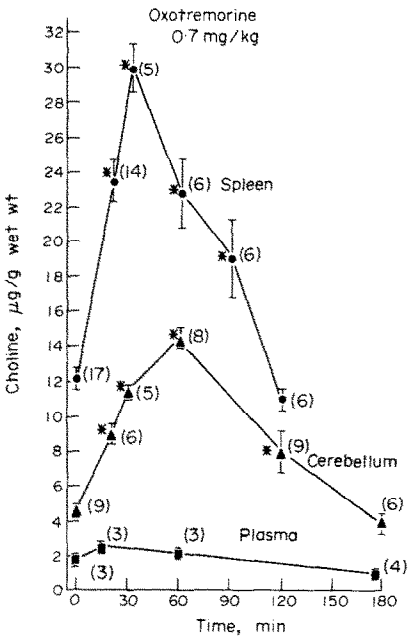

FIG. 1. Effect of oxotremorine on choline levels in mouse spleen, cerebellum and plasma with time. Oxotremorine oxalate was administered intraperitoneally and the tissues were removed for analysis at the times indicated. The 0 times refer to the tissue levels of untreated animals. The vertical bars represent S.E. of the means and the asterisks denote significance with respect to 0 time at $P < 0.01$. The number of animals used at each point is shown in parenthesis.

of this radiochemical method for acetylcholine is 1 ng, a concentration of 67 ng/g wet wt splenic acetylcholine would be detectable. Choline acetyltransferase activity was also not detectable in the mouse spleen (data not reported in detail) using the radiochemical method of McCaman and Hunt[16] whose lower limit of sensitivity is about 10^{-13} moles of acetylcholine formed per hr.[18]

Figure 2 shows the effect of physostigmine sulfate, 0·5 mg/kg, on choline levels in spleen, cerebellum and plasma. No effect on splenic choline was observed at any time. Cerebellar choline was doubled at 30 min after treatment, began to decline at 60 min and returned to normal by 150 min. Plasma levels were slightly increased only at 20 min after treatment.

TABLE 3. EFFECT OF OXOTREMORINE ON MOUSE SPLEEN ACETYLCHOLINE AND CHOLINE LEVELS

Drug	Dose (mg/kg, i.p.)	Time (min)	Acetylcholine (μg/g wet wt)	Choline (μg/g wet wt)
Saline		20	Nil* (<0·07)	12·80 ± 0·59 (17)
Oxotremorine	0·7	20	Nil	22·33 ± 1·63† (8)
	1·5	20	Nil	28·81 ± 0·92† (8)
	3·0	20	Nil	30·96 ± 0·94† (11)

Figures are mean values ± S.E. of mean.
Figures in parenthesis represent number of animals used.
Group comparison Student *t*-tests were performed with saline to each drug treatment as noted.
* The lower limit of sensitivity of this method is 1 ng acetylcholine which is equivalent to 67 ng/g wet wt of spleen.
† $P < 0.01$.

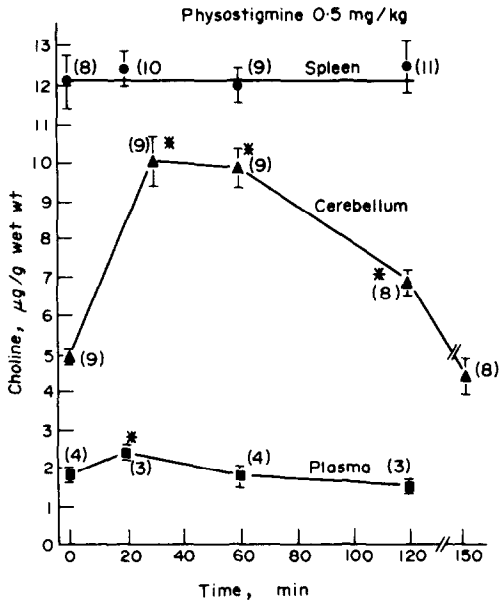

FIG. 2. Effect of physostigmine sulfate on choline levels in mouse spleen, cerebellum and plasma with time. See legend of Fig. 1 for details.

Table 4 shows that the oxotremorine and physostigmine induced increases in cerebellar choline were completely antagonized by atropine at a dose of 5 mg/kg. Atropine alone had no effect on cerebellar choline levels.

Atropine, at a dose of 5 mg/kg, also was able to completely block the increase in splenic choline produced by oxotremorine (Table 5), a finding which suggests that oxotremorine acts through muscarinic receptors to produce its activity.

TABLE 4. ATROPINE ANTAGONISM OF OXOTREMORINE AND PHYSOSTIGMINE INDUCED INCREASES IN CEREBELLAR CHOLINE

Drug	Dose (mg/kg, i.p.)	Time (min)	Cerebellar choline (μg/g wet wt)
Saline	—	50	5·49 ± 0·29 (6)
Atropine	5	50	6·18 ± 0·32 (6)
Physostigmine	0·5	20	8·13 ± 0·39* (6)
Atropine +	5	30	
Physostigmine	0·5	20	4·82 ± 0·78 (6)
Saline	—	50	5·76 ± 0·50 (5)
Atropine	5	50	6·58 ± 0·19 (5)
Oxotremorine	0·7	20	12·79 ± 0·64* (5)
Atropine +	5	30	
Oxotremorine	0·7	20	7·49 ± 0·67 (5)

Figures are mean values and S.E. of mean.
Figures in parenthesis represent number of animals used.
Analysis of variance—two factor factorial. Duncan's New Multiple Range test.
* $P < 0.01$ with respect to the saline treated group.

TABLE 5. ATROPINE ANTAGONISM OF OXOTREMORINE-INDUCED INCREASE IN MOUSE SPLENIC CHOLINE

Drug	Dose (mg/kg, i.p.)	Time (min)	Splenic choline (μg/g wet wt)
Saline		50	14·79 ± 1·52 (5)
Atropine	5	50	12·28 ± 1·18 (5)
Oxotremorine	0.7	20	29·18 ± 1·46* (5)
Atropine +	5	30	
Oxotremorine	0·7	20	18·47 ± 1·16 (5)

Values are mean ± S.E. of mean.
Figures in parenthesis represent number of animals used.
Analysis of variance—two factor factorial. Duncan's New Multiple Range test.
* $P < 0.01$ with respect to the saline treated group.

DISCUSSION

Physostigmine and oxotremorine share the property of increasing mouse whole brain acetylcholine and choline levels. Their mechanism of action in increasing acetylcholine must be different, however, because physostigmine is a powerful inhibitor of brain cholinesterase while oxotremorine is only a weak one.[4,9]

The data of Potter *et al.*[19] and Kaita and Goldberg[20] showing product inhibition of choline acetyltransferase suggested to us the possibility that choline was increased secondarily to feedback inhibition by the high acetylcholine concentration achieved by physostigmine.[10] However, DFP, a potent organophosphorous anticholinesterase did not affect whole brain choline at times when acetylcholine levels were substantially increased and furthermore, physostigmine increased choline levels in the cerebellum at doses which were without effect on acetylcholine levels. These data suggest that, at least under our conditions, either there is no feedback inhibition *in vivo* or that feedback inhibition of acetylcholine synthesis is not a necessary requirement for raising choline levels.

The increase in choline produced by physostigmine, therefore, must be related to its structure and its locus of action is probably neuronal because of the lack of activity on the spleen. The data further suggest that organophosphorous inhibitors of cholinesterase are preferable to physostigmine in kinetic studies on acetylcholine formation since mobilization of choline by physostigmine could compromise interpretation of results.

The high levels of choline produced by oxotremorine could lead to increased synthesis of acetylcholine since the normal brain concentration of choline, about 30–50 μM,[21] is about 10–15 times lower than the K_m for bovine caudate and rabbit brain choline acetyltransferase,[16,22] and approximately equivalent to the K_m for rat brain[19] suggesting that the enzyme is probably not saturated under normal conditions.

On the other hand, the decreased turnover of acetylcholine[23] or the retarded incorporation of choline into acetylcholine[11] may be the primary actions of oxotremorine, both of which could possibly lead to choline accumulation. However, since oxotremorine also increased choline levels in the spleen, it is most likely that the increase in choline is independent of the effect on acetylcholine synthesis.

The high tissue levels of choline resulting from oxotremorine treatment probably

reflect intracellular mobilization of choline rather than increased uptake of choline from the extracellular medium. It may be pertinent to recall in this respect that both physostigmine and oxotremorine increased plasma choline to a small extent at 20 min after administration but only oxotremorine increased choline levels in the spleen. Thus, uptake of choline from the circulation cannot account for oxotremorine's effect.

That atropine blocked the increase in choline induced by oxotremorine and physostigmine suggests that these agents act directly at muscarinic receptor sites for this biochemical action. This is evident even in a tissue such as the mouse spleen which, judging from our negative data on the presence of acetylcholine and choline acetyltransferase, probably is devoid of cholinergic innervation.

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