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GAS CHROMATOGRAPHIC EVALUATION OF THE EFFECTS OF SOME MUSCARINIC AND ANTI-MUSCARINIC DRUGS ON ACETYLCHOLINE LEVELS IN RAT BRAIN

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Abstract—The effects of some muscarinic and antimuscarinic drugs on brain acetylcholine levels have been re-evaluated using a specific gas chromatographic method for the analysis of acetylcholine as its volatile tertiary analog. The elevation of brain acetylcholine levels produced by oxotremorine has been demonstrated to be independent of the concomitant hypothermia, since the rise is not prevented by maintaining the animals in a thermally neutral environment in which the hypothermic effect is suppressed. Peripheral parasympathomimetic changes do not appear to be responsible for the alteration of brain acetylcholine because the quaternary analog, *N*-methyl oxotremorine, produces profound peripheral parasympathomimetic effects with no central cholinergic response and a minimal acetylcholine elevation. Similarly, while atropine sulfate is capable of abolishing the oxotremorine induced rise in acetylcholine, methylatropine, when given in a dose which antagonized all peripheral effects of oxotremorine, failed to inhibit the elevation of brain acetylcholine levels. It is concluded that the elevation of brain acetylcholine levels produced by oxotremorine is mediated by a direct central action.

SEVERAL investigators have reported changes in brain acetylcholine levels after administration of muscarinic and antimuscarinic drugs.[1–8] In each case, acetylcholine has been estimated by bioassay. Bioassays cannot always distinguish between acetylcholine and related compounds, and other tissue components may contribute to the acetylcholine-like activity measured or influence the sensitivity of the test object to acetylcholine.[9, 10] When the effect of drugs on acetylcholine levels is under investigation, additional complications arise because the presence of these drugs in tissue extracts may mimic, potentiate or reduce the response of the tissue to acetylcholine.

This report is concerned with a re-evaluation of the effects of some muscarinic and antimuscarinic agents on brain acetylcholine levels in the rat, using a specific chemical method which is free from interference by other pharmacologically active components of the tissue extract, whether endogenous or introduced as part of the experiment. This method is based on gas chromatographic estimation of dimethylaminoethyl acetate formed by the specific and quantitative *N*-demethylation of acetylcholine.[11, 12] A preliminary account of some of the results has appeared elsewhere.[13]

EXPERIMENTAL

Experimental animals. Male Sprague–Dawley rats, ranging in weight from 300 to

400 g, were maintained under standardized lighting and temperature conditions. At least a week was allowed between shipment of animals and their use for an assay and animals were killed at the same approximate time each day. The muscarinic and antimuscarinic drugs employed were prepared in normal saline and given by intraperitoneal injection (1 ml/kg), as were saline control injections.

Initial brain preparation. At the designated times, animals were decapitated, the brain was rapidly excised and immediately plunged into liquid nitrogen. This process ranged from about 15 to 20-sec duration. A pulverizing device, consisting of a stainless steel cylinder and a close tolerance stainless steel pestle, was also immersed in liquid nitrogen until equilibrated at that temperature. Each frozen brain was then thoroughly pulverized between the cold piston and cylinder bottom. The finely powdered brain was then transferred to a tared glass homogenizer (also chilled in liquid nitrogen to prevent thawing) and weighed.

Extraction procedure. The internal standardization, initial extraction, quaternary isolation with ammonium Reineckate, ion exchange, demethylation of the quaternaries and chloroform extraction were performed as described previously.[11, 12]

Gas chromatography. Analysis was done on an F&M 5750A dual column gas chromatograph equipped with dual flame ionization detectors and a Varian Associates Recorder (Model G-2000). Silanized glass columns (7ft:¼ in. O.D.) were packed with three parts 120/200 mesh Polypak I (F&M) and one part 100/200 mesh Gas Chrom Q (Applied Science Labs.), which was found to reduce the sensitivity of Polypak to temperature fluctuations. The liquid phase in preliminary work was 1% phenyldiethanolamine succinate (PDEAS, Analabs, Inc.). This was later replaced by 1% dodecyldiethylenetriamine succinamide synthesized in these laboratories.

Since Polypak has a high temperature coefficient of expansion, the carrier gas flow rate was found to be very sensitive to temperature changes. Consequently, chromatographic runs were performed isothermally with a column temperature of 165°. The flame detector and injection port temperatures were 200° and 205° respectively. The carrier gas was nitrogen, the flow being adjusted to 45 ml/min (80 psi). Hydrogen flow was maintained at 30 ml/min (26 psi) and air flow at 280 ml/min (30 psi), although air was later replaced by oxygen at 185 ml/min (20 psi), which doubled the sensitivity. All gases were dried by passage through drying tubes filled with molecular sieve (Linde, Type 5A).

RESULTS

Before investigating the effects of drugs on brain acetylcholine levels, it was of interest to determine whether endogenous acetylcholine would be influenced by procedures and conditions encountered in the laboratory. Two controls of this type were run: the effect of prior intraperitoneal injection of saline and the effect of a loud noise produced by mechanical vibration of the metal cage in which the animals were housed. Saline controls gave a mean level of 18·9 $\pm$ 0·6 nmole/g ($N = 14$) in comparison to a value of 19·1 $\pm$ 0·4 nmole/g ($N = 11$) for untreated animals ($t = 0{\cdot}27$, $P > 0{\cdot}1$). Exposure to a loud noise for 15 to 30 sec also produced no significant change (18·1 $\pm$ 0·5 nmole/g in seven animals; $t = 1{\cdot}56$, $P > 0{\cdot}1$). The changes to be described are therefore attributed to the effects of the administered drugs, rather than to the influence of handling or noise.

Oxotremorine was given intraperitoneally to groups of rats in graded doses ranging

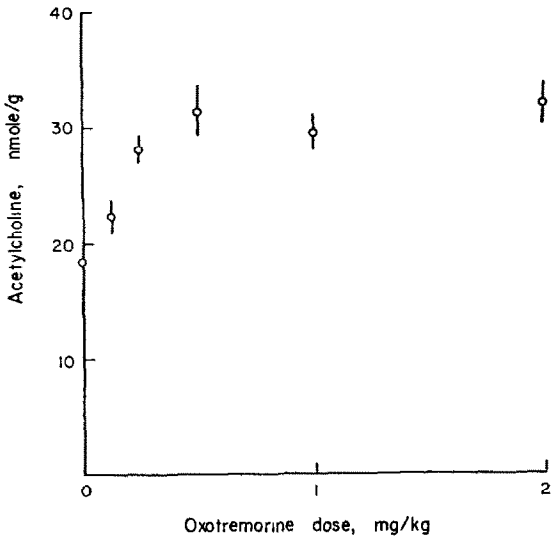

FIG. 1. Influence of graded doses of oxotremorine on rat brain acetylcholine 20 min after injection. Values represent the mean ± S. E.

from the threshold (0·125 mg/kg) required to produce obvious pharmacological effects (salivation, diarrhea, analgesia, tremor, hypokinesia, chromadacryorrhea) to a supramaximal dose (2 mg/kg). Fig. 1 shows that the brain acetylcholine level rose progressively as the dose increased, a significant increase being observed at the lowest dose tested. The largest dose (2 mg/kg) produced an elevation which was not significantly greater than that observed after 0·5 mg/kg. A number of chromatographic peaks are seen in brain extracts which have not yet been identified.[13, 14] This pattern was not altered in a reproducible manner after oxotremorine injection at any dose level. The time after oxotremorine injection at which the analysis was made had no significant influence on the elevation in brain acetylcholine level within the range 10–30 min.

One of the effects produced in rats by the systemic administration of oxotremorine in the doses used is a fall in body temperature.[15] Since cooling is known to inhibit acetylcholine release from subcellular organelles[16, 17] and would certainly be expected to interfere with normal acetylcholine turnover, it appeared possible that the elevation in total acetylcholine levels produced by the drug might be an indirect action secondary to the hypothermia. This possibility was tested by placing the rats in an environment of elevated temperature (32°) which separate experiments showed to prevent the normal hypothermic effect (Fig. 2). This treatment did not in itself influence brain acetylcholine levels, nor was the rise after oxotremorine significantly reduced in comparison to experiments conducted at room temperature (Table 1). It may be concluded that the increased acetylcholine levels produced by oxotremorine are not mediated by a fall in body temperature.

Since oxotremorine produces profound changes in the cardiovascular system at doses far lower than those used in the present experiments,[18] the possibility presented itself that the alterations in brain acetylcholine might be secondary to these peripheral changes. This possibility was examined in the following way: *N*-methyl atropine

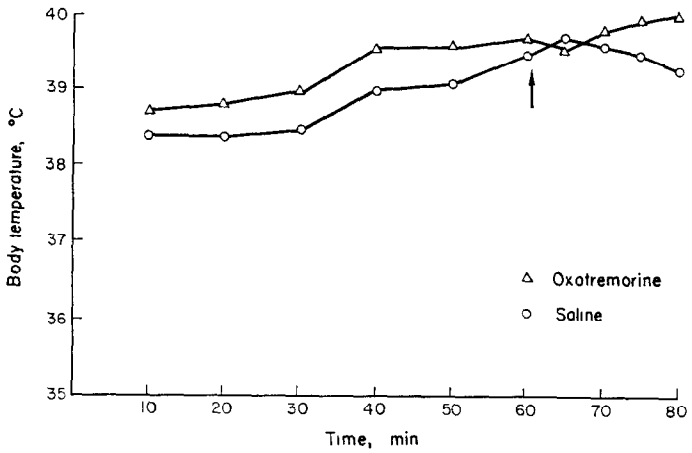

FIG. 2. Body temperature record of rats injected with oxotremorine (2 mg/kg, i.p.) and saline maintained in a constant temperature environment at 32°. Injections were made at the arrow.

TABLE 1. BRAIN ACETYLCHOLINE LEVELS (MEAN ± S. E.; nanomoles/gram) IN GROUPS OF RATS AFTER OXOTREMORINE AT TWO ENVIRONMENTAL TEMPERATURES*

Oxotremorine (mg/kg)	Environmental temperature (°)	
	20	32
0	18·9 ± 0·6 (14)	19·8 ± 1·0 (3)
2	32·1 ± 1·3 (6)	29·0 ± 1·9 (3)

* Both groups receiving oxotremorine showed a significant difference from controls receiving saline; differences between groups tested at isothermal and room temperatures were not significant ($P > 0·2$). Number of rats in each group is shown in parentheses.

TABLE 2. BRAIN ACETYLCHOLINE LEVELS IN GROUPS OF RATS SACRIFICED 20 min AFTER OXOTREMORINE, SHOWING THE EFFECTS OF PREMEDICATION WITH ATROPINE SULFATE OR ATROPINE METHYLBROMIDE, GIVEN INTRAPERITONEALLY 15 min BEFORE THE OXOTREMORINE*

Premedication	Dose of oxotremorine (mg/kg)		
	0	0·25	1·0
None	18·9 ± 0·6 (14)	28·1 ± 0·8 (8)	29·4 ± 1·0 (6)
Atropine sulfate 5 mg/kg	16·5 ± 1·0 (5)	17·9 ± 0·7 (10)	22·7 ± 1·7 (8)
Atropine methylbromide 5 mg/kg	19·2 ± 0·7 (8)	24·0 ± 0·7 (10)	24·5 ± 1·2 (10)

* Values are presented in nanomoles per gram as a mean ± S. E., and the number of rats in each group is indicated in parentheses.

blocks the cardiovascular muscarinic effects, but does not penetrate the blood–brain barrier to a significant degree and leaves the central actions of oxotremorine unchanged or even unmasked.[19] If the brain acetylcholine levels are increased purely as a secondary result of the peripheral actions of oxotremorine, *N*-methyl atropine should therefore prevent the increase. Table 2 shows that a highly significant rise was observed ($P < 0.001$; $P < 0.005$) after atropine methylbromide in response to oxotremorine at a dose of either 0·25 or 1·0 mg/kg, respectively, although at both doses the response was less than in unpremedicated animals ($P < 0.01$; $P < 0.005$). In contrast, atropine sulfate completely prevented the rise which normally occurs after 0·25 mg/kg oxotremorine ($P > 0.2$) and greatly reduced the response to 1 mg/kg. The levels seen after atropine alone are significantly lower than in control animals ($t = 2.11$, $P < 0.05$). These results are consistent with the view that the elevation of brain acetylcholine levels produced by oxotremorine is effected primarily by a direct central action of the drug, which is antagonized competitively by atropine. This inference was confirmed by some experiments with *N*-methyl oxotremorine, which produces the same peripheral effects as the tertiary amine[20] but lacks the central actions. The dose of 2·5 mg/kg which was used is equivalent in peripheral muscarinic activity to about 0·35 mg/kg of oxotremorine, which would be expected to cause a maximal rise in brain acetylcholine levels to about 30 nmole/g (cf. Fig. 1). In fact, the mean level of 10 animals was 20.9 ± 0.6 nmole/g, which is barely significant ($0.05 > P > 0.025$) when compared to the control level of 18.9 ± 0.6 ($N = 14$).

DISCUSSION

The work described here confirms several earlier publications in which bioassay was used to investigate the effects of muscarinic and antimuscarinic agents on brain acetylcholine levels.[1–8] The chemical method employed here is more specific and is free from interference by adventitious components of the brain extracts, including the drugs used in the experiments. Because bioassay is inherently susceptible to artifacts arising from these sources, some doubt existed regarding the validity of the changes reported. Independent evidence using a direct chemical procedure extends and provides unequivocal confirmation of some of the basic changes reported, and the present work also sheds some light on the mechanisms responsible.

Oxotremorine elevates total brain acetylcholine levels after doses which correspond in threshold and maximum effect to those required to produce gross motor disturbances. This elevation is not secondary to the hypothermia observed after oxotremorine since it is also seen in a thermally neutral environment in which no change in body temperature is elicited by the drug.

The rise in brain acetylcholine appears to be because of a direct action of oxotremorine on the central nervous system, since it cannot be prevented by a dose of atropine methylbromide sufficient to block all the peripheral effects, nor can it be duplicated by an equivalent dose of *N*-methyl oxotremorine, which reproduces the peripheral muscarinic actions of oxotremorine but lacks the central actions. However, it should be pointed out that the rise in brain acetylcholine induced by oxotremorine was somewhat less after premedication with atropine methylbromide than in control animals, and that 2·5 mg/kg *N*-methyl oxotremorine produced a small but statistically significant rise. These results might be explained by incomplete exclusion of quaternary

ammonium compounds from the central nervous system by the blood-brain barrier;[21] alternatively, it may be that the peripheral actions of these drugs exert a significant indirect influence on the brain acetylcholine level. In this connection it may be relevant that a large dose of atropine has been reported to lower the brain concentration of oxotremorine in comparison to unpremedicated animals.[22]

Atropine alone was found to lower brain acetylcholine levels, in confirmation of earlier observations using bioassay,[2-4, 6] while atropine methylbromide did not (Table 2). These results suggest that if this agent does pass the blood–brain barrier, it does not do so in significant amounts. The interaction between atropine and oxotremorine in regard to brain acetylcholine levels appeared to be grossly competitive in the sense that a small dose (0·25 mg/kg) of oxotremorine produced no significant change after atropine (5 mg/kg) premedication, while a larger dose of oxotremorine (1 mg/kg) broke through the atropine blockade. No such competition was observed between oxotremorine and *N*-methyl atropine. However, the data do not justify the conclusion that the antagonism between atropine and oxotremorine is strictly competitive in the sense that they interact with the same receptor; an additional mechanism has recently been proposed by Crossland and Slater[23] on the basis of bioassay studies of "free" and "bound" acetylcholine.

The cellular mechanisms by which these changes in acetylcholine levels are effected remain a matter of controversy, and no conclusions can be drawn from the present work. However, it seems likely that the freedom of this chemical microestimation procedure from interference by other drugs will render the technique a valuable means of elucidating these mechanisms, and it is now being applied to this purpose.

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