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EFFECTS OF OXOTREMORINE ON BRAIN ACETYLCHOLINE FORMATION *IN VIVO* AND *IN VITRO*

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Abstract—Under our experimental conditions rat brain normally contains 14·4 ± 1·3 (M ± SD) m μmole acetylcholine (AcCh)/g. If oxotremorine (OT), 2 mg/kg body wt. i.p., is given to rats, this increases the level of AcCh to 22·1 ± 2·3.

Homogenates of rat brain left for 15 min at 20° in the presence of physostigmine salicylate (10^{-5} M) contain 13·5 ± 1·4 (M ± SD) m μmole AcCh/g tissue. OT (10^{-5} M) added to the homogenate increases the AcCh level to 20·9 ± 3·4/g tissue.

Addition of 5–8 m μmole AcCh/g tissue to a homogenate of normal rat brain followed by hydrolysis of the free acetylcholine before incubation has no inhibitory effect on the formation of AcCh produced by OT *in vitro*.

The ability of OT to increase the AcCh level in a brain homogenate from rats pretreated with OT seems to be inversely proportional to its effect *in vivo*.

The amount of AcCh produced by OT either *in vivo* or *in vitro* does not exceed a certain limit. In combined experiments mirror-like time curves for the concentration of AcCh are obtained. This observation suggests that OT has a similar mechanism of action *in vivo* and *in vitro*.

The possibility for the presented results to be reconciled with an increased biosynthesis of AcCh has been discussed.

IN PREVIOUS experiments[1, 2] it has been shown that oxotremorine (OT) causes a rise in brain acetylcholine (AcCh) which coincides with the tremor period. Likewise OT (10^{-5} M) causes a rise in the total amount of AcCh when added to a brain homogenate. Possible explanations for this OT-effect have been discussed.[1–4]

The present work was undertaken in order to find out whether previous treatment of animals with OT affects the ability of the brain homogenate to increase the amount of AcCh *in vitro*. In this way it was hoped to throw light on common mechanisms of action as well as to be able to transfer conditions to a more easily handled *in vitro* system.

MATERIAL AND METHODS

Compounds used

Oxotremorine oxalate, 1(2'oxopyrrolidino)-4-pyrrolidino-2-butyne was synthesized by Farm. lic. Bo Karlén, Kgl. Farmaceutiska Institutet, Stockholm Va.

Acetylcholine iodide and physostigmine salicylate (eserine) were obtained commercially.

Determination of AcCh

AcCh was determined biologically by the frog rectus method, essentially according to Whittaker[5] and McIntosh and Perry[6] using an electromechanical recording technique.[2]

The brain was homogenized in 0·15 M NaCl (0·2 g tissue/ml) by means of an Ultra-Turrax homogenizer. AcCh was extracted by addition of perchloric acid to a final concentration of 0·4 M. After 30 min in the refrigerator at 8° the homogenate was centrifuged 15 min at 9000 *g* and 4°. The supernatant was filtered into a measuring cylinder and the volume noted. The perchlorate was precipitated by means of 5 N potassium carbonate at pH 5·7 and the supernatant transferred to a plastic centrifuge tube and left for about 20 min at −19°, whereupon it was centrifuged for 20 min at 15,000 *g* and 4°.

After centrifugation the supernatant was passed through a column containing Dowex 2 (200–400 mesh) equilibrated with Cl^-. The remaining AcCh was washed out with 5 ml distilled water and the volume of the combined eluates noted. The pH was adjusted to 6 before assay.

The columns can be used repeatedly if they are washed in the following sequence:

(1) 20 ml 1 M sodium hydroxyde
(2) dist. water (pH 5–6)
(3) 25 ml 1 M hydrochloric acid and
(4) dist. water (pH 5–6)

The columns were arranged according to Bertler, Carlsson and Rosengren.[7] The inner diameter of the column was 4 mm and the length occupied by the resin was 45 mm. The flow rate was 6–7 drops per minute.

In vivo–in vitro *experiments*

Sprague–Dawley rats weighing 250–300 g were injected with OT, 2 mg (base)/kg bodyweight i.p. At various times after the injection the animals were killed by a blow on the head. The brains were immediately removed, and homogenized in 0·15 M NaCl (0·2 g brain/ml). Four brains were used for each experiment.

Each homogenate was divided in four aliquots. In part 1 AcCh was determined without incubation while parts 2, 3 and 4 were left for 15 min in a water bath at 20° for hydrolysis of free AcCh. After hydrolysis, the AcCh content in part 2 was determined giving the AcCh level in the homogenate before incubation with OT. To part 3 eserine (10^{-5} M) and OT (10^{-5} M) were added and to part 4 only eserine (10^{-5} M). The latter was used as the incubation blank. The vessels were incubated at 20° for 15 min. Incubation and hydrolysis was stopped by addition of 1·2 M perchloric acid to a final molarity of 0·4 and AcCh determined.

Control experiments were performed in homogenates with various concentrations of hydrolysis products of free AcCh. Brains from animals, pretreated with saline, were homogenized in saline with 5–8 mμmole AcCh added per g tissue. The homogenates were left for 15 min at 20° without eserine before incubation with OT (10^{-5} M).

RESULTS

The results of the experiments are presented in Figs. 1 and 2. The normal level of AcCh in rat brain has been determined previously in a series of 56 tests.[2] The mean

value was 14·4 and the S.D. 1·3 mμmole/g tissue. Although the values in the literature vary due to different extraction techniques, strain and age of rats etc., Giarman and Pepeu e.g. have reported 14·9 m μmole AcCh/g rat brain.[8] Thirty minutes after the injection of 2 mg OT/kg body wt. rat brain contains 22·1 ± 2·3 m μmole/g tissue.[2]

The AcCh level in rat brain at various times (2–75 min) following the i.p. injection of 2 mg OT/kg body wt. was determined in a series of tests (Fig. 1 (a)). The maximal increase of AcCh was found around 20 min. Aliquots of the homogenates from which the curve represented in Fig. 1 (a) was obtained were left without eserine for 15 min at 20°. Under these conditions the level of AcCh per g tissue decreased to about 12 m μmole at all points studied along the time curve, (Fig. 1 (b)). When, on the other hand, another aliquot of a homogenate treated in this way was incubated with eserine and OT, the ability of OT to increase the acetylcholine level in the homogenate along the time curve was found to be inversely proportional to its effect *in vivo*. Seventyfive minutes after injection of OT the brain had completely regained its power to produce acetylcholine and the level after incubation with OT was about 20 m μmole acetylcholine per g tissue, Fig. 1 (b).

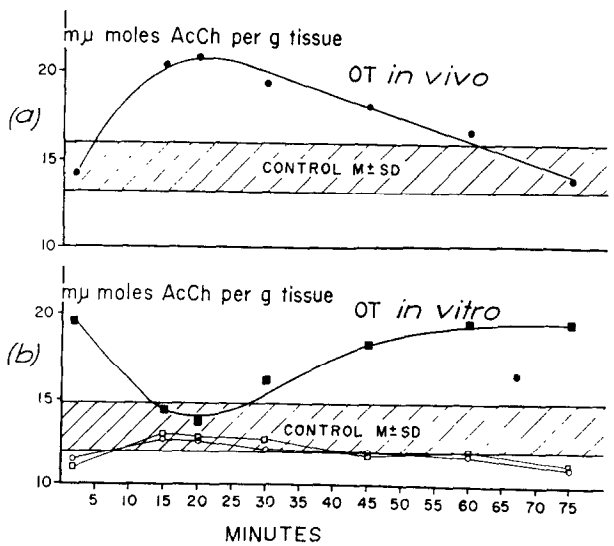

FIG. 1. Effect of OT on the brain AcCh level *in vivo* and added *in vitro* to a homogenate from the same tissue. The homogenate was left without eserine for 15 min at 20° C before incubation. Medium: 0·15 M NaCl. Incubation time: 15 min. Temperature: 20° C. All molarities in final concentration. ● 2 mg OT per kg bodyweight. ○ AcCh level before incubation. ■ After incubation with OT (10^{-5} M) in the presence of eserine (10^{-5} M). □ After incubation in the presence of only eserine (10^{-5} M). (a) Brain AcCh level in normal rats. Extraction with perchloric acid. Control M = 14·6 ± 1·4 mμmoles AcCh per g tissue. (b) AcCh level in brain homogenate from normal rats. Incubation during 15 min at 20° C in the presence of eserine (10^{-5} M). Control M = 13·5 ± 1·4 mμmoles AcCh per g tissue.

The AcCh content in normal rat brain homogenate left for 15 min at 20° in the presence of eserine (10^{-5} M) was determined in a series of 18 tests. The mean value was 13·5 and the S.D. ± 1·4 m μmole AcCh/g tissue. The AcCh content was also determined in normal rat brain homogenate left at 20° without eserine and analyzed at various time intervals (5–45 min). Under these conditions there is an initial rapid decrease, but after 5 min a relatively constant value of about 12 m μmole/g tissue is reached. It has previously been shown that the initial value of AcCh in a homogenate does not change in the presence of eserine (10^{-5} M) during incubation times up to 15 min.[4]

The AcCh content in rat brain homogenate after incubation with OT (10^{-5} M) in the presence of eserine (10^{-5} M) during 15 min at 20° was found to be 20·9 ± 3·4 m μmole/g tissue.[2] The rise in AcCh content in rat brain homogenate produced by the addition of OT in the presence of hydrolysis products of 5–8 m μmole AcCh added per g tissue, was determined in another series of experiments. The effect of OT was roughly the same as in a normal homogenate of rat brain, i.e. formation of 6–7 m μmole AcCh/g tissue (Fig. 2).

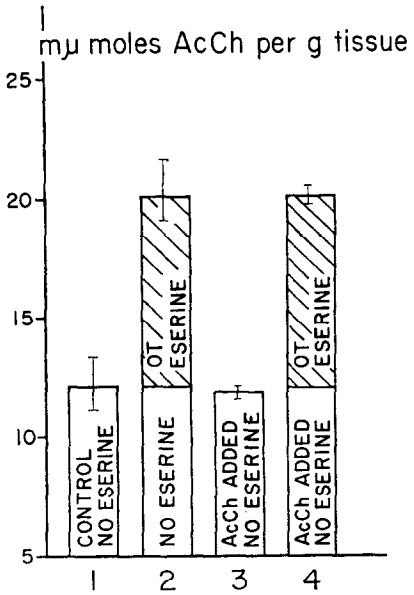

FIG. 2. Incubation experiment with rat brain homogenates. The homogenate was left for 15 min at 20° C without eserine before incubation to hydrolyze free AcCh. Medium: 0·15 M NaCl. Incubation time: 15 min. Temperature: 20° C. All molarities in final concentration. (1) Control. (2) Control + eserine (10^{-5} M) and OT (10^{-5} M). (3) Control + 5 mμmoles AcCh added per g tissue before hydrolysis. (4) Control + 5 mμmoles AcCh added per g tissue before hydrolysis and incubation with eserine (10^{-5} M) and OT (10^{-5} M). All analyses were performed in duplicate and the experiments were repeated at least twice. Extreme values have been indicated on the bars.

DISCUSSION

According to previously published results, AcCh is the only brain amine which can be appreciably influenced by muscarine-like tremorgenic agents during the period of tremor. The duration of tremor activity also corresponds roughly to the increase in brain AcCh[2]. The ability of OT to increase brain AcCh and, at the same time, to cause a well-sustained tremor undoubtedly is linked to a muscarine-like action in the central

nervous system, since it can be counteracted by atropine but not by atropine methylnitrate.[2, 9] Only tremorgenic agents with a muscarine-like activity produce a rise in brain AcCh *in vivo* or *in vitro*.[3] The increase is predominantly in free AcCh (Fig. 1 and to be published). The rise normally produced by addition of OT (10^{-5} M) to the homogenate can be prevented by either pretreatment of the intact animal or *in vitro* homogenate with atropine.

Previous results led to the hypothesis that tremorgenic agents of this kind have no direct effect but possibly act by mobilizing a precursor of AcCh from an otherwise undetected store.[2] The inability of OT to stimulate an *in vitro* system containing an acetone extract of cholineacetylase[10] does not exclude the possibility that it causes an increased biosynthesis of AcCh *in vivo*. In certain experiments, injection of tremorine, the precursor of OT, has been found to increase the cholineacetylase activity in a mouse brain homogenate.[11] One observation suggesting an increased synthesis of AcCh is the decrease in the amount of acetyl Coenzyme A after injection of OT.[12] The inhibition of the OT effect by hemicholinium *in vitro* may also constitute indirect proof of a biosynthetic effect, especially since the hemicholinium effect can be counteracted by choline.[4]

The results presented in this paper are also compatible with an increased biosynthesis *in vivo*. The amount of AcCh that can be produced does not however exceed a certain limit. When an animal has been treated with OT and the brain is taken out at the height of the OT effect, the brain homogenate does not respond to the addition of OT by an increase of the amount of AcCh. Shortly after the administration the homogenate still has this ability. It then loses it but regains its potential to accumulate AcCh along a curve which is a mirror image of the rise in acetylcholine concentration in the brain after administration of OT *in vivo*. The mirrorlike curves strongly suggest that OT has a similar mechanism of action both *in vivo* and *in vitro*. This would seem to be of importance in further experiments intended to explain the pharmacodynamics of centrally acting muscarine-like agents, especially since quarternary ammonium bases that do not penetrate the blood–brain barrier can be included in *in vitro* assays.

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