Br. J. Pharmac. (1972), **46**, 473–479.

Effects of some centrally acting drugs on acetylcholine synthesis by rat cerebral cortex slices

M. SHARKAWI

Département de pharmacologie, Faculté de médecine, Université de Montréal, Montréal, Qué., Canada

Summary

1. Brain cortex slices from rats injected i.p. with urethane (1 g/kg), chloral hydrate (350 mg/kg) or physostigmine (0·75 mg/kg) were examined for acetylcholine (ACh) content, cholinesterase (total enzyme) activity and formation of ^{14}C-ACh from carbon ^{14}-uniformly labelled glucose (U-^{14}C-D-glucose) in the presence of 0·01 mM physostigmine.

2. Slices from rats treated with urethane, chloral hydrate, or physostigmine contained significantly higher concentrations of ACh than slices from untreated animals.

3. Only slices from physostigmine-treated rats had a significantly lower cholinesterase activity.

4. Slices from urethane- or chloral hydrate-treated animals formed significantly less ^{14}C-ACh than slices from untreated or physostigmine-treated rats when incubated in 4 mM K^+ medium. In an ACh-releasing medium (31 mM K^+) slices from rats treated with urethane or chloral hydrate and slices from untreated rats formed similar amounts of ^{14}C-ACh.

5. Slices from rats treated with atropine (25 mg/kg) or pentylenetetrazol (75 mg/kg) had a similar ability to form ^{14}C-ACh as slices from untreated animals when incubated in either 4 or 31 mM K^+ medium.

6. These findings suggest that the intraneuronal ACh concentration is a limiting factor in the regulation of ACh synthesis.

Introduction

The concentration of acetylcholine (ACh) in rat brain varies inversely with the degree of cerebral activity. An elevated concentration of ACh is associated with the administration of anaesthetic agents (Tobias, Lipton & Lepinat, 1946; Richter & Crossland, 1949; Crossland & Merrick, 1954; Giarman & Pepeu, 1962). Whereas a decrease in ACh concentration is seen with the administration of the central stimulant pentylenetetrazol (Giarman & Pepeu, 1962; Kurokawa, Machiyama & Kato, 1963).

The process of ACh synthesis in rat cerebral cortex slices seems to be governed by the concentration of the neurotransmitter in cholinergic neurones. While ac-

celerated release of ACh stimulates the synthesis of the neurotransmitter, an increase in ACh concentration depresses it (Sharkawi & Schulman, 1969). There is also evidence that this feedback regulation is operative in mouse brain *in vivo* (Schuberth, Sparf & Sundwall, 1969).

In an attempt to examine further the relationship between ACh concentration and its synthesis, we injected rats with two types of drugs which increase ACh concentration in rat brain, namely, the two anaesthetics urethane and chloral hydrate and the cholinesterase inhibitor physostigmine, and studied ACh content and synthesis in cerebral cortex slices obtained from these rats. We have also extended these studies to pentylenetetrazol and atropine, both of which were reported to increase the release of ACh from the brain (Mitchell, 1963).

Methods

Male Sprague-Dawley rats weighing between 300–350 g were used in all experiments. The following drugs were injected i.p. in the doses stated: urethane 1 g/kg, chloral hydrate 350 mg/kg, pentylenetetrazol 75 mg/kg, atropine sulphate 25 mg/kg, physostigmine sulphate 0·75 mg/kg. The doses of atropine and physostigmine refer to the free base. Control animals received 0·9% NaCl w/v (saline) 1 ml/kg intraperitoneally.

Animals treated either with urethane or chloral hydrate were decapitated 60 min after drug administration while still under anaesthesia. Atropine and physostigmine-treated animals were decapitated 30 min after drug administration. Pentylenetetrazol-treated animals were decapitated while in a state of convulsion which started within 5 min after the injection of the drug. The brains were immediately removed from the skulls and placed in ice-cold saline. Cerebral cortex slices, 0·3–0·4 mm thick, were prepared as described by McIlwain & Rodnight (1962).

The synthesis of ^{14}C-ACh from U-^{14}C-D-glucose was studied by the method of Browning & Schulman (1968). Portions of 200 ± 10 mg (wet weight) of slices were placed in 50 ml vessels containing 3 ml of the incubation medium (pH 7·4). Two forms of incubation medium were used. The composition of the first medium was as follows (mM): NaCl, 130; KCl, 4; $CaCl_2$, 2; $NaHCO_3$, 25; U-^{14}C-D-glucose, 5. This medium will be referred to as '4 mM K^+ medium'. The composition of the second medium was as follows (mM): NaCl, 103; KCl, 31; $CaCl_2$, 2; $NaHCO_3$, 25; U-^{14}C-D-glucose, 5. This medium will be referred to as '31 mM K^+ medium'. Both media contained 0·2 mM physostigmine sulphate. The medium was equilibrated with 95% O_2 and 5% CO_2 and the vessels were shaken at 37° C in a Dubnoff shaker for 30 minutes.

At the end of the incubation period, the incubation vessels were cooled to 0° C. The slices were separated from the medium by centrifugation at $1{,}000 \times g$ for 10 minutes. The ^{14}C-ACh content of slices and media was estimated as described by Browning & Schulman (1968). This involves homogenization of the tissue in fresh incubation medium (pH 4) and subsequent heating in a boiling water bath for 10 min after the addition of ACh carrier. The mixture of labelled and unlabelled esters was purified by ion exchange, after which ACh was isolated as the chloroaurate salt. Acetylcholine chloroaurate was plated on Pyrex planchets and its ^{14}C content measured.

The pre-incubation ACh content of brain cortex was assayed on the frog rectus abdominis muscle sensitized with 0·01 mM physostigmine sulphate. Extraction of ACh was performed as described by McIntosh & Perry (1950).

Cholinesterase activity (combined activity of acetylcholinesterase and cholinesterase) was measured manometrically as described by DuBois & Mangun (1947). This entails the use of a system containing 0·3 ml of 0·1 M acetylcholine (free base) and 50 mg of homogenized cortex slices and sufficient calcium-free Ringer–bicarbonate buffer to make a final volume of 3 ml. The results were expressed as μl of CO_2 released per 50 mg tissue during 10 min at 38° C.

Results

Figure 1A shows that prior to incubation, the ACh concentration in brain cortex slices from urethane-, chloral hydrate-, and physostigmine-treated rats was significantly greater than that of untreated animals. Figure 1B shows that only physostigmine treatment resulted in a marked inhibition of cholinesterase activity.

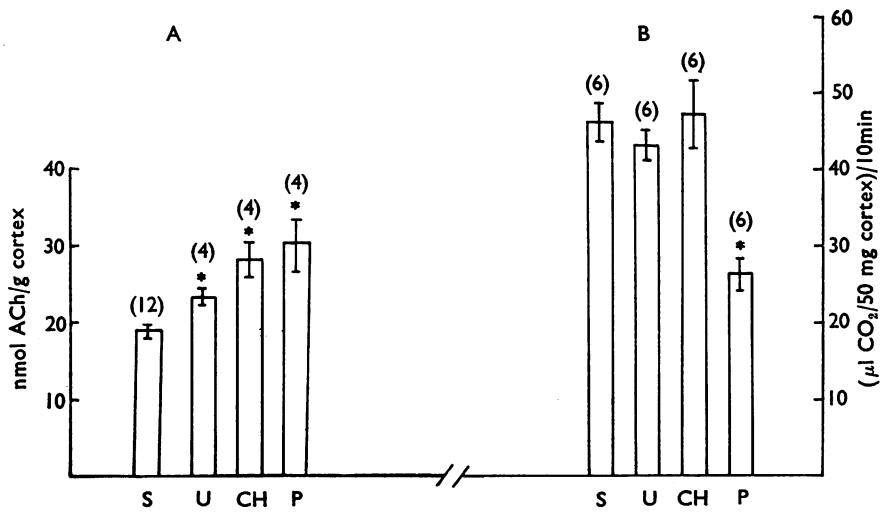

FIG. 1. Influence of the injection (i.p.) of urethane (1 g/kg), chloral hydrate (350 mg/kg) or physostigmine (0·75 mg/kg) on the acetylcholine (ACh) concentration (A) and activity of cholinesterase (combined activity of acetylcholinesterase and cholinesterase) (B) in rat cerebral cortex slices. (S) Control, (U) urethane, (CH) chloral hydrate, (P) physostigmine. Data represent means ± S.E.M., with number of samples in parentheses. Each sample came from an individual rat. * Significantly different from control ($P<0·05$).

Figure 2 shows that when slices from urethane- and chloral hydrate-treated rats were incubated in 4 mM K^+ medium, they formed less ^{14}C-ACh than slices from untreated animals. However, the amounts of ^{14}C-ACh found in the incubation medium were similar whether the slices came from treated or untreated animals. This was not the case when slices were incubated in an ACh-releasing medium (31 mM K^+ medium) where the amounts of ^{14}C-ACh found in slices from rats treated with urethane or chloral hydrate were about the same as those found in slices from untreated animals. The amounts of ^{14}C-ACh found in the 31 mM K^+ incubation medium

were also similar whether the slices came from treated or untreated animals but were greatly increased as compared to those found in the 4 mM K^+ medium. Finally, the figure shows that slices from physostigmine-treated rats formed similar amounts of ^{14}C-ACh as slices from untreated animals when incubated in 4 mM K^+ medium.

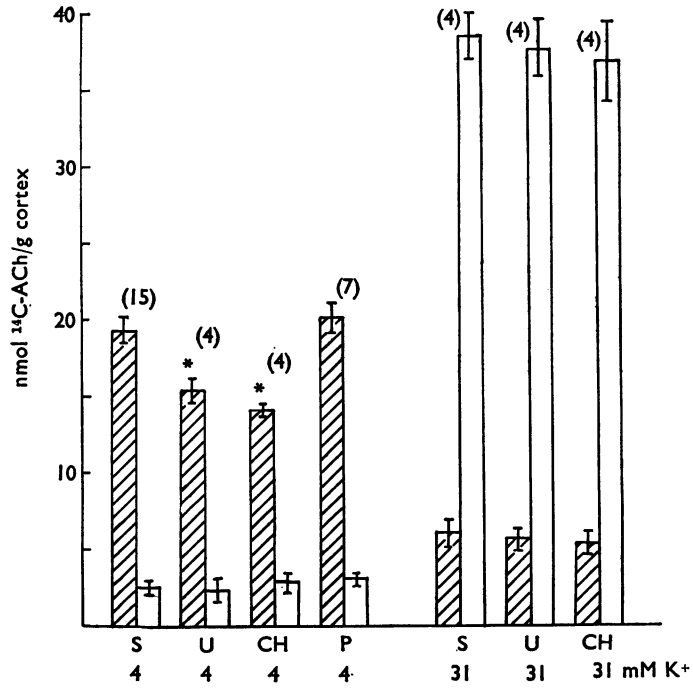

FIG. 2. Influence of the injection (i.p.) of urethane (lg/kg), chloral hydrate (350 mg/kg) or physostigmine (0·75 mg/kg) on ^{14}C-ACh formation by rat cerebral cortex slices. Portions of 200±10 mg (wet weight) of slices were incubated in 3 ml of 4 or 31 mM K^+ medium for 30 min at 37° C. (S) Control; (U) urethane; (CH) chloral hydrate; (P) physostigmine. Cross-hatched columns, ACh in slices; open columns, ACh in medium. Data represent means±S.E.M., with number of samples in parentheses. Each sample came from an individual rat. *Significantly different from control ($P<0·05$).

Cerebral cortex slices from rats treated with atropine or pentylenetetrazol formed about the same amounts of ^{14}C-ACh as did slices from untreated animals ($P>0·05$). This was the case whether slices were incubated in 4 or 31 mM K^+, as seen in Figure 3. The figure also shows that the increase in the amount of ^{14}C-ACh formed when the 31 mM K^+ medium was used was primarily due to ACh released into the incubation medium.

Discussion

Drug-induced increases in ACh concentration in brain could be consequent upon one or more of the following processes; (1) inhibition of acetylcholinesterase, (2) stimulation of ACh synthesis or (3) diminution of release of ACh from cholinergic neurones. The observed increase in ACh concentration in brain cortex of animals treated with urethane or chloral hydrate could not be due to inhibition of acetylcholinesterase since the enzyme activity was similar in drug-treated and control

animals. It seems rather unlikely that stimulation of synthesis could account for the observed increase in the ACh content in the brain of urethane- or chloral hydrate-treated animals since the administration of a similar drug, pentobarbitone, is associated with depression rather than stimulation of ACh synthesis in mice brain *in vivo* (Schuberth, *et al.*, 1969). Crossland (1953) has suggested that this increase in ACh concentration must be due to diminished release of the neurotransmitter from tissue containing it. There is direct evidence that anaesthetic agents do indeed reduce the release of ACh from nervous tissue (Mitchell, 1963; Beleslin & Polak, 1965; Celesia & Jasper, 1966). Reduced release of ACh could thus lead to an increase in its concentration within cholinergic neurones. Our recent finding that pentobarbitone administration was associated with a significant increase in the cytoplasmic ACh concentration in rat brain cortex (Vicas & Sharkawi, 1972) supports this idea. This increase in ACh concentration within neurones, by virtue of feedback inhibition, depresses the activity of choline acetyltransferase which is localized in the cell sap. The present observation that the increased pre-incubation ACh concentration in brain cortex slices of urethane- or chloral hydrate-treated animals was associated with a decrease in their ability to form ^{14}C-ACh could be due to feedback inhibition of choline acetyltransferase by the accumulated ACh. The fact that when an ACh releasing agent (31 mM K^+)

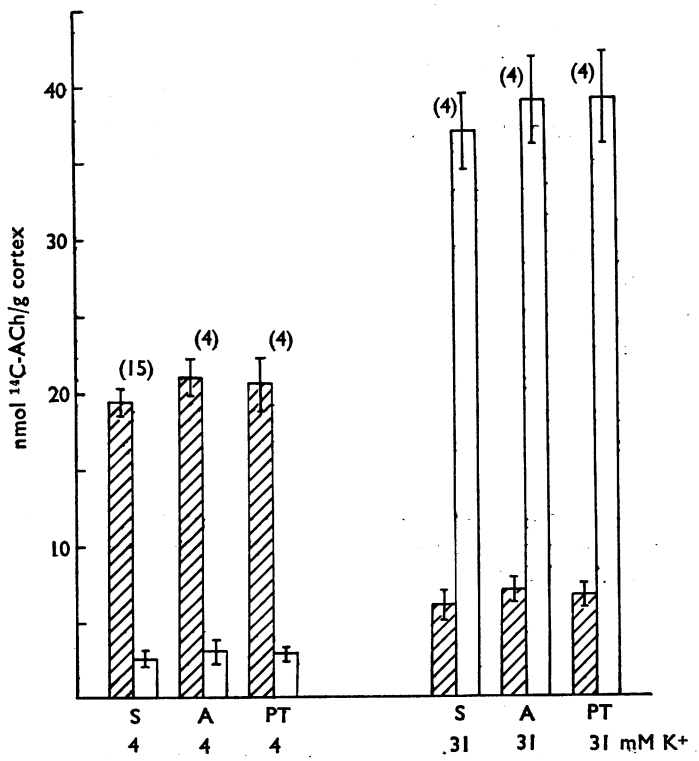

FIG. 3. Formation of ^{14}C-ACh by rat cerebral cortex slices from control, atropine-treated (25 mg/kg) or pentylenetetrazol-treated (75 mg/kg) animals. Portions of 200±10 mg (wet weight) of slices were incubated in 3 ml of 4 or 31 mM K^+ medium for 30 min at 37° C. (S) Control; (A) atropine; (PT) pentylenetetrazol. Cross-hatched columns, ACh in slices; open columns, ACh in medium. Data represent means±S.E.M., with number of samples in parentheses. Each sample came from an individual rat. Differences are not statistically significant ($P>0.05$).

was present in the incubation medium, slices from urethane- or chloral hydrate-treated animals formed similar amounts of ^{14}C-ACh as slices from control animals supports this view.

Inhibition of rat brain choline acetyltransferase by ACh has been demonstrated by Kaita & Goldberg (1969). They found that ACh, at a concentration of 10 mM, inhibited its own synthesis and that inhibition progressively increased as the concentration of ACh is increased. Choline acetyltransferase has been localized largely, if not entirely, in the cell sap (Fonnum, 1966). Thus, it would be expected that its activity is not depressed by increases in the extraneuronal ACh concentration produced by cholinesterase inhibitors. That this is the case is seen from our results with physostigmine.

Treatment of rats with atropine and pentylenetetrazol did not stimulate synthesis of ACh. Increased release of ACh from intact brains of several species has been ascribed to atropine (Mitchell, 1963 ; Celesia & Jasper, 1966). Theoretically, this should stimulate the process of synthesis of ACh as with K^+ which accelerates the release of ACh. However, the increase in brain ACh output associated with atropine administration is not necessarily due to increased release. Rather, it could represent that amount of ACh which would have normally interacted with muscarinic receptors, had they not been occupied by atropine. If this were the case, the lack of a stimulating effect by atropine on ACh synthesis is comprehensible. A similar mechanism has been invoked by Celesia & Jasper (1966) to explain the increase in ACh output from the cat brain after the administration of atropine. They related this increase in ACh output to blockade by atropine of binding mechanisms which are believed to account for a large part of the process of ACh inactivation independent of its hydrolysis by acetylcholinesterase. Such a mechanism could explain the observation that atropine, *in vitro*, increases the amount of ACh found in the incubation medium only when the release of the neurotransmitter from rat brain cortex slices is enhanced by high K^+ (Bertels, Meeuws & Polak, 1968) or by electrical stimulation (Bourdois, Mitchell & Szerb, 1971). However, it remains possible that the observed increase in ACh output from the intact brain by atropine administration is due to an active increase in the release of ACh from subcortical structures. This could explain the lack of an atropine effect on ACh synthesis by cortical tissue. This argument could also be invoked to explain the absence of a stimulating effect on ACh synthesis in cortical tissue by pentylenetetrazol administration.

The author wishes to thank Professor G. L. Plaa for his helpful comments on this manuscript. This work was supported by the Medical Research Council of Canada and the Magali Ducharme Foundation.

REFERENCES

Beleslin, D. & Polak, R. L. (1965). Depression by morphine and chloralose of acetylcholine release from the cat's brain. *J. Physiol., Lond.*, **177**, 411–419.

Bertels-Meeuws, M. M. & Polak, R. L. (1968). Influence of antimuscarinic substances on *in vitro* synthesis of acetylcholine by rat cerebral cortex. *Br. J. Pharmac. Chemother.*, **33**, 368–380.

Bourdois, P. S., Mitchell, J. F. & Szerb, J. C. (1971). Effect of atropine on acetylcholine release from cerebral cortical slices stimulated at different frequencies. *Br. J. Pharmac.*, **42**, 640–641*P*.

Browning, E. T. & Schulman, M. P. (1968). ^{14}C-acetylcholine synthesis by cortex slices of rat brain. *J. Neurochem.*, **15**, 1391–1405.

Celesia, G. G. & Jasper, J. J. (1966). Acetylcholine released from cerebral cortex in relation to state of activation. *Neurology*, **16**, 1053–1064.

Crossland, J. (1953). The significance of brain acetylcholine. *J. ment. Sci.*, **99**, 247–251.

Crossland, J. & Merrick, A. J. (1954). The effect of anaesthesia on the acetylcholine content of the brain. *J. Physiol., Lond.*, **125**, 56–66.

DuBois, K. P. & Mangun, G. H. (1947). Effect of hexaethyltetraphosphate on cholinesterase *in vitro* and *in vivo*. *Proc. Soc. exp. biol. Med.*, **64**, 137–139.

Fonnum, F. (1966). Is choline acetyltransferase present in synaptic vesicles? *Biochem. Pharmac.*, **15**, 1641–1643.

Giarman, N. J. & Pepeu, G. (1962). Drug-induced changes in brain acetylcholine. *Br. J. Pharmac. Chemother.*, **19**, 226–234.

Kaita, A. A. & Goldberg, A. M. (1969). Control of acetylcholine synthesis—the inhibition of choline acetyltransferase by acetylcholine. *J. Neurochem.*, **16**, 1185–1191.

Kurokawa, M., Machiyama, Y. & Kato, M. (1963). Distribution of acetylcholine in the brain during various states of activity. *J. Neurochem.*, **10**, 341–348.

McIlwain, H. & Rodnight, R. (1962). *Practical Neurochemistry*, 1st ed., p. 112. Boston: Little Brown and Company.

McIntosh, F. C. & Perry, W. L. M. (1950). Biological estimation of acetylcholine. In: *Methods in Medical Research*, ed. Gerard, R. W., vol. 3, pp. 78–92. Chicago: The Year Book Publ. Inc.

Mitchell, J. F. (1963). The spontaneous and evoked release of acetylcholine from the cerebral cortex. *J. Physiol., Lond.*, **165**, 98–116.

Richter, D. & Crossland, J. (1949). Variations in acetylcholine content of the brain with physiological state. *Am. J. Physiol.*, **159**, 247–255.

Schuberth, J., Sparf, B. & Sundwall, A. (1969). A technique for the study of acetylcholine turnover in mouse brain *in vivo*. *J. Neurochem.*, **16**, 695–700.

Sharkawi, M. & Schulman, M. P. (1969). Relationship between acetylcholine synthesis and its concentration in rat cerebral cortex. *Br. J. Pharmac.*, **36**, 373–379.

Tobias, J. M., Lipton, M. A. & Lepinat, A. (1946). Effect of anaesthetics and convulsants on brain acetylcholine content. *Proc. Soc. exp. biol. Med.*, **61**, 51–54.

Vicas, I. M. & Sharkawi, M. (1972). Synthesis of ^{14}C-acetylcholine by rat cerebral cortex. *Proc. Canad. Fed. biol. Soc.*, **15**, in press.

(*Received February 7, 1972*)