Br. J. Pharmac. (1971), **41**, 600–606.

Stimulating action of atropine on the release of acetylcholine by rat cerebral cortex *in vitro*

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Summary

1. In cortical slices from rat brain incubated in a medium containing the irreversible cholinesterase inhibitor, soman (0·005 mM) and a high concentration of KCl (25 mM), atropine exerts a stimulating action on the release of acetylcholine (ACh).

2. Two possible explanations for this action were examined. Atropine might expel ACh from the nerve endings by occupying its storage sites or it might prevent an inhibitory action of the released ACh on its further release by occupying muscarinic receptors at the presynaptic endings ; its action would then be a kind of 'disinhibition.'

3. The stimulating action of atropine on ACh release persisted during prolonged incubation (up to 3·5 h) provided choline was added to the medium. This finding would be difficult to explain if atropine acted by expelling ACh from its storage sites.

4. The stimulating action of atropine on ACh release was inhibited by oxotremorine and by methacholine, added to the medium in high concentrations. This finding is readily explained if atropine acts by 'disinhibition.'

Introduction

Atropine increases the release of acetylcholine (ACh) from incubated tissue slices of rat cerebral cortex. As the increased release is not secondary to an increased synthesis of ACh, the atropine must affect the release itself (Molenaar & Polak, 1970). In this paper two possible explanations for this action of atropine are examined.

Atropine might act by replacing ACh at the storage sites of the nerve endings, in which case the release should come to an end as soon as these sites are occupied by atropine. This possibility was examined by studying the release during prolonged incubation of the tissue slices. The other possibility, suggested earlier (Polak, 1967) is that the released ACh inhibits further release of ACh and this inhibition is antagonized by atropine. Its action would therefore be a kind of 'disinhibition.' Thus the addition of sufficient ACh to the incubation medium should inhibit the action of atropine. This is difficult to demonstrate as the added ACh interferes with the assay of the released ACh on the physostigmine treated leech preparation used for this purpose. The difficulty was overcome by using, instead of ACh, oxotremorine or acetyl-β-methylcholine (methacholine), two muscarinic agonists which do not cause the leech muscle to contract.

Methods

Two strains of female albino rats weighing 160–190 g were used, 'Glaxo' rats being used for the experiments on prolonged incubation and 'Brofo' rats for the experiments with oxotremorine and methacholine. The two strains were the same as in previous experiments done in this laboratory (Molenaar & Polak, 1970).

The methods for preparing, preincubating and incubating the cortical slices from the brain were the same as described previously (Molenaar & Polak, 1970). Incubation was in a medium containing a high (25 mM) concentration of KCl and the cholinesterase inhibitor soman (0·005 mM). Incubation was preceded by a pre-incubation period of 60 min in a medium which differed from the incubation medium in having a lower (4·7 mM) concentration of KCl and in not containing the drugs to be investigated.

For the prolonged incubation experiments in which the incubation medium was withdrawn and replaced every half hour the Erlenmeyer flasks containing the slices with the incubation medium had an opening at the bottom which was closed by a polyethylene stopper through which a hollow needle had been passed. The outer protruding end of the needle was connected by polyethylene tubing to a syringe so as to allow withdrawal and replacement of the medium without interrupting the incubation. A special device was needed to prevent plugging of the needle by pieces of tissue. A small cylindrical cage consisting of a frame of stainless steel covered with nylon gauze was placed vertically above the opening of the needle and fixed to the stopper.

The tissue slices were extracted according to the method of Elliott, Swank & Henderson (1950) and the ACh of the extracts and the incubation media was assayed against ACh perchlorate on the dorsal leech muscle pretreated with physostigmine. The details of the procedure were as described previously (Molenaar & Polak, 1970) but the ACh values are expressed in nmol/g of wet tissue.

Drugs

These were: atropine sulphate (Brocades); choline chloride (B.D.H.); methacholine chloride (Brocades); physostigmine sulphate (NBCo); soman synthesized by the Chemical Laboratory RVO-TNO; oxotremorine, the free base, kindly provided by Dr. B. C. Barrass from the Chemical Defence Establishment, Porton Down.

The concentrations of the sulphates of atropine and physostigmine are expressed as molar concentrations of the alkaloid moieties.

Results

Effect of atropine during prolonged incubation

The results of an experiment in which incubation with and without atropine was extended to 3·5 h are shown in Fig. 1. On incubation without atropine the amounts of ACh released in the successive half hour samples did not differ much though there was a slight decrease from $16 \pm 0{\cdot}3$ nmol/g in the first, to $14 \pm 0{\cdot}7$ nmol/g in the seventh half hour period. On incubation with atropine, however, there was a definite and progressive decrease from $44 \pm 2{\cdot}4$ nmol/g in the first to $23 \pm 1{\cdot}2$ nmol/g in the seventh half hour sample, and if only the stimulating effect of atropine (that

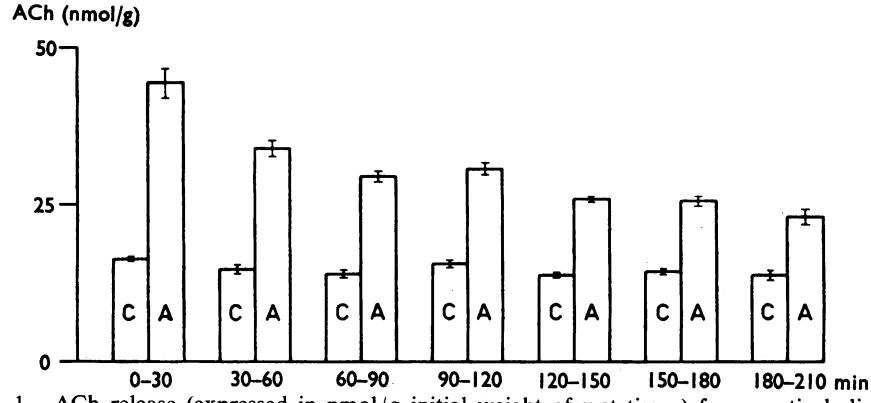

FIG. 1. ACh release (expressed in nmol/g initial weight of wet tissue) from cortical slices in seven successive 30 min periods obtained from eight portions of slices incubated simultaneously in separate vessels, four without (C) and four with (A) atropine (3×10^{-6}M). Each column represents the mean value from four vessels and shows the standard error of the mean.

TABLE 1. *The change in the increase in ACh release (nmol/g) produced by atropine in seven successive 30 min samples on incubation without (a) and with (b) the addition of choline to the incubation medium*

min	a	b
0–30	28	27
30–60	19	42
60–90	15	51
90–120	15	61
120–150	12	61
150–180	11	58
180–210	9	51

The figures are the differences between the release in the presence and the absence of atropine. (Same experiments as those of Figs. 1 and 2.)

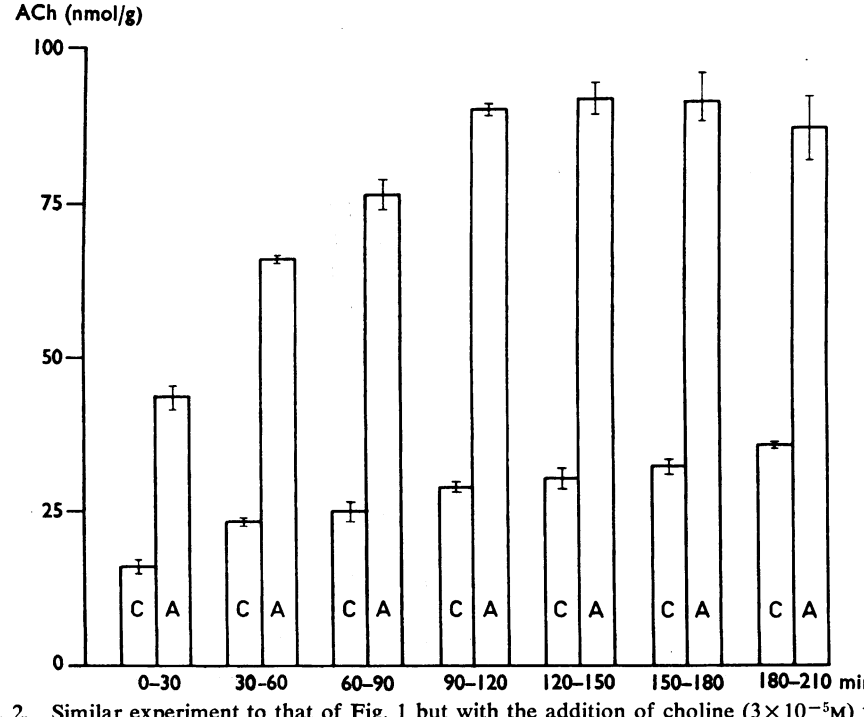

FIG. 2. Similar experiment to that of Fig. 1 but with the addition of choline (3×10^{-5}M) to the incubation medium in each vessel.

is, the increase in the release) is taken into account this increase became progressively smaller as seen from the results shown in Table 1. The increase amounted to 28 nmol/g during the first 30 min period but to only 9 nmol/g during the seventh 30 min period.

This progressive reduction of the stimulating action of atropine on ACh release could be attributed to choline lack since it did not occur when extra choline was added to the incubation medium. This is illustrated in Fig. 2. Prolonged incubation in the presence of 3×10^{-5}M choline actually led to an increase in ACh release both in the presence and in the absence of atropine. If only the increase produced by atropine is taken into account it became greater with time. As shown in Table 1 it amounted to 27 nmol/g in the first 30 min period and rose to 61 nmol/g in the fifth 30 min period and then fell slightly to 51 nmol/g in the seventh 30 min period, which is still nearly twice as great as that during the first 30 minutes.

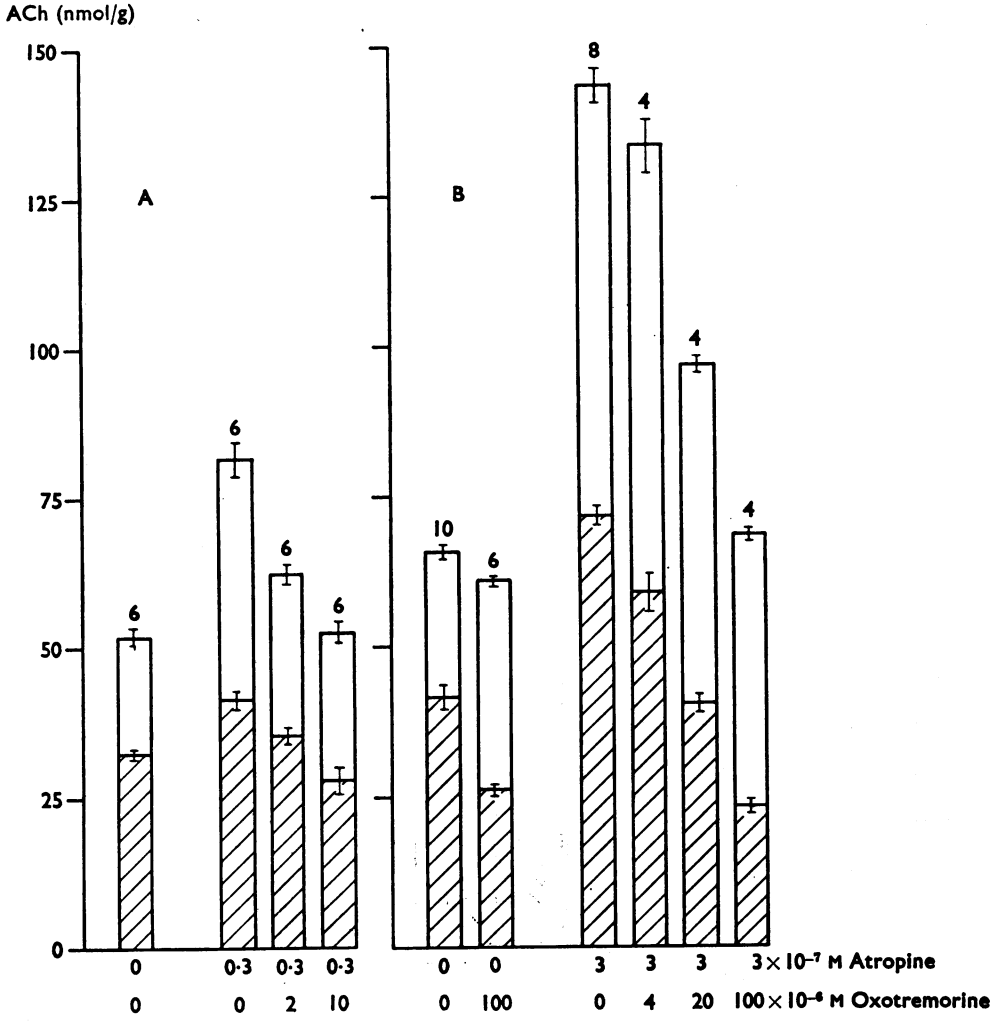

FIG. 3. ACh content (expressed as nmol/g initial weight of wet tissue) of cortical slices and incubation media after 1 h incubation with and without atropine and oxotremorine. (■), ACh extracted from the tissue slices; (□), ACh in the incubation medium. The figures on top of the columns give the number of observations from which the mean values were obtained.

Effect of oxotremorine and methacholine

Two effects were observed with oxotremorine. On incubation of the cortical slices without atropine the amount of ACh extracted from the slices was reduced and the amount in the incubation medium increased, but the sum of both was not significantly changed by the oxotremorine. On incubation in the presence of atropine its stimulating effect on the release was inhibited.

The effect of oxotremorine on the ACh content of the tissue in the absence of atropine is seen from a comparison of the first two columns of Fig. 3B. A similar effect was previously observed when physostigmine was added to the incubation medium containing soman, although physostigmine did not prevent the stimulating action of atropine (Bertels-Meeuws & Polak, 1968).

The antagonistic effect of oxotremorine on the increased ACh release produced by atropine is shown for two atropine concentrations in Fig. 3A and B. The results of these experiments suggest that the antagonism may be of a competitive nature because the effect of $0{\cdot}3 \times 10^{-7}$M atropine was abolished by 10×10^{-6}M oxotremorine (Fig. 3A), whereas the stronger effect of 3×10^{-7}M atropine was only reduced by 20×10^{-6}M but abolished by 100×10^{-6}M oxotremorine (Fig. 3B).

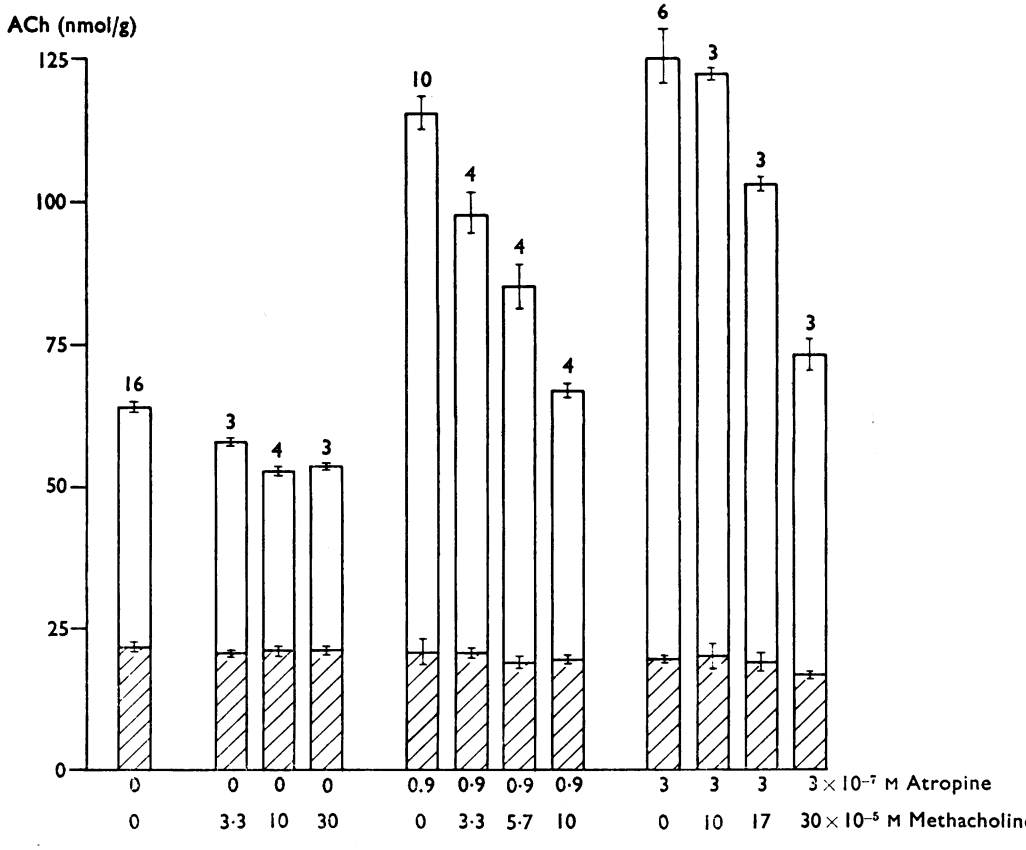

FIG. 4. ACh content (expressed as nmol/g initial weight of wet tissue) of cortical slices and incubation media after 1 h incubation with and without atropine and methacholine. Physostigmine (10^{-4}M) present in incubation medium. Other details same as Fig. 3.

With methacholine the same two effects as with oxotremorine were obtained. On incubation for 1 h without atropine, 10^{-4}M methacholine reduced the amount of ACh extracted from the tissue slices and increased the ACh in the medium by nearly the same amount.

In order to study the antagonism between methacholine and atropine without interference from the reduction in ACh content of the slices and the corresponding increase in the ACh content of the medium, the experiments were carried out in the presence of physostigmine (in addition to soman). In this case the changes were produced by the physostigmine and therefore occurred in the controls, also, that is, in the absence of methacholine (Fig. 4). As seen from the first four columns the methacholine had little effect in the absence of atropine since physostigmine was present in the media ; there was no significant reduction in the ACh content of the tissue slices but the release was somewhat inhibited. On the other hand the stimulating effect of atropine on the release of ACh was greatly inhibited. Again this effect seemed to be of a competitive nature, since, as seen from the last eight columns, 3·3, 5·7 and $10{\cdot}0 \times 10^{-5}$M methacholine inhibited and nearly abolished the stimulating effect of $0{\cdot}9 \times 10^{-7}$M atropine, whereas higher concentrations of methacholine (17·0 and $30{\cdot}0 \times 10^{-5}$M) were needed to antagonize 3×10^{-7}M atropine.

Discussion

Two possible explanations for the stimulating action of atropine on the release of ACh from incubated tissue slices were considered. Atropine was thought to act either by replacing ACh at the storage sites of the nerve endings, or by a kind of disinhibition, that is, by inhibiting an inhibitory effect of the released ACh on further release.

The results obtained on prolonged incubation would be difficult to explain on the assumption that atropine expels and replaces the ACh at its storage sites, because in that case, the effect of atropine should come to an end after a certain incubation period when the sites have become occupied by the atropine. A reduction in the amount of ACh released by the atropine was obtained on prolonged incubation but it was fully accounted for by lack of choline and no longer occurred when choline was added to the incubation medium. In the presence of choline, the amounts of ACh released by the atropine actually increased during the first 2·5 h of incubation and then decreased, but only slightly, so that even after 3·5 h they were still nearly twice those released during the first half hour. Choline not only increased the stimulating effect of atropine on the release, but also augmented the release which occurred without atropine. This suggests that during prolonged incubation of cortical slices the choline supplied by them is not sufficient to bring about optimal conditions for synthesis and release ; and the effect of this deficiency would naturally become even more pronounced when the release is stimulated by atropine.

On the other hand the assumption that the action of atropine can be looked upon as a kind of ' disinhibition ', counteracting an inhibitory effect of released ACh on the further release is supported by the results obtained with oxotremorine and methacholine. The stimulating effect of atropine on the release and synthesis of ACh is linked to the antimuscarinic property (Bertels-Meeuws & Polak, 1968). If atropine enhances ACh release from the nerve endings, it is to be expected that endogenous ACh has the opposite action. This implies the existence of a negative

feed back mechanism in which released ACh, by acting on presynaptic muscarinic receptors, inhibits the release of more ACh from the nerve endings (Polak, 1967). Due to technical limitations the effect of sufficiently high concentrations of added ACh could not be tested, but the atropine effect was antagonized by oxotremorine and methacholine, two strong muscarinic agents which do not interfere with the bio-assay of ACh on the leech muscle. The effective concentrations of oxotremorine and metacholine, however, were very high in comparison to the low concentrations in which atropine enhanced the release and synthesis of ACh, but this may merely imply a very high concentration of the released ACh at the presynaptic membrane.

Both oxotremorine and methacholine not only antagonized the action of atropine but in addition caused, in the absence of atropine from the incubation medium, a decrease in the ACh content of the cortical slices with a nearly corresponding increase in release. A similar effect was previously obtained with physostigmine which does not prevent the stimulating action of atropine on the release (Bertels-Meeuws & Polak, 1968). Since all three drugs inhibit ACh uptake by cortical slices, as shown for physostigmine by Polak & Meeuws (1966) and later by Polak (1969), for oxotremorine by Schuberth & Sundwall (1967), and for methacholine by Liang & Quastel (1969), this effect is readily explained by inhibition of reuptake of released ACh, and it is not linked to the atropine antagonism because the latter is not obtained with physostigmine.

I wish to thank Mrs. Anne Thiessen for her excellent technical assistance, Dr. M. Wijnans for the statistical calculations, Professor E. M. Cohen for stimulating discussions and Dr. B. C. Barrass for the oxotremorine.

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(*Received September* 21, 1970)