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Stimulation by atropine of acetylcholine release and synthesis in cortical slices from rat brain

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Summary

1. Cortical slices from rat brain were incubated in media containing the irreversible cholinesterase inhibitor soman and a high KCl concentration, and the release and synthesis of acetylcholine (ACh) were determined.

2. Atropine enhanced the release and synthesis of ACh.

3. Tetrodotoxin, a substance which blocks nervous conduction, did not influence the release and synthesis of ACh, in the absence or in the presence of atropine. Therefore the nerve endings are probably the site at which atropine acts when stimulating the release and synthesis of ACh.

4. Pretreatment of the slices with botulinum type A toxin partially blocked the release and synthesis of ACh and reduced the extra amounts of ACh released and synthesized under the influence of atropine.

5. Lowering the calcium or raising the magnesium concentration in the incubation medium reduced the release and synthesis of ACh and their enhancement by atropine.

6. Physostigmine decreased the total extractable ACh content of the slices during incubation in a 25 mM KCl containing medium. This decrease was nearly prevented when the release and synthesis of ACh were inhibited by omission of the calcium ions from the medium, but was enhanced by atropine.

7. The observations made with pretreatment by botulinum type A toxin, with changes in the calcium and magnesium concentration as well as with physostigmine, all support the theory that it is primarily the release of ACh which is enhanced by atropine and that its stimulating action on the synthesis results from the increased release.

Introduction

Atropine and hyoscine were shown to enhance the release of acetylcholine (ACh) from the surface of the exposed cerebral cortex of the cat, rabbit, and sheep, and from the superfused caudate nucleus of the cat when hydrolysis of ACh was prevented by a cholinesterase inhibitor (Mitchell, 1963 ; Szerb, 1964 ; Polak, 1965). A similar effect was observed in cortical slices from rat brain incubated with a cholinesterase inhibitor provided the incubation medium contained a high concentration of potassium which in itself caused a great enhancement of the release and synthesis of ACh. This was demonstrated as early as 1939 by Mann, Tennenbaum

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& Quastel. Atropine or other antimuscarinic agents added to such a medium in low concentration further greatly increased the release and synthesis of ACh (Polak & Meeuws, 1966; Bertels-Meeuws & Polak, 1968).

The experiments described in the present paper were carried out to obtain information about the mechanism of the atropine effect. For this purpose, the action of tetrodotoxin (TTX), botulinum type A toxin, physostigmine, and of changes in the calcium and magnesium concentration of the medium were examined on the synthesis and release of ACh in incubated cortical slices from rat brain. Some of the results have been communicated elsewhere (Polak, 1970).

Methods

Female albino rats (160–190 g) from two different strains, Brofo and Glaxo, were lightly anaesthetized with ether and decapitated. The brains were immediately removed from the skull. From each hemisphere one cortical slice thinner than 0·4 mm was cut at room temperature with a recessed glass guide and weighed on a torsion balance after removal of excess adherent fluid. The cortical slices were collected and stored for 20–60 min in an oxygenated phosphate buffered (pH 7·4) medium of the following composition (mM): NaCl 118·5; KCl 4·7; $CaCl_2$ 0·8; $MgSO_4$ 1·2; Na_2HPO_4 9·2; glucose 10; at room temperature (20°–25° C). The method is that described by McIlwain & Rodnight (1962).

A pre-incubation of 60 min preceded an incubation period of the same duration except when stated otherwise. In most experiments eight 25 ml vessels, each containing 56–201 mg of tissue and 2·5 ml of the pre-incubation medium, were simultaneously set up. After pre-incubation, the cortical slices were rinsed once with 2·5 ml of the medium used for incubation and then incubated in 2·5 ml.

The composition of the pre-incubation medium was (mM): NaCl 118·5; $NaHCO_3$ 24·9; KCl 4·7; $CaCl_2$ 2·5; $MgSO_4$ 1·2; KH_2PO_4 1·2; glucose 10; and the cholinesterase inhibitor soman 0·005. The composition of the incubation medium differed with regard to the KCl, $CaCl_2$ and $MgSO_4$ concentration. That of KCl was always 25 mM and that of the other two salts varied in different experiments as described in the text. No osmotic compensations were made for these changes. The incubation medium contained in addition the drugs whose effects were to be examined. Pre-incubation and incubation were carried out at 37° C under continuous shaking in an atmosphere of 95% oxygen and 5% carbon dioxide.

After incubation the fluid in which the slices had been incubated was collected in calibrated tubes, the slices were washed three times with 1·0 ml of fluid of the same composition as that of the pre-incubation medium, and the washings were added to the tubes. In order to determine the "total extractable" ACh content the slices were extracted by homogenization in an HCl-acidified pre-incubation medium according to the method of Elliott, Swank & Henderson (1950). In some experiments the cortical slices of one or two of the vessels were extracted immediately after pre-incubation to determine the ACh content of the tissue at zero time.

Extracts and media were frozen after adjustment of the volumes to 10 ml and of the pH to 4 and then stored at −12° C until assayed for ACh.

The ACh-like activity of the extracts and media was assayed against an ACh perchlorate standard solution on the dorsal leech muscle treated with physostigmine.

The values are expressed in terms of μg of the chloride per gram of tissue (initial wet weight). Suitable dilutions of alkali treated (pH near 14 for 120 min) and subsequently reneutralized extracts or of the media used for incubation were added to the standard solutions in order to correct as far as possible for substances other than ACh, which might influence the sensitivity of the assay preparation.

Experiments with botulinum type A toxin. To examine the effect of the toxin the procedure was slightly modified. For each experiment, thirty-two slices derived from sixteen rats (Glaxo) were trimmed so that the initial wet weight of each slice was between 30 and 35 mg. The slices were randomly distributed over two 100 ml Erlenmeyer vessels so that each vessel received sixteen slices. The vessels contained 10 ml of the pre-incubation medium with soman 0·005 mM. To one of them botulinum type A toxin was added, and to the other only the solvent of the toxin. Pre-incubation was carried out for 2 or 4 h under continuous shaking in an atmosphere of 95% oxygen and 5% carbon dioxide at 37° C. The following precautions against the toxin were taken during and after pre-incubation. The flow of humid 95% oxygen–5% carbon dioxide which was led through the vessels passed first a trap filled with cotton wool and then a trap filled with a solution of $Ca(OCl)_2$ (8%) before reaching the air of the fume hood in which pre-incubation was carried out. After pre-incubation the slices were rinsed five times with the phosphate buffered medium used for the preparation of the slices before they were randomly distributed over eight 25 ml vessels containing four slices per vessel. Subsequently they were rinsed once with the medium used for incubation and incubated in 2·5 ml for one hour. During the subsequent extraction the acidified tissue homogenates, instead of being stored at room temperature, were heated to between 90° and 100° C for 5 min so as to inactivate any botulinum toxin which might have been present. For the same reason the incubation media were similarly heated during 5 min after acidification to pH 4. In two experiments intraperitoneal injections into mice of either 0·5 ml pooled incubate or 0·5 ml pooled extract were found not to be lethal. For calculating the ACh/g of tissue the mean initial wet weight of the slices was used.

Drugs used. Atropine sulphate (Brocades), acetylcholine perchlorate (BDH), physostigmine sulphate (NBCo), soman (3,3-dimethyl 2-butyl methylphosphonofluoridate). The soman was synthesized by the Chemical Laboratory RVO-TNO, Rijswijk Z.H., The Netherlands. Crude botulinum type A toxin was kindly supplied to us by Dr. J. Keppie from the Microbiological Research Establishment, Porton Down, England. A stock solution was prepared in a medium containing 100 mM NaCl, 75 mM Na-acetate and 0·2% gelatin (pH 6·2) and stored at +4° C. The solution was not homogeneous. Dilutions were freshly made for each experiment. To obtain an indication of the potency of the toxin it was injected intraperitoneally into mice to determine the 96 h LD50 according to the method of Schantz (1964). The LD50 was found to be about 3 ng. Tetrodotoxin (Sankyo) was tested for its potency on several indirectly stimulated phrenic nerve-diaphragm preparations from rats. It produced total paralysis within 12 min in a concentration of 0·1 μg/ml and within 3 min in a concentration of 0·3 μg/ml.

Statistical evaluation. The statistical significance at the 5% level was established by Welch's *t* test. When the number of observations was smaller than six, Wilcoxon's rank sum test was used. For quotients and differences between results obtained in the presence and in the absence of atropine each observation in the presence of

atropine was paired at random with one observation in its absence, and the means ± S.E.M. of these quotients and differences were calculated. Bonferoni's *t* statistics were used for all multiple comparisons (Miller, 1966).

Results

Under comparable conditions, the amounts of ACh released and synthesized by slices from Glaxo rats were often greater than those from Brofo rats, whereas the relative magnitude of the stimulating effect of atropine on the release and synthesis of ACh was somewhat smaller. In previous experiments from this laboratory cortical slices from Brofo rats were used; however, all the effects described were also observed with Glaxo rats. The results illustrated in Table 1 and Fig. 3 were obtained from Glaxo, those in the other figures from Brofo rats.

Tetrodotoxin

According to Dudar and Szerb (1969), tetrodotoxin (TTX) reduces the release of ACh from the exposed cerebral cortex of the anaesthetized cat and abolishes the stimulating action of atropine on this release. When the toxin was examined on the cortical slices from rat brain it was found not to have these effects. This is illustrated in Fig. 1.

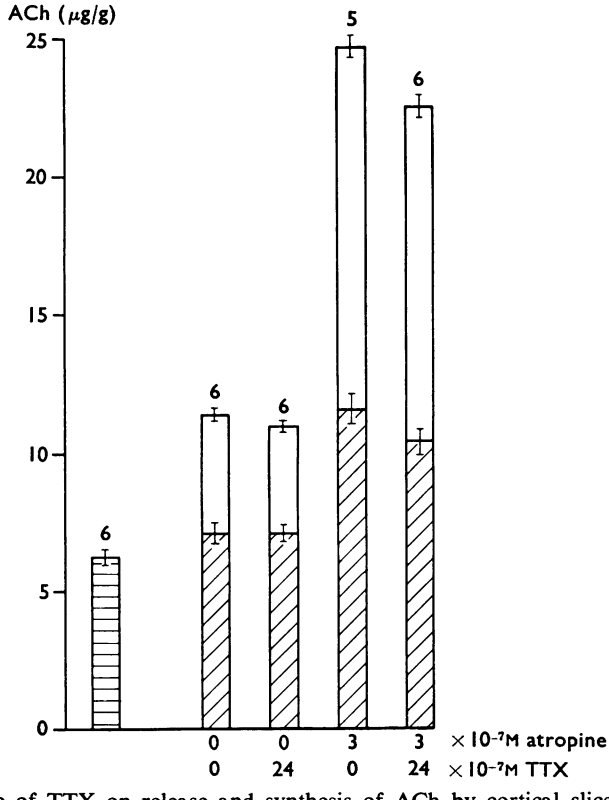

FIG. 1. Influence of TTX on release and synthesis of ACh by cortical slices from rat brain. The incubation period was 60 min, the medium contained 25 mM KCl and 0·005 mM soman. The concentrations of atropine and TTX are given below the columns. ≡, Extractable ACh content of the slices before incubation; ▨, extractable ACh content of the slices after incubation; □, ACh released during incubation. The released ACh ± S.E.M. is presented on top of and is separate from the extractable ACh ± S.E.M. after incubation. The numbers of experiments are indicated by the figures above the columns.

The first column shows the ACh content of cortical slices before incubation, and the other columns give the ACh in the slices and incubation media after 1 h incubation. The results shown in the second and third column were obtained without atropine. Approximately 5 μg/g ACh was synthesized during the 1 h incubation whether TTX had been added to the medium (third column) or not (second column). Thus TTX appears to have no effect on synthesis and release of ACh in incubated cortical slices from rat brain. The results represented by the fourth and fifth column were obtained in the presence of atropine ; they show that synthesis and release of ACh were greatly enhanced but that this effect of atropine was not inhibited by TTX. Thus TTX does not abolish the effect of atropine on synthesis and release of ACh in incubated cortical slices from rat brain.

The concentration of TTX used in the experiment of Fig. 1 was 0·8 μg/ml. Since the concentration of TTX topically applied by Dudar and Szerb to the surface of the cerebral cortex of the cat was much higher—10 μg/ml—this concentration was used for an experiment similar to that of Fig. 1. Again synthesis and release of ACh were not affected by the toxin whether atropine was present in the incubation medium or not.

Botulinum type A toxin

Pre-incubation of cortical slices for 2 h in a medium containing 20,000 mouse LD50/ml of botulinum type A toxin resulted in a depression of synthesis and release of ACh during incubation. The depression was not enhanced by prolongation of the pre-incubation to 4 h or by raising the botulinum toxin concentration to 250,000 mouse LD50/ml, but reducing the toxin concentration to 3,000 mouse LD50/ml led to a significantly smaller depression. Statistical analysis demonstrated that it was permissible to pool the results obtained with 20,000 LD50/ml (pre-incubation 2 or 4 h) and 250,000 LD50/ml (pre-incubation 2 h) since they were not significantly different. Table 1 gives the pooled results obtained under these conditions.

The upper half of Table 1 summarizes results obtained without botulinum toxin and shows that atropine increased the ACh in the incubation medium as well as in the cortical slices. The total yield of ACh from both sources was increased from 15·9 to 25·5 μg/g, emphasizing the strong stimulating effect of atropine both on release and synthesis. The lower half of Table 1 shows that after pre-incubation with botulinum toxin the amounts of ACh released into the incubation medium were greatly reduced. In the absence of atropine 3·1 instead of 6·1 μg/g and in its presence 4·8 instead of 12·5 μg/g were released into the medium during the 1 h incubation. So the release of ACh was greatly depressed by the pretreatment with toxin. Pretreatment with the toxin had no significant effect on the ACh content of

TABLE 1. *Influence of pretreatment with botulinum type A toxin on the release and synthesis of ACh by cortical slices incubated for* 1 *h in a* 25 *mM KCl containing medium with or without* 3×10^{-7}M *atropine (mean values* ± S.E.M.)

		No. of observations	ACh in μg/g: In incubation medium	In slices	In medium + slices
No botulinum toxin	No atropine	8	6·1±0·23	9·8±0·50	15·9±0·57
	Atropine	8	12·5±0·42	13·0±0·69	25·5±0·72
Botulinum toxin	No atropine	7	3·1±0·17	9·2±0·43	12·3±0·59
	Atropine	7	4·8±0·25	9·4±0·52	14·2±0·71

the cortical slices when the subsequent incubation was without atropine; with atropine, the increase in the ACh content no longer occurred. After incubation with atropine the ACh content was only 9·4 μg/g as compared with 9·2 μg/g, whereas the corresponding values for the untreated slices were 13·0 and 9·8 μg/g. From the total yield obtained from slices plus media, particularly when incubation was with atropine, it is evident that the toxin had a depressant effect not only on release but also on synthesis of ACh.

Ca ions

The $CaCl_2$ concentration of the pre-incubation medium was 0·8 mM instead of the usual 2·5 mM and that of the incubation medium was either 0, 0·8, 2·5 or 5·5 mM. The results obtained under these conditions are summarized in Fig. 2. They show that synthesis and release of ACh were greatest when the incubation medium contained 2·5 mM $CaCl_2$, and lowering its concentration to 0·8 mM

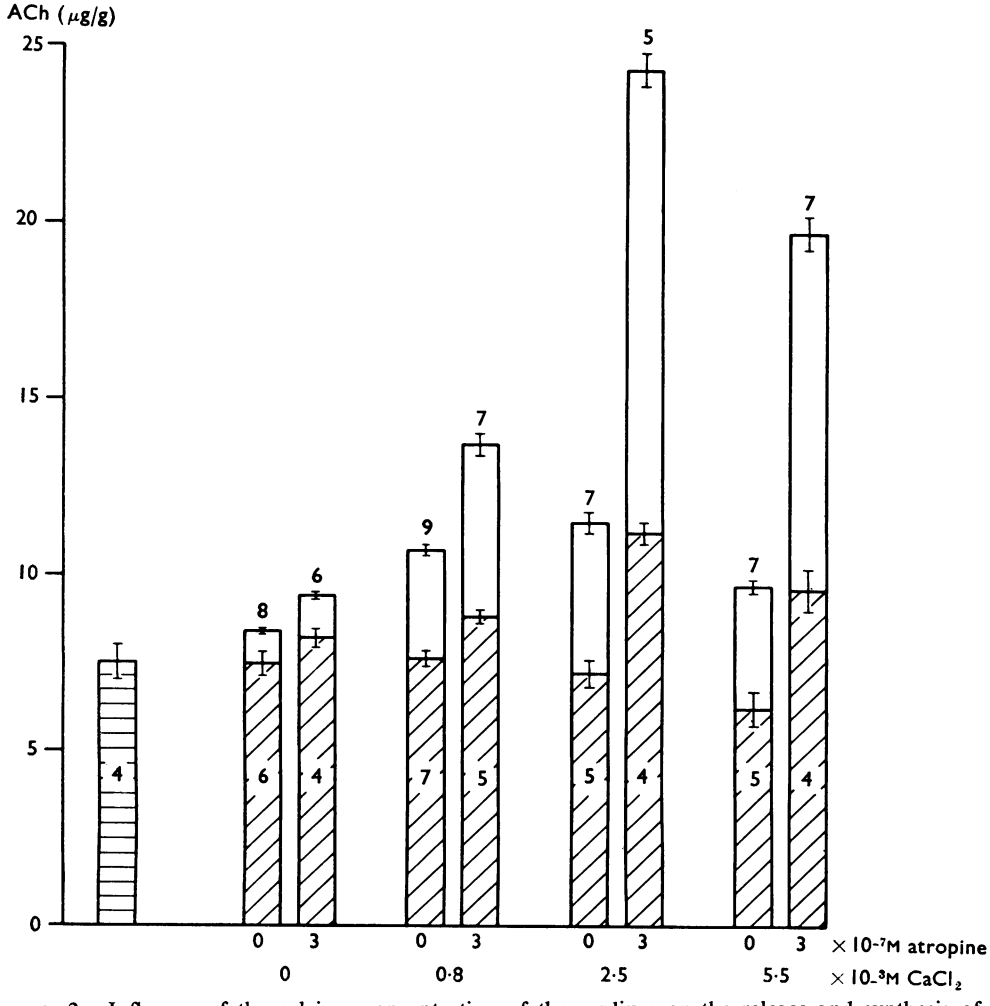

FIG. 2. Influence of the calcium concentration of the medium on the release and synthesis of ACh by cortical slices. The results are presented in the same way as in Fig. 1. The numbers of experiments for the released ACh are indicated by the figures above the columns and for the ACh content of the slices by the figures inside the columns. The concentrations of atropine and $CaCl_2$ are given below the columns.

resulted in a decrease of synthesis and release; there was a further decrease when the incubation medium contained no calcium. The effect was more pronounced in the presence than in the absence of atropine. Potassium and also calcium are therefore required to bring out the full stimulating effect of atropine. Raising the calcium concentration from 2·5 to 5·5 mM also decreased the synthesis and release of ACh but the stimulating action of atropine was little affected. At a 2·5 mM $CaCl_2$ concentration atropine increased the total yield of ACh from 11·5 to 24·3 μg/g (211%) and at a 5·5 mM concentration from 9·7 to 19·7 μg/g (203%). If the release into the incubation medium alone is taken into account it will be seen that atropine multiplied the release differently at different calcium concentrations. In media containing 0, 0·8, 2·5 and 5·5 mM $CaCl_2$ the multiplication factors were $1·3 \pm 0·10$ (six observations), $1·8 \pm 0·12$ (seven observations), $3·2 \pm 0·18$ (five observations) and $3·0 \pm 0·16$ (seven observations), respectively. All multiplication factors, except those obtained in 2·5 and 5·5 mM $CaCl_2$, were significantly different from one another ($P_2 < 0·05$).

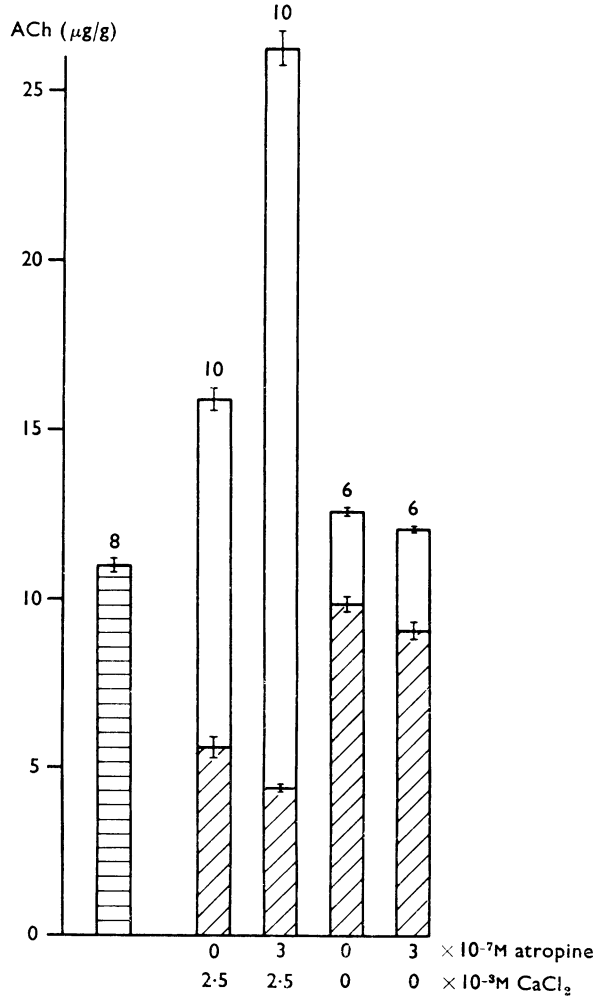

FIG. 3. Inverse relationship between the final extractable ACh content of the slices and the rate of ACh release. The results are presented in the same way as in Figs. 1 and 2. The incubation medium contained 8×10^{-4}M physostigmine.

When the incubation medium contained physostigmine in addition to soman the omission of calcium from the medium also reduced synthesis and release of ACh, and the stimulating effect of atropine was not only reduced but abolished. This is evident from the results shown in Fig. 3. They also show that the ACh content of the cortical slices was affected differently on incubation with physostigmine. In contrast to the experiments of Fig. 2 the ACh content of the cortical slices became greatly reduced on incubation in the presence of 2·5 mM $CaCl_2$. The content decreased from $11{\cdot}1 \pm 0{\cdot}21$ μg/g to $5{\cdot}6 \pm 0{\cdot}29$ μg/g and, when atropine was added to the incubation medium, even further to $4{\cdot}4 \pm 0{\cdot}11$ μg/g. In previous experiments with cortical slices from Brofo rats a similar decrease of the ACh content had been obtained but the enhancement of this effect by atropine was not noted (Polak & Meeuws, 1966; Bertels-Meeuws & Polak, 1968). A reduction of the ACh content of the cortical slices was also present when calcium had been omitted from the incubation medium, but the reduction was much smaller. When the ACh released is considered in connection with the ACh content of the slices an inverse relationship is evident: the greater the release the lower the content.

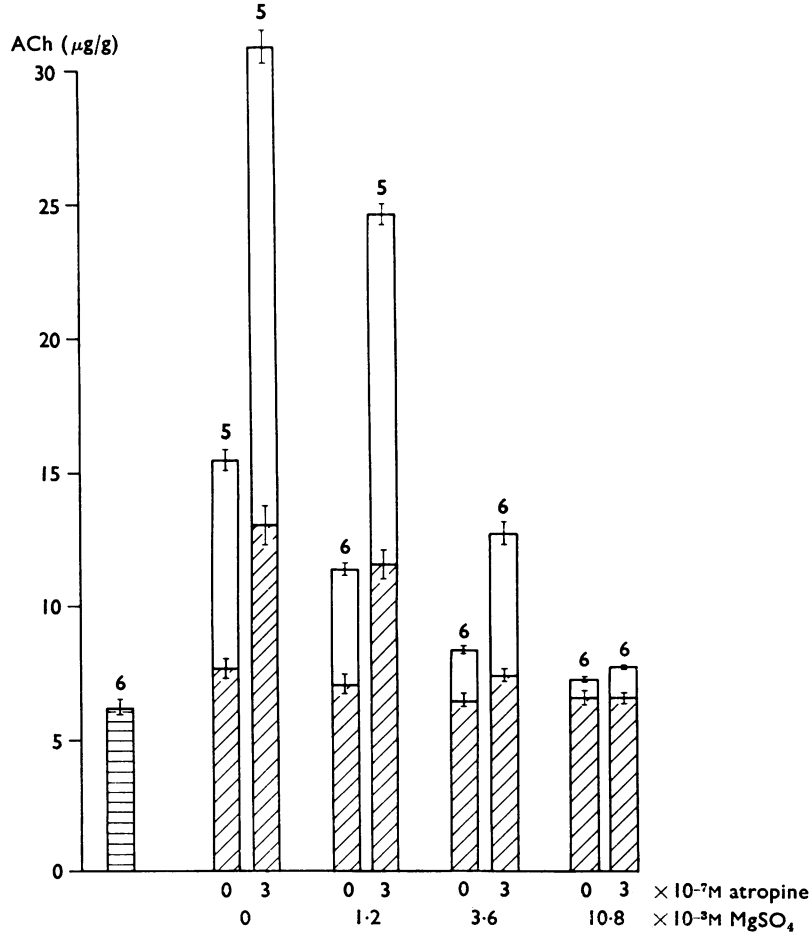

FIG. 4. Influence of the magnesium concentration of the medium on the release and synthesis of ACh by cortical slices. The results are presented in the same way as in Fig. 1. The concentrations of atropine and $MgSO_4$ are given below the columns.

Mg ions

The effect of $MgSO_4$ in the incubation medium was examined in the presence of 2·5 mM $CaCl_2$. As seen from the results given in Fig. 4 raising the Mg concentration progressively reduced synthesis and release of ACh. This occurred in the absence and in the presence of atropine, but up to a Mg concentration of 3·6 mM the stimulating action of atropine on the ACh release was little affected. The factor by which the atropine multiplied the release was $2·3 \pm 0·18$ (five observations) in the absence of Mg and $3·2 \pm 0·22$ (five observations) and $2·8 \pm 0·11$ (six observation) in the presence of 1·2 and 3·6 mM $MgSO_4$ respectively. These multiplication factors were not significantly different from one another. Synthesis and release of ACh were further reduced when the Mg concentration was raised to 10·8 mM, but the stimulating action of atropine on the ACh release was still present although it was now smaller; the factor by which the atropine multiplied the release was only $1·7 \pm 0·11$ (six observations), which is significantly smaller than the multiplication factors of 3·2 and 2·8 obtained in the presence of lower concentrations of $MgSO_4$, but not significantly different from the 2·3 obtained in its absence.

Discussion

The previous finding that atropine increases the release of ACh from and its synthesis by cortical slices from rat brain incubated in a Krebs-Ringer solution containing a high potassium concentration (Polak & Meeuws, 1966; Bertels-Meeuws & Polak, 1968) has been confirmed. This stimulating effect of atropine could not have resulted from an anti-cholinesterase action of atropine because the cholinesterase activity of the cortical slices was fully inhibited by the use of soman. Nor can it easily be explained by interference with the re-uptake of released ACh, a suggestion originally made to explain the increased release of ACh from the surface of the cerebral cortex or from the caudate nucleus obtained in *in vivo* experiments (Szerb, 1964; Polak, 1965). When this suggestion was made the possibility of increased synthesis of ACh by atropine was not taken into consideration since these *in vivo* experiments dealt only with release of ACh. In the cortical slices, however, atropine not only stimulates release but also synthesis of ACh, since the total yield of ACh—that is, the ACh present in the slices and released into the incubation medium—was increased after incubation with atropine. To explain this increased yield without increased synthesis, one would have to assume that in the absence of atropine the released ACh is at once taken up by the tissue and metabolized, and that atropine prevents this metabolism. When this possibility was investigated it was found that although cortical slices treated with soman or other organophosphorus anticholinesterases have the ability to take up large amounts of ACh added to the medium, this ACh was not metabolized in significant amounts and atropine in low concentration did not affect the uptake (Polak & Meeuws, 1966; Schuberth & Sundwall, 1967; Liang & Quastel, 1969a, b; Polak, 1969). Thus the increased yield signifies increased synthesis of ACh.

Another suggestion was made by MacIntosh (1963) to explain the finding that atropine stimulates the release of ACh from the exposed cerebral cortex of the living animal. According to this suggestion atropine would block cholinergic synapses which form a part of inhibitory neuronal circuits controlling the activity of the intra-cortical neurones from which the ACh is released. Recent findings by Dudar & Szerb (1969) appeared to support this hypothesis since they found that

TTX, a compound which blocks nervous conduction (Kao, 1966), applied to the exposed cerebral cortex of anaesthetized cats abolished the stimulating action of atropine. The same result was obtained when the TTX was applied in a solution containing 50 mM KCl. These results are different from those obtained in the present experiments with cortical slices from rat brain, in which TTX did not inhibit the stimulating action of atropine on the release and synthesis of ACh. This difference between the effect of TTX on the cat brain *in vivo* and on cortical slices from rat brain is at present unexplained. But, as far as cortical slices from rat brain are concerned, the inability of TTX to influence the stimulating effect of atropine restricts its site of action to the nerve endings from which the ACh presumably originates.

To attribute to atropine a stimulating action not only on release but also on synthesis of ACh may appear to be in contradiction to the finding that atropine has no influence on the synthesis of ACh by cell-free choline acetylase systems (Giarman & Pepeu, 1964; Schuberth, 1965). However, in cortical slices in which the cellular structures are more or less intact increased synthesis may be brought about indirectly by the increased release of ACh. This possibility was tested experimentally by investigating the effect of procedures which diminish the release, such as a reduction of the calcium concentration or an increase of the magnesium concentration of the incubation medium, or pre-incubation of the slices with botulinum type A toxin. If, under these conditions, atropine were to produce an increased ACh content of the cortical slices, it would be evidence for a direct stimulating action of atropine on the synthesis. It was found, however, that under all three conditions the stimulating effect of atropine on both the release and the synthesis of ACh was greatly reduced. This suggests that it is primarily the release which is enhanced by atropine and that its stimulating action on the synthesis results from the increased release. This suggestion is supported by results obtained with physostigmine.

It has been mentioned that ACh added to the incubation medium is taken up in large amounts by the cortical slices. This uptake was observed in the presence of soman with low and with high KCl concentrations and in the absence and in the presence of atropine in low concentrations. The uptake of added ACh, however, was strongly inhibited by physostigmine (Polak, 1969). Yet physostigmine appears to inhibit also the uptake of endogenous ACh released from cortical slices. Evidence for this effect of physostigmine is based on earlier observations (Polak & Meeuws, 1966; Bertels-Meeuws & Polak, 1968) which showed that the addition of physostigmine to incubation media containing a high KCl concentration resulted in a decrease of the total extractable ACh content of the slices, whereas the amounts of ACh recovered from the incubation medium were increased by the same amount. Consequently, the sum of released ACh and extracted ACh was not changed. Moreover, physostigmine did not influence the stimulating effect of atropine on the synthesis of ACh. These results are readily explained on the assumption that the ACh released from the slices is partly taken up by the tissue and that this re-uptake is inhibited by physostigmine. It has now been found that the decrease in the extractable ACh of the slices during incubation in a medium containing a high KCl concentration and physostigmine was inhibited when the release and synthesis of ACh were decreased by omission of the calcium ions from the medium. In contrast, the reduction of the ACh content in the slices was definitely enhanced by atropine. If atropine were to stimulate the synthesis primarily, one would expect the ACh content of the slices to increase and certainly not to decrease under its influence. However, if the

primary effect were on the release, a reduction could be expected. This reduction was actually found, but only in the presence of physostigmine, because in its absence it was probably masked by the re-uptake of released ACh.

The release of ACh from cortical slices resembles in many respects the spontaneous release of ACh at the neuromuscular junction which gives rise to miniature endplate potentials (MEPP's). Like the MEPP frequency (Elmquist & Feldman, 1965 ; Katz & Miledi, 1967) it is not affected by TTX. However, whereas botulinum toxin *in vitro* completely abolishes the MEPP frequency at the neuromuscular junction (Brooks, 1956), it only partly blocks the release of ACh from cortical slices. On the other hand, raised potassium concentrations in the medium, which strongly increase the MEPP frequency (Del Castillo & Katz, 1954 ; Liley, 1956 ; Hubbard, 1961) also stimulate the release of ACh from cortical slices, whereas reduction of the calcium or elevation of the magnesium concentration results in a decrease of the ACh release from slices in a way similar to the effects of these ions on the MEPP frequency (Hubbard, 1961 ; Gage & Quastel, 1966). Finally the release of ACh from the exposed cerebral cortex of anaesthetized cats has been shown to be affected by the calcium and magnesium concentration of the topically applied salt solutions (Randič & Padjen, 1967 ; Hemsworth & Mitchell, 1969) in about the same way as these ions affected the release from cortical slices in the present experiments.

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