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THE OUTPUT PER STIMULUS OF ACETYLCHOLINE FROM CEREBRAL CORTICAL SLICES IN THE PRESENCE OR ABSENCE OF CHOLINESTERASE INHIBITION

P.S. BOURDOIS[1] & J.F. MITCHELL[2]
Department of Pharmacology, University of Cambridge

G.T. SOMOGYI[3]
Department of Physiology and Biophysics, Dalhousie University, Halifax, N.S., Canada

J.C. SZERB
Department of Pharmacology, University of Cambridge, and Department of Physiology and Biophysics, Dalhousie University, Halifax, N.S., Canada

1 The release of endogenous acetylcholine (ACh) from cerebral cortical slices stimulated at 0.25, 1, 4, 16 and 64 Hz was measured in the presence either of physostigmine or of physostigmine and atropine.

2 Atropine potentiated the evoked release of endogenous ACh especially at low frequencies resulting in an output per stimulus which sharply declined with increasing frequency of stimulation, while in the absence of atropine the output of ACh per stimulus was low and fairly constant.

3 The evoked release of [^{3}H]-ACh per stimulus following the incubation of the slices with [^{3}H]-choline, as estimated by means of rate constants of the evoked release of total radioactivity, showed a frequency dependence similar to endogenous ACh when the two were tested under identical conditions.

4 In the absence of an anticholinesterase the evoked release of [^{3}H]-ACh per stimulus was dependent on frequency of stimulation in a similar way to that in the presence of physostigmine and atropine.

5 Results suggest that under physiological conditions, i.e. in the absence of an anticholinesterase, the release of ACh per stimulus decreases with increasing frequency of stimulation and that this decrease is due to a lag in the mobilization of stored ACh rather than in the synthesis of new ACh.

Introduction

There is information available on the relationship between the frequency of stimulation and the rate of release of acetylcholine (ACh) at several peripheral cholinergic terminals. For instance, in the superior cervical ganglion (Birks & MacIntosh, 1961) and at the neuromuscular junction (Potter, 1970) the volley output of ACh is constant in the physiological range of stimulation frequency, while in the cholinergic terminals of the intestine the volley output decreases with increasing frequency of stimulation (Paton, 1963; Knoll & Vizi, 1971).

No such information is available for cholinergic terminals in the cerebral cortex. In previous studies on the release of ACh from the cerebral cortex *in vivo*, the activity of cholinergic neurones was influenced through indirect means, such as drugs that stimulate or depress the central nervous system (Hemsworth & Neal, 1968; Szerb, Malik & Hunter, 1970) or by peripheral stimulation (Mitchell, 1963) which affects the cortical cholinergic neurones through multisynaptic pathways. In studies on the release of ACh *in vitro*, on the other hand, release was augmented either by increasing the potassium content of the

[1] Present address: Pfizer Laboratories, Amboise, France.
[2] Present address: Department of Pharmacology, University of Bristol.
[3] Present address: Department of Pharmacology, Semmelweiss Medical School, Budapest, Hungary.

medium (Mann, Tennenbaum & Quastel, 1939; Bertels-Meeuws & Polak, 1968) or by electrical stimulation at a single frequency (100 Hz) (Rowsell, 1954; Bowers, 1967).

This study was undertaken to investigate the output of ACh per stimulus from cholinergic terminals in cerebral cortical slices stimulated at different frequencies. The two methods used to measure ACh release, measurement of endogenous ACh release and that of preformed labelled ACh, each gave slightly different information. By measuring the release of endogenous ACh, the output per stimulus could depend both on the rate of release of preformed ACh and the rate of resynthesis of lost ACh. This method necessitated the use of a cholinesterase inhibitor which, as previous observations have shown (Szerb & Somogyi, 1973), depresses the release of ACh. The second method, in which the evoked release of labelled choline was measured (Somogyi & Szerb, 1972; Richardson & Szerb, 1974), avoided the use of a cholinesterase inhibitor, but gave information on the output of preformed ACh only. Results indicate that under physiological conditions the output of ACh per stimulus from the cerebral cortex is frequency-dependent over a wide range of frequencies in a similar way to the release of ACh from the cholinergic terminals of the guinea-pig ileum. Some of these results have been presented in a preliminary form (Bourdois, Mitchell & Szerb, 1971).

Methods

Measurement of endogenous ACh release

The preparation of cortical slices and the technique of superfusion and stimulation were similar to that described in the previous paper (Richardson & Szerb, 1974), with the following exceptions: the cortex was cut, after weighing, into 0.2 mm wide strips and the tissue obtained from both cortices (average weight 370 mg) was placed in a single perfusion bath without any preliminary incubation. The superfusion fluid consisted of Krebs solution equilibrated with 95% O_2 and 5% CO_2 and contained either physostigmine salicylate (0.2 mM) alone or the same amount of physostigmine with atropine sulphate (0.3 μM). Following 1 h superfusion, four 2.5 min samples were collected without stimulation and stimulation at 0.25, 1, 4, 16 and 64 Hz was started at the fifth sample. ACh was determined on strips of the dorsal longitudinal muscle of the leech (*Hirudo medicinalis*) (Dudar & Szerb, 1970). The leech muscle was bathed in dilute Krebs solution containing physostigmine 10^{-4} M but, instead of using water as a diluent as described previously, the Krebs solution was diluted with an equal volume of NaH_2PO_4 (1.45 mM) and $CaCl_2$ (0.18 mM). This reduced the spontaneous movements of the muscle. The contractions produced by the samples were antagonized by (+)-tubocurarine (1 μg/ml) to the same extent as those produced by added ACh and, when used in different dilutions, the samples gave regression lines similar to ACh. For these reasons the substance assayed in the samples was assumed to be ACh.

Estimation of evoked release of total radioactivity following incubation with [^{3}H]-choline

The method of preparation, incubation and superfusion of the cortical slices (0.4 mm wide strips) was the same as described in the previous paper (Richardson & Szerb, 1974). Incubation fluid always contained 50 μM, 2 μCi/ml [^{3}H]-choline. The superfusion fluid consisted of Krebs solution containing hemicholimium-3 (10 μM) either alone or with additional physostigmine or atropine or both in the same concentrations as used for measuring the release of endogenous ACh. The slices were superfused for 75 min before starting the collection of 20 2.5 min samples. At the end of the experiments, the slices were quantitatively removed and weighed. Radioactivity in the samples was counted as described before (Richardson & Szerb, 1974).

Stimulation was always started at the 6th sample, the maximum duration of stimulation never extending beyond the 10th sample. In order to obtain a quantitative estimate of the amount of radioactivity released as a result of stimulation, in every experiment the spontaneous efflux of the label was estimated and the difference between the observed and the expected spontaneous outflow was taken as the evoked release of radioactivity. In order to calculate the spontaneous efflux of radioactivity, it was assumed that it declines exponentially, an assumption which, although proven not to be entirely correct (see results section), made calculations relatively simple without introducing a major error. The exponential decline of spontaneous outflow was calculated by fitting a straight line by the least-squares method through the log d/min of the first five samples (before stimulation) and the last five samples (when the effect of stimulation presumably had subsided). The antilog of this line was subtracted from the corresponding observed values of samples 6-15 and the sum of these differences gave the evoked release of radioactivity. Results are expressed as pmol choline per g tissue, based on the specific activity of choline

added to the incubation mixture. All computations including the conversion of ct/min to d/min were carried out on data automatically punched on cards.

It has been shown in the previous paper (Richardson & Szerb, 1974) that the evoked release of [^{3}H]-ACh declines exponentially with time and that this evoked release of [^{3}H]-ACh is responsible for the increase in the efflux of total radioactivity following stimulation. Therefore, this increase in the release of total radioactivity following stimulation is referred to as the evoked release of [^{3}H]-ACh throughout the paper. The rate constant of the evoked release of [^{3}H]-ACh can be obtained from the slope of a simple semi-log plot using the following relationship between the evoked release of radioactivity, Δn, and the duration of stimulation, Δt:

$$\ln\left(1-\frac{\Delta n}{n_o}\right)=-k\Delta t \qquad (1)$$

where n_o is the total amount of [^{3}H]-ACh which can be released which was estimated to be 4116 ± 197 pmol/g (Richardson & Szerb, 1974). In order to compare the rate constant of evoked release of [^{3}H]-ACh with the output of endogenous ACh per stimulus it is necessary to have compatible units. In the time domain, k is defined as the fractional decrement of n per minute. The corresponding rate constant k', defined for convenience as the fractional decrement of n per 100 stimuli, is required. If the frequency of stimulation, in Hz, is f, then it follows that $k' = 100\,k/60\,f$. Thus the slope (k') of the linear equation (1), in terms of the number of stimuli delivered, can be obtained by

$$k'=\frac{\Sigma xy}{\Sigma x^2} \qquad (2)$$

and its standard error

$$s(k')=\sqrt{\frac{\Sigma y^2-k'\,\Sigma xy}{(n-1)\,\Sigma x^2}} \qquad (3)$$

where x = number of stimuli/100 and $y = \ln(1 - \Delta n/n_o)$.

Results

The release of endogenous acetylcholine

Since it is known that atropine enhances the release of ACh from cerebral cortical slices evoked by 25 mM potassium (Bertels-Meeuws & Polak, 1968) the release of ACh evoked by electrical stimulation was tested both in the absence and presence of atropine (0.3 μM). Atropine had no effect on the resting output of ACh prior to stimulation, this being 101.3 ± 6.6 pmol g^{-1} min^{-1} (n = 16) in the absence, and 94.6 ± 9.4 pmol g^{-1} min^{-1} (n = 13) in the presence of atropine. Electrical stimulation resulted in a well maintained increase in ACh release (Fig. 1), except in the case of stimulation at 64 Hz where the increase was less well sustained, probably due to the injurious effect of the large amount of current passed.

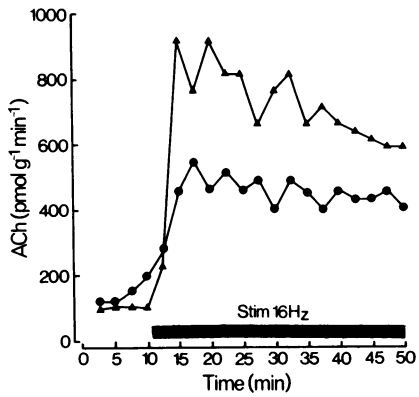

Fig. 1 The release from rat cerebral cortical slices of endogenous acetylcholine (ACh) in the absence (●) or presence (▲) of atropine sulphate (0.3 μM). Physostigmine present in both cases.

To estimate the evoked release of ACh due to stimulation at different frequencies, the average ACh content of the four pre-stimulation samples was subtracted from the average of the second to fifth samples following the start of stimulation. The first stimulated sample was not included since it contained, because of the dead-space, some of the superfusate exposed to the brain tissue prior to stimulation. From Fig. 2a it can be seen that the minute output rose with increasing frequencies of stimulation, with the exception of 64 Hz in the presence of atropine, and except at this highest frequency, atropine significantly increased the minute output. When the results are expressed as an increase in ACh release per stimulus (Fig. 2b) the greater potentiating effect of atropine on the release of ACh at low frequencies of stimulation becomes evident. In the presence of atropine, the output of ACh per stimulus was very high at 0.25 Hz and decreased rapidly with higher frequencies. In the absence of atropine, on the other hand, the output of ACh per stimulus was relatively unchanged between 0.25 and 4 Hz and dropped only at higher frequencies.

The release of labelled acetylcholine

The marked effect of the presence or absence of atropine on the relationship between frequency of

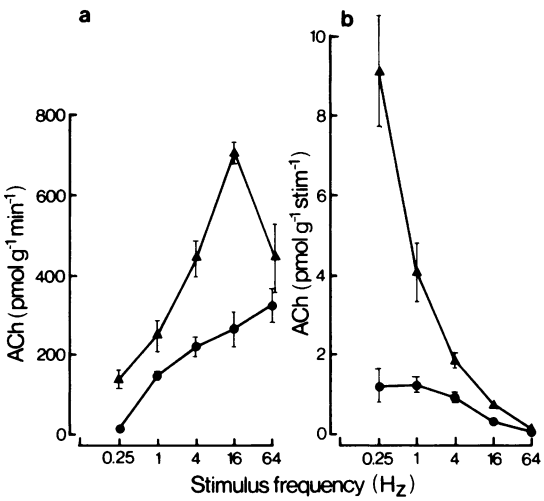

Fig. 2 (a) Increase in the minute output of endogenous acetylcholine (ACh) resulting from stimulation of rat cerebral cortical slices at different frequencies. Each point is the average of three observations. Vertical bars show s.e. mean. (b) The output per stimulus of endogenous ACh at different frequencies calculated from the minute output. (●) Physostigmine present; (▲) physostigmine and atropine present.

stimulation and the output of endogenous ACh per stimulus raised the question of which of these relationships obtained under physiological conditions, namely, in the absence of cholinesterase inhibition. Furthermore, by measuring the release of endogenous ACh it was impossible to establish whether the high output per stimulus at low frequencies in the presence of atropine was due to an increased contribution of newly synthesized ACh or to an enhanced mobilization of preformed ACh. Answers to these questions were sought through measuring the release of labelled ACh at various frequencies of stimulation, first in the presence of physostigmine or of physostigmine and atropine. If the release of labelled ACh under these conditions showed the same dependence on stimulation frequency as endogenous ACh, this dependence would be due to variation in the release of preformed ACh. In addition, measurement of the evoked release of total radioactivity was expected to give information on the effect of stimulation frequency on the output of ACh per stimulus in the absence of an anticholinesterase.

In order to obtain the rate constants of the evoked release of [^{3}H]-ACh by means of equations described in the methods section, stimulation at four different frequencies for each of two or three different durations was applied while the slices were superfused with four different solutions, namely, without the addition of either physostigmine or atropine, with physostigmine alone, with atropine alone, or with both physostigmine and atropine. It can be seen from Fig. 3a that without stimulation the observed efflux was slightly but significantly less (144 ± 53 pmol/g, $P < 0.02$) than the values of samples 6-15 which were calculated on the basis of an exponential decline of the efflux. Since it was much simpler to calculate the expected spontaneous outflow of radioactivity from a simple exponential equation, no account was taken in subsequent calculations of this small discrepancy between the observed and calculated spontaneous outflow. Figure 3b-d gives examples of the increasing effectiveness of longer periods of stimulation in releasing radioactivity. Figure 4a-d shows examples of the effect of the four superfusion solutions on the release of radioactivity resulting from the same stimulus parameters. The evoked release of radioactivity obtained with different stimulation parameters and superfusion fluids is summarized in Table 1. It can be seen that at all frequencies physostigmine depressed the evoked release of radioactivity as compared to controls while the presence of both physostigmine and atropine not only restored the evoked release to control levels, but the evoked release exceeded that in the controls. On the other hand, atropine alone hardly changed the evoked release of radioactivity as compared to the controls.

Results shown in Table 1 were plotted as the logarithm of the decrement of the initial [^{3}H]-ACh store against time or the number of stimuli applied, according to Equation (1), and the resulting linear regression lines were calculated according to Equation (2), as shown in Figure 5. It can be seen that points representing small amounts of evoked release are found randomly at both sides of the regression lines. However, points obtained with more prolonged stimulation generally fall above the regression lines, indicating that the rate constants decreased with time. This observation is at variance with the results described in the previous paper (Richardson & Szerb, 1974) which showed that the rate constant of evoked release of labelled ACh remained unchanged during stimulation of much longer duration than applied here. The probable reasons for this discrepancy are that, in experiments described in this paper, the evoked release of radioactivity was estimated as the difference between the expected spontaneous and the observed evoked efflux of radioactivity. If the collection of a large evoked release of the label was not complete before the 16th sample, the expected spontaneous release would have been overestimated and the difference between the

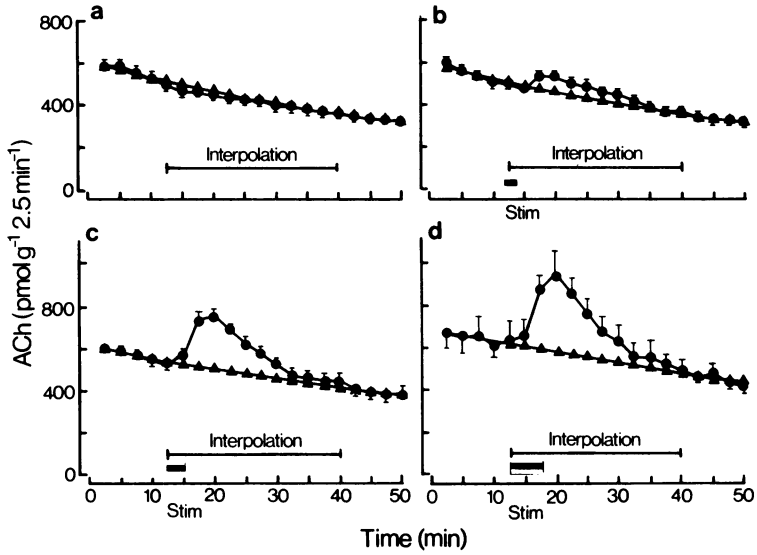

Fig. 3 The release from rat cerebral cortical slices of labelled acetylcholine (ACh) resulting from stimulation (Stim) with different number of pulses at 4 Hz. (a) No stimulation (n = 17); (b) 300 pulses (n = 6); (c) 600 pulses (n = 12); (d) 1200 pulses (n = 5). (●) Observed values with s.e. mean: (▲) expected spontaneous outflow. Interpolation encompasses the points from which the evoked release was calculated. (See methods section).

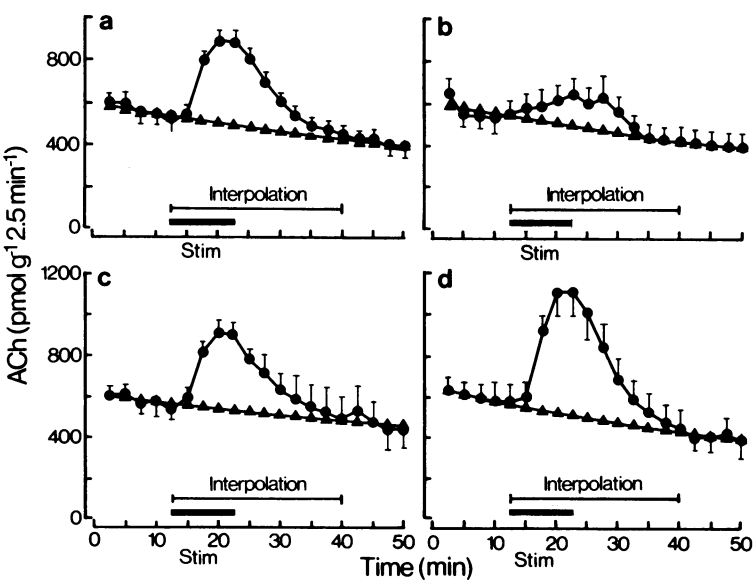

Fig. 4 The effect of physostigmine and of atropine alone or in combination on the evoked release from rat cerebral cortical slices of labelled acetylcholine (ACh) resulting from 600 pulses at 1 Hz (Stim). (a) Control (n = 9); (b) physostigmine (n = 5); (c) atropine (n = 4); (d) physostigmine + atropine (n = 6). Symbols as in Figure 3.

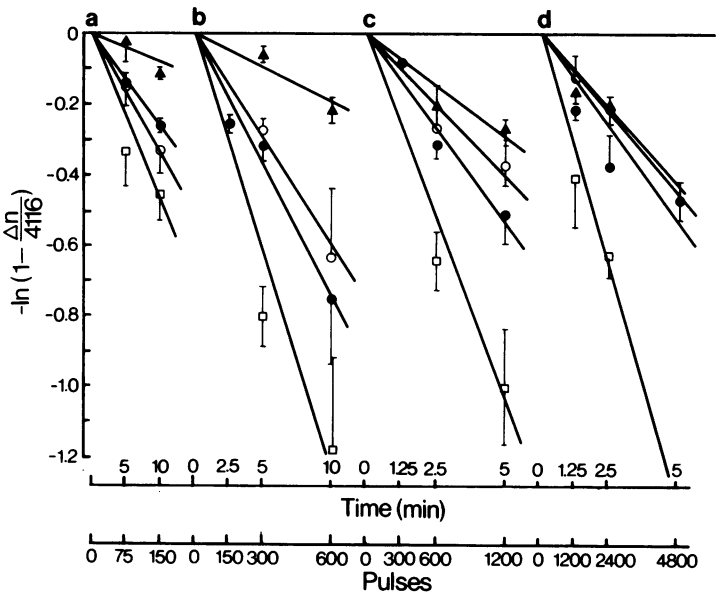

Fig. 5 The evoked release of [^{3}H]-acetylcholine (ACh) plotted as the logarithm of the decrement of initial [^{3}H]-ACh store against time or against number of stimuli. (•) Control; (▲) physostigmine; (○) atropine; (□) physostigmine and atropine. Vertical lines represent s.e. mean. Regression lines were calculated from Equation (2). (See methods section). (a) 0.25 Hz; (b) 1 Hz; (c) 4 Hz; (d) 16 Hz.

observed and expected efflux would have appeared to be less. Furthermore, the transient decline of [^{3}H]-choline efflux observed during an intensive release of [^{3}H]-ACh (Richardson & Szerb, 1974) would also reduce the evoked increase in the efflux of total radioactivity. Although this apparent decline in the rate constants with prolonged stimulation was consistently observed in these experiments, this discrepancy had very little effect on the slope of the linear regression lines which represent the rate constants of the evoked release of radioactivity.

Values of the rate constants of evoked release of radioactivity at various frequencies plotted

Table 1 The evoked release of radioactivity due to stimulation of different durations and frequencies

Frequency (Hz)	*0.25*		*1*			*4*			*16*		
No. of pulses	*75*	*150*	*150*	*300*	*600*	*300*	*600*	*1200*	*1200*	*2400*	*4800*
Drugs											
None	526	935	917	1099	1951	327	1093	1600	802	1247	1536
	±109	±71	±80	±133	±296	±34	±120	±208	±94	±299	±136
	(10)	(7)	(7)	(5)	(9)	(6)	(12)	(5)	(4)	(4)	(5)
Physostigmine	−12	437	–	223	797	–	756	966	620	746	–
	±202	±52	–	±81	±118	–	±112	±80	±101	±75	–
	(4)	(4)		(6)	(5)		(5)	(4)	(4)	(4)	
Atropine	665	1155	–	967	1797	–	942	1266	469	1045	–
	±109	±181	–	±91	±419	–	±162	±164	±218	±275	–
	(4)	(4)		(4)	(4)		(5)	(4)	(4)	(4)	
Physostigmine	961	1485	–	2245	2867	–	1929	2538	1323	1900	–
and atropine	±202	±172	–	±170	±335	–	±185	±276	±341	±146	–
	(5)	(6)		(4)	(6)		(4)	(4)	(4)	(4)	

Results are expressed in pmol/g (with s.e. mean) based on the specific activity of [^{3}H]-choline added to the incubation medium. Numbers of observations are in parentheses.

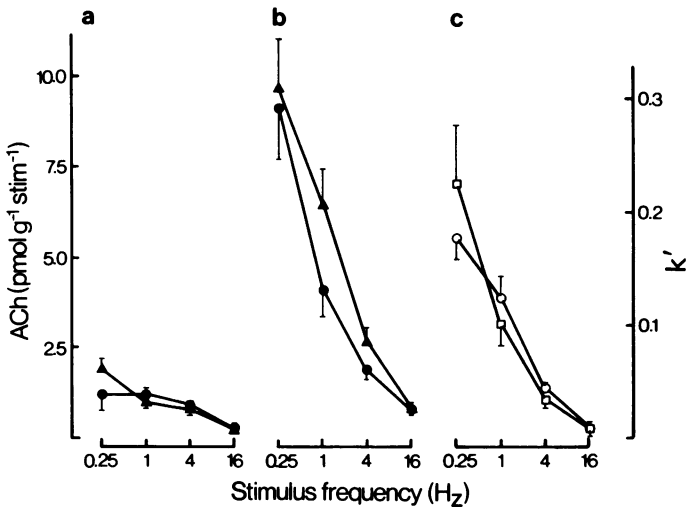

Fig. 6 Comparison of outputs from rat cerebral cortical slices of endogenous acetylcholine (ACh) per stimulus and rate constants per 100 stimuli (k'). (a) In presence of physostigmine; (b) physostigmine and atropine present; (c) no physostigmine. (•) Endogenous ACh; (▲) labelled ACh; (○) no atropine; (□) atropine. Vertical lines show s.e. mean for endogenous ACh and s.e. of k' as calculated from Equation (3). (See methods section).

against 100 pulses (k') are shown in Fig. 6 along with their standard errors as calculated from Equation (3) and these are compared with the output of endogenous ACh per stimulus taken from Figure 2. It can be seen from Fig. 6a and b that the rate constants of the evoked release of labelled ACh depended on the frequency of stimulation in the same way as did the output per stimulus of endogenous ACh measured under identical conditions. In the presence of physostigmine alone, both the output per stimulus and the rate constants were low and varied relatively little with stimulation at different frequencies, while in the presence of both physostigmine and atropine both were higher and were greatly dependent on the frequency of stimulation. In the absence of an anticholinesterase (Fig. 6c), the rate constants were somewhat smaller than in the presence of physostigmine and atropine but still varied markedly with the frequency of stimulation. Atropine alone did not significantly change the rate constants as compared to controls.

Discussion

The potentiating effect of atropine on the release of endogenous ACh evoked by electrical pulses is in many respects similar to the findings of Bertels-Meeuws & Polak (1968) on the effect of atropine on ACh release from cerebral cortical slices induced by high K^+ concentration in the incubation medium. They found that atropine enhanced ACh release induced by 25 mM but not by 50 mM K^+. In the present experiments, atropine produced a much greater increase in the release of endogenous ACh induced by low frequency than by high frequency stimulation. Since tetrodotoxin does not affect the potentiating effect of atropine on ACh release induced by K^+ (Molenaar & Polak, 1970) the effect of atropine is not due to the blockade of a cholinergic component of a neural intracortical negative feedback system but is more likely to be the result of a direct action on cholinergic terminals. However, experiments on the evoked release of labelled ACh show that atropine has very little effect on ACh release in the absence of a cholinesterase inhibitor. It appears, therefore, that the main action of atropine *in vitro* consists of overcoming the direct depressant effect of cholinesterase inhibition on ACh release. Results also show that the magnitude of the effects of physostigmine and atropine on cholinergic terminals depended on the frequency of stimulation. This observation makes it unlikely that the high output of ACh per impulse at low frequencies of stimulation was the result of repetitive discharges of cholinergic fibres. Had this been the case, physostigmine and atropine would have affected ACh release to about the same extent both at high and at low frequencies of stimulation.

The depressant effect of physostigmine on ACh release observed in the present experiments is shared by other anticholinesterases, both tertiary

and quaternary, and by a muscarinic parasympathomimetic agent, oxotremorine (Szerb & Somogyi, 1973) and, therefore, is likely to be the result of an action of ACh which accumulates extracellularly when cholinesterase is inhibited. It is not possible to ascribe a precise mechanism to the depressant effect of cholinesterase inhibitors on the evoked release of ACh. Muscholl (1970) and Fozard & Muscholl (1972) have described a similar inhibitory effect of muscarinic agents on the release of noradrenaline from adrenergic terminals in the heart. This depression was antagonized by atropine but again, atropine alone had no effect. Nor is it possible, at present, to explain the enhancement of the release of labelled ACh in the presence of both physostigmine and atropine above that observed in controls. Allen, Glover, Rand & Story (1972) observed a similar potentiating effect of a combination of ACh and atropine on release of [^{3}H]-noradrenaline from isolated arteries.

The potentiating effect of atropine on evoked ACh release from cortical slices in the presence of a cholinesterase inhibitor observed *in vitro* could account, under certain conditions, for the increase in ACh release observed *in vivo* following atropine administration (Mitchell, 1963; Szerb, 1964). For instance, Dudar & Szerb (1969) observed an enhancement by atropine of the release of ACh resulting from local stimulation of normal or acutely undercut cortices. However, there is evidence to suggest that, in addition to its direct effect on cholinergic terminals, atropine increases ACh release *in vivo* by blocking a cholinergic component of a recurrent inhibitory circuit (Dudar & Szerb, 1969; Szerb *et al.*, 1970).

The effect of frequency of stimulation on the output of endogenous ACh per stimulus and on the rate constants of release of labelled ACh was remarkably similar under two experimental conditions. This indicates that changes in the output of endogenous ACh per stimulus at different frequencies are not the result of a varying contribution of newly synthesized ACh to release but to a time-dependent mobilization of ACh stores already present.

The good agreement between outputs per stimulus and rate constants in conditions when both could be measured makes it likely that changes in the rate constants with different stimulation frequencies in the absence of an anticholinesterase also reflect changes in the output of endogenous ACh per stimulus without cholinesterase inhibition, although the latter cannot be measured. On this basis, it can be concluded that under physiological conditions, namely, when released ACh is hydrolysed, the output of ACh per stimulus decreases with increasing frequency of stimulation. This relationship between output per stimulus and stimulation frequency in the cerebral cortex, therefore, is similar to that observed in the guinea-pig ileum (Paton, 1963; Cowie, Kosterlitz & Watt, 1968; Knoll & Vizi, 1971).

The changes in the output of ACh per stimulus from cerebral cortical slices described here are similar but not as marked as the changes found by Mitchell (1963) in the output per stimulus from the cortex *in vivo* upon peripheral stimulation at various frequencies, where the output per stimulus decreased to an even greater extent with increasing frequencies of stimulation. Failure to transmit impulses through a series of synapses at high frequencies can account for this sharper decline in the output per stimulus.

It is known that iontophoretically applied ACh causes a long-lasting excitation of units in the deeper layers of the cortex (Krnjević & Phillis, 1963) or long-lasting inhibition in the more superficial layers (Jordan & Phillis, 1972). Therefore, a decrease in the output of ACh per stimulus with increasing frequencies of stimulation would result in a rather constant postsynaptic action of released ACh on cholinoceptive units in the cortex in spite of variations in the rate of firing of cholinergic neurones.

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