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THE RELEASE OF LABELLED ACETYLCHOLINE AND CHOLINE FROM CEREBRAL CORTICAL SLICES STIMULATED ELECTRICALLY

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1 In order to establish the origin of the increased efflux of radioactivity caused by electrical stimulation of cerebral cortical slices which had been incubated with [^{3}H]-choline, labelled choline and acetylcholine (ACh) collected by superfusion were separated by gold precipitation.

2 In the presence of physostigmine electrical stimulation (1 Hz, 10 min) increased the release of only [^{3}H]-ACh which was greatly enhanced by the addition of atropine.

3 Continuous stimulation in the presence of physostigmine resulted in an evoked release of [^{3}H]-ACh which declined asymptotically. This evoked release appeared to follow first-order kinetics with a rate constant which remained stable over the course of prolonged stimulation.

4 The rate constant for the evoked release of [^{3}H]-ACh with 1 Hz stimulation was three times greater in the presence of physostigmine and atropine than in the presence of physostigmine alone, while the size of the store from which [^{3}H]-ACh was released was nearly identical under these two conditions.

5 In the absence of physostigmine and atropine, stimulation caused the appearance of only [^{3}H]-choline in the samples.

6 Reduction of [^{3}H]-ACh stores before the application of physostigmine resulted in a reduced evoked release of total radioactivity, both in the absence or presence of physostigmine and atropine, and decreased the evoked release of [^{3}H]-ACh without affecting the release of [^{3}H]-choline.

7 Results suggest that electrical stimulation of cortical slices which had been incubated with [^{3}H]-choline causes the release of only [^{3}H]-ACh, both in the presence or absence of an anticholinesterase. The evoked increase in the efflux of total radioactivity is therefore a good measure of the release of [^{3}H]-ACh.

Introduction

When freshly prepared slices from the cerebral cortex of rats are incubated with labelled choline, part of the acetylcholine (ACh) formed during incubation is labelled (Collier, Poon & Salehmoghaddam, 1972; Molenaar, Polak & Nickolson, 1973). Superfusion of such incubated slices will result in an efflux of radioactivity and this efflux can be increased by electrical stimulation of the slices (Somogyi & Szerb, 1972). The observation that the increased release of radioactivity following stimulation could be prevented by absence of calcium in the perfusion fluid or by tetrodotoxin, suggested that the likely source of this increase was the release of labelled ACh from the slices (Somogyi & Szerb, 1972). In the present experiments, labelled ACh and choline released from cortical slices in the presence of an anticholinesterase were measured separately in order to establish the contribution of each to the resting and evoked release of radioactivity. The results obtained confirmed previous tentative conclusions; namely, that the evoked increase in radioactivity in the superfusion fluid, both in the presence or absence of an anticholinesterase, is the result of an increased release of ACh but not of choline.

Methods

Strips of rat cerebral cortex (0.4 mm wide) were prepared as described previously (Somogyi & Szerb, 1972) and were incubated at 37° C with constant bubbling with 95% CO_2 and 5% O_2 for 40 min in 50 ml Krebs solution containing choline (50 μM). When choline and ACh were separated in the superfusate, the incubation fluid contained 8 μCi/ml [^{3}H-methyl]-choline (Amersham-Searle, 16 Ci/mmol) or 2 μCi/ml if only the efflux of total

radioactivity was measured. After suction filtration, the slices were divided about equally (150-200 mg in each) between two perfusion baths (volume 1.5 cm^3 each), and the slices were superfused at the rate of 0.4 ml/min with Krebs solution that had been bubbled previously with 95% CO_2 and 5% O_2 and contained hemicholinium-3 (10 μM) to prevent the reuptake of released choline. The first sample was collected 75 min after the start of the superfusion, and eight 5 or 10 min samples were collected. Slices were stimulated at 1 Hz with square pulses of 40 mA, 5 ms duration and alternating polarity. At the end of the experiments the slices were quantitatively removed and weighed.

Labelled choline was separated from labelled ACh by the sequential precipitation method described by Collier & Katz (1971). To 1 ml of superfusate 0.2 ml of a mixture containing 1 mg phosphorylcholine and 3 mg ACh carrier was added, followed by 2 ml of saturated ammonium reineckate. The mixture was left in the refrigerator overnight, then was centrifuged and 1 ml water was added to the precipitate, which was shaken with 400 mg ion exchange resin (AG2-X8, Cl-form, Bio-Rad Laboratories). The precipitate was washed with 1 ml water, and 1 ml of the combined supernatant was mixed with 0.5 ml of 10% $NaAuCl_4 . 2H_2O$ (BDH). After overnight refrigeration, the mixture was centrifuged, and after separation the precipitate was dissolved in 1.5 ml of water. For clearing the resulting solutions the precipitate was shaken with 100 mg silver metal powder and the supernatant with 200 mg. With every batch of sequential precipitation samples, duplicate standard samples containing known amounts of labelled choline and ACh were run, and the labelled ACh and choline contents of the samples were calculated by means of simultaneous equations from the distribution of the known choline and ACh in the standard samples of that batch. Throughout the experiments, the percentage precipitated (± s.d.) by reineckate was 98.0 ± 0.9 of choline, 96.2 ± 0.8 of ACh, and gold chloride precipitated 12.8 ± 5.0% of choline and 75.9 ± 3.1% of ACh. However, the proportion of choline and ACh precipitated by gold chloride in the two standard samples of any one day differed always by less than 2% of the total added, and the larger overall variability was due to the day to day variation in the percentage precipitated by gold chloride.

The radioactivity was measured by placing 0.2 ml of the sample in 10 ml toluene containing 0.4% 2-(4-Tert. butylphenyl)-5-(4-biphenyl)-1, 3, 4, oxadiazol (Packard Instrument Co.) and 4 ml ethoxyethanol (Baker) and the resulting homogeneous mixture was counted for 10 minutes. The counts were converted to d/min by the external channels ratio method. The radioactivity released was expressed in the form of pmol choline or ACh per g tissue, determined in every experiment from the radioactivity of the incubation medium and the amount of choline added to the incubation medium. This method of expressing the results seems to be justified since it has been shown (Somogyi & Szerb, 1972) that with 50 μM [^{3}H]-choline added to the incubation medium dilution of the label by endogenously formed choline is insignificant.

In order to measure the rate constant of the perfusion system, 50 μl [^{3}H]-choline was injected into the superfusion bath directly during perfusion at the usual rate. On this occasion, brain slices were replaced by glass beads of a volume corresponding to 180 mg brain tissue and 1 min samples were collected for the following 20 minutes.

The composition of the Krebs solution was (mM): NaCl, 120; $CaCl_2$, 2.6; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; glucose 10. When used, atropine (0.3 μM) and physostigmine (0.2 mM) were added to the superfusion fluid. Drugs used were: hemicholinium-3 bromide (Aldrich), physostigmine (eserine) salicylate (Sigma), atropine sulphate (Merck).

Results

The largest part of the radioactivity released spontaneously in the presence of physostigmine (0.2 mM) and atropine (0.3 μM) consisted of choline (Fig. 1), but the amount of choline in the superfusate declined during the 40 min observation period. On the other hand, the spontaneous efflux of ACh was nearly stable during this period and therefore represented an increasing portion of the total radioactivity released. The sum of labelled choline and ACh accounted for 75-77% of the total radioactivity in the samples; the remainder, not precipitated by reineckate, probably consisted of choline metabolites such as phosphorylcholine and betaine (Snyder, Yamamura, Pert, Logan & Bennett, 1973). The identity of the radioactivity not precipitated with reineckate was not investigated any further.

Stimulation at 1 Hz during 10 min in the presence of physostigmine (0.2 mM) increased the efflux of labelled ACh only, without affecting that of choline (Figure 2a). If, in addition to physostigmine, atropine (0.3 μM) was also present, the effect of stimulation on the efflux of total radioactivity was greatly increased (Figure 2b). This increase was entirely due to the greatly enhanced evoked release of labelled ACh. Not only did choline efflux fail to increase as a result of

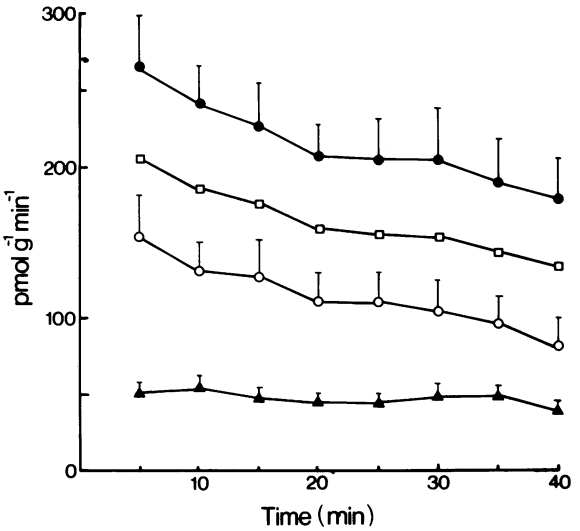

Fig. 1 The spontaneous release of labelled choline and acetylcholine (ACh) in the presence of physostigmine (0.2 mM) and atropine (0.3 μM) (n = 6) from rat cerebral cortical slices. (●) Total radioactivity; (▲) labelled ACh; (○) labelled choline; (□) sum of labelled ACh and choline. In this and subsequent figures vertical lines denote the s.e. mean.

stimulation, it declined temporarily during or just after the period when the release of labelled ACh was the highest. The efflux of radioactivity not precipitated by reineckate was not increased by stimulation.

If stimulation was continued for more than 10 min, the amount of labelled ACh appearing in the sample did not remain constant but declined (Figure 3a and b). In the absence of atropine, when the rate of ACh release was lower, more prolonged stimulation was necessary to obtain a decline than in the presence of atropine. This observation indicated that labelled ACh was released from a finite store. Hence the kinetics of release might be characterized by the amount of stored labelled ACh, n_o, and a rate constant, k, for release.

Assuming that the rate constant, k, does not vary significantly during the period of stimulation, the amount of labelled ACh in the tissue, n, changes in time according to

$$\frac{dn}{dt} = -kn \qquad (1)$$

Labelled ACh is released into the perfusion chamber, which is perfused at a constant flow rate.

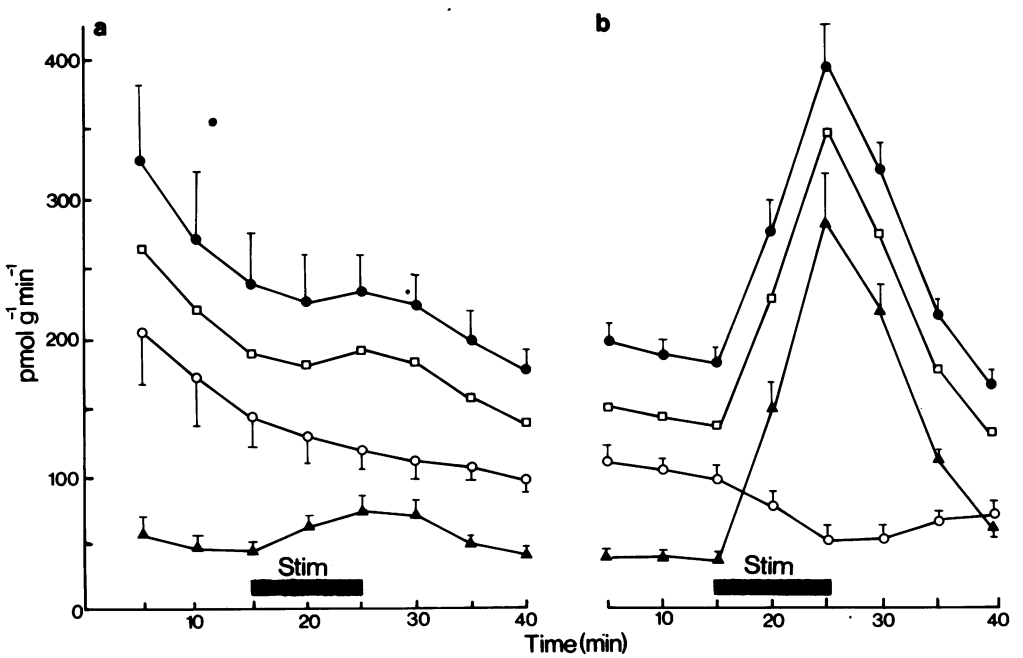

Fig. 2 The effect of stimulation (Stim) at 1 Hz for 10 min on the release of labelled acetylcholine (ACh) and choline from rat cerebral cortical slices, (a) in the presence of physostigmine (0.2 mM) (n = 6); (b) in the presence of physostigmine (0.2 mM) and atropine (0.3 μM) (n = 6). Symbols as in Figure 1.

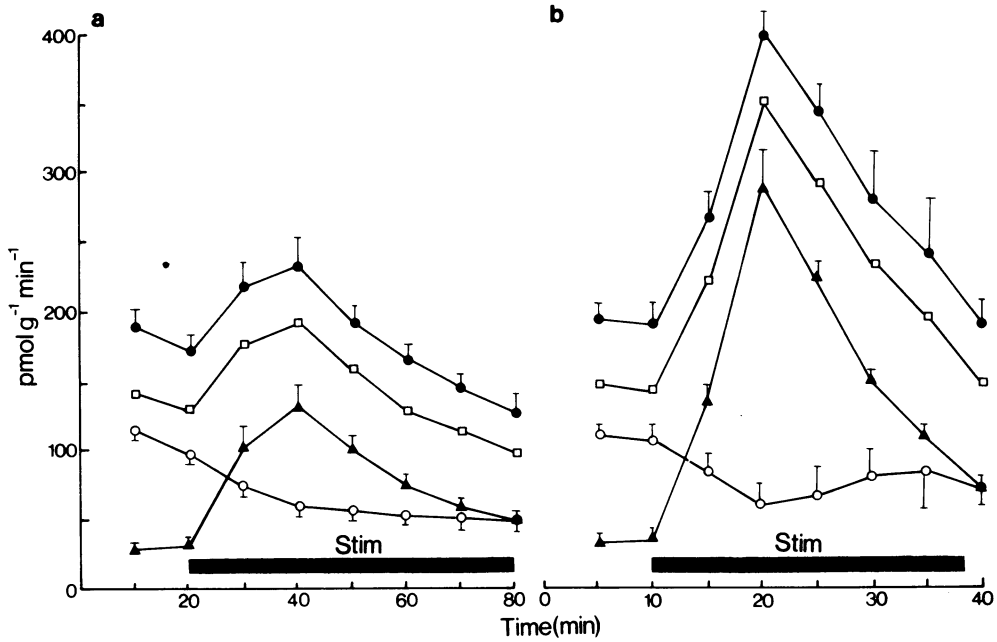

Fig. 3 Effect of continuous stimulation (Stim) at 1 Hz on the release of labelled choline and acetylcholine (ACh) from rat cerebral cortical slices, (a) in the presence of physostigmine (0.2 mM) (n = 5); (b) in the presence of physostigmine (0.2 mM) and atropine (0.3 μM) (n = 5). Note the different time scales in a and b. Symbols as in Figure 1.

Therefore, the amount of ACh in the chamber, n', is characterized by

$$\frac{dn'}{dt} = kn - k_p n' \qquad (2)$$

where k_p is the rate constant of the perfusion system and was found experimentally to be 0.264 min^{-1}. The rate at which ACh appears in the vials collecting the perfusate is

$$\frac{dm}{dt} = k_p n' \qquad (3)$$

where m is the total of ACh collected up to time t. It is m, not n or n' that is the measured datum.

The following equation gives the solution for this two-compartment sequential system:

$$m(t) = n_o + \frac{k_p\, k\, n_o}{k_p - k}\left[\frac{1}{k_p}\, e^{-k_p(t+T)} - \frac{1}{k} e^{-k(t+T)}\right] \qquad (4)$$

where n_o is the amount of releasable labelled ACh present in the tissue at the start of the stimulation.

The origin of the time scale in Equation (4) is not the onset of stimulation but, for mathematical convenience, $t = 0$ in Equation (4) corresponds to the time at which the collection of the first sample following the start of stimulation was completed. In this scale, $t = -T$ is the time when evoked ACh first appears in the collected vial, i.e., $m(-T) = 0$. This appearance occurred some time after the start of stimulation because ACh released into the perfusion chamber was siphoned down a length of tubing to the collection vials, thus creating a time delay. Unlike k_p, the time delay varied slightly from experiment to experiment and was, therefore, determined from the time course of the data using Equation (4). Data in Fig. 4, nevertheless, are plotted with the time scale relative to events occurring in the tissue and not in the collection vials.

It should be noted that Fig. 3 is actually a histogram, the ordinates representing the average rate at which labelled ACh was collected during a set time period. To obtain a continuous function of the amount of evoked labelled ACh collected (m) up to time (t) the amount of ACh collected in one period minus the average amount collected in one period at rest was added sequentially as shown by the plotted points in Figure 4. Each individual experiment was fitted to Equation (4) using standard linearization techniques to obtain a Gaussian least squares fit by means of a computer. For each experiment, the deviation of the fitted curve from the six data points was negligible (~1%). The continuous curve of Fig. 4 was plotted

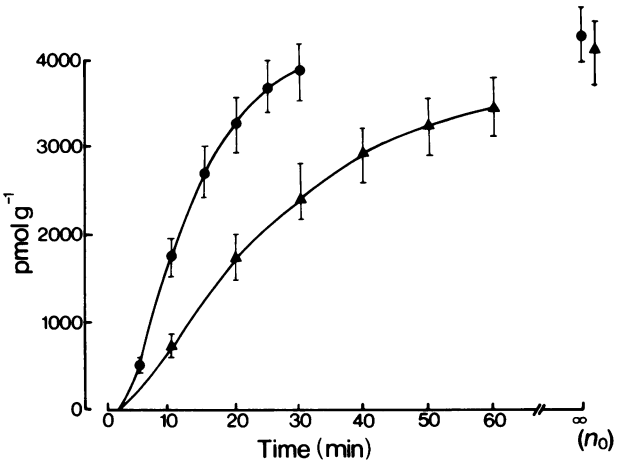

Fig. 4 The evoked release from rat cerebral cortical slices of acetylcholine (ACh) plotted cumulatively in the presence of physostigmine (0.2 mM; ▲) physostigmine (0.2 mM) and atropine (0.3 μM; ●). Lines are drawn through the average points calculated from each experiment from Equation (4) by multiple regression analysis. The values for n_0 were calculated in the same way. Time scale begins with the start of the stimulation.

from the average of the curves obtained from each experiment. Average values obtained from individual experiments for k, and n_0 and T in Equation (4) are shown in Table 1. It can be seen that while the rate constants for release in the absence or presence of atropine differed by a factor of 3, n_0, the amount of labelled ACh releasable by stimulation, was nearly identical under the two conditions. The difference in the values of T reflects the different collection periods employed in the two sets of experiments, 10 min in the absence and 5 min in the presence of atropine, making the collection period minus T approximately 1.6 min in both, which is the delay in the collection system.

Labelled acetylcholine release in the absence of an anticholinesterase

While the above results clearly show that in the presence of physostigmine electrical stimulation causes an increased release of only labelled ACh, it is necessary to obtain evidence as to the source of the evoked release of radioactivity in the absence of an anticholinesterase such as observed by Somogyi & Szerb (1972). As shown in Fig. 5, in the absence of both physostigmine and atropine, practically all radioactivity released and precipitated by reineckate was recovered in the choline fraction, both at rest and following stimulation. In order to see whether the evoked release of labelled choline in the absence of an anticholinesterase originated from released labelled ACh hydrolyzed after release, or whether labelled choline was released independently from ACh, the size of releasable labelled ACh stores was reduced before the addition of an anticholinesterase. Following this, in the presence of physostigmine and atropine, the contribution of labelled choline and ACh to the total radioactivity released was estimated. These results were compared with the effect of the same treatments on the amount of total radioactivity released in the absence of an anticholinesterase.

Two methods were used to decrease selectively

Table 1 Estimated values of constants describing the evoked release of labelled acetylcholine (± s.e. mean)

Drug in superfusion fluid (n)	*k (min⁻¹)*	*n_0 (pmol g⁻¹)*	*T (min)*
Physostigmine (5)	0.0397 ±0.0064	4023 ±344	8.351 ±0.477
Physostigmine + atropine (5)	0.1185 ±0.0153	4209 ±230	3.468 ±0.091

the releasable labelled ACh stores: the first was based on the observation of Bhatnagar & MacIntosh (1967) and of Collier *et al.* (1972) that ACh synthesis in brain slices can proceed without the addition of extraneous choline, presumably by the utilization of choline released from the slices during incubation (Browning, 1971; Collier *et al.*, 1972). Furthermore, the ACh content of brain slices increases rapidly only in the first 30 min of incubation (Sattin, 1966; Lefresne, Guyenet & Glowinski, 1973). Therefore, if brain slices were incubated for 30 min without the addition of any labelled or cold choline followed by the usual 40 min incubation with [^{3}H]-choline (50 μM), much of the ACh synthesized during incubation will not be labelled, while the uptake of labelled choline, unrelated to ACh synthesis, would remain

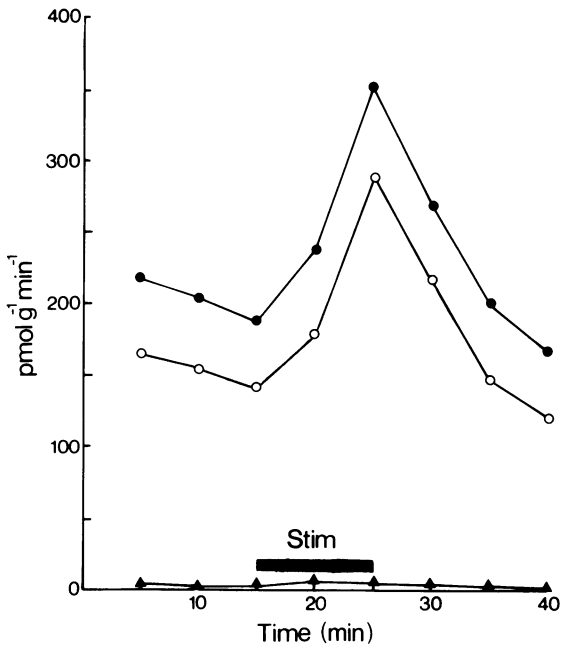

Fig. 5 The release from rat cerebral cortical slices of labelled choline and acetylcholine (ACh) in the absence of physostigmine or atropine. Each point is the average of two observations. Stimulation (Stim) and symbols as in Figure 1.

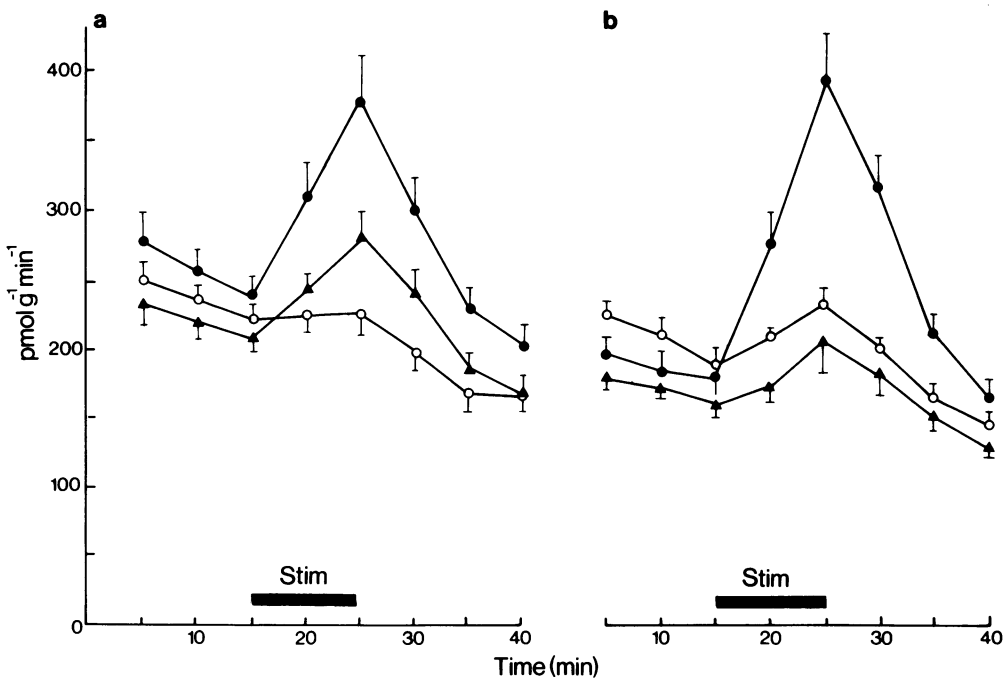

Fig. 6 The effect of preincubation and prestimulation on the release of total radioactivity from rat cerebral cortical slices (a) in the absence of physostigmine or atropine, and (b) in the presence of physostigmine (0.2 mM) and atropine (0.3 μM). (●) Control, (a) n = 8; (b) n = 6; (▲) 30 min preincubation without added cold or labelled choline, (a) n = 6; (b) n = 4; (○) 10 min prestimulation at 16 Hz, (a) n = 6; (b) n = 6. Stimulation (Stim) at 1 Hz for 10 minutes.

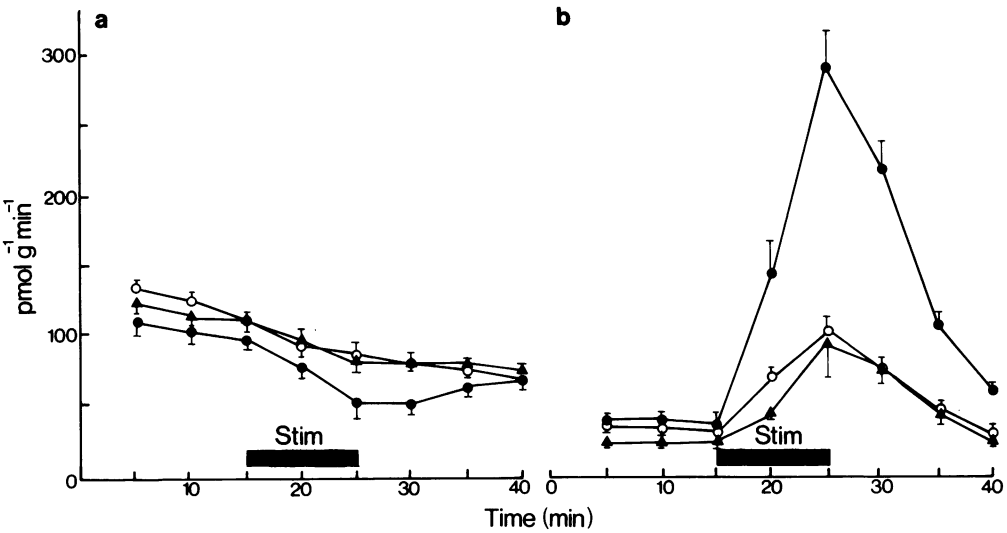

Fig. 7 (a) The effect of preincubation and prestimulation on the release of labelled choline and (b) of labelled acetylcholine from rat cerebral cortical slices. Symbols and number of experiments as in Figure 6b.

approximately the same as under the usual conditions of incubation. The second method, aimed at depleting labelled ACh stores was to stimulate the slices for 10 min at 16 Hz while superfused without physostigmine, followed by the usual collection of samples and stimulation at 1 Hz in the presence of physostigmine and atropine. Figure 6a and b show that these two forms of pretreatment reduced the increase in the release of total radioactivity in the absence of physostigmine and there was a similar reduction in the evoked release of total radioactivity in the presence of physostigmine plus atropine. As Fig. 7 shows, this reduction was not due to a smaller efflux of labelled choline but to the lesser evoked release of labelled ACh. The resting release of labelled ACh was not reduced significantly by prestimulation, but was decreased significantly ($P < 0.01$) by preincubation.

Discussion

Results clearly show that the increased efflux of radioactivity following electrical stimulation is the result of an enhanced release of labelled ACh when measured in the presence of an anticholinesterase. Although in the absence of an anticholinesterase, stimulation resulted in an increased amount of labelled choline appearing in the samples, it is probable that this evoked release of labelled choline originates from the release of labelled ACh which is hydrolyzed subsequently by cholinesterase. The evidence for this is the reduced evoked release of total radioactivity in the absence of an anticholinesterase and of labelled ACh in the presence of physostigmine under two conditions in which labelled ACh stores were selectively reduced. All these observations support the tentative conclusions put forward by Somogyi & Szerb (1972) that the increased release of radioactivity from cortical slices stimulated by electrical pulses is the result of an enhanced release of labelled ACh.

In the presence of physostigmine, labelled choline efflux was not increased by stimulation. On the contrary, it declined transiently whenever the evoked release of ACh was high. Since, in all experiments, hemicholinium-3 (10 μM) was present in the superfusion fluid, uptake of released choline was largely prevented and the amount of labelled choline collected reflected the amount released. However, under conditions when ACh stores were rapidly depleted some of the released labelled choline could have been recaptured by cholinergic endings due probably to an incomplete blockade of the re-uptake of choline by hemicholinium-3. Collier & MacIntosh (1969) described the recapture of choline derived from the hydrolysis of released ACh in the superior cervical ganglion in the absence of an anticholinesterase. This recapture halved the total evoked efflux of radioactivity as compared to the evoked efflux in the presence of physostigmine. Apart from this

transient decline in choline efflux, the release of choline both at rest and during stimulation showed an initial rapid and a later slower decline similar to that observed by Cooke & Robinson (1971).

The presence of physostigmine reduced the increase in the evoked release of total radioactivity as compared to that without physostigmine (Fig. 2a and 6a) while, in the presence of both physostigmine and atropine, the evoked release of total radioactivity was greatly enhanced as compared to that in the presence of physostigmine alone, and exceeded somewhat the evoked increase observed in the absence of both physostigmine and atropine (Figure 6 and b). This confirms previous observations on the effect of anticholinesterase and of atropine on the evoked release of radioactivity from cerebral cortical slices (Szerb & Somogyi, 1973). This effect is more fully discussed in the following paper (Bourdois, Mitchell, Somogyi & Szerb, 1974). However, experiments presented here show clearly that the potentiating effect of atropine on the evoked release of radioactivity in the presence of physostigmine is solely due to an enhanced release of labelled ACh.

Additional conclusions concerning the nature and size of the stores from which labelled ACh is released are suggested by the present observations. Since a considerable reduction of labelled ACh stores by stimulation did not reduce the spontaneous efflux of labelled ACh, it is likely that labelled ACh released spontaneously and by stimulation originates from two different stores. This conclusion is supported by the observation that atropine potentiates the release of ACh evoked by stimulation but not the amount of ACh released spontaneously. Much of the spontaneously released ACh might originate from damaged cholinergic nerve endings and the release of ACh from these is not expected to be influenced by stimulation. For these reasons, the resting release was not included in calculating the size of releasable labelled ACh stores.

The extremely good fit of the observed data to the values provided by the model indicate that the evoked release of labelled ACh follows first order kinetics governed by a rate constant which remains stable over a prolonged period of time. Furthermore, the observation that n_o, the initial store from which labelled ACh is released by stimulation, is almost identical under conditions where the rate constant varies three-fold makes it likely that the size of this store is relatively unaffected during the period of observation by factors, other than stimulation, such as spontaneous release or deterioration of the preparation. The observed average value of $n_o = 4116 \pm 197$ pmol g^{-1} is very similar to the value observed by Molenaar *et al.* (1973), who found 3800 pmol g^{-1} of labelled ACh in cortical slices following incubation with labelled choline (10 μM). However, as they emphasize, their experimental conditions were not optimal for the synthesis of labelled ACh because the incubation with labelled choline was preceded by a preliminary incubation with an anticholinesterase.

This estimate of the size of the store from which labelled ACh is released by stimulation allows the calculation of the rate constant of the evoked release of labelled ACh. For this purpose, it is necessary to measure only the total evoked increase in the efflux of radioactivity resulting from stimulation for a period of time since it has been shown here that this increase reflects well the evoked release of labelled ACh.

Stimulation for a period of time Δt will release an amount of labelled ACh $\Delta n = n_o - n$, where n_o is the initial store of labelled ACh and n the amount of labelled ACh which remains after stimulation.

Equation (1) can be immediately integrated to give

$$n = n_o e^{-k\Delta t} \tag{5}$$

which can be rewritten as

$$\ln\left(1 - \frac{\Delta n}{n_o}\right) = -k\Delta t \tag{6}$$

According to Equation (6), therefore, plotting $\ln(1 - \Delta n/n_o)$ against the duration of stimulation will give a straight line which passes through the origin at zero time and has a negative slope, the value of which is the rate constant of the release. Use of this relationship was made in estimating the rate constant of labelled ACh release due to stimulation at various frequencies and in the presence or absence of an anticholinesterase as described in the following paper (Bourdois *et al.*, 1974).

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