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STIMULATION, BY INHIBITION OF (Na^+-K^+-Mg^{2+})-ACTIVATED ATP-ASE, OF ACETYLCHOLINE RELEASE IN CORTICAL SLICES FROM RAT BRAIN

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SUMMARY

1. A study has made of the effect of (Na^+-K^+-Mg^{2+})-activated membrane ATP-ase inhibitors on the acetylcholine release from the terminals of enteric nerves and from cortical slices.

2. The resting output of acetylcholine from slices of rat cortex was not affected by tetrodotoxin or by noradrenaline, indicating the lack of propagated activity during rest. Furthermore, there was an output of acetylcholine in the absence of Ca.

3. The resting acetylcholine output from cortical slices was increased by (*a*) addition of ouabain or (*b*) administration of sodium *p*-hydroxymercuribenzoate (PHMB), (*c*) sodium withdrawal and (*d*) Ca replacement by Ba^+.

4. Omission of Ca in the presence of 1 mM ethyleneglycol-bis-(*β*-aminoethyl-ether)-*N,N'*-tetra-acetic acid (EGTA) did not affect the increase of acetylcholine release by the inhibition of (Na^+-K^+-Mg^{2+})-activated ATP-ase induced by ouabain or by PHMB, but reduced that due to Na removal.

5. Ouabain increased acetylcholine release promptly.

6. Mg-excess (9·3 mM), noradrenaline and adrenaline were capable of reducing the increase of acetylcholine release from cortical slices evoked by ouabain, PHMB or by Ca replacement by Ba, but not by Na removal.

7. A possible role for (Na^+-K^+-Mg^{2+})-activated ATP-ase in the release of acetylcholine is discussed. It is suggested that the effect of Ca and Mg ions on acetylcholine release might be attributed to their ability to inhibit and activate the membrane ATP-ase, respectively.

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INTRODUCTION

It has been shown (Birks, 1963; Elmquist & Feldman, 1965; Birks & Cohen, 1968), that digitalis glycosides are capable of increasing the frequency of occurrence of miniature end-plate potentials which represent an increase in the resting release of acetylcholine from motor-nerve terminals. Birks (1963) also showed that ouabain releases acetylcholine from perfused sympathetic ganglia. However, Paton, Vizi & Zar (1971) showed that not only ouabain but also other conditions known to lead to inhibition of (Na^+-K^+-Mg^{2+})-activated ATP-ase, were able to enhance the acetylcholine output from nerve terminals in the guinea-pig ileum. The present study was undertaken to examine the effect of inhibition of (Na^+-K^+-Mg^{2+})-activated ATP-ase on acetylcholine release from isolated cortical slices and to compare this with release from guinea-pig ileum. Since Baker (1971) presented convincing evidence that the movement of Ca is mainly determined by the magnitude and direction of the electrochemical gradient for Na it seemed interesting to determine whether external Ca ions are vital for the effect of inhibitors of membrane ATP-ase on acetylcholine release.

In addition, a study was made of the effect of Mg-excess and of noradrenaline and adrenaline on the acetylcholine output augmented by inhibition of membrane ATP-ase.

METHODS

Rats weighing 140–180 g were killed by stunning. The brains were immediately removed from the skull. A slice of cortex (weighing 120–200 mg and less than 1·2 mm thick) was cut from each hemisphere. Two to three slices were incubated at 36° C in glass chamber of 2 ml. containing Krebs solution gassed with 5 % CO_2 in oxygen. The acetylcholine released from the cortical slices was collected in the presence of physostigmine sulphate (2×10^{-6} g/ml.) and assayed on guinea-pig ileum as described by Paton & Vizi (1969). The pre-incubation period with physostigmine sulphate was 60 min.

In other series of experiments longitudinal muscle strips of guinea-pig ileum were used. The preparation of the strips and the method of electrical stimulation were the same as described by Paton & Vizi (1969). The strips were suspended in Krebs solution at 36° C in an organ bath of 3·5 ml., bubbled with 95 % O_2 and 5 % CO_2. Physostigmine (2×10^{-6} g/ml.) was added to the Krebs solution to prevent enzymic hydrolysis of acetylcholine. Since physostigmine sulphate requires time for its complete action to develop, a period of preincubation of 60 min was allowed. In some experiments an intermittent stimulation technique was used (Knoll & Vizi, 1970, 1971) in order to study the acetylcholine output by the first shocks at a high frequency of stimulation.

The acetylcholine output was expressed as ng/g.min; the acetylcholine output per pulse was calculated as described by Paton & Vizi (1969) and expressed as ng/g.volley.

The composition of normal Krebs solution and its modifications were as follows. (*a*) Normal Krebs solution (mM), NaCl 113; KCl 4·7; $CaCl_2$ 2·5; KH_2PO_4 1·2; $MgSO_4$

1·2; $NaHCO_3$ 25; glucose 11·5. (*b*) Na-substituted solution contained equimolar amounts of sucrose or LiCl in place of NaCl; the $NaHCO_3$ was replaced with $KHCO_3$, KCl and KH_2PO_4 were omitted. (*c*) Ca-free solutions were made by omitting $CaCl_2$. (*d*) For barium-Krebs solution, $CaCl_2$ was replaced by $BaCl_2$, and $MgCl_2$ replaced an equimolar amount of $MgSO_4$. Before the assay of such solutions for acetylcholine content, barium was precipitated by adding Na_2SO_4 to a concentration of 15 mM in the assay fluid, after shaking the fluid was centrifugated and the overflow used. (*e*) Mg-Krebs solution was prepared by adding $MgCl_2$ as much as required. (*f*) Li-Krebs solution was made by replacing NaCl by equimolar LiCl. In all assays, the standard acetylcholine solutions were made in appropriately modified solution. When equilibrated with a mixture of 95 % O_2 and 5 % CO_2 the solutions had a pH between 7·1 and 7·3.

Drugs. The drugs used were: acetylcholine chloride (B.D.H.; unless otherwise stated values given in the paper are given as the acetylcholine salt, wt.: 181·7); physostigmine sulphate (Burroughs Wellcome); (−)-noradrenaline bitartrate (Koch Light); (±)-adrenaline bitartrate (Burroughs Wellcome); ouabain (B.D.H.); Na-*p*-hydroxymercuribenzoate (Sigma); 2,4-α-dinitrophenol, LiCl (Fluka) and KCN (Fluka).

A Ca buffer system was employed in order to omit Ca from the solution. Since ethyleneglycol-bis-(β-aminoethyl-ether)-*N*,*N*′-tetra-acetic acid (EGTA, Fluka) possesses a low Mg-EGTA formation constant, EGTA buffer system was used when the calcium was omitted, and normal Mg level was required.

Concentrations of the drugs are given in moles or in terms of their salts, as indicated in the text.

RESULTS

Spontaneous release of acetylcholine from rat cortex

The spontaneous release of acetylcholine in the presence of physostigmine sulphate averaged 6·6 ng/g.min (36·2 p-mole/g.min), and varied between 1·5 and 11·4 ng/g.min in twenty-one slices. The output was very constant throughout experiments which lasted 2–3 hr. These results were obtained on slices of rat cortex which had been pre-incubated for 1 hr in eserinized Krebs solution. Polák & Meeuws (1966) obtained an average acetylcholine output from the same preparations of 13·1 ng/g.min. The higher output in their experiments can be attributed to the use of soman (pinacolylmethylphosphonofluoridate), another type of cholinesterase inhibitor. However, the output in Bowers' (1967) experiments, 8·8 ng/g.min in the presence of physostigmine is in good agreement with the present work. As can be seen from Table 1, neither tetrodotoxin, nor noradrenaline had any effect on the acetylcholine release. Both were found (Paton *et al.* 1971) to reduce the resting output by 50–80 % from the nerve terminals of longitudinal muscle strip of guinea-pig ileum, where propagated activity always contributes to the amount of acetylcholine released during rest. Ca removal in the presence of EGTA (1 mM) caused only a slight reduction in output (Table 1). On the other hand, when the concentration of K in the Krebs solution was increased to 24 mM by replacement of NaCl, there was an increase in the release of acetylcholine.

This effect is consistent with observations on other tissues, for example, longitudinal muscle strip (Paton *et al.* 1971), ganglion (Brown & Feldberg, 1936) and neuromuscular junction (Liley, 1956). Mann, Tennenbaum & Quastel (1939) and Polák & Meeuws (1966) also showed that high external K releases acetylcholine from cortex slices. In a more detailed study Vizi, Illés, Rónai & Knoll (1972) showed that there is 3 to 4 power relation between $[K^+]_o$ and acetylcholine release which is in agreement with that observed by Liley (1956) for the neuromuscular junction and by Paton *et al.* (1971) for the nerve terminals of Auerbach's plexus.

TABLE 1. Acetylcholine release from slices of the rat cortex. n = number of experiments; n.s. = not significant, $P > 0.05$. $^+$ collection period, 10 min; $^{++}$ NaCl was replaced by equimolar concentration of LiCl; $^{+++}$ the glucose was withdrawn and replaced by NaCl. The acetylcholine release was measured for 70 min and the highest output in a 10 min collection period was taken into account in each experiment. For experiments with cyanide the acetylcholine output was measured for 90 min and the highest 10 min output is given

	n	ACh release$^+$, ng/g.min (mean ± S.E.)	Significance (P)
1. Control (Krebs soln.)	21	6·6 ± 0·3	
2. (−)-Noradrenaline, 10^{-6} g/ml.	3	5·9 ± 0·5	2:1 n.s.
3. Adrenaline, 10^{-6} g/ml.	3	5·8 ± 0·7	3:1 n.s.
4. Tetrodotoxin, 5×10^{-7} g/ml.	5	6·3 ± 0·6	4:1 n.s.
4. Ca^{2+}-lack + EGTA, 1 mM	6	4·1 ± 0·3	5:1 < 0·01
6. Mg^{2+}-excess, 9·3 mM	4	4·9 ± 0·4	6:1 < 0·05
7. K^+-excess, 24 mM	3	24·5 ± 1·6	7:1 < 0·01
8. Li^+-Krebs^{++}	4	12·4 ± 0·8	8:1 < 0·01
9. Ca^{2+}-lack + Li^+-Krebs	3	8·2 ± 0·3	9:8 < 0·01
10. KCN, 10^{-3} M	3	7·0 ± 0·5	10:1 n.s.
11. Glucose-free Krebs^{+++}	4	6·0 ± 0·6	11:1 n.s.

If NaCl was replaced by LiCl the acetylcholine output was significantly enhanced (Table 1), although, on replacement of NaCl by sucrose there was only a slight increase in the output. Withdrawal of Ca from the Krebs solution significantly reduced the effect of Li.

Metabolic inhibitors, such as KCN and dinitrophenol (DNP) did not enhance the acetylcholine release from the cortical slices (Table 1 and Fig. 1), although a limited increase by DNP was observed after some minutes (Fig. 1).

Increase by ouabain of acetylcholine output from cortical slices

It was shown that ouabain increases the acetylcholine release from the nerve terminals of cholinergic nerves in longitudinal muscle strips of guinea-pig ileum (Paton *et al.* 1971) and increases the frequency of occurrence of m.e.p.p.s (Elmquist & Feldman, 1965; Birks, 1963; Birks &

Cohen, 1968). In these experiments ouabain in a concentration of 2×10^{-5} M also increased the acetylcholine output from the cortex slices of rats from $6{\cdot}3 \pm 0{\cdot}2$ to $53{\cdot}6 \pm 3{\cdot}3$ ng/g.min (S.E. of mean, Fig. 2*a*).

Omission of Ca from the incubation medium in the presence of 1 mM-EGTA did not prevent the effect of ouabain on acetylcholine release from slices of cortex (Fig. 2*b*), although the output was smaller.

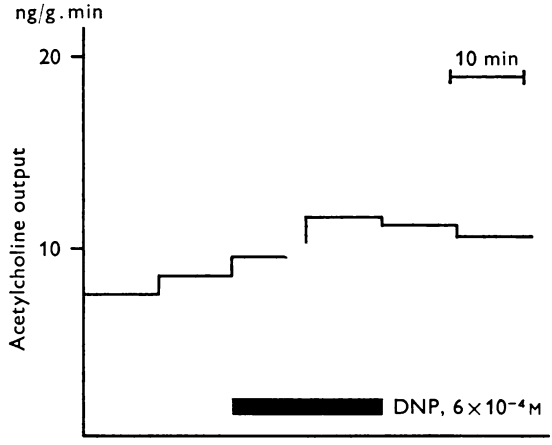

Fig. 1. The effect of 2,4-dinitrophenol (DNP) on the acetylcholine output from slices of the rat cortex. Average of two experiments.

A similar observation was made on the longitudinal muscle strip preparation (Fig. 3). Before applying ouabain, Ca withdrawal and 1 mM-EGTA significantly reduced the resting acetylcholine output (from $53{\cdot}5 \pm 5{\cdot}2$ to $10{\cdot}7 \pm 3{\cdot}6$ ng/g.min) and inhibited the output in response to stimulation at 10 Hz; the output of $810{\cdot}0 \pm 31{\cdot}6$ ng/g.min was reduced to $23{\cdot}0 \pm 4{\cdot}1$ ng/g.min, indicating the high Ca dependence of acetylcholine output evoked by stimulation. Ouabain (2×10^{-5} M), however, was still able to enhance the acetylcholine output. In control experiments ouabain in a concentration of 2×10^{-5} M increased the acetylcholine output during a 1-min collection period from $60{\cdot}5 \pm 8{\cdot}6$ to $350{\cdot}5 \pm 41{\cdot}6$ ng/g.min (S.E. of mean; $n = 4$).

Since the nerves of the longitudinal muscle strip of guinea-pig ileum possess the advantage of being readily accessible to externally applied substances and releases relatively large amounts of acetylcholine, they provide an opportunity for studying the acetylcholine output during a short collection period (Fig. 4). Ouabain, 2 ± 10^{-4} M, increased the acetylcholine output, immediately after its administration. The highest rate of release was reached in the first 15-sec sample collected.

The effect of Na deficiency on the action of ouabain was studied. Ouabain, 2×10^{-5} M, in the absence of Na in the external medium failed to increase the acetylcholine output from slices of the rat cortex which had previously been exposed to Na-free Krebs solution for 10 min. Output in Na-free solution was 24·5 ng/g.min, while output in Na-free solution with ouabain was 26·4 ng/g.min. However, when Na was withdrawn and

ouabain was added to the bath immediately, an increased acetylcholine release was observed for a short period (1 min). The output of acetylcholine reached 20·5 ng/g.min in the absence of Na, and 27·8 ng/g.min when ouabain (2×10^{-5} M) was also added. In the following periods, however, there were no differences between the Na-free and Na-free plus

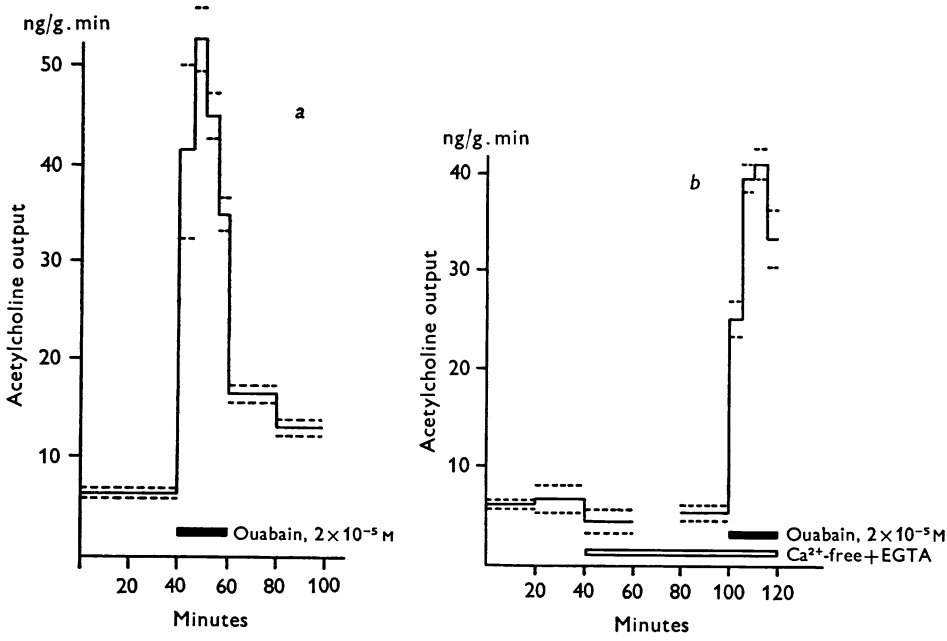

Fig. 2. Increase of acetylcholine output from cortical slices of the rat by ouabain in the presence and absence of Ca ions. (*a*) Time course of ouabain action. Abscissa: time in min. Ordinate: acetylcholine output in ng/g.min. The values of acetylcholine output are the means of four experiments with identical schedules. Dashed lines indicate S.E. (*b*) The effect of ouabain on acetylcholine output from cortical slices which had been exposed to 1 mM-EGTA in Ca-free Krebs solution. Means of three experiments with identical schedules. Note the effectiveness of ouabain in Ca-free solution. The expected Ca concentration when no Ca is added is about $5{\cdot}8 \times 10^{-10}$ M (Hubbard, Jones & Landau, 1968*b*).

ouabain treated slices. The impairment of synthesis by Na deficiency (Birks, 1963; Paton *et al.* 1971) probably affects the estimate of the acetylcholine-releasing capacity of ouabain in the absence of sodium, since it would be expected that the release would have been higher had the tissue not become depleted of acetylcholine. This is, in fact, the case in control experiments (see e.g. Fig. 2*a*).

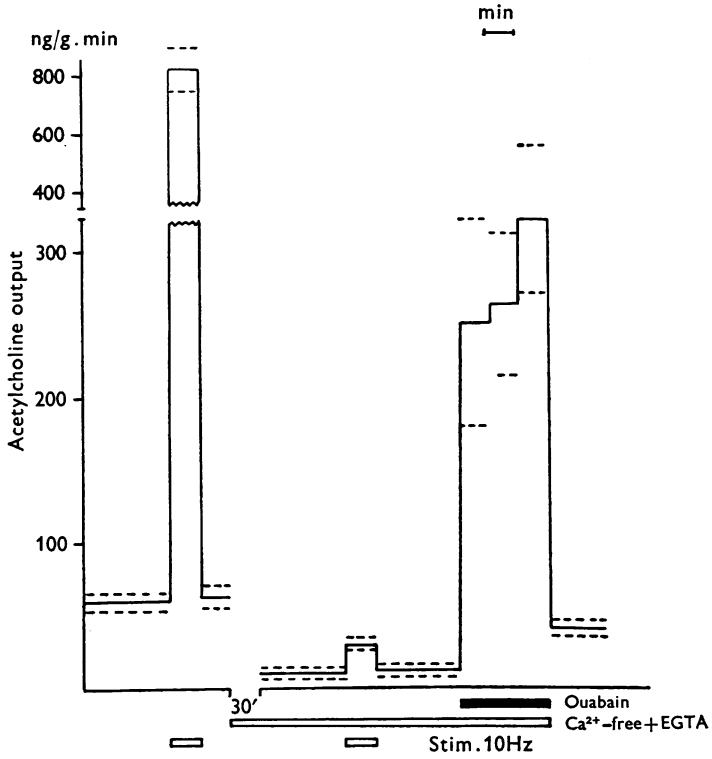

Fig. 3. Increase by ouabain (2×10^{-5} M) of acetylcholine release from the longitudinal muscle strip of the guinea-pig ileum in the absence of Ca ions. The values of acetylcholine release (ng/g.min) are the means of three identical experiments. Dashed lines indicate S.E. There was a 30 min period during which the acetylcholine output was not measured, as indicated on the abscissa. Stimulation period was 1 min at 10 Hz. Note that although the output in response to stimulation was completely blocked by omission of Ca in the presence of 1 mM-EGTA, ouabain still caused an increase.

Increase by Na-p-hydroxymercuribenzoate of acetylcholine output in cortical slices from rat brain

PHMB in a concentration of 5×10^{-4} M also enhanced the acetylcholine release from the nerve terminals of rat cortical slices (Fig. 5*a*). The resting output was increased from $4{\cdot}5 \pm 0{\cdot}5$ to $26{\cdot}5 \pm 1{\cdot}8$ ng/g.min. However, when Ca was withdrawn and EGTA (1 mM) was added to the bath, PHMB (5×10^{-4} M) was still able to enhance the acetylcholine release (Fig. 5*b*); the highest output observed was $21{\cdot}8 \pm 3{\cdot}1$ ng/g.min.

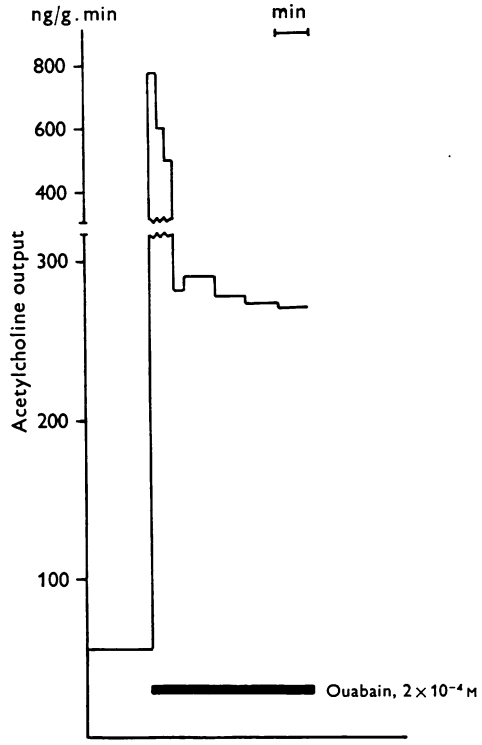

Fig. 4. Time course of ouabain (2×10^{-4} M) effect on acetylcholine output from the longitudinal muscle strip of the guinea-pig ileum. Five strips were in the organ bath of 3·5 ml. The value of acetylcholine output is the mean of two experiments with identical treatment schedules. Note the immediate action of ouabain. In the first minute there were four collection periods, each of 15 sec.

Increase of acetylcholine output by Na withdrawal from cortical slices of rats

On switching from normal Krebs solution to Na-free solutions, the basal release of acetylcholine invariably increased at first but subsequently fell (Fig. 10*a*). In one experiment the acetylcholine release in Na-free medium was measured over a period of 2½ hr; the output fell to normal value by 120 min. This is probably due to the impairment of synthesis by Na deficiency. When the Ca was omitted (two experiments) from the medium, Na withdrawal was still able to enhance acetylcholine release from cortex slices. The highest outputs observed were 19·8 and 16·3 ng/g.min, respectively. A similar observation was made by Paton *et al.* (1971) on the longitudinal muscle strip of guinea-pig ileum.

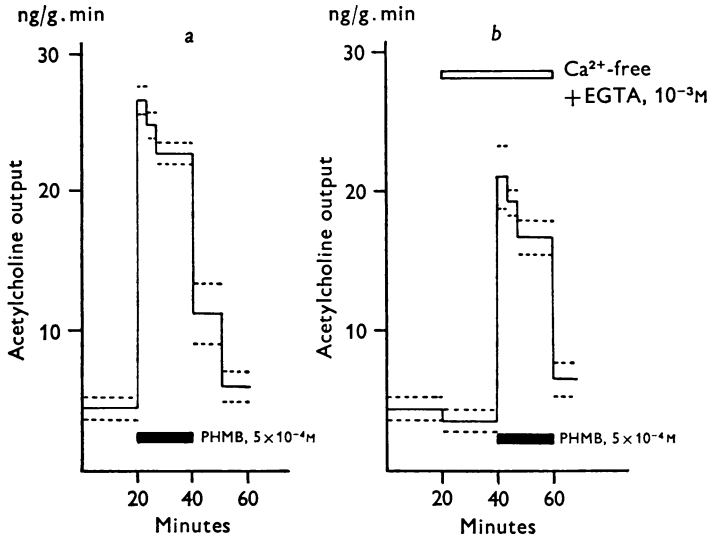

Fig. 5. Increase of acetylcholine output from cortical slices of the rat by *p*-hydroxymercuribenzoate (PHMB) in the presence and absence of Ca-ions. (*a*) Time course of PHMB (5×10^{-4} M). Three identical experiments. Dashed lines indicate S.E. (*b*) The effect of PHMB (5×10^{-4} M) on the acetylcholine output from cortical slices which had been exposed to 1 mM-EGTA in Ca-free (Ca^{2+}-free) Krebs solution. Three experiments. Mean values of acetylcholine output, S.E.

Reduction by noradrenaline and magnesium of acetylcholine output

In a previous paper (Paton & Vizi, 1969) we described the inhibitory action of noradrenaline and adrenaline on acetylcholine release from parasympathetic nerves. Noradrenaline or adrenaline reduced the transmitter output during rest or at a low frequency of stimulation. The acetylcholine output by sustained stimulation at high frequencies (5–20 Hz) was not decreased by noradrenaline. However, noradrenaline and adrenaline were effective when the frequency of stimulation was higher, but was given short bursts of 2–20 shocks in a train (Knoll & Vizi, 1970, 1971; Vizi & Knoll, 1971). Mg, which is believed to antagonize the action of Ca (Hubbard, 1961; Hubbard, Jones & Landau, 1968*a*, *b*; Elmquist & Feldman, 1965), also inhibited the acetylcholine released by stimulation of the longitudinal muscle strip (Fig. 6). The inhibition by Mg excess (9·3 mM) was very similar to that by noradrenaline: reduction was observed at low (0·1 Hz), but not at high (10 Hz) frequencies of stimulation. The resting release was also reduced, from $64{\cdot}2 \pm 8{\cdot}1$ to $11{\cdot}4 \pm 3{\cdot}5$ ng/g.min. The reduction is significant, $P < 0{\cdot}01$. Mg^{2+}-excess (9·3 mM) reduced the acetylcholine output at 0·1 Hz stimulation from 11·3 to 3·0 ng/g.volley.

However, it did not affect the output induced by 10 Hz stimulation; 1·37 ng/g.volley was the output in the normal Krebs solution (1·2 mM-mg^{2+}) and 1·29 ng/g.volley in the Mg^{2+}-enriched media (9·3 mM-Mg^{2+}). The total outputs during stimulation at 10 Hz for 1 min were 882·0 ± 35·0 and 785·0 ± 16·0 ng/g.min, respectively (Fig. 6). A similar correlation between frequency of stimulation and Mg concentration has been observed by Cowie, Kosterlitz & Watt (1968).

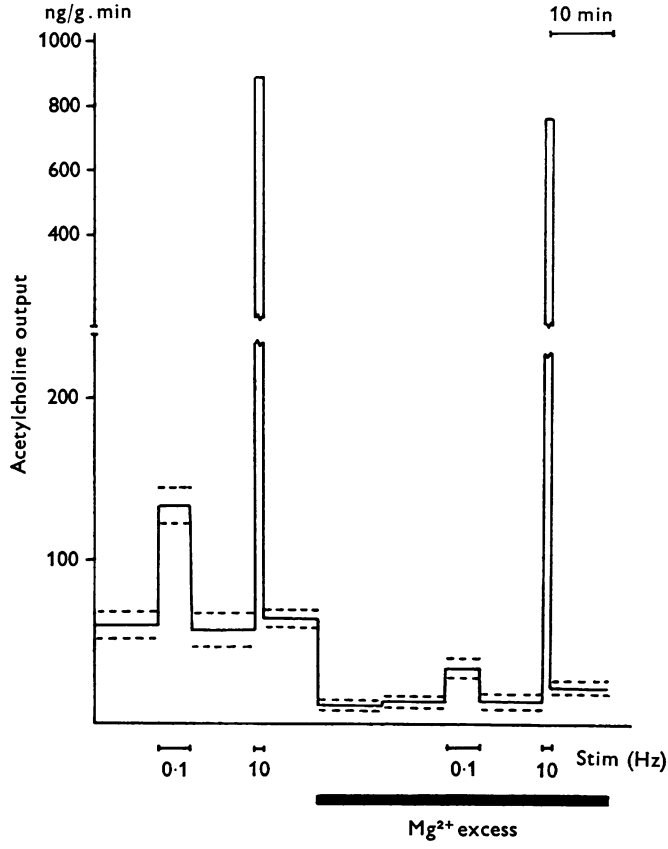

Fig. 6. Effect of Mg^{2+}-excess (9·3 mM) on acetylcholine output from the longitudinal muscle strip under resting condition, or stimulation at 0·1 or 10 Hz as indicated. Mean of three experiments; dashed lines indicate S.E. Stimulation periods were 5 min at 0·1 Hz and 1 min at 10 Hz. Note the relative ineffectiveness of Mg^{2+}-excess during stimulation at 10 Hz.

However, excess of Mg (9·3 mM) depressed the acetylcholine output induced by a train of five pulses applied at intervals of 100 msec (10 Hz), but when the number of pulses per train was increased to 60, there was no longer a significant difference between the outputs of acetylcholine obtained

in normal and in Krebs solution containing 9·3 mM-Mg^{2+} (Table 2). A similar observation was made with noradrenaline (Knoll & Vizi, 1970, 1971; Vizi & Knoll, 1971).

Having established the similarity in effect on acetylcholine output between noradrenaline and Mg excess, the question arose as to their actions on acetylcholine output induced by different conditions all known to lead to inhibition of membrane ATP-ase in nervous tissue. Fig. 7 illustrates that the acetylcholine release increased by ouabain (2×10^{-5} M) was reduced by noradrenaline (2×10^{-6} g/ml.) or by Mg excess (9·3 mM). When cortical slices were exposed to ouabain for 20 min, the acetylcholine release was as much as $28{\cdot}2 \pm 1{\cdot}5$ ng/g.min; however, noradrenaline (Fig. 7*b*) or Mg^{2+} excess (Fig. 7*c*) reduced the output. The reductions were significant: $P < 0{\cdot}01$ in both cases. PHMB in a concentration of 5×10^{-4} M also enhanced the acetylcholine release from cortical slices of rats (Fig. 8). After 20 min preincubation with adrenaline (10^{-6} g/ml.), which otherwise failed to reduce the resting output of cortex slices (Fig. 8*b* and Table 1), the effect of PHMB was significantly reduced (Fig. 8*b*). Mg^{2+} excess also proved to be effective in preventing the increase of acetylcholine output by PHMB (Fig. 8*c*).

Paton *et al.* (1971) showed that when Ba replaces Ca in the medium the resting release of acetylcholine from longitudinal muscle strips increases markedly. A similar result was observed in the rat cortex in the present experiments. Ca replacement with Ba increased the transmitter output from $4{\cdot}6 \pm 0{\cdot}5$ to $19{\cdot}5 \pm 2{\cdot}5$ ng/g.min (Fig. 9). Mg^{2+} excess (9·3 mM) was fully effective in inhibiting the acetylcholine output augmented by Ba. It is interesting that the resting output was not affected (Table 1 and Fig. 9*b*) by Mg^{2+}. Adrenaline, 10^{-6} g/ml., also reduced the increased output, although to a lesser extent (Fig. 9*c*).

Na withdrawal caused an increase of acetylcholine release from cortical slices of rats (Fig. 10*a*). Furthermore, Na withdrawal increased the release to the same extent in normal and excess Mg (Fig. 10*b*). Noradrenaline 10^{-6} g/ml., did not reduce the output induced by Na withdrawal.

DISCUSSION

Many investigators have used isolated slices of cerebral cortex to study acetylcholine release (e.g. Mann, Tennenbaum & Quastel, 1939; McIlwain & Joanny, 1963; Polák & Meeuws, 1966; Okamoto & Quastel, 1970; Vizi & Knoll, 1972). This method makes it possible to study the effect of drugs on cholinergic nerve endings separate from cell bodies (Hebb, Krnjević & Silver, 1963; Shute & Lewis, 1967). Such cortical slices maintain their

TABLE 2. Inhibitory effect of Mg^{2+} excess (9·3 mM) on the output per volley of acetylcholine induced by electrical stimulation of 10 Hz

Condition	No. of trains	Total no. of shocks	Total ACh output (ng/g)	Presumed resting output during stimulation period* (ng/g)	Calculated ACh output by a train (ng/g.train)	Volley output (ng/g volley)	Significance (P)
(a) Intermittent stimulation: trains of 5 pulses, train frequency: 0·1 Hz							
Krebs soln.	60	300	1850·0	401·0	241·0	4·83 ± 0·2	< 0·01
Mg excess (9·3 mM)	60	300	915·0	92·0	13·7	2·7 ± 0·1	
(b) Intermittent stimulation: trains of 60 pulses, train frequency: 0·1 Hz							
Krebs soln.	5	300	508·0	64·5	88·7	1·47 ± 0·2	> 0·1
Mg excess (9·3 mM)	5	300	431·0	26·5	80·9	1·34 ± 0·3	
(c) Sustained stimulation: 600 pulses, 10 Hz for 1 min							
Krebs soln.	1	600	905·0	62·5	842·5	1·4 ± 0·2	> 0·1
Mg excess (9·3 mM)	1	600	855·5	16·5	839·0	1·39 ± 0·1	

* Calculated from the preceding resting period.

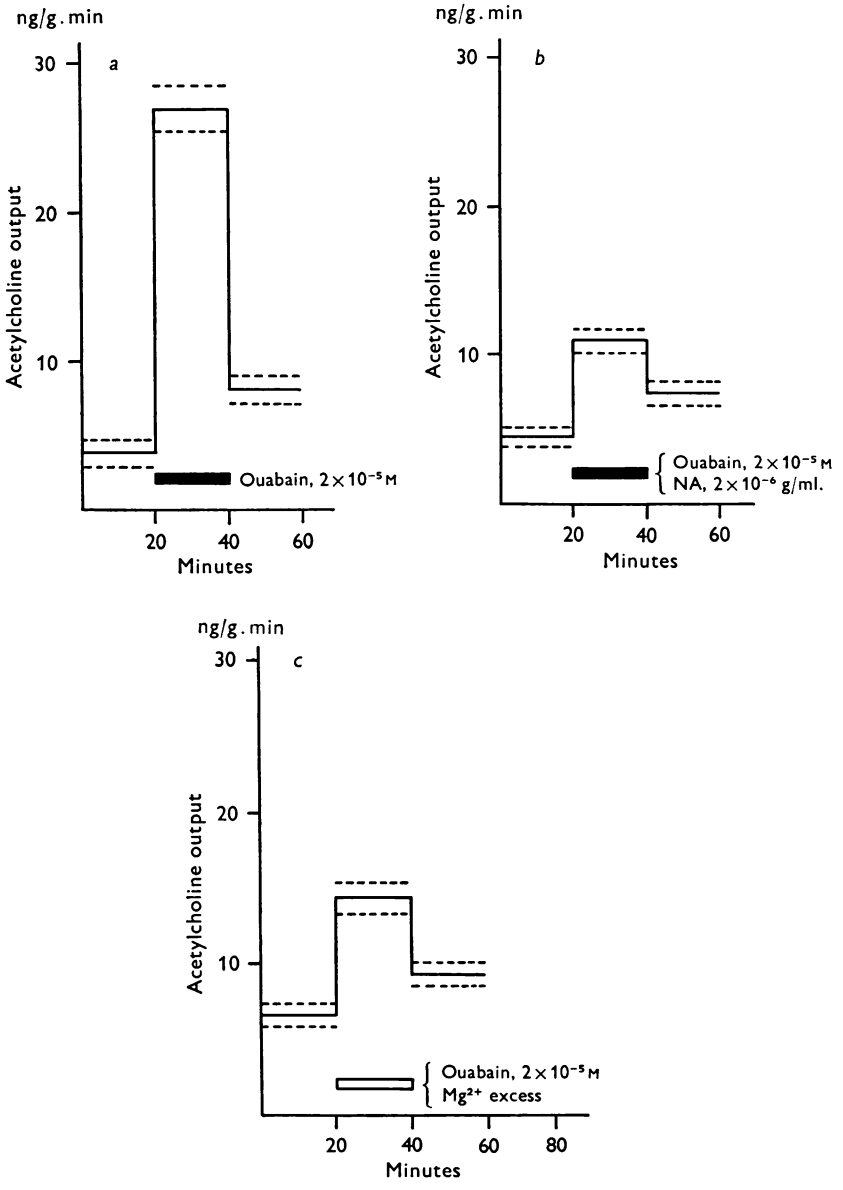

Fig. 7. Reduction by noradrenaline or Mg^{2+} excess of acetylcholine output produced by ouabain. Cortical slices from the rat. (*a*) Acetylcholine release by ouabain, (*b*) acetylcholine output by ouabain in the presence of noradrenaline (NA, 2×10^{-6} g/ml.) (three experiments), (*c*) Acetylcholine output by ouabain in the medium containing 9·3 mM-Mg (four experiments).

metabolic activity (Krebs, 1950) and their K content (Creese, & Taylor, 1967) for several hours.

Tetrodotoxin did not reduce the resting output of acetylcholine from cortical slices indicating that the spontaneous release is not due to propagated activity as is partly the case in Auerbach's plexus (Paton *et al.* 1971). Furthermore, noradrenaline and adrenaline, which were found to be

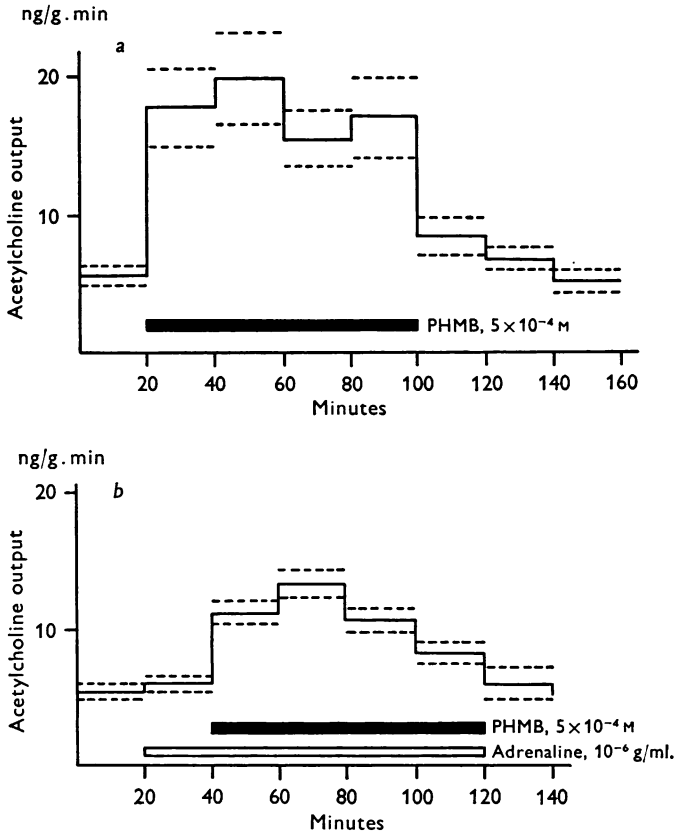

Fig. 8*a* and *b*. For legend see opposite page.

able to inhibit the acetylcholine release from parasympathetic nerve due to propagated activity (Paton & Vizi, 1969; Knoll & Vizi, 1970; Paton *et al.* 1971; Vizi, 1968; Vizi & Knoll, 1971), failed to reduce the resting acetylcholine output from cortical slices. This preparation, in fact, provides an opportunity for 'pure' spontaneous (resting) release to be examined. This release is likely to be quantal and the results can be compared with studies of m.e.p.p. frequency changes at the motor end-plate. K excess augmented the acetylcholine release. Lack of Ca or Mg^{2+} excess only slightly reduced

the output. When LiCl replaced NaCl in the Krebs solution there was an increase in acetylcholine release; however, LiCl was ineffective in Ca^{2+}-free media. Since Li increases the intracellular concentration of Ca as shown by Baker, Blaustein, Hodgkin & Steinhardt (1967), it is likely that an increase in intracellular Ca^{2+} is responsible for the increased release of transmitter.

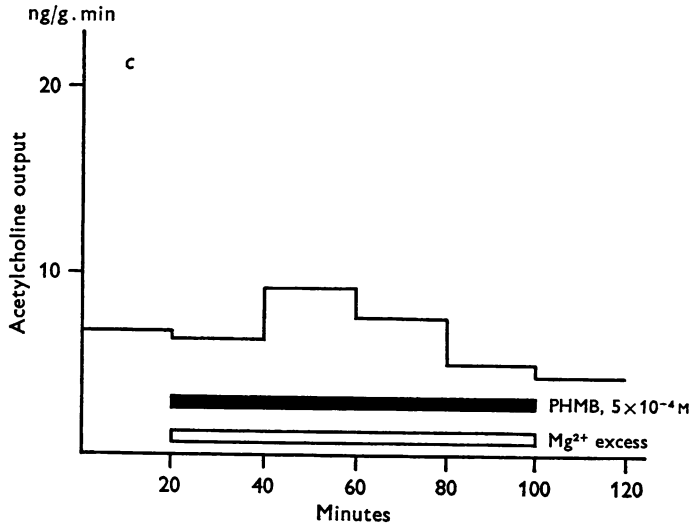

Fig. 8. Increase of acetylcholine output from the cortex slices of the rat by Na salt of *p*-hydroxymercuribenzoate (PHMB). (*a*) The effect of PHMB in the concentration of 5×10^{-4} M. Three experiments with identical schedules. Mean ± S.E. (*b*) Acetylcholine output by PHMB in the presence of adrenaline (10^{-6} g/ml.). Four experiments. Dashed lines indicate S.E. (*c*) Acetylcholine release by PHMB in Krebs solution containing 9·3 mM-Mg. The values of acetylcholine output (ng/g.min) are the means of two experiments with identical schedules. Dashed lines indicate S.E.

In a preceding paper (Paton *et al.* 1971) it has been shown, that a variety of experimental conditions known to lead to inhibition of (Na^+-K^+-Mg^{2+})-activated ATP-ase was able to induce acetylcholine release from parasympathetic nerves. Ouabain, PHMB, Na or K removal were capable of enhancing release. The present experiments show that using similar conditions the acetylcholine output from slices of rat cortex is also increased. However, it is also known, that all the conditions used are able, as a consequence of the inhibition of membrane ATP-ase, to change the intracellular concentration of Na^+, K^+ and Ca^{2+}. The reduction in sodium pump activity leads to a gain of $[Na^+]_i$, a loss of $[K]_i$ and possibly changes in the intracellular concentration of Ca^{2+}, together with a fall of resting potential.

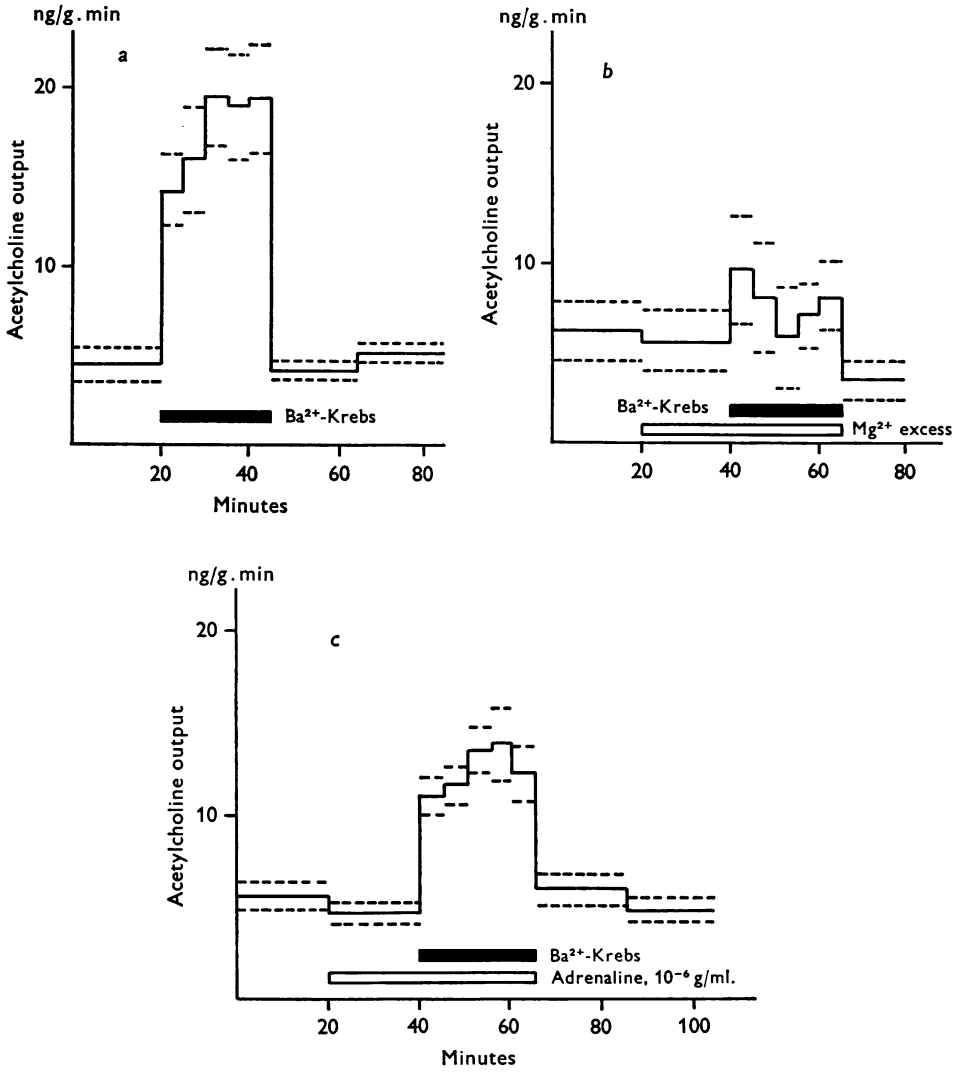

Fig. 9. Effect of Ca omission and its substitution with Ba on acetylcholine resting output from cortex slices of the rat. For procedure of barium extraction from the Krebs assay, see Methods. (*a*) Acetylcholine output in Ba^{2+}-Krebs. $CaCl_2$ was substituted by aequimolar $BaCl_2$. Three experiments. Mean ± S.E. (*b*) Acetylcholine output in Ba^{2+}-Krebs solution in the presence of high Mg (9·3 mM). $MgSO_4$ was used and increased to a final concentration of 9·3 mM. Three experiments. Mean ± S.E. (*c*) Acetylcholine output in Ba^{2+}-Krebs solution in the presence of adrenaline (10^{-6} g/ml.). Four experiments with identical treatment schedules. Mean values of acetylcholine output, S.E.

The role of depolarization. During ouabain and PHMB treatment there is an accumulation of Na and loss of K at the nerve terminals resulting from inhibition of the Na pump. These ionic changes lead to depolarization of the nerve terminals, which in turn could give rise to the increased acetylcholine release. However, the kinetic study of ouabain action (Fig. 4) shows that the increase of acetylcholine release promptly appears after administration of ouabain. In spite of the large surface area/volume of

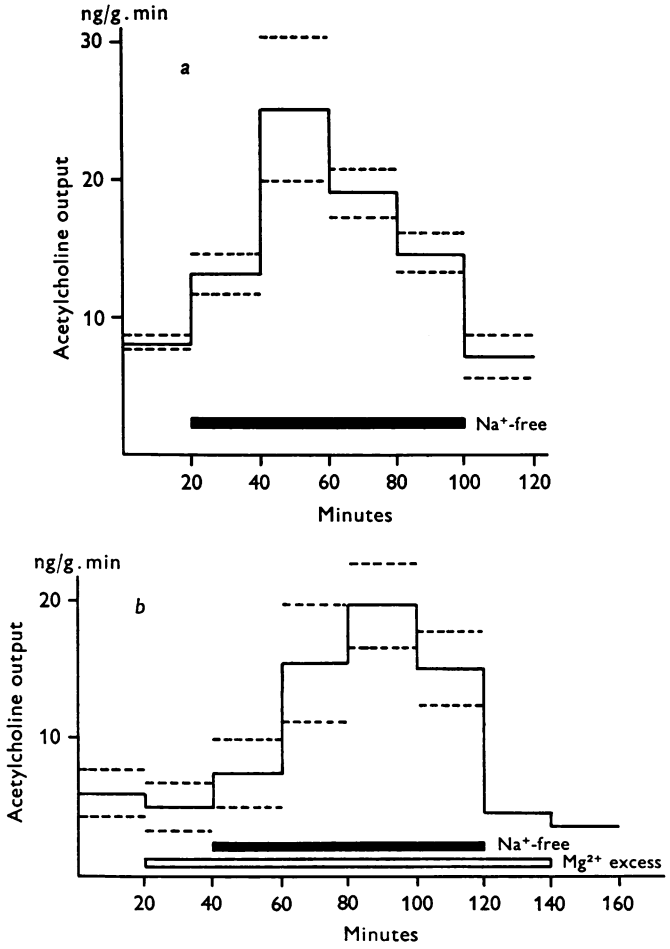

Fig. 10. Increase of acetylcholine output by total Na deprivation. Cortex slices of the rat. Na was replaced by sucrose. For details see Methods. (*a*) Time course of acetylcholine release during omission of Na ions. Three experiments. Means ± S.E. (*b*) Acetylcholine output during Na deprivation in Krebs solution containing Mg in a concentration of 9·3 mM. The values of acetylcholine output are the means of three experiments with identical schedules.

cells, it seems unlikely that ionic movements within 15 sec of ouabain application could be sufficient to lead to significant depolarization. After inhibition of the Na pump the changes in ionic content and the concomitant depolarization develop slowly, although the output increased quickly at first and subsequently fell. Furthermore, although there is yet no evidence that Ca^{2+} replacement by Ba^{2+} should cause a depolarization, Ba also augmented the transmitter release. Also, no depolarization could be expected to result from the withdrawal of Na^+, but a considerable increase of acetylcholine release was observed.

The role $[Na^+]_i$. The $[Na^+]_i$ accumulated by the inhibition of membrane ATP-ase could be responsible for enhanced acetylcholine release, as suggested by Birks (1963). However, Katz & Miledi (1967) showed that Na ions are not necessary at all for acetylcholine liberation. Furthermore, Rahamimoff (1970) also found that when Ca^{2+} replaces Na^+ acetylcholine is still liberated spontaneously and extra release is evoked by depolarization of the terminals. Na withdrawal, which is likely to reduce the intracellular Na concentration, has been found to increase the acetylcholine output from the parasympathetic nerves (Paton *et al.* 1971). In my experiments this procedure also augmented the release of acetylcholine from cortical slices. Li, which also reduces intracellular Na^+ concentration, enhanced the release of acetylcholine. These data indicate that an augmentation of intracellular Na is not essential in the increase of acetylcholine output. The accumulation of $[Na^+]_i$ seems to be important in the effect of ouabain, since the omission of $[Na^+]_o$ reduced the effectiveness of ouabain. If there is a quantitative relation between the extent of inhibition of (Na^+-K^+-Mg^{2+})-activated ATP-ase and the transmitter release the removal of Na^+ and ouabain should be additive. This was certainly not the case in these experiments. However, it should be kept in mind that the $[Na^+]_i$ plays an important role in maintaining the normal rate of synthesis for acetylcholine (Birks, 1963; Paton *et al.* 1971; Vizi *et al.* 1972). In addition, the readily mobilized stores (Birks & McIntosh, 1961; Paton & Vizi, 1969; Collier & McIntosh, 1969) play an important role in the amount of acetylcholine available for release. Thus, the fact that ouabain was not able to increase further the acetylcholine release from the cortical slices which had been augmented by Na removal might be attributed to the lack of freshly synthesized acetylcholine which is in turn mainly available to be released (Collier & McIntosh, 1969). Furthermore, the omission of glucose, which also leads to uptake of Na^+ in brain slices (Okamoto & Quastel, 1970), did not increase the acetylcholine output (Table 1).

The role of Ca ions. The important role of Ca ions in transmitter release is generally accepted (cf. Rubin, 1970). But how Ca^{2+} acts is not known with certainty. In the absence of Ca the action potential still invades the nerve

terminals but fails to induce release of transmitter (Katz, 1969). The main role of Ca is to mediate between the presynaptic depolarization and the transmitter release. It has been suggested (Dodge & Rahamimoff, 1967; Hubbard *et al.* 1968*a*, *b*), that Ca affects release of acetylcholine by combination with receptor sites in the nerve terminals and that Mg^{2+} and Ca^{2+} compete for these specific sites (Hubbard *et al.* 1968*a*, *b*; Rahamimoff, 1970). Katz & Miledi (1967, 1969) showed that the inward movement of Ca is important in the release of acetylcholine. Birks & Cohen (1968) put forward the Na^+ entry hypothesis (Birks, 1963) and suggested that the digoxin-induced increase in m.e.p.p. frequency arises from an acceleration by intracellular Na^+ of Ca influx. This idea is in agreement with the findings that the Ca influx is accelerated not only by ouabain (Baker, Blaustein, Hodgkin & Steinhardt, 1969; Stahl & Swanson, 1969) and by the action potential (Katz & Miledi, 1965, 1967; Miledi & Slater, 1966), but also by maintained depolarization (Hodgkin & Keynes, 1957) all known to increase acetylcholine release.

Baker and his colleagues have shown that on the axons of *Loligo forbesi* and *Maia squinado* (Baker *et al.* 1967, 1969; Baker & Blaustein, 1968) Ca^{2+} entry is increased by increasing the intracellular Na concentration or lowering external Na concentration. This was the case on isolated nerve terminals, as well (Blaustein & Weisman, 1970). This can be the case in our experiments when the (Na^+-K^+-Mg^{2+})-activated ATP-ase is inhibited by some procedure. It was also shown (Swanson, 1968; Stahl & Swanson, 1969) that ouabain increases the intracellular Ca^{2+} concentration of cortex slices. The question arises now, whether the Ca^{2+} influx is the critical step in the increase of acetylcholine release in consequence of the inhibition of membrane ATP-ase. The experiments presented in this paper provides some evidence that $[Ca^{2+}]_o$ is not essential in the acetylcholine release when a membrane ATP-ase inhibitor is used. Ca removal in the presence of 1 mM-EGTA failed to prevent the acetylcholine output augmented by ouabain or by PHMB, which are known to inhibit the activity of (Na^+-K^+-Mg^{2+})-activated ATP-ase. In the medium lacking both Na and Ca there is an increase of acetylcholine release from enteric cholinergic nerve terminals (Paton *et al.* 1971). In considering the role of Ca ions in the release of acetylcholine by ouabain and by PHMB the question arises as to whether the amount of intracellular Ca or whether the amount of Ca passing through the membrane is important. Since DNP and CN, which increase dramatically the intracellular concentration of Ca by releasing it from mitochondria (Baker, 1971) and also increase the Na content of cortical slices (Okamoto & Quastel, 1970), failed to augment reasonably the acetylcholine release from cortical slices (Table 1, Fig. 1), the effect of Ca seems to be located in the membrane. This seems to be sup-

ported by the findings of Miledi & Slater (1966) who showed that the intracellular injection of Ca ions fails to cause transmitter release previously suspended by omission of $[Ca^{2+}]_o$. These observations could not, however, lead to the conclusion that Ca is not at all involved in the acetylcholine release when a membrane ATP-ase inhibitor is used, because there is a large internal store of Ca in the mitochondria and possibly also in the membrane and the extent to which the intracellular Ca is bound may well depend on the intracellular concentration of Na^+ and K^+. It is rather suggested that the Ca released may take part additionally in the release of acetylcholine by inhibition of membrane ATP-ase (Skou, 1957; Somogyi & Vincze, 1962; Somogyi, 1964).

The role of inhibition of (Na^+-K^+-Mg^{2+})-activated ATP-ase in the release mechanism of acetylcholine. Paton *et al.* (1971) presented evidence that conditions known to inhibit membrane ATP-ase augmented the acetylcholine release. They put forward the hypothesis that under physiological condition calcium may act by inhibiting the membrane ATP-ase provoking release of acetylcholine. Baker (1971) presented convincing evidence that the movement of Ca is mainly determined by the magnitude and direction of the electrochemical gradient for Na. The changes in nervous activity of membrane lead to changes in the concentration of Ca inside the cell. The late phase of Ca influx (Baker, Hodgkin & Ridgway, 1970; Baker, 1971) resembles the Ca mechanism involved in transmitter release (Katz & Miledi, 1967, 1969) and can also be connected with the inhibition of membrane ATP-ase. The inward movement of calcium should transiently impair the activity of (Na^+-K^+-Mg^{2+})-activated ATP-ase which in turn would make the membrane ready for release mechanism, for exocytosis. Possibly, the activity of membrane ATP-ase provides energy for maintaining membrane stability. The inhibition of (Na^+-K^+-Mg^{2+})-activated ATP-ase by Ca can to some extent be reversed by an increase in the Mg concentration (Skou, 1960) and also by an increase in Na concentration (Portius & Repke, 1962). These data fit very well with our knowledge on the Ca/Mg and Ca/Na antagonism in transmitter release.

Ca removal in the presence of EGTA inhibited both the spontaneous and stimulated acetylcholine output from parasympathetic nerve terminals. However, the release evoked by ouabain was little affected; in this respect the results were clearly different from those obtained in the presence of Mg excess. In this preparation Mg excess had a similar effect to noradrenaline, it reduced the resting output evoked by a low frequency of sustained stimulation, or by brief stimuli at high frequencies.

Both Mg and noradrenaline reduced, but never inhibited totally, the increase of acetylcholine output in response to the inhibition of (Na^+-K^+-Mg^{2+})-activated ATP-ase by ouabain, PHMB and by Ba. However, the

output evoked by Na withdrawal was not affected either by Mg^{2+} or by noradrenaline. Mg is capable of stimulating the membrane ATP-ase and there is a great similarity in action between Mg and noradrenaline or adrenaline; therefore it is suggested that their inhibitory effect on acetylcholine release may be similar in mechanism.

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