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STUDIES OF THE EFFECTS OF AGENTS WHICH ALTER CALCIUM METABOLISM ON ACETYLCHOLINE TURNOVER IN THE RAT DIAPHRAGM PREPARATION

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1 We measured the effects of agents, which are thought to alter the Ca^{2+} concentration in cells, on tissue and released acetylcholine and choline of rat diaphragm using a gas chromatographic mass spectrometric assay for acetylcholine and choline.

2 Lanthanum and ouabain increased the resting output of acetylcholine, while 4-aminopyridine enhanced the evoked output of acetylcholine.

3 Guanidine first increased and then depressed acetylcholine release.

4 Theophylline, dinitrophenol and the ionophore, A-23187, had no measurable effect on acetylcholine release and tetraethylammonium inhibited the release of acetylcholine.

5 Dinitrophenol caused a highly significant increase of the tissue and released choline.

6 None of these agents increased the tissue acetylcholine content. Tetraethylammonium caused a large decline of tissue acetylcholine while ouabain and guanidine caused smaller reductions of acetylcholine in diaphragm.

7 These results are discussed in relation to the hypothesis that intraterminal Ca^{2+} activity regulates the synthesis of acetylcholine in diaphragm nerve endings.

Introduction

We recently postulated that the dual actions of the presynaptic neurotoxin, β-bungarotoxin on acetylcholine (ACh) release and synthesis in the rat diaphragm preparation were due to a substantial rise in the concentration of ionized calcium in the cytosol of nerve terminals (Gundersen, Newton & Jenden, 1980; Gundersen, Jenden & Newton, 1981). A wide variety of agents and treatments are known to cause an enhancement of transmitter release in nerve-muscle preparations (see Ginsborg & Jenkinson, 1976). In many instances the mechanism of this enhancement has been attributed to a rise in the nerve ending Ca^{2+} activity. For example, in electrophysiological studies, Thesleff and colleagues (Cull-Candy, Lundh & Thesleff, 1976; Lundh, Leander & Thesleff, 1977) used 4-aminopyridine (4-AP), tetraethylammonium (TEA), guanidine and the calcium ionophore, A-23187, to promote the Ca^{2+}-dependent release of transmitter in rat neuromuscular preparations. By analogy, we have monitored the effect of these and other agents on tissue and released ACh of the rat diaphragm using a gas chromatographic mass spectrometric (GCMS) assay for ACh.

Methods

Diaphragm preparation

The experimental protocol has been described in detail elsewhere (Gundersen *et al.*, 1981). Briefly, hemidiaphragms were dissected from male Sprague-Dawley rats (90 to 150 g) and fan-shaped segments (50 to 100 mg) with an intact costal margin were pinned in a temperature-regulated organ bath (36 to 38°C) containing 3.5 ml of oxygenated Krebs bicarbonate medium. When ACh release was to be measured, the tissue was equilibrated for 30 min in a medium containing physostigmine (15 μM). Other additions to or modifications of the Krebs solution are indicated in the text.

Depending on which drug was being tested, we measured its effects on either resting or evoked ACh release. Resting release was measured during 15 min collection periods with 1 min intervals between suc-

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cessive measurements. Evoked release was measured during indirect supramaximal stimulation for 10 min periods with 3 min rests between collections. Stimulation frequencies are given in the text.

Two types of experiments were usually performed with each agent. First, release measurements were made on separate preparations with or without the drug present. These experiments allowed a comparison between preparations of the effects of the drug on ACh release and on tissue ACh content. A second set of experiments was performed to confirm the effects of the drug on ACh release. In these trials the control rate of ACh output from a diaphragm was compared to ACh release from the same preparation with the drug present. This latter type of experiment is more sensitive for small changes in ACh output as it compensates for intrinsic differences in release rates among preparations.

At the end of each experiment the diaphragm tissue was cut free of the costal margin and placed in a tared tube containing 2.5 ml of 1.N formic acid in acetone (3:17, v/v) and internal standards of ACh and choline. The tissue was weighed and ACh and choline were extracted by a slight modification of the procedure outlined earlier (Gundersen *et al.*, 1981). We determined that 100% of the ACh and more than 95% of the choline are extracted from the diaphragm by the formic acid-acetone solution within 5 h at 4°C. Thus the tissue extractions were completed within 6 to 15 h rather than the 16 to 24 h reported previously.

Determination of acetylcholine and choline

ACh and choline were measured by an isotope dilution procedure using gas chromatographic mass spectrometry (GCMS) (Jenden, Roch & Booth, 1973; Gundersen *et al.*, 1981). The results for released ACh and choline are normalized to the wet weight of the diaphragm segment. Tissue ACh and choline content are given as pmol/mg wet wt. of the diaphragm segment. The values are the mean ± s.e. and the significance of the results was evaluated by the paired or unpaired Student's *t* test, as appropriate.

A modification of the normal assay protocol was necessary in the experiments with TEA. To ensure the complete extraction from aqueous samples of ACh and choline it was necessary to increase the volume of the ion pair extractant. Otherwise TEA, a quaternary amine, prevented the extraction of measurable quantities of ACh and choline.

Solutions and chemicals

The normal Krebs bicarbonate medium contained, (mM): NaCl 138, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, KH_2PO_4 0.4, $NaHCO_3$ 12 and glucose 11. A Tris-buffered Krebs was used in some experiments; it had the same composition as the Krebs bicarbonate medium except that the $NaHCO_3$ and KH_2PO_4 were omitted and Tris-HCl (20 mM) was used. The Krebs bicarbonate medium was equilibrated with 95% O_2, 5% CO_2 to a pH of 7.2 to 7.4, while the Tris-buffered Krebs was adjusted to pH 7.4 with 1 N NaOH. The Tris-buffered Krebs was aerated with 100% O_2. Modifications of the composition of these media are given in the text.

The calcium ionophore, A-23187, was provided by the Eli Lilly Company and was prepared as a stock solution (1 mg/ml) in ethanol. Guanidine hydrochloride, ouabain, lanthanum chloride, 2,4-dinitrophenol and theophylline were obtained from the Sigma Chemical Company (St. Louis, MO, U.S.A.). Tetraethylammonium bromide and 4-aminopyridine were purchased from Aldrich Chemical Company (Milwaukee, WI, U.S.A.).

Results

Eight agents that are thought to affect nerve ending Ca^{2+} metabolism were tested for their effects on tissue ACh content and ACh release in the rat phrenic nerve-diaphragm preparation. The doses of the agents employed and the time of exposure of the tissue to the drugs were selected to maximize the potential effects on the Ca^{2+} concentration in the nerve terminal cytosol.

Calcium ionophore, A-23187

The calcium ionophore, A-23187, increases the frequency of miniature endplate potentials (m.e.p.ps) in mammalian nerve-muscle preparations (Kao, Drachman & Price, 1976; Cull-Candy *et al.*, 1976). We used several different procedures to evaluate the effects of this compound on resting ACh release and diaphragm ACh content. In all experiments, Ca^{2+} was omitted from the Krebs solution during the treatment with ionophore to ensure the binding of the ionophore by the tissue (Selinger, Eimerl & Schramm, 1974).

Two different experiments using A-23187 (10 μM) are recorded in Figure 1. In the first series, the diaphragm was pretreated for 30 min with ionophore and resting ACh output was measured in two successive periods. Using this protocol, neither the output of ACh nor the tissue content of ACh were changed relative to controls treated with vehicle only (Figure 1a). In the second series, the efflux of ACh was measured while the ionophore was in the Ca^{2+}-free Krebs solution, and in the second period ionophore was absent and Ca^{2+} was present at 4 mM. Cull-Candy *et al.* (1976) observed a large increase in m.e.p.p. frequency in ionophore-treated preparations that were exposed to elevated Ca^{2+}. As in the first series, ACh release from ionophore-treated preparations was not signifi-

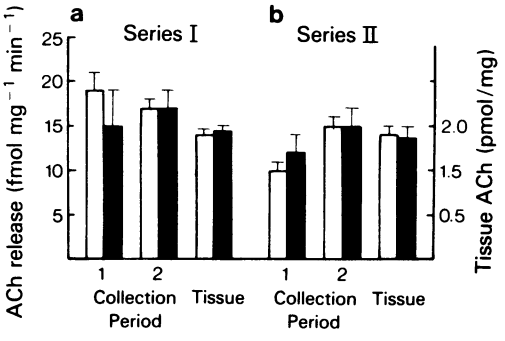

Figure 1 Action of the ionophore, A-23187, on resting acetylcholine (ACh) release and diaphragm ACh content. (a) Series I: Hemidiaphragms were equilibrated (30 min) with or without the ionophore, A-23187 (10 μM), in a Krebs medium with physostigmine (15 μM) and no added $CaCl_2$. Vehicle only was added to controls so that the final ethanol concentration was the same (0.5%) as in experimentals. After two measurements of resting ACh output in a normal Krebs medium with physostigmine (15 μM) and $CaCl_2$ (2 mM), the tissue content of ACh was determined. Results for ACh output and for tissue ACh content are given as the mean of 5 separate experiments; vertical lines show s.e. Solid columns are experimental preparations, open columns are controls. (b) Series II: Hemidiaphragms were equilibrated for 30 min in a Krebs solution with no added $CaCl_2$. During the first collection period, the calcium ionophore, A-23187 (10 μM) was added to experimental preparations (solid columns) and vehicle only (ethanol, to a concentration of 0.5%) to controls. The second collection was made in a Krebs solution supplemented with $CaCl_2$ (4 mM). Physostigmine (15 μM) was present throughout. Results are given as the mean of 7 separate experiments; vertical lines show s.e.

cantly different ($P > 0.50$) from controls during either collection period (Figure 1b). However, in all instances the output of ACh into solutions with 4 mM $CaCl_2$ was significantly greater ($P < 0.01$ by the paired t test) than the output in solutions with no added $CaCl_2$ (Figure 1b). Tissue ACh was unaffected by A-23187 relative to controls (Figure 1b).

In light of the contrast between our results and electrophysiological studies of ionophore action (Kao *et al.*, 1976; Cull-Candy *et al.*, 1976) we examined more critically the effects of ionophore on resting ACh release. As indicated in the first entry of Table 2, the resting release of ACh from control diaphragm preparations is quite stable (±3 fmol mg^{-1} min^{-1}) within the time frame of these experiments. Thus one can compare release measurements of preparations before and during drug treatment as a sensitive means of evaluating the action of an agent on release. Table 1 contains two such experiments. In the first experiment (Table 1: II) we compared the output of ACh when ethanol alone or ionophore and ethanol were present. In the third entry of Table 1, we tested the effects of ionophore and of Ca^{2+}-supplemented solutions. Under no circumstances did ionophore-containing solutions significantly increase ACh output. As anticipated from Figure 1, spontaneous ACh release in Ca^{2+} (4 mM) supplemented solutions was significantly greater ($p < 0.01$ by the paired t test) than that in 'Ca^{2+}-free' solutions. Moreover, none of the manipulations of Experiments II and III (Table 1) had any detectable effect on tissue ACh compared to diaphragms kept in normal Krebs media (Table 1, Experiment I). From these results we conclude that A-23187 does not measurably affect either the resting output of ACh or the tissue transmitter content of rat diaphragm.

Table 1 Effect of Ca^{2+} ionophore on acetylcholine (ACh) release and tissue ACh content

		ACh release (fmol mg^{-1} min^{-1})						*Tissue ACh* (pmol/mg)
Experiment	n	1	2	3	4	5	6	
I Control	4	15 ± 1	15 ± 1	14 ± 1	14 ± 1	13 ± 1	12 ± 2	1.49 ± 0.10
II N, N, E, E, I, I	7	13 ± 3	13 ± 2	12 ± 1	10 ± 1	14 ± 3	13 ± 1	1.65 ± 0.05
III N, N, I, I, H, H	6	14 ± 1	15 ± 1	14 ± 2	12 ± 2	20 ± 1	19 ± 1	1.72 ± 0.08

After a 30 min equilibration period, resting ACh output was measured during six successive 15 min collection periods using modified Krebs solutions containing physostigmine (15 μM). In control experiments (I) the standard Krebs solution with 2 mM $CaCl_2$ was used. In experiments II and III, Ca^{2+}-free Krebs solutions (no added $CaCl_2$) were used during the 30 min equilibration and then paired collections were made in Krebs media modified according to the following code: N = no further modification of the Ca^{2+}-free Krebs; E = ethanol to a final concentration of 0.5% was added; I = ionophore, A-23187 (10 μM), dissolved in ethanol (final concentration, 0.5%) was added; H = $CaCl_2$ (4 mM) was added to the solution. Tissue ACh was measured at the end of the experiment. Results are the mean ± s.e. of n experiments.

Guanidine

Lundh *et al.* (1977) found that guanidine caused maximal enhancement of both the muscle twitch and the endplate potential amplitude using concentrations of the drug above 3 mM and with Ca^{2+} concentrations of 4 mM or higher. In the following experiments we used guanidine (4 mM) and $CaCl_2$ (4 mM).

In Experiment I of Table 2, diaphragms were equilibrated with or without guanidine and then stimulated for three 10 min periods, followed by a 15 min rest collection. As indicated (Table 2, Experiment I), the presence of guanidine caused a progressive decline of the evoked release of ACh. By the third period of stimulation, ACh release was 50% of control. This decline is significant at $P < 0.01$. Moreover, resting ACh output (period 4) was inhibited by guanidine ($P < 0.02$ relative to control). The drop of ACh output may reflect the reduction in tissue ACh content caused by guanidine. After the paradigm of Experiment I (Table 2), the ACh of guanidine-treated diaphragms was only 64% of control, a difference that is significant at $P < 0.01$.

Since guanidine enhances ACh release as measured by electrophysiological criteria (e.g. Lundh *et al.*, 1977), we tested the effects of this drug using short term conditions in which the reduction of tissue ACh (Table 2, Experiment II) might be avoided. Diaphragms were stimulated (10 Hz) for two periods in high Ca^{2+} Krebs and then for two periods in the same medium containing guanidine (4 mM). During the first period of stimulation in guanidine-containing Krebs there was a clear cut stimulation ($P < 0.01$ by the paired t test) of ACh release (Table 2, Experiment II). However, the ACh output during the following period in guanidine (period 4) was significantly lower ($P < 0.01$ by the paired t test) than during period 3 and it was only slightly higher than output in periods without guanidine. These data argue that during high frequency stimulation guanidine has a short-lived stimulatory effect on ACh release which subsides as a likely consequence of reduced ACh stores in the tissue.

Additional confirmation of these results was obtained in experiments in which preparations were stimulated according to the protocol of Table 2 (part I) but with physostigmine omitted from the Krebs solution. Under these circumstances the ACh content of controls was 1.88 ± 0.06 pmol/mg ($n = 6$) and after guanidine (4 mM) the level was 1.47 ± 0.09 pmol/mg ($n = 7$). The reduction caused by guanidine is significant at $P < 0.01$. However, guanidine (4 mM) did not affect the ACh content of diaphragms that were treated for 1 h at rest (Gundersen, unpublished observations). Thus, the inhibitory effects of this drug on ACh metabolism became apparent during high frequency stimulation.

4-Aminopyridine

Several groups (Simpson, 1978; Horn, Lambert & Marshall, 1979) have supported the hypothesis of Lundh *et al.* (1977) that 4-aminopyridine (4-AP), in addition to its action on K^+ channels, may increase the level of Ca^{2+} in nerve terminals. This effect presumably contributes to the marked facilitation of evoked transmitter release caused by 4-AP.

We tested the actions of 4-AP on ACh output from the diaphragm using two different doses (10 μM and 100 μM) and two different stimulation frequencies (0.5 and 5.0 Hz). At 10 μM we observed no stimulation by

Table 2 Effect of guanidine on released and tissue acetylcholine (ACh)

		ACh release (fmol mg^{-1} min^{-1})				*Tissue ACh* (pmol/mg)
Experiment	n	1	2	3	4	
I						
Control	7	91 ± 3	81 ± 4	78 ± 5	23 ± 2	2.11 ± 0.08
Guanidine	8	80 ± 3	64 ± 8**	48 ± 4*	13 ± 2*	1.36 ± 0.08*
II	6	81 ± 2	78 ± 3	102 ± 5	87 ± 3	1.55 ± 0.19

Experiment I Hemidiaphragms were equilibrated with or without guanidine (4 mM) for 30 min in a Krebs solution with Ca^{2+} (4 mM) and physostigmine (15 μM). ACh release was then measured during three periods (1–3) of stimulation (10 Hz for 10 min). After a 15 min rest collection (period 4) tissue ACh was measured. The results are the mean ± s.e. of n experiments.

Experiment II Diaphragm segments were equilibrated (30 min) in a physostigmine-containing (15 μM) Krebs solution with Ca^{2+} (4 mM). The first two collection periods (1 and 2) used the same Krebs solution with indirect stimulation (10 Hz). In periods 3 and 4 the Krebs medium was supplemented with guanidine (4 mM) and the nerve was stimulated at 10 Hz. The results are the mean ± s.e. of n separate experiments.

Results differ from control at *$P < 0.001$; **$P < 0.05$.

4-AP of resting or evoked ACh release (Gundersen, unpublished observation). In experiments in which 100 μM 4-AP was used a significant enhancement of ACh output occurred at either 0.5 or 5.0 Hz (Table 3). Again, we used two different experimental protocols. In the first trials we measured ACh output of diaphragms incubated with or without 4-AP (100 μM); in the confirming series we measured the evoked output from diaphragms for two periods without 4-AP and then for two periods with 4-AP present. Regardless of which protocol was employed, evoked ACh output in the presence of 4-AP was significantly greater than release from control preparations (Table 3). However, 4-AP did not affect the resting release of ACh that was measured in period 4 (Table 3: parts I and II); nor did this drug significantly change the tissue ACh content. Thus, 4-AP appears to enhance impulse-evoked ACh output, and the increased demand for releasable ACh is presumably supplied by a more rapid synthesis of ACh so that the tissue ACh content is unchanged.

Tetraethylammonium

TEA, like 4-AP, has been postulated to have actions on Ca^{2+} metabolism of nerve endings in addition to those caused by its known inhibition of K^+ conductance (Lundh *et al.*, 1977). In our first set of experiments we measured the effects of TEA (2 mM) on tissue ACh content only. In a normal Krebs medium without physostigmine the diaphragm ACh content of six control preparations stimulated at 5 Hz for three 10 min periods and then rested for 15 min was 1.20 ± 0.08 pmol/mg. When diaphragms were treated throughout with TEA (2 mM) we measured a highly significant decline ($P < 0.01$) of tissue ACh to 0.42 ± 0.04 pmol/mg ($n = 6$). This action of TEA resembles that of hemicholinium-3 (HC-3), a potent inhibitor of choline transport and ACh synthesis in the diaphragm (Potter, 1970; Gundersen *et al.*, 1981).

The effects of TEA on ACh release were measured in experiments similar to those of Table 3. Relative to control, TEA (2 mM) depressed the release of ACh from diaphragm during the first period of stimulation. In three experiments the initial output of ACh after TEA was 19 ± 2 fmol mg^{-1} min^{-1} vs 44 ± 3 fmol mg^{-1} min^{-1} for control. However, during the second and third periods of stimulation, ACh release from the drug-treated muscles fell progressively below the normal resting level of output (15 to 20 fmol mg^{-1} min^{-1}) while the control rate of release remained between 40 and 50 fmol mg^{-1} min^{-1}. At the end of these experiments the ACh content of TEA-treated diaphragms was 0.48 ± 0.02 fmol/mg which is significantly less ($P < 0.001$) than controls (1.76 ± 0.17 pmol/mg). In summary, these data show that TEA inhibits transmitter release and this decline of ACh output is a probable consequence of the reduction by TEA of tissue ACh.

$LaCl_3$

Although La^{3+} blocks the stimulus-dependent release of ACh in frog muscle, concentrations above 10 μM

Table 3 Effect of 4-aminopyridine (4-AP) on tissue and released acetylcholine (ACh)

Experiment	n	*ACh release* (fmol mg^{-1} min^{-1}) 1	2	3	4	*Tissue ACh* (pmol/mg)
I 0.5 Hz						
Control	4	30 ± 3	30 ± 3	32 ± 2	18 ± 3	2.09 ± 0.12
4-AP	5	50 ± 5*	40 ± 5	42 ± 2*	17 ± 2	2.32 ± 0.20
II 5 Hz						
Control	4	59 ± 7	59 ± 8	59 ± 5	19 ± 1	1.82 ± 0.13
4-AP	6	93 ± 7*	86 ± 4*	93 ± 7*	17 ± 1	1.85 ± 0.11
III 0.5 Hz	4	29 ± 3	26 ± 2	46 ± 8*	54 ± 11*	2.06 ± 0.08
5 Hz	4	59 ± 10	59 ± 11	79 ± 11*	78 ± 17*	2.01 ± 0.19

Experiments I and II ACh output was measured using diaphragm preparations that had been equilibrated (30 min) with or without 4-AP (100 μM) in a standard Krebs with physostigmine (15 μM) and stimulated at 0.5 or 5.0 Hz. Evoked release was measured in periods 1–3 and resting output in period 4.
Experiment III Diaphragms were indirectly stimulated (0.5 or 5.0 Hz) for periods 1 and 2 in a physostigmine-containing Krebs without 4-AP and then during periods 3 and 4 in a medium supplemented with 4-AP (100 μM). Tissue ACh was measured at the end of all experiments. All results are given as the mean ± s.e. of n experiments.
* ACh output in media containing 4-AP is significantly different from output in control solutions at $P < 0.01$ by the unpaired t test (experiments I and II) and by the paired t test (experiment III).

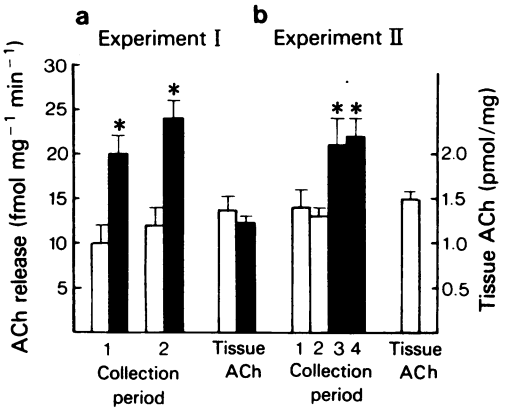

Figure 2 Effect of La^{3+} on tissue and released acetylcholine (ACh). (a) Experiment I: Diaphragm segments were equilibrated (20 min) in the Tris-modified medium with physostigmine (15 μM). Resting ACh output was measured during two consecutive periods with (solid columns) or without (open columns) La^{3+} (0.2 mM) added to the bath solution. Tissue ACh was measured at the end of the experiment. Results are the mean of four separate experiments; vertical lines show s.e. (b) Experiment II: Diaphragm segments were equilibrated (30 min) in the Tris modified Krebs medium with physostigmine (15 μM). During the first two collection periods, resting ACh output into the same medium was measured. In the third and fourth periods the medium was supplemented with La^{3+} (0.2 mM). Tissue ACh was measured at the end of the experiment. Results are the mean of four experiments; vertical lines show s.e. * ACh output in the presence of La^{3+} is significantly different from control at $P < 0.01$ by the unpaired t test (Experiment I) and paired t test (Experiment II).

promote, within 30 min, a transient, massive increase of m.e.p.p. frequency (Blioch, Glagoleva, Liberman & Nenashev, 1968; Debassio, Schnitzler & Parsons, 1971). The mechanism of these actions is not known, though it might involve the inhibition of specific Ca^{2+} transport processes. An inhibition by La^{3+} of Ca^{2+} extrusion from nerve endings may result in an increase in the intracellular Ca^{2+} concentration and rise of m.e.p.p. frequency (DeBassio *et al.*, 1971).

We tested the actions of $LaCl_3$ (0.2 mM or 1.0 mM) on diaphragm ACh content and release. To prevent the precipitation of La^{3+}, we used a modified Krebs medium containing Tris-HCl (20 mM) and no $NaHCO_3$, KH_2PO_4 or $CaCl_2$. The first set of experiments in Figure 2 demonstrate clearly the stimulatory effect of $LaCl_3$ on the 'resting' output of ACh. ACh release in the La^{3+}-containing medium was nearly twice the control rate of efflux. This stimulation by La^{3+} was confirmed in the experiments in which hemidiaphragms served as their own controls (Figure 2b). La^{3+} (0.2 mM) caused a two fold increase in the release of ACh from the diaphragm.

La^{3+} did not significantly alter the tissue ACh content in the first set of experiments of Figure 2. Similarly, in preparations treated with $LaCl_3$ (1 mM) in the Tris-modified Krebs medium without physostigmine a small decline (significance level, $P < 0.30$) of tissue ACh was observed (data not shown). These results clearly indicate that $LaCl_3$ does not cause an accumulation of ACh in rat diaphragm.

Theophylline

Theophylline has been shown to potentiate neuromuscular transmission in the rat diaphragm prep-

Table 4 Effect of theophylline on tissue and released acetylcholine (ACh)

Experiment	n	*ACh release* (fmol mg^{-1} min^{-1}) 1	2	3	4	*Tissue ACh* (pmol/mg)
I						
Control	6	62 ± 4	59 ± 6	56 ± 7	17 ± 1	2.37 ± 0.37
Theophylline	6	65 ± 6	62 ± 7	64 ± 10	19 ± 4	2.26 ± 0.22
II	6	46 ± 8	41 ± 4	42 ± 2	45 ± 3	2.00 ± 0.13

Experiment I Diaphragm segments were equilibrated for 30 min in physostigmine-containing Krebs solution with or without theophylline (1 mM). After three periods during which evoked ACh release (5 Hz) was measured and a final rest period, the tissue ACh content was determined.
Experiment II Diaphragm segments were equilibrated for 30 min in a normal Krebs solution with physostigmine (15 μM). The phrenic nerve was then stimulated (5 Hz) for four consecutive periods and ACh output was measured. In periods 3 and 4 the Krebs medium was supplemented with theophylline (1 mM). Tissue ACh was measured at the end of the experiment.
Results are the mean ± s.e. of n experiments.

aration (Singer & Goldberg, 1970). This has been postulated to be due to an effect of theophylline on the modulation by cyclic nucleotides of Ca^{2+} availability in nerve endings (Singer & Goldberg, 1970). We tested the effect of theophylline (1 mM) on the output of ACh evoked by indirect stimulation (5 Hz). As shown in Table 4, theophylline had no significant effect on ACh release either during indirect stimulation (periods 1 to 3) or at rest (period 4). Moreover, this agent had no detectable effect on the tissue content of ACh. Again, we performed experiments to determine whether this initial procedure might have failed to demonstrate a small effect of theophylline on release. Diaphragms were first stimulated (5 Hz) for two periods without theophylline and then for two periods with the drug present. However, even this paradigm did not reveal a change of ACh output when theophylline was added to the Krebs medium. Therefore, using these experimental procedures we were unable to detect any effect of theophylline on ACh turnover in diaphragm.

2,4-Dinitrophenol

2,4-Dinitrophenol (DNP), an uncoupler of oxidative phosphorylation, causes a marked increase of m.e.p.p. frequency and an inhibition of evoked ACh release at the neuromuscular junction (Kraatz & Trautwein, 1957). Glagoleva, Liberman & Khashaev (1970) proposed that the stimulation of spontaneous release caused by uncoupling agents was due to Ca^{2+} liberation from mitochondria. The resting output of ACh that we measured in diaphragm was apparently unaffected by DNP (50 μM) (Table 5). This was also true for experiments in which diaphragms served as their own controls (Table 5, Experiment II).

Tissue ACh was raised slightly, though not significantly ($P > 0.10$) by a 60 min exposure to DNP (50 μM) (Table 5). This result contrasts sharply with that obtained by Beach, Vaca & Pilar (1980) in experiments on iris preparations in which DNP inhibited the synthesis of [^{3}H]-ACh from medium [^{3}H]-cho-

Table 5 Effect of 2,4-dinitrophenol (DNP) on tissue and released acetylcholine (ACh)

		ACh release (fmol mg^{-1} min^{-1})				*Tissue ACh* (pmol/mg)
Experiment	n	1	2	3	4	
I						
Control	4	12 ± 2	11 ± 2	10 ± 2	11 ± 1	1.49 ± 0.17
DNP	4	14 ± 2	13 ± 2	14 ± 2	15 ± 3	1.90 ± 0.21
II	4	13 ± 0	12 ± 1	14 ± 1	12 ± 1	1.76 ± 0.17

Experiment I Diaphragm segments were equilibrated (30 min) in a normal Krebs medium with physostigmine (15 μM) after which four 15 min measurements of resting ACh output were made with or without DNP (50 μM). Tissue ACh was assayed at the end of the experiment.
Experiment II After equilibrating a diaphragm for 30 min in normal Krebs with physostigmine (15 μM) there were two 15 min collections using the same medium. In periods 3 and 4 DNP (50 μM) was added to the Krebs solution and resting output was measured. Tissue ACh was determined at the end of the experiment.
Results are the mean ± s.e. of *n* experiments.

Table 6 Action of ouabain on tissue and released acetylcholine (ACh)

		ACh release (fmol mg^{-1} min^{-1})				*Tissue ACh* (pmol/mg)
Condition	n	1	2	3	4	
Ouabain	8	16 ± 2	37 ± 4*	43 ± 4*	39 ± 2*	1.22 ± 0.11
Control	6	16 ± 2	16 ± 2	13 ± 2	15 ± 1	1.79 ± 0.12

Diaphragm segments were incubated for 30 min in a normal Krebs medium with physostigmine (15 μM). Four 15 min resting release collections were taken in physostigmine-containing Krebs with or without ouabain (0.1 mM). Tissue ACh was measured at the end of the experiment.
* The difference between ouabain-treated and control is significant at $P < 0.001$.

line. If iris preparations respond to DNP in the same fashion as diaphragm, then the results of Beach *et al.* (1980) may be explained by the large increase in choline efflux from muscle that is caused by DNP (see below).

Ouabain

It has been proposed that ouabain, an inhibitor of Na^+, K^+-ATPase, enhances ACh release from mammalian motor nerve terminals by increasing intracellular Ca^{2+} (Elmqvist & Feldman, 1965). In our experiments (Table 6) the output of ACh from unstimulated diaphragms was significantly increased ($P < 0.001$) by ouabain (0.1 mM) during all but the first collection period. Ouabain also caused a reduction (25%) of tissue ACh content that was significant at $P < 0.01$. However, this reduction of tissue ACh may be due to the large increase of ACh turnover caused by ouabain. In fact, in the preparations of Table 6 the sum of the released plus tissue ACh after ouabain (3.2 pmol/mg) was significantly greater ($P < 0.05$) than controls (2.6 pmol/mg). Thus, ouabain actually increased the net production of ACh though the tissue ACh content was significantly depressed.

Experiments were performed that were similar to those shown in Table 6 except that 2 h incubation was used. Ouabain (0.1 mM) caused an unabated increase of ACh output over this period. Release remained at approximately three times the control rate of 14 fmol mg^{-1} min^{-1}. Moreover, tissue ACh content at the end of this period was depleted by ouabain to about the same extent as in Table 6. After a 2 h incubation in a physostigmine-containing Krebs solution, ouabain-treated preparations contained 0.99 ± 0.14 pmol ACh/mg ($n = 4$) while the control ACh content was 1.30 ± 0.06 pmol/mg ($n = 4$). These results emphasize the fact that ouabain enhances ACh efflux without causing a severe depletion of tissue ACh stores comparable to that seen with TEA.

Choline content and release in diaphragm

Some of the procedures described in this paper had small but reproducible effects either on choline efflux from diaphragm or on the tissue content of choline. For instance, Ca^{2+}-free media and solutions supplemented with guanidine caused a decline of choline efflux and a reduction of tissue choline content (Gundersen, unpublished observations). However, DNP-containing media had an unprecedented effect on choline content and release. As shown in Figure 3, DNP (50 μM) caused a progressive increase of choline output that approached three times the control level after 1 h. Moreover, diaphragms that were exposed to DNP for 1 h contained 2.5 to 3 times as much choline as paired hemidiaphragm segments taken from the same rat (Figure 3). These effects of DNP may be mediated by a reduction of ATP levels in the tissue and a decline in the activity of choline kinase.

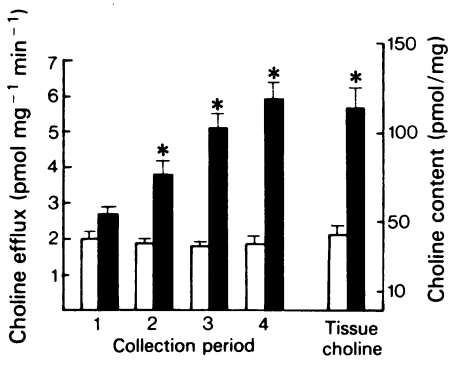

Figure 3 Effect of 2,4-dinitrophenol (DNP) on tissue and released choline. Hemidiaphragm segments were equilibrated for 30 min in a normal Krebs solution with physostigmine (15 μM). After four collection periods (15 min) with (solid columns) or without (open columns) DNP (50 μM) the tissue content of choline was assayed. The results are the mean of the same set of 6 experiments for which the acetylcholine data are given in Table 4; vertical lines show s.e. * Results differ significantly from control at $P < 0.001$.

Discussion

We proposed that β-bungarotoxin caused the accumulation of ACh in rat diaphragm as a consequence of raising the level of Ca^{2+} in the cytosol of phrenic nerve endings (Gundersen *et al.*, 1980; 1981). As one test of this hypothesis we have investigated the effects on diaphragm ACh content of a series of compounds that are believed to influence Ca^{2+} concentrations in cells. The conditions (e.g. dose, time of exposure) of the individual experiments were designed to maximize the possibility of obtaining an effect of the agent on tissue ACh content. However, our results were uniformly negative in that none of the compounds caused a significant accumulation of ACh in diaphragm. This fact suggests that we revise our postulate: either the effect of β-bungarotoxin on ACh content is not mediated by Ca^{2+}, or β-bungarotoxin has a unique action in increasing Ca^{2+} that is not reproduced by the agents we tested. At present, we cannot distinguish between these two possibilities except to suggest that before the Ca^{2+} hypothesis of β-bungarotoxin action is dismissed, it would be desirable to measure directly the effect of this toxin on the distribution of Ca^{2+} in cholinergic nerve endings.

While most of the compounds that were tested had no effect on tissue ACh, TEA and guanidine reduced ACh content. The effects of guanidine were observed only after a relatively sustained exposure of the preparation to the drug using high frequency stimulation in Krebs medium with raised Ca^{2+}. This effect was not observed during shorter periods of stimulation or at rest. Our data do not provide an explanation for this finding though we speculate that guanidine might compromise the supply of precursors for ACh synthesis. A similar explanation holds for the action of TEA. This agent caused a reduction of tissue ACh comparable to that seen with HC-3 (Potter, 1970; Gundersen *et al.*, 1981), an effect that is most probably due to an inhibition of choline transport (Simon, Mittag & Kuhar, 1975).

We obtained mixed results from the effects of the various agents on ACh release. While La^{3+}, 4-AP and ouabain stimulated ACh output, neither DNP nor the calcium ionophore had any detectable effect on resting ACh release, and theophylline did not enhance the evoked output of ACh. One explanation for our failure to detect an effect of either the calcium ionophore or DNP on resting release is that m.e.p.ps, the frequency of which is normally used as a criterion of a stimulatory effect of a drug on ACh release, account for less than 5% of the resting output of ACh from diaphragm (e.g. Fletcher & Forrester, 1975; see also Katz & Miledi, 1977, for a discussion of non-quantal ACh release). Thus, agents which cause large changes in m.e.p.p frequency might have barely detectable effects on the total resting ACh release that we measure. From the mean and standard deviation for the resting release of ACh in these experiments, we have calculated that an increase of ACh output of approximately 40% would be significant at the $P < 0.05$ level. This explains why DNP did not measurably affect resting ACh release, because this drug generally causes less than a ten fold change of m.e.p.p. frequency (Kraatz & Trautwein, 1957) which may have been obscured by the large non-quantal efflux of ACh. However, this explanation does not suffice in the case of A-23187. Cull-Candy and colleagues (1976) observed more than a 100 fold increase of m.e.p.p. frequency using this ionophore. Our failure to obtain an effect of A-23187 on ACh output may reflect methodological differences in the use of this compound.

The reasoning of the preceding paragraph may extend to the results obtained with theophylline. While this drug has small but reproducible effects on quantal ACh output (Singer & Goldberg, 1970), the changes it induces may be too subtle to be detected by measuring net ACh efflux. Finally, in contrast to results of electrophysiological studies (Cull-Candy *et al.*, 1976; Lundh *et al.*, 1977), TEA inhibited ACh release from diaphragm. Our results were obtained under circumstances in which TEA caused a marked inhibition of ACh synthesis and this is the most likely explanation for this difference in findings.

DNP caused a substantial increase of choline efflux from diaphragm preparations (Figure 3). If a similar phenomenon occurs in iris preparations, it might account for (on the basis of substrate dilution) the apparent inhibition by DNP of [^{3}H]-choline uptake and [^{3}H]-ACh synthesis in these preparations (Beach *et al.*, 1979). In fact, we observed a slight rise of tissue ACh after DNP which clearly demonstrates that this drug does not inhibit ACh synthesis in diaphragm preparations.

The results we obtained with ouabain might also be used to clarify certain findings of Beach *et al.* (1980). While they concluded that ouabain inhibited ACh synthesis by depleting the electrochemical gradients for the Na^+-coupled uptake of [^{3}H]-choline, our data suggest that one action of ouabain is to enhance ACh turnover. This drug (at 0.1 mM) clearly does not decrease net ACh production, because the sum of released plus tissue ACh after 1 or 2 h of ouabain treatment is significantly greater than control. A similar result is obtained in Krebs solutions with high K^+ (Gundersen, unpublished observations), which leads us to suggest that the effects of ouabain on ACh turnover may be mediated in large part by a sustained reduction of membrane potential which does not significantly depress ACh synthesis in this preparation.

The apparent resistance to the metabolic poisons, ouabain and DNP, of the ACh synthesizing machinery in the phrenic nerve-diaphragm preparation may reflect specializations in this tissue for maintaining ACh content under conditions of stress. We are currently investigating, in more detail, the interactions of metabolic inhibitors and β-bungarotoxin with the processes of ACh synthesis and release in rat diaphragm with the aim of elucidating the mechanism regulating ACh turnover in this preparation.

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