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OXOTREMORINE DOES NOT ENHANCE ACETYLCHOLINE RELEASE FROM RAT DIAPHRAGM PREPARATIONS

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We have reinvestigated the dramatic effect of oxotremorine on acetylcholine release from the rat diaphragm reported by Das, Ganguly & Vedasiromoni (1978), using a rigorous gas chromatographic mass spectrometric/isotope dilution method for identification and measurement of acetylcholine and choline. Oxotremorine (10 μM) causes no significant change in the spontaneous or evoked (1 or 10 Hz) release or in the tissue levels of acetylcholine or choline.

Introduction Recently, Das, Ganguly & Vedasiromoni (1978) reported a 30 to 60 fold increase in resting and evoked (1 Hz) acetylcholine (ACh) release from rat phrenic nerve diaphragm preparations that had been treated with the muscarinic agonist, oxotremorine. Using a gas chromatographic mass spectrometric (GCMS) assay for ACh, we have found that oxotremorine (10 μM) produces no significant change in spontaneous or evoked release of ACh or in tissue ACh content. At 100 μM this drug caused a small reduction of tissue ACh.

Methods Our experimental protocol was similar to that of Das *et al.* (1978). Fan-shaped segments of the left or right hemidiaphragm of 100 to 150 g male rats were dissected with the costal margin intact. The tissue was equilibrated for 30 min at 36 to 38°C in 3.5 ml of Krebs bicarbonate medium (composition in mM: NaCl 138, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, KH_2PO_4 0.4, $NaHCO_3$ 12, glucose 11 and physostigmine sulphate, 0.015) gassed with 95% O_2 and 5% CO_2. Resting or evoked release of ACh was then measured during six consecutive collection periods (10 or 15 min). Oxotremorine was added to the Krebs medium at the times indicated in Table 1. Indirect stimulation of the preparation was achieved with a suction electrode using supramaximal pulses (4 to 6 V, of 0.1 ms duration). At the end of the experiment the diaphragm tissue was cut free of the rib margin, blotted lightly and placed in 2.5 ml of 1 N formic acid in acetone (3:17, v/v) containing deuterium-labelled internal standards of ACh and choline. The tissue was weighed and then extracted overnight as described previously (Gundersen, Jenden & Newton, 1980). A GCMS assay was used to measure ACh and choline in all samples (Jenden, Roch & Booth, 1973; Freeman Choi & Jenden, 1975; Gundersen *et al.*, 1980). Quantitation was based on the relative ion current at m/e 58 (unlabelled) and m/e 64 (internal standards); m/e 71 was monitored to confirm the identity of the compounds measured. Results for both tissue and released ACh are normalized to the wet weight of the diaphragm tissue (weight range: 55 to 90 mg) and are given as means ± s.e. The significance of the results was evaluated using the paired or unpaired Student's *t* test, as appropriate. Oxotremorine was prepared as previously described (Cho, Haslett & Jenden, 1961; Bebbington & Shakeshaft, 1965), and crystallized as the oxalate salt.

Results To assess the effects of oxotremorine on ACh release, we first obtained a series of release measurements in control preparations. As indicated in the first row of Table 1, the evoked (1 Hz) output of ACh fell slightly over the course of four consecutive periods of stimulation while the resting output of ACh was unchanged from the beginning (period 1) to the end (period 6) of the experiment. When oxotremorine (10 μM) was added to the Krebs medium for the second half of the experiment (Table 1, row 2, periods 4 to 6), the evoked release of ACh during periods 4 and 5 increased slightly, but not significantly, relative to the corresponding control output (Table 1, row 1). Similarly, when comparisons are made within a row (i.e. output measured before and after addition of oxotremorine, 10 μM), no significant increase of ACh release was seen during stimulation (1 Hz) after the addition of oxotremorine. Resting output was also unchanged. When the concentration of oxotremorine was raised to 100 μM (row 3) ACh output was not increased during periods of stimulation, and the resting release of ACh was significantly depressed ($P < 0.01$) relative to the control rate (Table 1, row 1) or to the rate measured in the absence of the drug (Table 1, period 1, row 3).

An effect of oxotremorine (10 μM) on ACh output was also sought during high frequency (10 Hz) stimulation of the phrenic nerve (Table 1, row 4). No significant effect of this compound on ACh release was detected. Output of ACh in the presence of oxotre-

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Table 1 Effect of oxotremorine on acetylcholine released into medium and remaining in the tissue

Treatment	n	Period ACh released (fmol mg^{-1} min^{-1}) Rest 1	Stimulation 2	3	4	5	Rest 6	Tissue ACh (pmol/mg)
1 Hz								
(1) Control	5	17 ± 3	33 ± 3	33 ± 5	28 ± 2	27 ± 3	19 ± 4	2.22 ± 0.31
(2) Oxotremorine (10 μM)	4	18 ± 3	32 ± 3	33 ± 6	36 ± 8	34 ± 6	17 ± 2	2.10 ± 0.17
(3) Oxotremorine (100 μM)	4	13 ± 2	31 ± 3	25 ± 4	27 ± 8	21 ± 5	8 ± 2	1.57 ± 0.22
10 Hz								
(4) Oxotremorine (10 μM)	6	16 ± 3	80 ± 8	78 ± 11	76 ± 9	74 ± 9	19 ± 3	2.29 ± 0.15

Hemidiaphragm preparations were equilibrated for 30 min in a normal Krebs medium with physostigmine (15 μM). Then, two 15 min rest collections (periods 1 and 6) were bracketed with four periods (2 to 5) of indirect stimulation (1 or 10 Hz) in all of which, released ACh and choline were measured. In rows 2 to 4, oxotremorine (10 or 100 μM) was added to the medium during periods 4 to 6 only. Tissue ACh and choline were assayed at the end of the experiment. Results are the mean ± s.e. of n experiments.

morine (periods 4 to 6) was not significantly different from the appropriate control periods (1 to 3).

The ACh content of the diaphragm was not significantly affected by oxotremorine (10 μM) relative to controls that received no drug (Table 1). However, at 100 μM, oxotremorine caused a small decrease of ACh content ($P < 0.05$ relative to control).

Oxotremorine did not alter the tissue concentration of choline or its rate of release in these experiments. The rate of choline output from control preparations ranged from 1.5 to 2.6 pmol mg^{-1} min^{-1}, while the output after oxotremorine (10 or 100 μM) was 1.4 to 2.3 pmol mg^{-1} min^{-1}. Similarly, tissue choline content of controls averaged 56 ± 9 pmol/mg, while those exposed to oxotremorine (10 μM) contained 61 ± 7 pmol/mg.

Discussion There is evidence that oxotremorine and other muscarinic agents inhibit the release of ACh in brain slices (Polak, 1971) and synaptosomes (Weiler & Jenden, 1977), myenteric plexus (Kilbinger & Wagner, 1975) and ciliary ganglion (Johnson, Beach, Alanis & Pilar, 1977), but apparently not from the perfused superior cervical ganglion of the cat (Kato, Collier, Ilson & Wright, 1975). In contrast, oxotremorine has been reported to produce a substantial increase in resting and stimulation-induced release from the rat phrenic nerve-diaphragm preparation (Das *et al.*, 1978). Das and his colleagues (1978), using the dorsal muscle of the leech to bioassay ACh, reported that in the presence of 10 μM oxotremorine, the evoked (1 Hz) release of ACh rises to 1.3 nmol in 15 min, a value about 60 fold more than control. This greatly exceeds the normal ACh content of freshly dissected rat diaphragm preparations (1.2 pmol/mg wet wt.; Gundersen, Jenden & Newton, 1980), and approaches the V_{max} (60 pmol mg^{-1} protein min^{-1}; Gundersen, Jenden & Newton, 1980) of choline acetylase in the preparation. It appeared possible that this remarkable result was an artefact arising from the use of a bioassay, and the effect of oxotremorine on the isolated rat diaphragm preparation was therefore reinvestigated using an unequivocal chemical means of measurement. No significant effect on resting or stimulation-induced release was found in the presence of 10 μM oxotremorine; our data suggest that 100 μM oxotremorine may reduce the release rate. The tissue level of ACh was also unchanged by 10 μM oxotremorine and was reduced moderately by 100 μM oxotremorine. Oxotremorine did not affect the rate at which choline was released.

We have tried to duplicate the experiments of Das *et al.* (1978) as exactly as possible; the difference in our results is not due to a difference in the medium, temperature, size of rats, or stimulation conditions. It seems most likely that the remarkable effect reported by Das *et al.* (1978) is an artefact arising from reliance on bioassay for measurement of ACh. While bioassay has proved a reliable method for ACh measurement in many circumstances, it is open to question on the grounds of specificity, especially when other drugs are used in the experiment. The GCMS/isotope dilution assay employed in the present work not only allows precise measurement but simultaneously provides rigorous identification of the compounds measured.

We conclude that oxotremorine causes no significant stimulation of resting or evoked release of ACh in the rat phrenic nerve-diaphragm preparation, which like the superior cervical ganglion of the cat (Kato *et al.*, 1975) appears to lack presynaptic muscarinic receptors modulating release.

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