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Effect of choline on the release of acetylcholine and on its tissue stores in the phrenic nerve-diaphragm preparation treated with dyflos

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The release of acetylcholine from the guinea-pig phrenic nerve-diaphragm preparation treated with different concentrations of dyflos during four successive stimulation periods, was detected. At 38°, after 50 and 200 μg/ml of dyflos, the amount of acetylcholine released fell abruptly after the first stimulation period; a less evident decrease in the output was observed from the preparations preincubated with 500 and 1,000 μg/ml of dyflos. A reasonably constant release was detected at 28° after 1,000 μg/ml of dyflos; at this temperature the initial output was about 40% of that at 38°. Choline chloride (3 μg/ml) added to the perfusion fluid prevented the decreasing output only in the preparations incubated with the anticholinesterase at concentrations of 500 and 1,000 μg/ml. Dyflos did not affect the time course of acetylcholine release at 28°, the initial output of acetylcholine and the release at rest under any experimental conditions or the tissue stores of acetylcholine at the end of the fourth period of stimulation. A probable impairment by dyflos of the choline transfer system is discussed.

THE rat and guinea-pig phrenic nerve-diaphragm preparation, treated with neostigmine or physostigmine, maintains a steady release of acetylcholine in response to repeated stimulation periods (Straughan, 1960; Matthews & Quilliam, 1964); no significant increase in the acetylcholine released is detectable when choline is added to the perfusion fluid (Straughan, 1960). In the cat superior cervical ganglion kept in the usual Locke solution containing physostigmine, a decreasing output has been observed (Birks & McIntosh, 1961); the release becomes reasonably constant in choline-supplemented perfusion fluid (Birks & McIntosh, 1961; Matthews, 1963). The acetylcholine released from the guinea-pig phrenic nerve-diaphragm preparation pretreated with diisopropylfluorophosphonate (dyflos; DFP) gradually decreases during successive stimulation periods (Beani, Bianchi & Ledda, 1964). We now report the results of experiments made to examine the effect of choline on acetylcholine release and on its tissue stores in the guinea-pig phrenic nerve-diaphragm preparation treated with various concentrations of dyflos.

Experimental

All the experiments were made on the guinea-pig phrenic nerve-diaphragm preparation according to the method previously described (Beani & others, 1964); the preparations (1/4 of the whole hemidiaphragm) were kept in oxygenated Tyrode solution with glucose 2 g/litre, at 28° and 38°. The nerve was stimulated by supramaximal rectangular impulses 0·1 msec duration, at 50 shocks/sec. In a few control experiments neostigmine 10^{-5} was used as a cholinesterase inhibitor, the preparations being preincubated with the drug for 150 min before the experiment. The acetylcholine released was assayed on the leech dorsal muscle kept in

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Locke solution diluted 1 to 1·4 with distilled water containing physostigmine 10^{-5} and morphine hydrochloride 2×10^{-5} (Murnagham 1958).

In most experiments we used different concentrations of dyflos (50, 200, 500 and 1,000 μg/ml) to inhibit the esterase; the method of estimating the acetylcholine release was that of Beani & others (1964).

The experiments consisted of four successive 10 min stimulation periods, with 10 min intervals of rest; a 3 min conditioning stimulation period (Straughan, 1960) at 50/sec was given before the first collecting period.

When the effect of choline was tested, one preparation was kept in normal perfusion fluid and the contralateral one, from the same animal, in choline supplemented Tyrode solution. The tissue acetylcholine was extracted from the control and from the choline-treated preparations at the end of the fourth stimulation period, using the method of Beani, Bianchi & Ledda (1962). The drugs used were: acetylcholine chloride, Roche; neostigmine, Roche; dyflos, Boots; choline chloride, Merck; physostigmine sulphate, B.D.H.

Results

The release of acetylcholine from neostigmine-treated preparations. As previously described by Straughan (1960), a steady release was observed when neostigmine 10^{-5} was employed as esterase inhibitor. The average release (3 experiments) was 36·8 ng in the first stimulation period, 39 ng in the second, 40·1 ng in the third and 39·7 ng in the fourth period. The samples collected in rest periods were not assayed.

The release of acetylcholine from dyflos-treated preparations. The results of these experiments are reported in Table 1. At 38°, after dyflos 50 μg/ml, the amount of acetylcholine released fell abruptly after the first

TABLE 1. ACETYLCHOLINE RELEASED (NG ± S.D.) DURING FOUR SUCCESSIVE PERIODS OF STIMULATION AT 50/SEC FOR 10 MIN, FROM GUINEA-PIG PHRENIC NERVE-DIAPHRAGM PREPARATIONS PERFUSED EITHER WITH TYRODE SOLUTION OR WITH CHOLINE (3 μG/ML) SUPPLEMENTED TYRODE SOLUTION, AND PREINCUBATED WITH DIFFERENT CONCENTRATIONS OF DYFLOS

Temp. °C	Dyflos µg/ml	No expts	Groups	Stimulation periods			
				1st	2nd	3rd	4th
38	1,000	10	controls	31·8 ± 8·8	29·3 ± 8·2	24·1 ± 8·8	19·7 ± 5·7
		6	choline	30·5 ± 4·8	31·4 ± 4·9	28·3 ± 4·4	27·2 ± 5·9*
	500	7	controls	32·5 ± 5·1	27·1 ± 7·4	21·0 ± 6·3	17·0 ± 9·4
		7	choline	34·4 ± 7·3	34·2 ± 6·6	29·7 ± 7·3	27·1 ± 5·9†
	200	6	controls	29·5 ± 9·6	20·4 ± 8·2	12·0 ± 3·4	11·8 ± 3·5
		—	—	—	—	—	—
	50	5	controls	28·1 ± 7·5	14·6 ± 2·6	11·6 ± 3·9	10·3 ± 2·8
		6	choline	25·0 ± 7·4	18·0 ± 7·4	14·2 ± 6·8	14·2 ± 5·1
28	1,000	5	controls	13·3 ± 5·5	13·9 ± 5·2	13·7 ± 4·7	10·0 ± 3·0
		5	choline	13·9 ± 5·8	15·0 ± 7·4	14·4 ± 7·2	12·4 ± 6·3

* = Statistically different ($0{\cdot}05 > P > 0{\cdot}02$) from the control group.
† = Statistically different ($P = 0{\cdot}05$) from the control group.

stimulation period so that in the fourth period it was only 36·6% of the amount released in the first period; a similar time course of transmitter output was observed after 200 μg/ml, the acetylcholine released in the last period being 40% that of the first period.

The percentage decrease was less evident in preparations preincubated with dyflos, 500 and 1,000 μg/ml: it was 52·3 and 61·9% respectively. At 28°, after dyflos, 1,000 μg/ml, the acetylcholine release was almost constant. The amount released during the first stimulation period was about 40% of that released at 38° in the same period.

Effect of choline on dyflos-treated preparations. Choline chloride 3 μg/ml added to the perfusion fluid improved the transmitter output (see Table 1).

At 38°, the acetylcholine released in the fourth period was 57% of that released in the first period in the preparations incubated with dyflos, 50 μg/ml; it was 78·7 and 89·1% respectively in those incubated with 500 and 1,000 μg/ml. The difference between the average release of the controls and of the treated preparations was statistically significant only in the fourth period, with the 500 and 1,000 μg/ml amounts employed The effect of choline on preparations pretreated with 200 μg/ml of dyflos was not tested because the output detected was similar to that after 50 μg/ml.

Choline had no significant effect on the time course of acetylcholine release in preparations incubated at 28°, where a steady release was present even in normal Tyrode solution; nor did choline affect the amount of transmitter released in the first stimulation period both at 28° and at 38° or the release at rest under every experimental condition (values not given).

Acetylcholine tissue stores. The results of these experiments are reported in Table 2. Both at 28° and at 38° there was no difference between the acetylcholine tissue stores in the control and in the choline-treated preparations, at the end of the fourth stimulation period.

TABLE 2. TOTAL TISSUE ACETYLCHOLINE (NG/G OF FRESH TISSUE) AT THE END OF THE FOURTH PERIOD OF STIMULATION AT 50/SEC, IN HEMIDIAPHRAGMS PERFUSED EITHER WITH TYRODE SOLUTION OR WITH CHOLINE SUPPLEMENTED TYRODE SOLUTION, AND PREINCUBATED WITH DYFLOS 1,000 μG/ML FOR 150 MIN (Every value is the mean of 10 experimental points.)

Temp. °C	Groups	Guinea-pig weight (g ± s.d.)	Hemidiaphragm weight (mg ± s.d.)	Acetylcholine (ng ± s.d.)
28	controls	253 ± 17	193 ± 30	455 ± 106
	choline	253 ± 17	198 ± 21	470 ± 51
38	controls	256 ± 26	200 ± 24	181 ± 61
	choline	256 ± 26	203 ± 20	189 ± 67

Discussion

As previously reported by Straughan (1960), a constant transmitter release was observed in neostigmine-treated phrenic nerve-diaphragm preparations during successive stimulation periods; this suggests that all the elements necessary for an efficient acetylcholine synthesis are available in the preparation itself.

Under identical experimental conditions, the acetylcholine released in the first stimulation period in dyflos-treated preparations was almost equal to that in neostigmine-treated preparations; nevertheless it decreased in the subsequent collecting periods. Dyflos concentrations of 500 μg/ml or more are necessary to ensure a complete and irreversible esterase inhibition (Beani & others, 1964); the release in preparations preincubated with 50 or 200 μg/ml was low, irregular and unaffected by choline. It is remarkable that dyflos, in concentrations as high as 1,000 μg/ml, did not affect the nerve conduction; neither irregular muscle responses to the nerve stimulation nor an acetylcholine release lower than that detected after lower concentrations was observed.

The decreasing output in dyflos-treated preparations was detectable at 38°, but was much less evident at 28°; at this incubation temperature the initial acetylcholine output was about 40% of the amount released at 38°. This is in agreement with the observation of Straughan (1960) who used neostigmine as inhibitor. This finding suggests that dyflos, which is not a pure esterase inhibitor (Holmstedt, 1959), can cause some metabolic damage which may also affect acetylcholine synthesis when the experimental conditions (high temperature and stimulation rate) seriously deplete the reserves of the nerve terminals. It seems likely that this action of dyflos is an impairment of the enzymatic process of the choline transfer to the site of acetylcholine synthesis as it is counteracted by the presence of choline in the medium.

Choline affects the output of acetylcholine but not its tissue stores. This observation agrees with the idea that the extracted tissue acetylcholine after esterase inhibitors is, for the most part, the fraction not readily available for the release provoked by nerve stimulation (Birks & McIntosh, 1961). It is evident that this fraction increases in hypothermia because tissue acetylcholine at 28° is 2·5 times higher than at 38° (Table 2).

Dyflos appears to cause some damage in the metabolic pathways of acetylcholine synthesis; nevertheless we believe that its use in a choline supplemented medium is more suitable than that of reversible esterase inhibitors in experiments made to examine whether a drug is able to modify the transmitter release from motor nerve terminals. Neostigmine or physostigmine, which must be kept in the bath throughout the experiment, may react with acetylcholine receptors (Nachmansohn, 1959) and alter the effect of the drug under investigation.

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