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THE EFFECTS OF CHOLINE AND OTHER FACTORS ON THE RELEASE OF ACETYLCHOLINE FROM THE STIMULATED PERFUSED SUPERIOR CERVICAL GANGLION OF THE CAT

BY

E. K. MATTHEWS*

From the Department of Pharmacology, Medical College of St. Bartholomew's Hospital, London, E.C.1

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When the superior cervical ganglion of the cat was perfused with Locke solution, the amount of acetylcholine released into the perfusate decreased during successive periods of repetitive stimulation of the preganglionic nerve. Addition of choline to the perfusion fluid prevented this decrease. Choline also significantly increased ($P<0.01$) the initial output of acetylcholine. In contrast, variation of the physostigmine concentration or of the pCO_2 and pH of the perfusion fluid had no statistically significant effect ($P>0.05$) upon the initial release of acetylcholine.

The classical technique for perfusion of a sympathetic ganglion with an artificial saline medium, Locke solution, was first described 30 years ago by Kibjakow (1933). Since then Locke solution, essentially of original composition (Locke, 1901), has enjoyed almost exclusive and unquestioned popularity for perfusion of the superior cervical ganglion of the cat. The findings of Birks & MacIntosh (1961), however, pointed to the failure of simple Locke solution to support the turnover of ganglionic acetylcholine at the rapid rate necessary for a well-maintained output of acetylcholine and have been confirmed by my studies reported here.

The present work was undertaken to compare the liberation of acetylcholine from the intermittently stimulated superior cervical ganglion of the cat during perfusion with Locke solution, either alone or enriched with choline, and to examine the action of physostigmine and of CO_2 tension (pCO_2) and pH upon acetylcholine release.

METHODS

Cats of either sex weighing between 1.6 and 4.5 kg were anaesthetized first with ethyl chloride and ether and then with intravenous chloralose (80 mg/kg). The right superior cervical ganglion was isolated for perfusion *in vivo* in the manner of Kibjakow (1933), as described by Feldberg & Gaddum (1934), with the modifications of Perry (1953). The perfusion fluid was Locke solution of the original formula as used by Emmelin & MacIntosh (1956), but with twice the amount of glucose: NaCl 9.0 g, KCl 0.42 g, $CaCl_2$ 0.24 g, $NaHCO_3$ 0.5 g and glucose 2.0 g, dissolved in distilled water to 1 l. The pH of this solution, after bubbling either with pure oxygen or with a mixture of 2% carbon dioxide and 98% oxygen, was measured at 20° C with a glass electrode. Physostigmine sulphate (5×10^{-6} or 1×10^{-5} g/ml.) was added to all perfusion fluids. In some experiments, choline chloride (1×10^{-6} g/ml.) also

* Present address: Department of Physiology, McGill University, Montreal, Canada.

was added to the perfusion fluid. Supramaximal stimulation by rectangular pulses of 0.5 msec duration at 10 shocks/sec was applied through a fluid electrode to the preganglionic cervical sympathetic nerve. The nerve was stimulated for the initial 3 min of a 6 min period of collection of perfusate, the remaining 3 min being allowed to ensure complete collection of the acetylcholine released during stimulation. Such 6 min periods will be referred to as "stimulation periods." The perfusion flow rate was maintained as near the optimum level of 0.3 ml./min as possible. The samples of perfusate were collected into vessels at 0° C during successive 6 min periods and then stored at −14° C until assayed. Their content of acetylcholine was measured by comparison with standard acetylcholine chloride solutions using the rat blood pressure preparation introduced by Straughan (1958, 1959) as modified by Matthews (1961). The standard acetylcholine solutions were prepared in Locke solution removed from the reservoirs at the end of a perfusion experiment and were stored with the samples of collected perfusate, under identical conditions. Locke solutions containing choline (10^{-6} g/ml.) did not depress the rat blood pressure and did not interfere with the normal depressor response to injected acetylcholine.

The identification of the depressor substance in perfusate samples as acetylcholine was based on the following criteria. In the absence of an anticholinesterase (physostigmine) in the fluid perfusing the stimulated ganglion no acetylcholine-like activity was apparent upon the rat blood pressure. Previous injection of atropine (200 μg) abolished the depressor activity of both the perfusate samples and the standard acetylcholine solutions used for comparison. The depressor effects of both perfusate samples and standard acetylcholine solutions were abolished concurrently after treatment with alkali (0.17 M-sodium hydroxide) at room temperature for 10 min, but remained undiminished after similar treatment with acid (0.17 M-hydrochloric acid). Acetylcholine outputs are expressed in ng of the chloride.

RESULTS

Effect of physostigmine. Although the concentrations of physostigmine in Locke perfusion fluids used by other workers to inhibit ganglionic cholinesterase have varied over a considerable range, for example from 2×10^{-6} g/ml. (Feldberg & Gaddum, 1934) to 1×10^{-4} g/ml. (Feldberg & Vartiainen, 1934), there has been little systematic study of the effect of the concentration of physostigmine upon acetylcholine release. Birks & MacIntosh (1961) found that a five-fold increase in physostigmine concentration, from 4×10^{-6} to 2×10^{-5} g/ml., did not affect initial acetylcholine outputs, but they did only five experiments in each group and the acetylcholine content of the two groups of ganglia differed considerably. Therefore further data relevant to this point were obtained.

In 35 experiments in which the physostigmine concentration was 5×10^{-6} g/ml. the initial acetylcholine output (mean and standard error) was 47.2 ± 2.6 ng, whilst in thirteen experiments in which the physostigmine concentration was 10^{-5} g/ml. the output was 49.4 ± 5.2 ng (Table 1). As there was no significant difference ($P > 0.05$) between these two results, it was concluded that a concentration of physostigmine of 5×10^{-6} g/ml. was adequate for maximal recovery of the acetylcholine released during periods of nerve stimulation. The oxygenated Locke solution used for these experiments contained no choline.

Effect of pCO_2 *and pH.* Locke solution contains sodium bicarbonate as its only buffer and, when bubbled with pure oxygen, was found to have a *p*H of about 8.7 (range 7.9 to 9.0). The effect upon initial acetylcholine output of increasing the pCO_2 of Locke solution, and hence lowering its *p*H, was tested in six experiments. The *p*H of Locke solutions containing physostigmine varied between 6.6

TABLE 1

EFFECT OF PHYSOSTIGMINE CONCENTRATION, CHOLINE, AND *p*H ON THE MEAN INITIAL ACETYLCHOLINE OUTPUT DURING SUPRAMAXIMAL STIMULATION OF GANGLIA PERFUSED WITH LOCKE SOLUTION

*p*H values are means. Acetylcholine outputs are means with standard errors for the initial 3 min period of stimulation at 10 shocks/sec

Perfusion fluid					
	Physostigmine (g/ml.)	Equilibrated with	*p*H	No. of expts.	Acetylcholine output (ng)
Locke solution	1×10^{-5}	O_2	8·7	13	49·4±5·2
Locke solution	5×10^{-6}	O_2		35	47·2±2·6
Locke solution+choline (1×10^{-6} g/ml.)	5×10^{-6}	O_2		15	69·1±7·0
Locke solution	1×10^{-5} or 5×10^{-6}	2% CO_2 in O_2	6·9	6	56·6±8·7

and 7.1 (mean 6.9) when equilibrated with a gas mixture of 2% carbon dioxide and 98% oxygen. The initial acetylcholine output (mean and standard error) upon stimulation during perfusion with such solutions was 56.6±8.7 ng (Table 1) which was slightly, but not significantly ($P>0.05$), greater than the mean values of 47.2 and 49.4 ng obtained with Locke solution gassed with pure oxygen.

Effect of choline. Experiments were made with choline-free or choline-supplemented oxygenated Locke solutions containing physostigmine (5×10^{-6} g/ml.).

Each experimental cycle consisted of two successive 6 min stimulation periods followed by one 6 min rest period without stimulation. This cycle, repeated consecutively three times, constituted one experiment and gave a total of six stimulation and three rest periods.

When normal oxygenated Locke solution containing physostigmine but no choline was used for perfusion, the acetylcholine output from the ganglion steadily decreased with each successive period of nerve stimulation, as depicted by the lower regression line in Fig. 1. There was no tendency for the output to recover in the third and fifth stimulation periods which were preceded by rest periods.

As Bligh (1952) had found the normal choline content of cat plasma to be of the order of 10^{-6} g/ml., the influence of perfusion of this concentration of choline chloride in oxygenated Locke solution containing physostigmine was tested next. The effect of choline was clear. The acetylcholine output was greater than before, even in the first stimulation period, and it did not decrease when the stimulation was repeated (Fig. 1, upper line). Ganglia perfused with choline-supplemented Locke solution released, over the six stimulation periods, about twice as much acetylcholine as those perfused with unmodified Locke solution, namely 423±45.1 ng as against 198±36.1 ng (means and standard errors, n=3 and n=6 respectively). This difference in total output was statistically significant ($P<0.01$).

The acetylcholine output during the initial stimulation period was also measured in a number of other experiments with choline-free and choline-supplemented Locke solution, each solution containing physostigmine (5×10^{-6} g/ml.) (Table 1). For the whole series, the initial output (mean and standard error) was 69.1±7.0 ng in

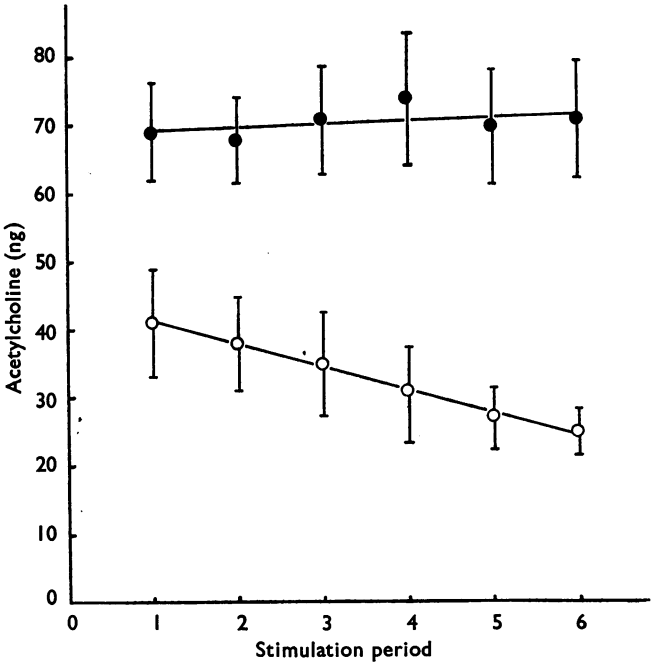

Fig. 1. Acetylcholine output in successive stimulation periods from ganglia perfused with either Locke solution ○ (six experiments) or choline-supplemented Locke solution ● (three experiments). Physostigmine (5×10^{-6} g/ml.) was present throughout. Each point plotted represents the mean amount of acetylcholine released by preganglionic nerve stimulation during each period of 3 min at 10 shocks/sec: the vertical bars indicate the standard error of each mean. For definition of "stimulation period" see text. The best fitting straight lines through the plotted points were determined by regression analysis.

experiments with choline present, and 47.2 ± 2.6 ng in experiments with choline absent. This difference, too, was significant ($P<0.01$).

DISCUSSION

The results show that the output of acetylcholine from the superior cervical ganglion perfused with normal Locke solution containing physostigmine was not well maintained in experiments in which the ganglion was subjected to repeated short bursts of stimulation separated by periods of rest. For maintenance of the output at the initial level it was necessary to add choline to the perfusion fluid.

Two interpretations of the increase in the initial acetylcholine output with choline-supplemented Locke perfusion fluid are possible. Choline might either directly enhance the release of acetylcholine or act indirectly to promote acetylcholine synthesis. A direct effect of choline upon acetylcholine release was not detected by Birks & MacIntosh (1961), and this suggested that most of the immediate effect of choline in the present experiments might be attributed to a prompt increase in acetylcholine synthesis. Yet the effect was clearly discernible in the first stimulation period, that is at a time when ganglionic acetylcholine stores would hardly have been much depleted. On balance therefore, it seems probable that choline exerts

some direct influence upon acetylcholine release, at least initially. With repeated stimulation some additional factors may come into play. Thus if, as Perry (1953) has proposed, choline arising from hydrolysis of released acetylcholine is normally used by the preganglionic nerve endings to conserve their store of acetylcholine available for synaptic release, then it might be expected that, during perfusion with normal Locke solution containing physostigmine but no choline, the acetylcholine output would show a progressive decline with repeated stimulation, for released acetylcholine would be preserved and removed from the synaptic region. This contention is supported by my finding of a gradual decrease in acetylcholine output with successive periods of stimulation. On the other hand, the observations of Birks & MacIntosh (1961) might be taken to suggest that this decrease in output is but a reflection of the sub-optimal conditions for acetylcholine synthesis prevailing in the ganglion perfused with Locke solution, since the small store of endogenous choline at the nerve endings might well, in the absence of replenishment from the extracellular plasma, be progressively depleted. This depletion would lead, in turn, to a corresponding gradual fall in the amount of acetylcholine released.

On the basis of either hypothesis it might be anticipated that, if choline were added to such a fluid in the concentration of 10^{-6} g/ml., which is approximately that usually found in plasma (Bligh, 1952), then the output of acetylcholine would be maintained because provision of an exogenous source of choline could make good any lost in the perfusate as acetylcholine or, alternatively, any denied to the nerve endings by the absence of plasma from their environment. The experiments described here have demonstrated clearly that provision of an extracellular supply of choline will maintain the acetylcholine output at a reasonably stable level in successive stimulation periods.

In contrast with the large effect of choline upon acetylcholine output, neither doubling the physostigmine concentration nor raising the pCO_2 of the perfusion fluid and hence lowering its *p*H from 8.7 to 6.9, significantly affected acetylcholine release from the ganglion perfused with normal Locke solution. The absence of a *p*H effect confirms and extends the finding of Emmelin & MacIntosh (1956), but it should be noted that these authors lowered the *p*H of their Locke solutions by a change of buffer system from " bicarbonate-Locke " to " phosphate-Locke " rather than by directly increasing the pCO_2 of " bicarbonate-Locke " as was done in the present study. Caldwell (1956) has pointed out that, although in many cells and tissues the intracellular *p*H often appears to follow slowly, if at all, quite large changes in the *p*H of their environment, extracelluar pCO_2 rapidly influences intracellular *p*H. It seems therefore that extracellular pCO_2 may be of much greater importance than extracellular *p*H, *per se*. Nevertheless, my findings, together with those of Emmelin & MacIntosh (1956), point to the conclusion that ganglionic acetylcholine release is, at least initially, largely independent of the *p*H or pCO_2 of the perfusing Locke solution.

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