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Investigation of the mechanism of action of oxotremorine on the guinea-pig isolated ileum preparation

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Summary

1. The effects of oxotremorine on the guinea-pig ileum have been investigated to determine whether any component of its action is due to acetylcholine release.

2. Log dose-response curves to oxotremorine and acetylcholine were similar and both drugs were competitively antagonized by atropine.

3. Mipafox potentiated acetylcholine but not oxotremorine.

4. No evidence of an indirect component of the action of oxotremorine was obtained using various pharmacological procedures (tetrodotoxin, morphine, cooling, procaine and hemicholinium-3).

5. Oxotremorine had no effect on acetylcholine release from mipafox treated ileum but decreased the release from physostigmine treated ileum.

6. It is concluded that oxotremorine acts directly on muscarinic receptors and not by releasing acetylcholine.

Introduction

Holmstedt (1967) has postulated that the rise in brain acetylcholine concentrations, after oxotremorine injection, is due to acetylcholine release. On the other hand, Jenden (1968) suggested that the increase in brain acetylcholine concentrations following oxotremorine is due to a decreased release of acetylcholine. It is difficult to obtain direct proof for either of these theories by determining the amounts of acetylcholine released from various parts of the brain. Thus it seemed that more information could be gained by studying the interaction between oxotremorine and the cholinergic system on a simpler peripheral model.

On isolated intestinal preparations oxotremorine is a powerful muscarinic agonist (Cho, Haslett & Jenden, 1962), and György, Pfeifer & Kenyeres (1970) found that oxotremorine releases acetylcholine into the bath fluid of the rat isolated intestine preparation treated with physostigmine. The present work was designed to show whether oxotremorine contracts the guinea-pig isolated ileum preparation by a direct action on muscarinic receptors of the smooth muscle or indirectly by the release of acetylcholine.

Methods

Segments of non-terminal ileum from male albino guinea-pigs were used. They were bathed in Tyrode solution, kept at $37 \pm 1°$ C and aerated with oxygen. Con-

tractions were recorded either isotonically on a smoked drum using a frontal writing lever or isometrically on a potentiometric recorder using a force displacement transducer. Resting tension applied to the ileum was 1 g. For transmural stimulation, the electrode in the lumen was made positive and the inter-electrode distance was 4 ml. A pulse width no greater than 0·5 ms was used, the voltage was adjusted to be supramaximal, and the frequency of stimulation was 0·2 Hz.

Collection of acetylcholine released into the bath fluid

The ileum was washed at 10 min intervals for 1 h before treatment with a cholinesterase inhibitor. The ileum was then soaked either in Tyrode solution containing mipafox (10 μg/ml) for 75 min and washed at 10 min intervals for 1 h or bathed in Tyrode solution containing physostigmine (10 μg/ml) for 30 min before any collections were made. The volume of each sample was noted. At the end of an experiment the ileum was blotted gently and weighed. Samples of bath fluid were stored at $-10°$ C along with acetylcholine standards prepared in Tyrode solution to act as controls for acetylcholine stability during storage.

Assay of bath fluid samples

The assay preparation used was the leech dorsal muscle strip. The strip, 2×40 mm, was suspended in diluted Tyrode solution containing physostigmine and morphine (Murnaghan, 1958). Contractions were recorded isometrically. Test solutions were brought to room temperature and diluted 1:1·4 with distilled water. Assays were performed by bracketing the response of a test solution with responses to known doses of acetylcholine. The acetylcholine solutions were also prepared in diluted Tyrode solution.

When oxotremorine was present in the test solution, the standard acetylcholine solution contained the same concentration of oxotremorine. Some assays were also performed on the guinea-pig isolated ileum preparation.

The active substance from the ileum was identified as acetylcholine in the following manner: (1) there was close agreement between the results of assays using leech dorsal muscle and guinea-pig ileum preparations; (2) changes in tension produced by the bath fluid could be completely blocked by tubocurarine, 10 μg/ml; and (3) the active substance in the bath fluid was unstable in alkali.

Drugs

The following drugs were used: acetylcholine chloride (B.D.H.); atropine sulphate (B.D.H.; hemicholinium-3 (Aldrich); histamine acid phosphate (B.D.H.); mipafox (NN di-*iso*propylphosphorodiamidic fluoride) (L. Light & Co.); morphine sulphate (B.D.H.); nicotine acid tartrate (B.D.H.); oxotremorine, physostigmine salicylate (B.D.H.); procaine hydrochloride (B.D.H.); tetrodotoxin citrate (Sankyo); *d*-tubo-curarine chloride (Duncan, Flockhart & Evans). With the exception of mipafox all drug concentrations are expressed as weight of base per ml. Concentrations of mipafox are expressed as weight of salt per ml.

Statistical methods

Non-parametric statistical tests were used to determine the significance of the difference between two groups of results. When the data had been obtained from

related samples, for example the same piece of ileum, the Wilcoxon matched pairs signed ranks test was used. Where the samples were not so closely related, a two tailed Mann Whitney 'U' test was used. These tests were applied according to Siegel (1956).

Results

Comparison of dose-response curves to oxotremorine and acetylcholine

In seven experiments, using isotonic recording, oxotremorine caused a dose dependent contraction of the ileum similar to that produced by acetylcholine. The mean ED50s were 14·86 ng/ml (0·072 μM) for oxotremorine and 12·71 ng/ml (0·0874 μM) for acetylcholine. The slopes of the lines calculated for the best fit through all the points lying between an ED20 and an ED80 were 76·34 and 72·46% of the maximum response for a 10-fold change in concentration of oxotremorine and acetylcholine, respectively.

Effect of atropine on responses to acetylcholine, oxotremorine and nicotine

Maximum responses to acetylcholine, oxotremorine and nicotine were determined on the guinea-pig ileum under isometric conditions. The maximum response to nicotine was always 40–50% less than those to acetylcholine or oxotremorine, which were similar. The preparation was washed frequently for 15 min and doses of the three agonists added which produced responses approximately 20 and 80% of their respective maxima. The ileum was then incubated with atropine, 0·57 ng/ml, for 15 minutes. On subsequent testing doses of oxotremorine and acetylcholine were found which again gave responses of 20 and 80% of their original maxima. With nicotine the maximum response was depressed and doses giving 20 and 80% of the new maximum response were tested. This procedure was repeated with increasing doses of atropine. Throughout the experiment the maximum response to acetylcholine and oxotremorine did not change but the maximum response to nicotine became progressively less. In a control experiment the three agonists were administered in the same order (and at the same intervals) but no atropine was added to the Tyrode solution. The magnitude of the responses did not change. In all experiments doses of nicotine were interposed between at least two doses of either oxotremorine or acetylcholine to avoid the possibility of tachyphylaxis.

With oxotremorine and acetylcholine, atropine caused a parallel rightward shift of the log dose-response lines, plotted from the 20 and 80% response. The pA_2 and pA_{10} values calculated by the method of Arunlakshana & Schild (1959) were 9·36 and 8·46 for acetylcholine and 9·4 and 8·3 for oxotremorine. The rightward shift of the nicotine log dose-response curve was not parallel and therefore no pA values were calculated for this compound.

Effect of mipafox on log dose-response curves to acetylcholine and oxotremorine

Using isotonic recording, log dose-response curves were obtained to acetylcholine and oxotremorine. The ileum was then incubated with mipafox (10 μg/ml) for 75 min and after 1 h of washing log dose-response curves to acetylcholine and oxotremorine were repeated. In each of six experiments the log dose-response curve to acetylcholine was shifted to the left. Measured at the 50% response level, the mean leftward shift was 0·31 log units. Statistical examination of these results

using the Wilcoxon matched pairs signed ranks test showed that this shift was significant ($P=0.05$). With oxotremorine, mipafox caused a small rightward shift in three experiments, a small leftward shift in two experiments and no change in another experiment. The mean shift, 0·03 log units to the left, was not significant.

Effect of drugs and cooling on oxotremorine induced contractions of the ileum

The drugs used were morphine, tetrodotoxin, procaine and hemicholinium-3. Control responses were obtained using isometric recording to transmural stimulation and doses of oxotremorine, acetylcholine and histamine which gave submaximal responses (approx. ED70). The ileum was then exposed to the test procedure and the same stimulation and doses of oxotremorine, acetylcholine and histamine were repeated in the presence of the test drug or in the case of cooling, when the temperature of the bath had been lowered to 20° C. The results are summarized in Table 1. Although the response to oxotremorine was depressed by procaine, 10 μg/ml, neither this drug nor the other test procedures reduced the response to oxotremorine to a significantly greater extent than that to acetylcholine or histamine.

Effect of oxotremorine on acetylcholine release from the ileum

The rate of accumulation of acetylcholine in the bath fluid of the ileum pretreated with either physostigmine or mipafox was used as a measure of the rate of acetylcholine release from the ileum. In all experiments a 10 min collection period was used. The 'resting rate' of acetylcholine release was determined immediately before the ileum was exposed to oxotremorine. Two doses of oxotremorine were used—(1) 16 ng/ml (an ED50 dose) and (2) 130 ng/ml (a dose which just gave a maximum response). The results are presented in Table 2.

TABLE 1. *Effect of tetrodotoxin, morphine, cooling, procaine and hemicholinium-3 on responses of the guinea-pig ileum to oxotremorine, acetylcholine, histamine and transmural stimulation*

Treatment	Pre-treatment time	Mean response as % of control: Oxo-tremorine	Acetyl-choline	Histamine	Transmural stimulation
Tetrodotoxin, 0·1 μg/ml	10 min	97	92	92	0*
Morphine, 1 μg/ml	10 min	100	91	86	39*
Cooling to 20° C	30 min	75	49	70	0*
Procaine, 10 μg/ml	10 min	29	44	55	44
Procaine, 1 μg/ml	10 min	85	93	87	90
Hemicholinium-3, 1 mg/ml	15 min	57†	69	108	90

Each result is the mean of six experiments except for hemicholinium-3 which is the mean of four experiments.
* Significantly lower than acetylcholine and histamine (Mann Whitney 'U' test; $P=0.05$). † Significantly lower than histamine (Mann Whitney 'U' test; $P=0.05$) but not significantly different from acetylcholine.

TABLE 2. *Effect of oxotremorine on acetylcholine release ((ng/g ilium)/10 min) into the bathing fluid of the guinea-pig isolated ileum preparation treated with a cholinesterase inhibitor*

Concentration of oxotremorine	Mipafox, 10 μg/ml		Physostigmine 10 μg/ml	
0	198	N.S.	673	$P=0.05$
16 ng/ml	128		413	
0	146	N.S.	617	$P=0.05$
130 ng/ml	175		396	

N.S., not significant. P=probability level, using Wilcoxon matched pairs signed ranks test. Each value is the mean of at least five experiments.

In a second series of experiments the 'resting rate' of acetylcholine release was determined immediately before a 10 min period of transmural stimulation (0·2 Hz). In the presence of physostigmine, 10 μg/ml, the mean 'resting rate' of release from six experiments was (350 ng/g ileum)/10 min, which increased to (479 ng/g ileum) /10 min during the stimulation period. This increase was significant (P=0·05) when examined by the Wilcoxon matched pairs signed ranks test. When mipafox, 10 μg/ml, was used no such increase in acetylcholine release could be detected.

Discussion

The action of acetylcholine on the guinea-pig isolated ileum preparation is predominantly on muscarinic receptors whereas the action of nicotine is predominantly on nicotinic receptors with subsequent release of acetylcholine (Paton & Zar, 1968). If a significant part of the action of oxotremorine on the guinea-pig ileum is due to release of acetylcholine, then it would be expected that oxotremorine would behave more like nicotine than acetylcholine. In fact this was not the case. The antagonism of acetylcholine and oxotremorine by atropine was similar and of a competitive nature whereas the antagonism of nicotine by atropine did not fulfil the criteria for competitive antagonism. This similarity between oxotremorine and acetylcholine is further emphasized by the log dose-response curves to these compounds with respect to their slopes and maximum responses. In contrast the maximum response to nicotine was always less than that to either acetylcholine or oxotremorine.

If oxotremorine releases acetylcholine, it would be expected that treatment of the ileum with a cholinesterase inhibitor would cause a leftward shift of the log dose-response curve to oxotremorine. It did not do so although Brownlee & Johnson (1963) found that after treatment of the ileum with mipafox, log dose-response curves to acetylcholine and nicotine were shifted to the left.

Several agents which inhibit the release of acetylcholine from nerve endings in the ileum were also used in an attempt to detect an indirect component in the action of oxotremorine.

Cooling the ileum to a temperature at which responses to nerve stimulation were abolished reduced the responses to acetylcholine and histamine. This suggests that cooling also was affecting the responsiveness of the smooth muscle of the ileum. Procaine reduced the responses to oxotremorine, acetylcholine, histamine and transmural stimulation. There was no indication of a selective effect on transmural stimulation. These findings are not in agreement with those of Harry (1962) and Johnson (1963) but Ogura, Mori & Watanabe (1966) have found that procaine reduced the response of the ileum to acetylcholine and histamine in doses that did not abolish the response to transmural stimulation, and Weston (1968) found that responses of the ileum to bradykinin and methacholine were also reduced by procaine.

Hemicholinium-3 reduced the responses of the ileum to acetylcholine and oxotremorine by a similar amount. Since responses to histamine and transmural stimulation were least affected, hemicholinium-3 may have been exerting an atropine-like action as reported by Bertolini, Greggia & Ferrari (1967).

The two most effective agents in separating transmural stimulation from acetylcholine and histamine were tetrodotoxin and morphine. Neither had an effect on the response of the ileum to oxotremorine.

There is evidence, however, that tetrodotoxin (Gershon, 1967) and morphine (De la Lande & Porter, 1967) inhibit acetylcholine release arising from nervous activity but do not affect the spontaneous release. Thus these results do not exclude the possibility that oxotremorine may be increasing the spontaneous release of acetylcholine. If oxotremorine releases acetylcholine by such a mechanism, the release of acetylcholine into the bath fluid of ileum treated with a cholinesterase inhibitor should be increased. When mipafox was used, there was no significant difference between the 'resting release' and the release in the presence of oxotremorine. When physostigmine was used, oxotremorine decreased the release of actylcholine. Under similar conditions, transmural stimulation increased the release of acetylcholine from the ileum, showing that the methods used were capable of detecting increased acetylcholine release.

Thus in contrast to results reported for the rat isolated urinary bladder preparation and rat gut (György *et al.*, 1970), it is concluded that oxotremorine acts directly on muscarinic receptors of the guinea-pig ileum preparation, and not via acetylcholine release.

In relation to the interactions of oxotremorine and acetylcholine on the central nervous system, these results give no support to the theory that oxotremorine increases the amount of acetylcholine in the brain by release of this substance (Holmstedt, 1967). Jenden (1968) has postulated that the rise in brain acetylcholine might be due to prevention of the release of acetylcholine resulting from stimulation of a feedback mechanism by the action of oxotremorine on muscarinic receptors. There is no inconsistency between the results obtained here and the latter theory.

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