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Conditions affecting the interaction of cholinergic agents on the longitudinal muscle of the guinea-pig ileum—unexpected effects with hexamethonium

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Affinity constants were determined for atropine and *N*-methyl atropine, using both acetylbetamethylcholine and carbaminoylcholine as agonists, on plexus-containing and plexus-free preparations of the guinea-pig ileum. Hexamethonium (1×10^{-4}M) decreased the apparent affinity constant of atropine on the plexus-free preparation when carbaminoylcholine but not acetylbetamethylcholine was the agonist. With the latter agonist both antiacetylcholine drugs were equiactive. Hexamethonium's selective action was associated with a significant shift to the left of the log dose-response curve to carbaminoylcholine on the plexus-free preparation. While others have observed that hexamethonium may be atropine-like, samples of hexamethonium have been reported as being contaminated with a "depolarizing compound", which may account for the results presented in this study. Nevertheless, the present results are consistent with an allosteric rather than a direct binding mechanism in the blocking action of atropine. The observation that the agonist, acetylbetamethylcholine, was not similarly affected by hexamethonium (or any possible contaminant) suggests that agents with primarily muscarinic activity may not be so affected. This study is another example emphasizing the care needed in interpreting data obtained with the aid of "pharmacological tools".

This study was begun to investigate the action of atropine and its quaternary analogue, *N*-methyl atropine, on both plexus-containing and plexus-free preparations of the longitudinal muscle of the guinea-pig ileum *in vitro*. The affinity constants for the antagonists were determined using two different agonists, carbaminoylcholine and acetylbetamethylcholine. Hexamethonium was also employed in some of the experiments since Rathbun & Hamilton (1970) had observed that its presence decreased the apparent affinity constant of gallamine triethiodide as an antimuscarinic on the cat heart *in vivo*.

MATERIALS AND METHODS

Preparations

Preparation of plexus-containing longitudinal muscle strips of guinea-pig ileum

Longitudinal muscle strips with Auerbach's plexus attached (plexus-containing preparations) were prepared according to Paton & Zar (1968) and 2–3 cm lengths were mounted in a 10 ml organ bath in Krebs solution (mM; KCl 4·72; $CaCl_2$ 2·56; $MgSO_4 \cdot 7H_2O$ 0·58; KH_2PO_4 1·2; NaCl 118; $NaHCO_3$ 25, dextrose 10, in distilled

water) at 37 ± 0·5° and gassed with 5% CO_2 in oxygen. Isotonic contractions were recorded with a Physiograph isotonic myograph transducer with a load of 200–300 mg. Drugs were applied automatically by means of a Vickers Instrument Automatic Bioassay Unit. Preparations were allowed to equilibrate for 45 min before any addition of agonist using a regular 5 min cycle: —0–45 s, in-flow valve opened, drug solution pre-warmed, drug in; 45–75 s, drug contact; 75–85 s, wash; 85–300 s, resting period.

Preparation of plexus-free longitudinal muscle strips of the guinea-pig ileum

A modification of the technique of Paton & Zar (1968) was used in which greater tension was applied during the stripping. Strips so obtained have been shown to be free of nerves as well as ganglia (Norberg, 1964; Paton, 1964; Gabella, 1970).

Further evidence that the preparation was ganglion-free was obtained by adding 1×10^{-4}M nicotine at the beginning and the end of each experiment. A very few preparations that did not respond to nicotine at the outset of an experiment did so at the end; the data obtained from such preparations have not been included in the results.

Experimental procedures

Two series of experiments were undertaken. These were identical in the two series except that in one hexamethonium (1×10^{-4}M) was present in both the wash and the agonist solutions.

A high and a low concentration of agonist was chosen from the linear portion of the log concentration-response curve. The high dose was twice the low. A log concentration-response line was then established from a mean of four estimations at each concentration. The antagonist was then introduced into both the wash and agonist solutions. Then two higher concentrations of agonist which gave similar responses to those used in the absence of antagonist were selected and repeated until stable equilibrium responses were obtained. Four additions of each of these doses were randomly applied, recorded and used to establish a second concentration-response curve. With these data the dose ratio could be calculated according to the equation formulated for competitive antagonists at equilibrium (Edinburgh Staff, 1970).

After calculating the dose ratio by this method the affinity constant (K) for each antagonist was calculated using the following equation (Schild, 1949):

$$K = \frac{\text{dose ratio}—1}{\text{molar concentration of antagonist}}$$

Affinity constants were determined for atropine and *N*-methyl atropine using carbaminoylcholine as the agonist, and for atropine using acetylbetamethylcholine as well as carbaminoylcholine as the agonists. The affinity constants were determined in the presence and absence of 1×10^{-4}M hexamethonium. Similar experiments were made using plexus-free preparations except that the affinity constant for *N*-methyl atropine was determined using both agonists.

Statistical methods

Regression lines were calculated by the method of least squares and the significance of the difference between two slopes and the 95% confidence intervals of regression

lines were determined as described by Wonnacott & Wonnacott (1972). Student t values were also determined for unpaired data for comparisons of affinity constants, using the pooled variance for small samples as outlined by Goldstein (1964). As such multiple t-tests on pairs of samples may give misleading results, duplicate calculations were performed according to the method of Scheffé, the results of which, although discussed as over-conservative by Armitage (1971), who considers the t-test appropriate for studies of the kind presented, are included to allow the reader to derive his own conclusions.

Materials

The following drugs and chemicals were used: carbaminoylcholine chloride (Carbachol, BDH), acetylbetamethylcholine chloride (Sigma), nicotine hydrogen tartrate (BDH), hexamethonium bromide (lot 42754L: K and K Laboratories), atropine sulphate (BDH), *N*-methyl atropine bromide (K and K). The constituents for Krebs solution and saline were all of Reagent Grade and were dissolved in distilled-deionized water.

RESULTS

Affinity constants

The affinity constants for both atropine and *N*-methyl atropine are shown in Table 1 and statistical comparisons in Table 2. When carbaminoylcholine was the agonist, the affinity constant determined for atropine in the presence of hexamethonium (1×10^{-4}M) was significantly lower by the simple t-test ($P < 0{\cdot}025$), but not by the Scheffé test, in both plexus-containing and plexus-free preparations [compare groups (1)–(2) and (3)–(4)]. The affinity constant for *N*-methyl atropine was significantly lower by both tests ($P < 0{\cdot}005$) in the presence of hexamethonium in the plexus-containing preparations [groups (5)–(6)] but not the plexus-free preparations. With acetylbetamethylcholine as the agonist, no significant difference ($P > 0{\cdot}1$) was found

Table 1. *Affinity constants ($k \times 10^9$) for atropine and* N-*methyl atropine on the longitudinal muscle of the guinea-pig ileum.*

	Carbaminoylcholine as the agonist							
	Plexus-containing				*Plexus-free*			
	No hexamethonium		Hexamethonium (1×10^{-4}M)		No hexamethonium		Hexamethonium (1×10^{-4}M)	
Atropine	(1)	$1{\cdot}8 \pm 0{\cdot}25$* (n = 11)	(2)	$0{\cdot}9 \pm 0{\cdot}06$ (n = 11)	(3)	$1{\cdot}9 \pm 0{\cdot}22$ (n = 11)	(4)	$1{\cdot}2 \pm 0{\cdot}14$ (n = 7)
N-Methyl atropine	(5)	$3{\cdot}4 \pm 0{\cdot}27$ (n = 9)	(6)	$1{\cdot}9 \pm 0{\cdot}14$ (n = 7)	(7)	$0{\cdot}6 \pm 0{\cdot}10$ (n = 9)	(8)	$0{\cdot}9 \pm 0{\cdot}09$ (n = 5)
	Acetyl-β-methylcholine as the agonist							
Atropine	(13)	$1{\cdot}8 \pm 0{\cdot}15$ (n = 2)	(14)	$1{\cdot}4 \pm 0{\cdot}37$ (n = 2)	(9)	$1{\cdot}4 \pm 0{\cdot}07$ (n = 5)	(10)	$1{\cdot}2 \pm 0{\cdot}10$ (n = 5)
N-Methyl atropine		—		—	(11)	$1{\cdot}2 \pm 0{\cdot}12$ (n = 5)	(12)	$1{\cdot}1 \pm 0{\cdot}26$ (n = 5)

* Mean affinity constants ± s.e.

Table 2. *Affinity constants: statistical comparisons between experimental groups.*

Groups	t_1*	t_2**	Degrees of Freedom d.f.	Probability *P*	Groups	t_1*	t_2**	d.f.	*P*
1–2	3·920	3·613	20	<0·005	9–10	0·587	1·772	8	>0·1
3–4	2·703	2·788	16	<0·025	9–11	0·587	1·586	8	>0·1
1–3	0·439	0·281	20	>0·1	10–12	0·293	0·352	8	>0·1
2–4	1·158	1·947	16	>0·05	7–11	2·000	3·301	12	<0·005
5–6	5·792	4·910	14	<0·005	8–12	0·587	0·776	8	>0·1
7–8	1·000	1·549	12	>0·1	3–9	1·730	2·455	14	<0·05
5–7	11·067	9·486	16	<0·005	4–10		0·232	10	>0·1
6–8	3·175	5·928	10	<0·005	13–14		1·077	2	>0·1
1–5	6·639	4·114	18	<0·005	13–9		2·490	5	>0·05
2–6	3·861	6·180	16	<0·005	14–10		0·501	5	>0·1
3–7	5·394	5·294	18	<0·005	1–13		0·225	11	>0·1
4–8	0·952	2·057	10	>0·05	2–14		1·212	11	>0·1
11–12	0·293	0·321	8	>0·1					

* The minimum requirement for significance between two groups based on pooled variance of all groups (except 13 and 14 which had only one degree of freedom) by the Schaeffé method at a probability level of 0·05 is a critical *t* value of 4·57. Values of t_1 which are underlined meet this requirement.

** Values of t_2 are determined by the simple Student *t*-test using the pooled variance of the groups being compared and the degrees of freedom indicated. The "*P* values" tabulated refer to t_2 and are considered by the authors as a valid comparison according to the statistical treatise of Armitage (1971).

between affinity constants determined for both atropine and *N*-methyl atropine either in the presence or absence of hexamethonium.

With carbaminoylcholine as agonist, *N*-methyl atropine was more active than atropine on the plexus-containing preparation [compare groups (1)–(5) (both tests) and (2)–(6) (simple *t*-test only)], whereas in plexus-free preparations *N*-methyl atropine was actually somewhat less active than atropine in the absence of hexamethonium [groups (3)–(7) (both tests)] and not significantly different in the presence of hexamethonium [groups (4)–(8) (both tests)]. With plexus-free strips and acetylbetamethylcholine as the agonist there was no significant difference between the affinity constants determined for the two antagonists [groups (9)–(11) and (10)–(12)].

The difference in type of preparation did not affect affinity constants obtained for atropine [compare groups (1)–(3), (2)–(4), (13)–(9) and (14)–(10)]. However, the presence or absence of plexus greatly affected the affinity constants determined for *N*-methyl atropine [compare groups (5)–(7) (both tests) and (6)–(8) (simple *t*-test only)].

The effect of hexamethonium on carbaminoylcholine and acetylbetamethylcholine dose-response curves

To attempt to explain the varied results with the two agonists in the presence or absence of hexamethonium, we measured the effect of hexamethonium on the agonist dose-response curves for carbaminoylcholine using plexus-containing or plexus-free preparations. Although not significantly altering the dose-response curves for plexus-containing preparations, hexamethonium caused a shift to the left of the dose-response curve with the plexus-free preparation. No significant difference in the slope of the

two lines was found ($P > 0{\cdot}2$) but when 95% confidence limits were calculated and plotted (Wonnacott & Wonnacott, 1972), it was found that the two lines did not overlap. The two curves were significantly different ($P < 0{\cdot}001$), hexamethonium causing a shift to the left of approximately 0·3 log units (Fig. 1). Hexamethonium caused no significant change in the dose-response curve obtained for acetylbetamethylcholine on the plexus-free longitudinal muscle strips.

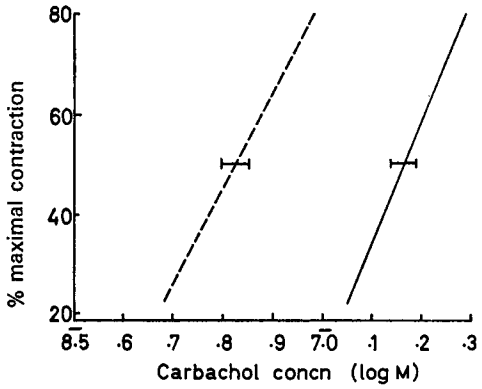

FIG. 1. Regression lines for the log dose-response curves for carbachol in the absence of hexamethonium (solid line) and in the presence of 1×10^{-4}M hexamethonium (broken line). The 95% confidence intervals about the 50% response are indicated.

DISCUSSION

Subsequent to these studies, we learned of the "atropinic" actions of hexamethonium found by Barlow, Franks & Pearson (1972), findings which are opposite to our present results and which would indicate a marked difference in the activity of the samples of hexamethonium employed. Recently Blackman (1973, personal communication) observed that "nearly all samples of hexamethonium bromide are contaminated with a depolarizing compound, presumably a monoquaternary, which can stimulate the . . . gut". Stephenson (1958) also mentioned that spontaneous activity sometimes produced by hexamethonium could be blocked by mepyramine.

Further experiments (Durham & Hamilton, 1973, unpublished results) using a different lot of hexamethonium indicate it to be atropinic as described by Barlow & others (1972). The chance observation with one batch of hexamethonium has, however, shed more insight into the binding properties of carbaminoylcholine and acetylbetamethylcholine and their antagonism to the effect of atropine than that of pure hexamethonium.

The study has demonstrated that the affinity constants determined for atropinic compounds using the Schild Equation yielded inconstant, often significantly different values. However, a closer scrutiny of the conditions under which different affinity constants were found allows the selection of conditions under which a claim for true constancy is valid: the plexus-free preparation in which hexamethonium was not employed and in which acetylbetamethylcholine was the agonist.

In the plexus-containing ileum of the guinea-pig, there are ganglion cells upon which an agonist with mixed nicotinic and muscarinic agonist potency may act and which might be blocked by the anti-nicotinic "pharmacological tool", hexamethonium, or indeed quaternary ammonium atropinic compounds such as *N*-methyl atropine. The

presence of muscarinic receptors in parasympathetic ganglia, similar to those now believed to be present in sympathetic ganglia (Trendelenburg, 1966), is still in dispute, although Roszkowski (1961) claimed they do not exist. Other factors which might lead to erroneous interpretation of the above results are the existence of sympathetic nerve endings (which Norberg, 1964, did not observe on the plexus-free, longitudinal muscle preparation) and the greater thickness of the plexus-containing preparation which offers more binding sites, both "specific" and "non-specific", in addition to a greater diffusion barrier.

The reciprocal interactions of sympathomimetic and parasympathomimetic drugs on cholinergic and noradrenergic nerve endings respectively, recently reviewed by Muscholl (1970), point to the necessity of simplifying the experimental procedure. Moreover, the recent controversy regarding the hexamethonium-sensitive, presynaptic release of acetylcholine from the guinea-pig ileum by such drugs as carbaminoylcholine, but not by acetylbetamethylcholine, has not yet been settled (Chiou & Long, 1969a,b; Marshall, 1971).

What was most striking in this study was not the variability but the lack of difference in the affinity constants for atropine and *N*-methyl atropine when acetylbetamethylcholine was the agonist. This in spite of the fact that it is generally believed that *N*-methyl atropine was 2–3 times as active [e.g. Edinburgh Staff, 1970; and our own results on plexus-containing preparations—compare groups (1) and (5)].

The higher affinity constant for *N*-methyl atropine when tested on the plexus-containing preparation, with carbaminoylcholine as agonist, might simply be a reflection of its anti-nicotinic action on the parasympathetic ganglia.

If it is conceded that the investigation was aimed at elucidating the effect of hexamethonium on the preparations and thus according to Armitage (1971) the simple *t*-tests are valid, then the present study has identified an interesting action of hexamethonium. The decreased apparent affinity constants of atropinic agents observed in the presence of hexamethonium suggest that it might be acting directly or indirectly to decrease the binding of atropine. If this was by direct interference at an identical site, then this should have occurred with either agonist. The significant shift to the left of the log dose-response curve of carbaminoylcholine, but not of acetylbetamethylcholine, on the plexus-free preparation again suggests some specific and significant interaction between carbaminoylcholine and hexamethonium at a smooth muscle receptor site.

Moreover, the lack of interaction between acetylbetamethylcholine and the same batch of hexamethonium as used in the parallel carbaminoylcholine studies strongly suggests that the competitive action of atropine is mediated through an allosteric rather than a direct binding mechanism. Chiou & Long (1969a,b) claimed that, in addition to stimulating the muscarinic receptor, carbaminoylcholine has major ganglion stimulating action and, additionally, a possible nerve-ending stimulant component which was blocked by hexamethonium. The apparent discrepancy in our results when hexamethonium was added to plexus-containing preparations stimulated by carbaminoylcholine may be explained by assuming an algebraic sum of the net effect of ganglion and neuronal blockade of acetylcholine release, which displaces the dose-response curve to the right, and the selective potentiating effect of the sample of hexamethonium on the response to carbaminoylcholine, shown on the plexus-free preparation, which displaces the curve to the left. This explanation, however, may not be entirely acceptable since Marshall (1971) has refuted the claim

of Chiou and Long that the prime action of carbaminoylcholine is the presynaptic release of acetylcholine from ganglia and nerve endings.

Hexamethonium has generally been regarded as a ganglion-blocker with very little effect at the muscarinic receptor. The results of this study are consistent with the hypothesis that some samples of hexamethonium may be interacting at similar sites to carbaminoylcholine at the muscarinic receptor. The site must be post-synaptic because these effects have been demonstrated on plexus-free preparations (Norberg, 1964; Paton, 1964; Gabella, 1970). Also, where acetylcholine is involved either as an intrinsically released transmitter or as a pharmacologically applied agonist, the weak anticholinesterase activity of hexamethonium should be borne in mind (Lullman, Ohnesorge & others, 1971; Gandiha, Green & Marshall, 1972). Yet other studies have suggested that the action of hexamethonium might not be entirely ganglionic (Greenberg, Kosterlitz & Waterfield, 1970; Gandiha & others, 1972; Ferry & Marshall, 1973).

Koshland (1960), Belleau (1965) and Wilson (1967) have prompted the hypothesis that the function of active sites can be influenced by drugs bound to neighbourhood or "side receptors". We may then consider such a receptor arrangement with more than one possible site for the action of acetylcholine-like agonists and their antagonists. The non-equivalance of acetylbetamethylcholine and carbaminoylcholine, with regard to our sample of hexamethonium, on the plexus-free preparation blocked by atropine suggests either that the two agonists act at different sub-sites or that the postulated contaminant in this hexamethonium had a greater affinity than carbaminoylcholine for these sites.

Should some agonists be capable of producing metaphilic changes, as suggested by Rang & Ritter (1969a,b; 1970), then it is possible that acetylbetamethylcholine is less efficacious in this respect than carbaminoylcholine. These concepts have been considered by some as an explanation for the atropine anomaly; i.e. atropine and the agonist are likely not to be acting upon the same receptors but upon adjacent receptors (Goldstein, Aronow & Kalman, 1968). Evidence that atropine is capable of combining with a distinct but adjacent site to that which binds eserine on the acetylcholinesterase molecule has been provided by the elegant studies of Kato & Yung (1971).

The identification of the contaminant of hexamethonium and knowledge of its pharmacology seem to be mandatory to throw more light on the interaction of cholinergic agents on the muscarinic receptors of guinea-pig smooth muscle.

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