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Some effects of pilocarpine and acetylcholine on the responses of the guinea-pig vas deferens to hypogastric nerve stimulation and transmural stimulation and applied (−)-noradrenaline

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Enhancement by acetylcholine and by pilocarpine of the contractile responses of the guinea-pig isolated vas deferens to hypogastric nerve and transmural stimulation and to exogenous (−)-noradrenaline was examined. At high concentration (600 nM) acetylcholine enhanced responses in the order: hypogastric nerve > transmural > exogenous (−)-noradrenaline. Pilocarpine produced a similar maximal degree of enhancement with all three stimuli. All these effects were reversibly inhibited by 1·4 μm atropine, a concentration which alone did not affect responses to the stimuli. Pilocarpine (50–400 μM) gave a concentration-related inhibition of the enhancement of responses to hypogastric nerve stimulation by 600 nM acetylcholine.

It has been reported that acetylcholine is capable of enhancing the contractile responses obtained to hypogastric nerve and transmural stimulation in guinea-pig and rat isolated vasa deferentia, at doses which do not themselves cause contraction of the muscle and that the enhancement can be blocked by low concentrations of atropine (Sjöstrand, 1961; Takikata, 1972; Rossignol, Valette & Massé, 1965; Graham, Katib & Spriggs, 1968; Sjöstrand & Swedin, 1968).

Our study confirms these findings and shows that the effects are not ascribable, solely, to the enhancement of noradrenaline effect at the post-synaptic membrane.

Pilocarpine is a muscarinic agonist (Volle, 1963), whose main therapeutic use is in the treatment of glaucoma (Weber, 1877; Ringer & Gould, 1875; Kolker & Hetherington, 1970). It has been shown to be a partial agonist at muscarinic receptors on various tissues; rat ileum, frog heart, cat blood pressure (van Rossum, 1961), rabbit fundus (Furchgott & Bursztyn, 1967), and guinea-pig ileum (Waud, 1969). Rand & Varma (1970) have shown in the rabbit isolated ear artery preparation that it enhances vasoconstriction during sympathetic stimulation at high frequencies (20 Hz). However, Steinsland, Furchgott & Kirkepar (1973) found inhibition of the vasoconstriction at low frequencies of stimulation (1–4 Hz) in this preparation. Pilocarpine also causes potentiation of responses to sympathetic nerve stimulation in the cat superior cervical nerve-nictitating membrane preparation (Trendelenburg, 1956) and has secondary pressor effects on the cat blood pressure ascribed to stimulation of sympathetic nerves (Trendelenburg, 1961).

This study investigated the effects of pilocarpine and acetylcholine in modifying adrenergic stimulation of the guinea-pig isolated vas deferens.

MATERIALS AND METHODS

Albino guinea-pigs 400–600 g, were killed by neck dislocation and bleeding. Vasa deferentia with hypogastric nerve attached were dissected according to Huković (1961) for hypogastric nerve stimulation. For the transmural stimulation and applied (—)-noradrenaline experiments the vas was set up without the hypogastric nerve. The tissue was mounted on an electrode which incorporated facilities for hypogastric nerve and transmural stimulation via platinum wires, and placed in a 25 ml overflow, isolated organ bath containing Krebs-Henseleit solution, aerated with 5% CO_2 in oxygen and maintained at 32°. A "Square One" S.69 square wave pulse generator was used for electrical stimulation.

Hypogastric nerve stimulation was by using supramaximal (50 V) shocks of 0·1 ms pulse width at 5–50 Hz. 10 s trains of stimuli were given at all times. A 3 min time cycle with two changes of bath fluid was used. Drugs were added to the bath in 0·1 ml volumes 1 min before stimulation unless otherwise stated. Transmural stimulation was by using supramaximal (100 V) shocks of 0·1 ms pulse width at 5–50 Hz. 10 s trains were again used and the general procedure outlined for hypogastric nerve stimulation was followed. (—)-Noradrenaline (10–100 μM) contractions were induced with 1 min contact time in a 4 min cycle with three changes of bath fluid. Other drugs were added to the bath in 0·1 ml volumes 1 min before (—)-noradrenaline.

All responses were recorded isotonically on a smoked drum kymograph with a frontal writing lever; load 1 g, magnification × 15. Initially concentration- or stimulus-effect relations to the applied stimuli were obtained. Two sub-maximal responses were then selected, the smaller being a contraction 15–20% of the maximum height obtained for the tissue and caused by a stimulus of frequency x; the other being elicited by twice the frequency x: hence '2x response'. Regular responses to these two were obtained before addition of acetylcholine or pilocarpine to the bath. These were added as described above, and the height of contraction caused by the x stimulus was compared to the response obtained to 2x stimulus before their addition and expressed as a percentage of this response. Responses to x and 2x were allowed to return to normal levels after acetylcholine or pilocarpine had been washed from the bath, and regular responses to these again obtained before any further addition of acetylcholine or pilocarpine. Responses to the 4x stimulus were also obtained at regular intervals to ensure that the responses to x and 2x were still submaximal.

The results were analysed using Student's *t*-test.

Drugs used were: acetylcholine chloride, pilocarpine hydrochloride, atropine sulphate (BDH), (—)-noradrenaline bitartrate (Koch-Light). All drugs were made up in 0·9% w/v sodium chloride. Concentrations given are final bath concentrations.

RESULTS

Effect of acetylcholine and pilocarpine on responses to hypogastric nerve stimulation

Within a concentration range of 10–600 nM, acetylcholine increased the height of contraction to a selected sub-maximal frequency of stimulation by up to fivefold. A concentration-effect relation was obtained, the responses being expressed as a percentage of the response to twice the selected frequency in the absence of acetylcholine (Fig. 1). The enhancement of responses had not reached a maximum at the concentrations of acetylcholine used; however, higher concentrations of acetylcholine

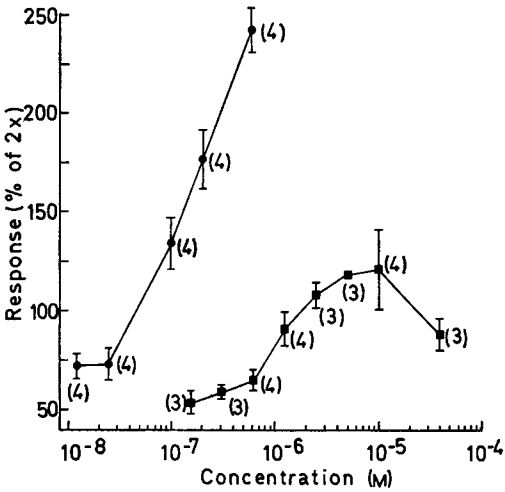

FIG. 1. Concentration-effect curves of enhancement of contractile responses of the isolated vas deferens of guinea-pig to stimulation of the hypogastric nerve by : ● acetylcholine; ■ pilocarpine. Responses to stimulus x Hz in presence of drug given as percentage of initial responses to 2x in the absence of drug. n in parentheses = no. of experiments. Each point is the mean and vertical bars indicate ± s.e.

consistently caused contraction of the muscle. At no time were the responses increased to a height greater than the maximum achieved by nerve stimulation alone.

Pilocarpine, even in high concentrations (10 mM) was not able to cause contraction of the muscle. Concentrations between 1 and 400 μM increased the height of contraction obtained to nerve stimulation by up to two-threefold. A maximal effect was seen at 100 μM. With higher concentrations a reduction in the degree of enhancement was seen (Fig. 1). The maximum effect achieved with pilocarpine was significantly lower than that obtained with 600 nM acetylcholine ($P < 0{\cdot}001$).

Atropine, 1·4 μM, had no effect on contractions elicited by nerve stimulation when added to the bath 90 s before stimulation. However, if it was added to the bath at this time and followed by either pilocarpine (100 μM) or acetylcholine (100 nM), which are known to cause enhancement of responses to hypogastric nerve stimulation, a total inhibition of this enhancement was seen, (n = 3 for each drug). This inhibitory effect was reversible, as the initial response obtained to pilocarpine or acetylcholine could be repeated after normal washing and recovery procedures.

Effect of acetylcholine and pilocarpine on responses to transmural stimulation

Concentrations of acetylcholine from 10–600 nM enhanced the responses obtained to transmural stimulation up to more than threefold. The increase in the size of a selected sub-maximal response is shown in Fig. 2, expressed as a percentage of the response to twice that frequency in the absence of acetylcholine. Maximum responses had again not been reached with acetylcholine concentrations which did not themselves cause contraction of the muscle. The effect obtained with a concentration of 600 nM on transmural stimulation was significantly less than that obtained with the same concentration with hypogastric nerve stimulation ($P < 0{\cdot}005$).

Pilocarpine concentrations in the range 6–800 μM increased responses by up to two-

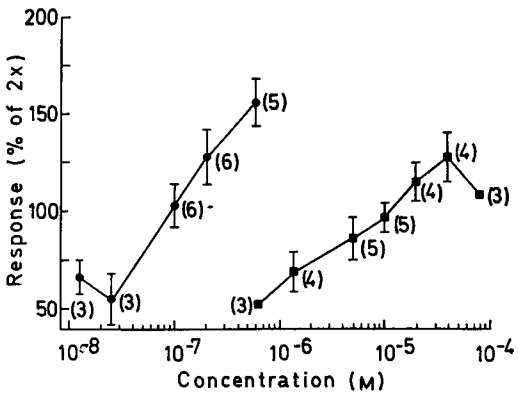

FIG. 2. Concentration-effect curves of enhancement of contractile responses of the isolated vas deferens of guinea-pig to transmural stimulation by: ● acetylcholine; ■ pilocarpine. Responses to stimulus x Hz in presence of drug given as percentage of initial responses to 2x in the absence of drug. n in parentheses = no. of experiments. Each point is the mean and vertical bars indicate ± s.e.

threefold. Maximum effects were reached at a concentration of 400 μM (Fig. 2). The maximum enhancement by pilocarpine of responses to hypogastric nerve or transmural stimulation were not significantly different ($P > 0{\cdot}80$). However, there did appear to be a slight separation of the concentration effect curves, with that to transmural stimulation being to the right.

A reduction in the degree of enhancement with high concentrations, similar to that described for hypogastric nerve stimulation, was seen. The maximum effect seen with acetylcholine (600 nM) on transmural stimulation was not significantly different from the maximum effect seen with pilocarpine (400 μM) $P > 0{\cdot}15$).

Atropine, 1·4 μM was without effect on responses to transmural stimulation when added to the bath 90 s before stimulation. When added at this time and followed by either pilocarpine (100 μM) or acetylcholine (100 nM), which are known to cause enhancement of responses to transmural stimulation, it caused a total inhibition of enhancement, (n = 3 for each drug). As with hypogastric nerve stimulation, this effect was reversibile.

Comparison of effects

To enable a comparison of the effects of acetylcholine and pilocarpine to be made, pE_2 values were calculated for each compound for each method of stimulation. The pE_2 value is the negative logarithm of the molar concentration of a drug required to enhance the response to the x stimulus to the height of the response to the stimulus 2x and is analogous to the pPy value described by Edge (1967). The values were taken from individual concentration-effect curves and the mean and standard error of the mean calculated, (Table 1). It is apparent that the pE_2 in this context enables comparison of effects of low concentrations of the drugs to be made (see Table 2) and that a somewhat different pattern is seen to that described for high concentrations. Clearly acetylcholine is more potent than pilocarpine, but at these levels no significant difference is found between its effects on responses to transmural and hypogastric nerve stimulation.

Table 1. *pE_2 values obtained from contractions of the guinea-pig isolated vas deferens to different electrical stimuli in the presence of acetylcholine or pilocarpine.* pE_2 is defined as the negative logarithm of the molar concentration of drug required to enhance the response to stimulus of frequency x Hz to that obtained with 2x in the absence of drug.

Drug	Stimulus	Mean pE_2 value (n in parentheses)	± s.e. mean
Pilocarpine	Hypogastric	5·675 (5)	0·136
Pilocarpine	Transmural	5·159 (6)	0·147
Acetylcholine	Hypogastric	7·281 (6)	0·088
Acetylcholine	Transmural	7·049 (7)	0·124

Table 2. *Statistical comparisons of pE_2 values obtained from contractions of the guinea-pig isolated vas deferens to different electrical stimuli in the presence of acetylcholine or pilocarpine.*

Comparison made between:	Probability
Acetylcholine hypogastric: acetylcholine transmural	$> 0{\cdot}20$
Acetylcholine hypogastric: pilocarpine hypogastric	$< 0{\cdot}001$
Acetylcholine transmural: pilocarpine transmural	$< 0{\cdot}001$
Pilocarpine transmural: pilocarpine hypogastric	$< 0{\cdot}05$

Effect of acetylcholine and pilocarpine on applied (−)-noradrenaline responses

Acetylcholine, 10–600 nM, caused a slight enhancement of response. Up to a two-fold increase was seen. The increase in the size of response to a selected sub-maximal concentration of noradrenaline is shown in Fig. 3 as a percentage of the response to twice that concentration of noradrenaline in the absence of acetylcholine. The effect obtained with acetylcholine (600 nM) was significantly lower than that seen with the same concentration on hypogastric nerve or transmural stimulation ($P < 0{\cdot}001$ and $< 0{\cdot}01$ respectively).

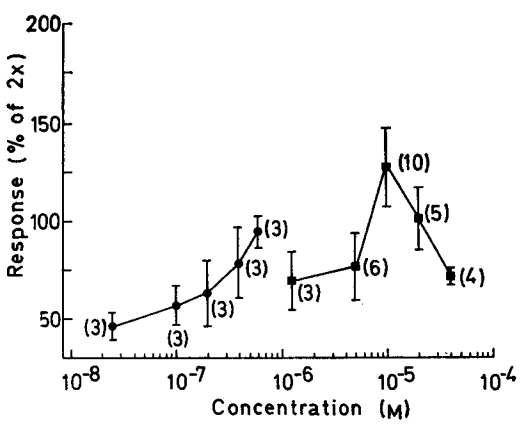

FIG. 3. Concentration-effect curves of enhancement of responses of the isolated vas deferens of guinea-pig to exogenously applied (−)-noradrenaline by: ● acetylcholine; ■ pilocarpine. Responses to concentration x of (−)-noradrenaline in the presence of acetylcholine or pilocarpine expressed as percentage of initial responses to 2x in the absence of drug. n in parentheses = no. of experiments. Each point is the mean and the vertical bars indicate ± s.e.

Pilocarpine, 10–400 μM, was found to cause a more marked enhancement of responses than acetylcholine (Fig. 3). Maximal effects were achieved with a 100 μM concentration. The degree of enhancement observed was not significantly different from that seen with pilocarpine (100 μM) on either hypogastric nerve or transmural stimulation ($P > 0{\cdot}90$ and $> 0{\cdot}30$ respectively). A similar reduction in the degree of enhancement was seen with high concentrations of pilocarpine.

Atropine, 1·4 μM, was without effect on responses to applied (−)-noradrenaline, when added to the bath 90 s beforehand. However, if atropine was added at this time and followed by either pilocarpine (100 μM) or acetylcholine (600 nM), which are known to cause enhancement of the responses to (−)-noradrenaline, a total inhibition of this enhancement was seen, (n = 3 for each drug). This inhibition was found to be reversible.

Effect of pilocarpine on acetylcholine enhancement of responses to hypogastric nerve stimulation

In Fig. 4 the effects of increasing concentrations of pilocarpine on the enhancement produced by acetylcholine (600 nM) are seen. In these experiments the effect of acetylcholine alone on hypogastric nerve stimulation was first obtained. After normal washing and recovery procedures this response was repeated in the presence of pilocarpine added to the bath 30 s before the acetylcholine. Pilocarpine reduced the observed effect of acetylcholine. The inhibition seen was reversible as the original response obtained with acetylcholine could be repeated after normal recovery procedures.

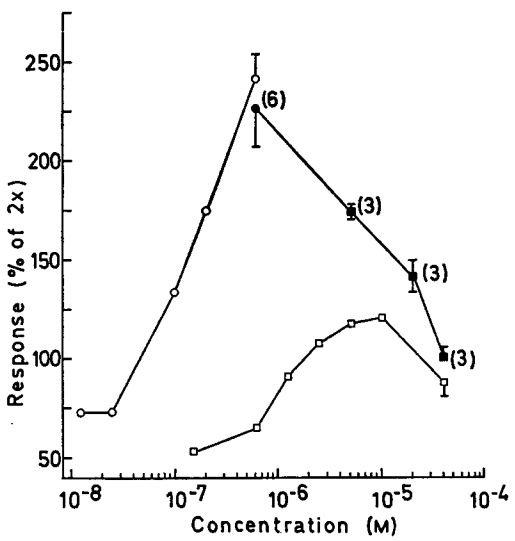

FIG. 4. Concentration-effect curve showing reduced enhancement of contractile responses of the guinea-pig isolated vas deferens to hypogastric nerve stimulation by 600 nM acetylcholine in the presence of increasing concentrations of pilocarpine. Responses to stimulus x Hz in presence of drugs given as percentage of initial responses to 2x in the absence of drugs. ○ acetylcholine concentration-effect curve (see Fig. 2). □ pilocarpine concentration-effect curve (see Fig. 2). ● response in presence of 600 nM acetylcholine without pilocarpine. ■ responses in presence of 600 nM acetylcholine plus various concentrations of pilocarpine. n in parentheses = no. of experiments. Each point is the mean and the vertical bars indicate ± s.e.

DISCUSSION

The results confirm the observations of others that acetylcholine enhances the responses obtained to electrical stimulation of the guinea-pig vas deferens (Sjöstrand, 1961; Takikata, 1972; Rossignol, Valette & Massé, 1965; Sjöstrand & Swedin, 1968). The degree of enhancement which occurred was concentration-dependent.

Acetylcholine also enhanced slightly the responses obtained to applied (−)-noradrenaline. Similar enhancement of (−)-noradrenaline responses has been observed with rabbit ear artery preparations (Rand & Varma, 1970; Steinsland & others, 1973). Bell (1967) found that choline also could slightly enhance applied (−)-noradrenaline responses of the guinea-pig vas deferens and ascribed this to a direct action of choline on muscarinic receptors on the muscle membrane, or an effect of choline in enhancing the effect of endogenous acetylcholine, to cause a depolarization of the membrane and increased excitability of the tissue. This effect was blocked by atropine.

Sjöstrand (1971), using vas deferens-hypogastric nerve preparations with the sucrose gap technique, showed that the main action of exogenous acetylcholine was to cause a depolarization of the smooth muscle membrane associated with a concomitant increase in excitability of the organ to nerve stimulation and also to affect calcium ion fluxes in the tissue and consequently to affect contraction processes.

In the experiments performed here, although the pE_2 values for acetylcholine in low concentrations were similar for hypogastric nerve stimulation and transmural stimulation, at higher concentrations responses to the three stimuli were enhanced to different degrees. The effect on responses to (−)-noradrenaline is probably due to direct action on the muscle, but the additional enhancement with transmural or hypogastric nerve stimuli presumably involves other mechanisms.

The innervation of the vas deferens, via the hypogastric nerve trunk, is mainly of sympathetic adrenergic nature and ganglia, pre- and post-ganglionic fibres have been found (Graham, & others, 1968; Ferry, 1967). There is also evidence for a cholinergic component (Bhargava, Kar & Parmar, 1965; Ohlin & Strömblad, 1963) and fibres containing acetylcholinesterase have been demonstrated using histochemical methods (Gosling & Dixon, 1972; Bell & McLean, 1967). In the vas the outer longitudinal and inner circular muscle layers both have a rich adrenergic innervation along their whole length. There is a sparse cholinergic innervation to the circular muscle and a very few fibres to the outer longitudinal layer, (Gosling & Dixon, 1972) and also to the mucosa (Bell & McLean, 1967). Transmission of impulses in the longitudinal muscle is reported to be solely adrenergic (Furness, 1974).

Hypogastric nerve stimulation, therefore, probably involves fibres of adrenergic and cholinergic origin. However, in our experiments, the cholinergic component would not appear to be involved in normal responses to hypogastric nerve stimulation as, in the presence of atropine, no change in height of contraction was seen. Transmural stimulation presumably involves mainly post-ganglionic effects (Birmingham & Wilson 1963). Responses to transmural stimulation were again unaffected by atropine This supports the observations of Graham & others (1968).

The difference in effects, seen with the same high concentrations of acetylcholine between hypogastric nerve and transmural stimulation may, therefore, be due to ganglionic effects of acetylcholine in the former case. All these effects appear to be muscarinic in nature, as the enhancement is totally inhibited by atropine and, therefore, the results are consistent with the suggestion of the presence of muscarinic receptors in sympathetic ganglia (Jones, 1963; Trendelenburg, 1966).

The difference between the effects of acetylcholine on responses to transmural stimulation and to applied (−)-noradrenaline might be due to increased release of noradrenaline from sympathetic nerve ending during transmural stimulation. This has been suggested as a possible mechanism in the rabbit ear artery preparation (Rand & Varma, 1970), where low doses of acetylcholine enhance the effects of sympathetic nerve stimulation. Alternatively, the difference might reflect an effect on penetration of exogenous (−)-noradrenaline which would not operate when endogenous noradrenaline is released near its site of action. Recent work (Furness, 1974) using electronmicroscopy suggests that the adrenergic nerves lie in invaginations in the muscle membrane, and are protected by a basement membrane which might provide a barrier to the free diffusion of exogenous noradrenaline. However, antagonism of the effect by atropine suggests that a direct effect on muscle muscarinic receptors is more likely.

Penetration of exogenous (−)-noradrenaline can be improved by using stripped vasa (Birmingham, 1970). However, this would probably also affect the penetration of acetylcholine and pilocarpine in the tissue and hence the comparison of results between modes of stimulation would be less valid.

Pilocarpine appears to behave in a fairly similar manner with all three stimuli. Although a small significant difference was seen between the pE_2 values, all three stimuli gave maximal responses at similar concentrations. The enhancement of responses to exogenous (−)-noradrenaline suggests an effect on the muscle similar to that proposed for acetylcholine. With other stimuli other mechanisms may be involved but obviously only to a slight degree.

In concentrations similar to those giving maximal intrinsic enhancement of responses to the three stimuli, pilocarpine gave a concentration-related reversible inhibition of the enhancement by acetylcholine of responses to hypogastric nerve stimulation, caused by the maximum used concentration of acetylcholine. Thus it is probable that at sites where acetylcholine has effects leading to enhancement of responses but pilocarpine effects are negligible or absent, e.g. ganglia and nerve-endings as suggested above, pilocarpine has an antagonistic function. It is not, however, possible to deduce at present whether this is a specific effect at muscarinic receptors.

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