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PHARMACOLOGICAL CHARACTERIZATION OF THE PRESYNAPTIC α-ADRENOCEPTORS REGULATING CHOLINERGIC ACTIVITY IN THE GUINEA-PIG ILEUM

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1 The presynaptic α-adrenoceptors located on the terminals of the cholinergic nerves of the guinea-pig myenteric plexus have been characterized according to their sensitivities to α-adrenoceptor agonists and antagonists.

2 Electrical stimulation of the cholinergic nerves supplying the longitudinal muscle of the guinea-pig ileum caused a twitch response. Clonidine caused a concentration-dependent inhibition of the twitch response; the maximum inhibition obtained was 80 to 95% of the twitch response. Oxymetazoline and xylazine were qualitatively similar to clonidine but were about 5 times less potent. Phenylephrine and methoxamine also inhibited the twitch response but were at least 10,000 times less potent than clonidine.

3 The twitch-inhibitory effects of clonidine, oxymetazoline and xylazine, but not those of phenylephrine or methoxamine, were reversed by piperoxan (0.3 to 1.0 μg/ml).

4 Lysergic acid diethylamide (LSD) inhibited the twitch response, but also increased the basal tone of the ileum. Mepyramine prevented the increase in tone but did not affect the inhibitory action of LSD. Piperoxan or phentolamine only partially antagonized the inhibitory effect of LSD.

5 Phentolamine, yohimbine, piperoxan and tolazoline were potent, competitive antagonists of the inhibitory effect of clonidine with pA_2 values of 8.51, 7.78, 7.64 and 6.57 respectively.

6 Thymoxamine was a weak antagonist of clonidine; it also antagonized the twitch-inhibitory effect of morphine. Thus, its effect against clonidine is probably not mediated specifically at presynaptic α-adrenoceptors.

7 Labetalol, itself, depressed the twitch response but did not antagonize the inhibitory effect of clonidine on the residual twitch.

8 The results demonstrate that the presynaptic α-adrenoceptors in the guinea-pig ileum are of the same type as those located presynaptically in sympathetically innervated tissues. They are α_2-adrenoceptors and are different from those located postsynaptically.

Introduction

The guinea-pig ileum receives parasympathetic and sympathetic innervations. The preganglionic, parasympathetic nerves synapse primarily in the myenteric (Auerbach's) plexus, a dense network of short, mainly cholinergic postganglionic fibres. Stimulation of these nerves releases acetylcholine which, in turn, causes contraction of the longitudinal muscle fibres. The longitudinal muscle receives only a sparse adrenergic innervation; instead, the adrenergic nerve terminals form a network around the intramural neurones (for references see reviews by Furness & Costa (1974) and Wilkberg (1977)). Stimulation of the adrenergic nerves inhibits cholinergic nerve activity and thus reduces responses to cholinergic nerve stimulation; the adrenergic-inhibitory effect is reduced by phentolamine (Kröneberg & Oberdorf, 1974).

The twitch response of the guinea-pig ileum to low frequency stimulation of the cholinergic nerves is also inhibited by adrenaline, noradrenaline and isoprenaline. The maximum inhibition caused by adrenaline or noradrenaline is greater than that produced by isoprenaline and differs from it in being accompanied by a reduction in the release of acetylcholine from the cholinergic nerves. β-Adrenoceptor blockade greatly reduces or abolishes the twitch inhibition caused by isoprenaline but has little or no effect on either the twitch-inhibition or the reduction in acetylcholine release caused by adrenaline. On the other

hand, α-adrenoceptor blockade prevents both actions of adrenaline, but has little effect on isoprenaline. Noradrenaline-induced twitch inhibition is reduced by α- or β-adrenoceptor blockade, but its effects on acetylcholine release are prevented only by α-antagonists. From these observations it has been concluded that, in the guinea-pig ileum, twitch-inhibition caused by β-adrenoceptor stimulation is the result of a direct inhibitory action on the longitudinal muscle, whereas the twitch inhibition caused by α-adrenoceptor stimulation is primarily the result of a reduction in the release of acetylcholine, although a small component of the inhibition may be mediated via α-adrenoceptors on the smooth muscle (Anderson & Lees, 1976). The α-adrenoceptors involved in regulating acetylcholine release are thought to be located presynaptically at the postganglionic cholinergic nerve terminals (Paton & Vizi, 1969; Kosterlitz, Lydon & Watt, 1970; Knoll & Vizi, 1971). This inhibitory mechanism is analogous to that which modulates the release of noradrenaline from sympathetic nerve terminals (Langer, 1974).

It has recently been shown that pre- and postsynaptic α-adrenoceptors in sympathetically innervated tissues are different. In particular, presynaptic α-adrenoceptors are less sensitive than postsynaptic receptors to the agonist actions of phenylephrine and methoxamine (Starke, 1972; Starke, Endo & Taube, 1975; Steinsland & Nelson, 1975; Drew, 1976; 1977a), and to the antagonist actions of phenoxybenzamine (Dubocovich & Langer, 1974), labetalol (Blakely & Summers, 1977) and thymoxamine (Drew, 1976; 1977a). Langer (1974) has suggested the notation α_1 for post- and α_2 for presynaptic α-adrenoceptors. The present experiments were carried out to characterize the α-adrenoceptors present in the guinea-pig ileum. A preliminary account of the results has been presented to the British Pharmacological Society (Drew, 1977b).

Methods

Male or female guinea-pigs (Duncan Hartley-Porcellus) weighing 300 to 400 g were killed by cervical dislocation and the small intestine was removed. The 10 cm nearest to the ileocaecal junction was discarded. After carefully washing out the luminal contents, segments of ileum, 2 to 3 cm long were selected from the terminal portion and suspended under an initial tension of 0.5 to 1.0 g in Krebs solution at 37°C in a 50 ml gut bath. The composition of the Krebs solution was (mmol/l): Na^+ 143.4, K^+ 5.9, Mg^{2+} 0.6, Ca^{2+} 1.3, Cl^- 124.5, $H_2PO_4^-$ 1.2, SO_4^{2-} 0.6, HCO_3^- 25; and glucose 11.1. The solution contained propranolol (0.3 μg/ml; 1.2 μmol/l) to block β-adrenoceptors and was bubbled continuously with 5% CO_2 and 95% O_2. Intramural nerves were stimulated electrically with supramaximal square wave pulses, 1 ms in duration, delivered at a frequency of 0.1 Hz from a Farnell physiological stimulator connected to two platinum electrodes placed on either side of the ileum. Contractions of the longitudinal muscle were recorded isometrically, with a Dynamometer UF 1 2 oz strain gauge, and displayed on a Devices chart recorder.

Measurement of α-agonist potency

When twitch responses to transmural stimulation had become constant, one of the agonists was added to the bathing fluid in a cumulative-concentration schedule. The interval between successive doses was adjusted to allow the effect of each dose to develop fully. The concentration of each agonist required to reduce the twitch responses by 50% was determined. At the end of each experiment piperoxan (0.3 to 1 μg/ml; 1.3 to 4.3 μmol/l) was added to the bathing fluid. If piperoxan reversed the twitch-inhibition it was assumed that the agonist had exerted its effect *via* α-adrenoceptors.

Effects of clonidine on contractile responses to acetylcholine

In three unstimulated preparations the effect of clonidine on contractile responses to acetylcholine was examined. A concentration-response curve to acetylcholine was first established; then a submaximal dose (30 ng/ml; 0.2 μmol/l) producing about 80% of the maximum attainable response, was administered at 4 min intervals until tissue sensitivity became constant. Clonidine (1 to 10 ng/ml; 4.3 to 43 nmol/l) was then added to the bathing fluid and the response to acetylcholine was measured 2 min later.

Measurement of α-antagonist potency

Initial experiments showed that the inhibitory effects of repeated administration of clonidine (0.3 to 30 ng/ml; 1.3 to 130 nmol/l) were reproducible over a period of 3 to 4 h. Therefore the following experimental protocol was observed in the subsequent experiments. A concentration-response curve to clonidine was first established. Exposure to clonidine was then discontinued; when the twitch responses had recovered, the antagonist was added to the bathing fluid. The concentration response curve to clonidine was repeated 15 min later in the presence of the antagonist. This procedure was repeated on two further occasions with progressively higher concentrations of antagonist. Clonidine dose-ratios were determined

from the concentrations causing 50% of the maximum inhibition of the twitch response in the absence and in the presence of each concentration of antagonist. Results were expressed in the form of a Schild plot (Arunlakshana & Schild, 1959), and the pA_2 and slope of the regression line were calculated.

In some experiments, morphine was used instead of clonidine to inhibit the twitch response. Morphine, like adrenaline, inhibits the release of acetylcholine from the cholinergic nerves of the myenteric plexus (Paton, 1957; Paton & Aboo Zar, 1968) but its effects are mediated specifically *via* presynaptic opiate receptors, (Kosterlitz & Watt, 1968; Vizi, 1974). In preliminary experiments it was found that the second of two consecutive concentration-response curves to morphine (0.1 to 100 ng/ml; 0.35 to 350 nmol/l) was always displaced about 3 to 5 fold to the right of the first curve ($n = 6$). Thereafter there was little or no further shift of repeated morphine dose-response curves. Accordingly, in subsequent experiments, the first curve was ignored and only the second to the fifth concentration-response curves were used to determine the specificity of the antagonists.

Drugs

The following drugs were used; acetylcholine chloride (BDH), atropine sulphate (BDH), clonidine hydrochloride (Boehringer Ingelheim), labetalol (5-[1-hydroxy-2-[(1-methyl-3-phenylpropyl)amino]ethyl]-salicylamide hydrochloride; AH 5158, Allen and Hanburys), lysergic acid diethylamide tartrate (Sandoz), (±)-methoxamine hydrochloride (Burroughs Wellcome), mepyramine maleate (May and Baker), morphine hydrochloride (MacFarlan Smith), naphazoline nitrate (Ciba), oxymetazoline hydrochloride (Merck), phentolamine mesylate (Ciba), (−)-phenylephrine hydrochloride (Koch-Light), piperoxan hydrochloride (May and Baker), tolazoline hydrochloride (Ciba), thymoxamine hydrochloride (Warner), xylazine (2-(2,6-dimethylphenylamino)-4-H-5,6-dihydro-1,3-thiazin hydrochloride; BAY-1470, Bayer AG) and yohimbine hydrochloride (Sigma). All drugs were dissolved in 0.9% w/v NaCl solution (saline) or distilled water immediately before use. Concentrations mentioned in the text refer to the base.

Results

Electrical stimulation of the guinea-pig ileum at 0.1 Hz produced a twitch response. The peak tension developed in each response was generally between 1 and 3 g, and there was little variation in individual preparations over 3 to 4 h. Atropine (1 μg/ml; 3.5 μmol/l) abolished the responses, confirming that they were produced by cholinergic nerve stimulation. Hexamethonium (10 to 30 μg/ml; 50 to 150 μmol/l) reduced the responses by only about 10% showing that the nerves involved were mainly postganglionic.

Effects of α-adrenoceptor agonists on twitch responses

Clonidine (0.1 to 30 ng/ml) produced a concentration-dependent reduction in the twitch response of the ileum, but rarely abolished it; the maximum inhibition obtained was 80 to 95% (Figure 1). Twitch responses recovered rapidly after the tissue was washed with clonidine-free Krebs solution. Tissue sensitivity to repeated administration of clonidine remained constant over a period of 3 to 4 h; in seven experiments, the mean concentrations of clonidine producing 50% of the maximum inhibition in four consecutive concentration-response curves were 1.62, 1.70, 1.76 and 1.56 ng/ml respectively (similar results have been reported recently by Hughes, Kosterlitz, Robson & Waterfield, 1978). The maximum twitch inhibition produced by clonidine remained constant. In two experiments hexamethonium (10 and 30 μg/ml) did not alter the responsiveness to clonidine, which indicates that its site of action is probably the postganglionic cholinergic nerve terminal.

Oxymetazoline and xylazine also reduced the twitch response. The maximum inhibition produced by these compounds was similar to that produced by clonidine but these agents were about 5 times less potent than clonidine. Phenylephrine and methoxamine were much less effective in reducing the twitch response, being at least 10,000 times less potent than clonidine. At the highest concentration tested (300 μg/ml; 1.5 mmol/l) methoxamine produced about the same maximum inhibition as clonidine, whereas phenylephrine reduced responses only by 60 to 70%.

Piperoxan (0.3–1 μg/ml) reversed the inhibitory effects of clonidine (Figure 1), oxymetazoline and xylazine, but not those of phenylephrine or methoxamine. The concentration of each agonist producing 50% reduction in the twitch response is given in Table 1. Results are expressed in mol/l to enable accurate comparison of drug potencies.

Lysergic acid diethylamide (LSD, 0.01 to 3 μg/ml; 0.03 to 10 μmol/l) exerted two effects; it increased the basal tone of the ileum and reduced the size of the twitch response by up to 60% (Figure 2). The effects occurred at similar concentrations but pretreatment with mepyramine (10 to 100 ng/ml; 35 to 350 nmol/l) prevented the LSD-induced increase in basal tone without affecting its twitch-inhibitory effect. This shows that the twitch-inhibition was not simply a consequence of the increased basal tone. The inhibition of the twitch elicited by LSD in the presence of mepyramine was only partially antagonized by piperoxan (0.3 to 1 μg/ml) or phentolamine (1 μg/ml; 3.6 μmol/l).

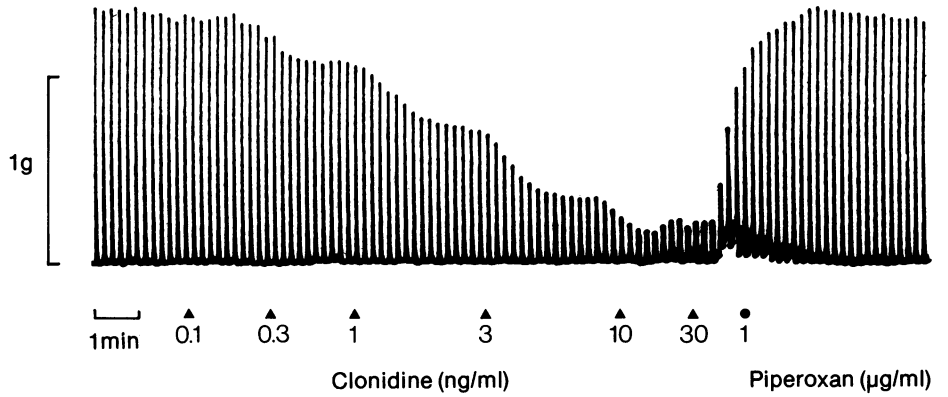

Figure 1 The twitch-inhibitory effect of clonidine in the transmurally stimulated guinea-pig ileum (0.1 Hz; 1 ms, supramaximal voltage), and its reversal by piperoxan. Clonidine was added to the bathing fluid in a cumulative-concentration schedule.

The effect of clonidine on contractile responses to acetylcholine

Acetylcholine (30 ng/ml) caused a rapid and sustained contracture of the ileum, with a peak tension similar to that produced by electrical stimulation, about 1.5 to 2.5 g. Clonidine (1 to 10 ng/ml) did not alter the responsiveness of the ileum to acetylcholine.

Interactions between clonidine and α-adrenoceptor antagonists

Phentolamine (0.01 to 1 μg/ml; 0.036 to 3.6 μmol/l), piperoxan (0.03 to 0.3 μg/ml; 0.13 to 1.3 μmol/l) and tolazoline (0.1 to 1 μg/ml; 0.62 to 6.2 μmol/l) given alone produced a small (up to 30%) but sustained increase in the size of the twitch response. Thymoxa-

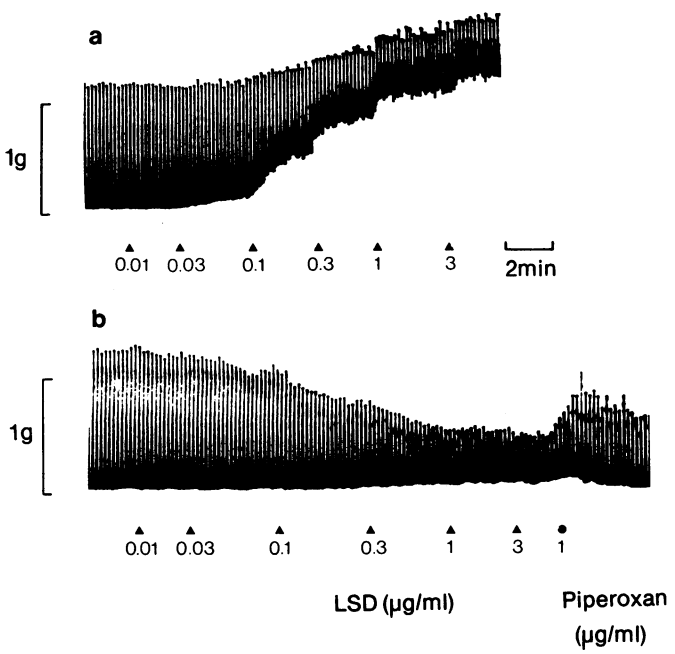

Figure 2 The effect of lysergic acid diethylamide (LSD) on the twitch response of the transmurally stimulated guinea-pig ileum. LSD was added to the bathing fluid in a cumulative concentration schedule. (a) LSD alone; (b) LSD in the presence of mepyramine (10 ng/ml). Note the poor reversal by piperoxan.

mine (1 to 10 μg/ml; 3.6 to 36 μmol/l) caused larger increases in the twitch response (up to 50%) and this effect was also sustained. In contrast, yohimbine (0.03 to 0.3 μg/ml; 0.085 to 0.85 μmol/l) caused only a concentration-dependent reduction of the twitch response; labetalol (0.3 μg/ml; 0.9 μmol/l) also inhibited the twitch. The effects of the antagonists on the twitch response are summarised in Table 2.

Phentolamine, piperoxan, yohimbine and tolazine caused parallel displacements to the right of the clonidine concentration-response curve; the maximum inhibitory effect of clonidine was unaltered. Schild plots gave linear regressions, and their slopes were close to unity, suggesting that these compounds exert competitive antagonism at the presynaptic α-adrenoceptors in the ileum.

In contrast, thymoxamine only weakly antagonized the responses to clonidine and the slope of the Schild plot was significantly less than unity, which suggested this effect of thymoxamine might involve an action other than blockade of presynaptic α-adrenoceptors. This suspicion was confirmed, in other experiments, by the finding that thymoxamine antagonized the twitch-inhibition caused by morphine. Thymoxamine (1, 3 and 10 μg/ml) caused the morphine (0.1 to 100 ng/ml) concentration-response curve to be displaced 2.45-, 3.49- and 3.92-fold to the right ($n = 5$); for clonidine-induced inhibition the values were 2.60, 4.93 and 8.25 respectively ($n = 9$). Thus, only the highest concentration caused a greater shift of the concentration-response curve to clonidine than to morphine. In contrast, neither piperoxan (0.03 to 0.3 μg/ml) nor phentolamine (0.01 to 1.0 μg/ml) antagonized the effects of morphine.

Although labetalol, 0.3 μg/ml, itself reduced the twitch response by 19 to 43% (see Table 2), the residual twitch was inhibited by clonidine over the same concentration range that was effective in untreated preparations. It is likely that the twitch-inhibitory effect of labetalol alone, was the result of the combination of the membrane stabilizing effect of labetalol with that exerted by the propranolol already in the Krebs solution (see Discussion). Thus, in order to investigate the interaction between clonidine and a higher concentration of labetalol (1 μg/ml; 3 μmol/l), propranolol was omitted from the Krebs solution in an attempt to minimize the inhibitory effect of labetalol on the twitch response. Under these conditions, labetalol reduced the twitch by 20 to 57% ($n = 3$), but the effect of clonidine on the residual twitch response was unaffected.

The pA_2 values for the antagonists and the slopes of regression lines of the Schild plots are given in Table 3.

Table 1 Concentrations of α-adrenoceptor agonists required to reduce by 50% (IC_{50}) the twitch response of the electrically stimulated guinea-pig ileum

Agonists	n	*IC_{50} (95% confidence limits)*
Clonidine	7	9.6 (7.4–12.6) nmol/l
Xylazine	6	48.2 (40.9–56.8) nmol/l
Oxymetazoline	6	43.4 (22.3–84.1) nmol/l
Phenylephrine*	4	216.7 (29.8–1575) μmol/l
Methoxamine*	5	576.7 (390–853) μmol/l

* Inhibitory effect not reversed by piperoxan (see text)

Table 2 Effects of α-adrenoceptor antagonists, alone, on the size of the twitch response of the electrically stimulated guinea-pig ileum

Antagonist	n	*Mean % change† in twitch-size in the presence of antagonist at the stated concentrations*						
		0.01	0.03	0.1	0.3	1	3	10 *μg/ml*
Phentolamine	6	+5		+9		+2		
Yohimbine	6		−9	−13	−25			
Piperoxan	5		+12	+22	+29			
Tolazoline	7			+10	+15	+29		
Thymoxamine	9					+42	+48	+26
Labetalol	6				−31	−37*		

In seven control preparations, in which no antagonist was administered the twitch size immediately prior to each of 4 consecutive clonide concentration-response curves varied by less than ±5%.

* in the absence of propranolol, $n = 3$.

† mean change expressed as % of average twitch amplitude before exposure to antagonist

Discussion

The twitch response of the guinea-pig ileum to low frequency stimulation of the intramural nerves was inhibited by all the α-adrenoceptor agonists examined. The inhibitory effect of clonidine was clearly mediated presynaptically because it did not alter the responsiveness of the ileum to exogenous acetylcholine. Thus, clonidine probably reduces the twitch response to nerve stimulation by reducing acetylcholine release, as has been shown previously for xylazine (Vizi, 1974). Oxymetazoline probably causes twitch-inhibition via the same mechanism because piperoxan antagonized the inhibitory effect of all three agonists. The very low potency of phenylephrine and methoxamine at inhibiting the twitch is consistent with their ineffectiveness at reducing acetylcholine release (Paton & Vizi, 1969) and the inhibition caused by very high concentrations of both drugs is unlikely to be mediated via presynaptic α-adrenoceptors since piperoxan did not antagonize their effects. Thus, the order of potency of the agonists in causing twitch inhibition, clonidine > xylazine ≏ oxymetazoline ≫ phenylephrine > methoxamine, clearly indicates that presynaptic α-adrenoceptors in the guinea-pig ileum are of the α_2-type. This was confirmed by the finding that phentolamine, yohimbine, piperoxan and tolazoline were potent antagonists of the twitch-inhibitory action of clonidine, whilst thymoxamine and labetalol were weak antagonists. The low potency of labetalol in blocking the presynaptic α_2-adrenoceptors is in contrast to its effect at postsynaptic α_1-adrenoceptors where it exerts profound blockade at the concentrations used in the present experiments (Kennedy & Levy, 1974). Furthermore, the antagonist action of thymoxamine seems to be largely non-specific because the inhibitory effect of morphine was also antagonized. In summary, the orders of potency of the α-agonists and antagonists show that the presynaptic α-adrenoceptors on the cholinergic nerves of the guinea-pig myenteric plexus are of the same type as those on the terminals of the sympathetic nerves supplying the rabbit heart (Starke, 1972), rat heart (Drew, 1976) and rabbit pulmonary artery (Starke *et al.*, 1975; Borowski, Starke, Ehrl & Endo, 1977), and of the motor nerves supplying the rat vas deferens (Drew, 1977a).

The actions of LSD at α-adrenoceptors are complicated. It has been shown to be a potent agonist at presynaptic α_2-adrenoceptors in the rat heart (Drew, 1976), rat vas deferens (Ambache, Dunk, Verney & Zar, 1973; Hughes, 1973), rat anococcygeus muscle and dog retractor penis muscle (Ambache, Killick, Srinivasan & Zar, 1975), but a weak agonist, or even an antagonist, at postsynaptic α_1-adrenoceptors (Ambache *et al.*, 1975; Drew, 1976). Hughes (1973) has reported that LSD inhibits the twitch response of the guinea-pig ileum by a presynaptic agonist action, which suggests that α_2-adrenoceptors are involved. However, Ambache *et al.* (1975) found that LSD blocked presynaptic α-adrenoceptors in the ileum, which suggests they are of the α_1-variety. In the present experiments LSD was found to be a weak inhibitor of the twitch response and the inhibition was only partly antagonized by α-adrenoceptor antagonists. Further work is obviously needed to clarify the mechanism whereby LSD reduces the twitch response of the guinea-pig ileum; a 'neurone blocking' action similar to that observed in the rat vas deferens and anococcygeus muscle (Gillespie & McGrath, 1975) may be responsible.

The contractile effect of LSD noted in these experiments has also been observed by Ambache *et al.* (1975), and it seems to be mediated via H_1-receptors since it was prevented by pretreatment with mepyramine in concentrations previously shown to have little or no effect on muscarinic or tryptaminergic receptors in this tissue (Cambridge & Holgate, 1955; Harry, 1963; Brown & Quilliam, 1965). It is not known whether the effect of LSD on H_1-receptors is direct or indirect.

In the present experiments, some of the α-adrenoceptor antagonists potentiated the twitch response, an effect which might be thought to reflect the abolition of presynaptic inhibition caused by endogenous noradrenaline released from the sympathetic nerve ter-

Table 3 Potencies of α-adrenoceptor antagonists against clonidine at the presynaptic α-adrenoceptors in the electrically stimulated guinea-pig ileum

Antagonist	n	pA_2 *(95% confidence limits)*	*Slope (95% confidence limits)*
Phentolamine	6	8.51 (8.26–8.70)	1.23 (1.14–1.32)
Yohimbine	6	7.78 (7.52–8.05)	1.02 (0.80–1.23)
Piperoxan	5	7.64 (7.43–7.84)	1.11 (0.99–1.22)
Tolazoline	7	6.57 (6.31–6.81)	1.04 (0.90–1.19)
Thymoxamine	9	6.12 (5.61–6.63)	0.60 (0.38–0.82)
Labetalol	6	<5.5	—

minals during transmural stimulation. This seems unlikely, however, because the effects of the antagonists on the twitch did not correlate with their abilities to antagonize responses to clonidine, and because Dzoljic (1967) found that the potentiating effect of tolazoline persisted in preparations removed from reserpine-treated animals. He attributed the potentiation to an anticholinesterase action, which might be valid for tolazoline but is unlikely to hold for the other agonists since their potentiating effects were apparent at concentrations below those required to inhibit significantly cholinesterase activity (Boyd, Chang & Rand, 1960). Whatever the mechanism involved, it may be the same as that which causes potentiation of the twitch responses of the rat vas deferens to motor nerve stimulation (Drew, 1977a).

The twitch-inhibitory effect of labetalol may result from a membrane-stabilizing action on smooth muscle cells, because in concentrations above 10^{-6} M, β-adrenoceptor antagonists such as propranolol and pronethalol are known to reduce the contractile responses of the guinea-pig ileum to acetylcholine and histamine (Raper & Wale, 1968) and labetalol does the same (Kennedy, personal communication).

The failure of labetalol to block the presynaptic α-adrenoceptors may explain why it does not cause diarrhoea in man at therapeutic dose levels. In contrast, phentolamine and tolazoline block the presynaptic α-adrenoceptors and both compounds are reported to cause diarrhoea in man (Nickerson, 1970). This effect may result from the loss of a physiological modulatory action of endogenous catecholamines, mediated via the presynaptic α-adrenoceptors in the ileum, with a resultant enhancement of vagal activity and increase in intestinal motility.

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