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INDEPENDENT CHOLINERGIC AND ADRENERGIC MECHANISMS IN THE GUINEA-PIG ISOLATED NERVE VAS DEFERENS PREPARATION

BY

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Huković (1961) described the responses of the guinea-pig isolated vas deferens to hypogastric nerve stimulation. Anatomically the vas deferens seems to be exclusively sympathetically innervated. The hypogastric nerve is predominantly composed of non-myelinated C fibres (Burnstock & Holman, 1961). The presence of catechol amines (predominantly noradrenaline) has been demonstrated in the guinea-pig vas deferens (Sjöstrand, 1962b; Falck, 1962). Appropriately high doses of adrenergic blocking agents prevent the responses of the isolated vas deferens to nerve stimulation (Boyd, Chang & Rand, 1960). Contrary to the finding of Boyd *et al.* (1960) that 0.1 μg/ml. of atropine reduced the responses to hypogastric nerve stimulation, Chang & Rand (1960) reported that high concentrations of atropine (3.0 μg/ml.) had no such effect. Treatment of the animal with reserpine diminishes the effect of hypogastric nerve stimulation and the reduction can be reversed by noradrenaline (Huković, 1961). Bretylium and guanethidine block the response to nerve stimulation (Boyd, Chang & Rand, 1961).

There is evidence for a cholinergic mechanism in sympathetic nerves to many organs including the vas deferens (Boyd *et al.*, 1960). Choline acetylase (Ohlin & Strömblad, 1963) and cholinesterase (Boyd *et al.*, 1960) have been demonstrated in the vas deferens. The results obtained with the cholinesterase inhibitors (physostigmine and neostigmine) also support a cholinergic mechanism in the sympathetic innervation to this organ (Boyd *et al.*, 1960; Bentley, 1962; Riley & Maanen, 1962; Burn & Weetman, 1963; Ohlin & Strömblad, 1963). Furthermore, the existence of ganglia in the vicinity of the vas deferens demonstrated pharmacologically (Sjöstrand, 1962a; Ohlin & Strömblad, 1963; Bentley & Sabine, 1963; Birmingham & Wilson, 1963) as well as histologically (Ohlin & Strömblad, 1963) does not support the view of a cholinergic mechanism (the cholinergic link) causing the release of adrenergic transmitter as suggested by Burn & Rand (1960).

The present work was undertaken to study the role of adrenergic and cholinergic mechanisms in the guinea-pig isolated hypogastric nerve vas deferens preparation.

METHODS

The vas deferens of the guinea-pig with the hypogastric nerve was prepared according to the method described by Huković (1961) and suspended in an organ-bath of 150 ml. capacity containing Krebs solution

at 30° C. A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the solution. The hypogastric nerve trunk was stimulated about 2 cm from the vas deferens by rectangular pulses of 2 msec duration at 20 shocks/sec for 4 sec at intervals of 50 sec by an electronic stimulator (Techno). The stimulation period was kept constant by an automatic timing device. The voltage varied between 2.0 to 8.0 V in different preparations to obtain maximal contractions of uniform character. The contractions of the vas deferens were recorded by a frontal-writing lever. A total of 162 guinea-pig isolated nerve vas deferens preparations was used in the present study. Results with each drug were obtained from the increase or decrease in the height of contractions obtained in at least three separate vas deferens preparations.

Reserpine (5 mg/kg) in twenty-eight animals or guanethidine (10 mg/kg) in ten animals was injected intraperitoneally daily for 2 days before an experiment.

The following drugs were used in the study: acetylcholine chloride, carbachol chloride, physostigmine salicylate, tetramethoquin (MER-31, Merrell), demecarium bromide (Humorsol, Merck Sharpe & Dohme), atropine sulphate, noradrenaline bitartrate, adrenaline hydrochloride, (±)-normetanephrine hydrochloride, (±)-metanephrine hydrochloride, 3-methoxy-4-hydroxymandelic acid, 3,4-dihydroxymandelic acid, dexamphetamine sulphate, (−)-amphetamine sulphate, tranylcypromine (Smith Kline & French), pheniprazine (Lakeside Laboratories), pyrogallol and phenoxybenzamine (Dibenzyline, Smith Kline & French). Doses of the drugs are given as concentrations of the salt per ml. of the bath solution.

RESULTS

Effects of drugs acting through cholinergic mechanisms

Cholinesters. Acetylcholine (0.1 to 0.5 μg/ml.) or carbachol (0.5 to 2 μg/ml.) potentiated the response of the isolated vas deferens to nerve stimulation. Higher doses of acetylcholine produced irregular contractions whereas carbachol elicited prolonged and regular contractions of increased amplitude.

Anticholinesterases. Tetramethoquin and demecarium bromide, known to possess anticholinesterase activity (Kuhn, Maanen & Ketteler, 1959), were compared with physostigmine on this preparation (Fig. 1). Each drug, in bath concentrations of 1 μg/ml., potentiated the response of the vas deferens to nerve stimulation. Subsequent addition of a higher dose of these agents inhibited the response. With demecarium inhibition occurred with 3 to 5 μg/ml.

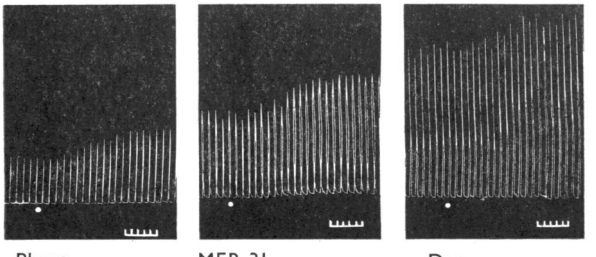


Fig. 1. Potentiating effects of anticholinesterase agents, physostigmine (Physo), tetramethoquin (MER-31) and demecarium bromide (Dec), each in a final bath concentration of 1 μg/ml., on the hypogastric nerve-vas deferens preparation. The records are from three separate experiments. Time scale, 1 min.

Effects of drugs acting through adrenergic mechanisms

Noradrenaline and adrenaline. Noradrenaline or adrenaline (1 μg/ml.) appreciably augmented the contractions elicited by nerve stimulation. Higher concentrations raised the baseline and elicited spontaneous contractions.

Inhibitors of enzymes which metabolize catechol amines. The monoamine oxidase inhibitors, pheniprazine (0.5 to 10 μg/ml.) and tranylcypromine (0.1 to 5.0 μg/ml.), and the catechol-*o*-methyl transferase inhibitor, pyrogallol (1.0 to 5.0 μg/ml.), independently potentiated the response. More than 10 μg/ml. of tranylcypromine and pyrogallol inhibited the response of the vas deferens to nerve stimulation. Pyrogallol (1.0 to 5.0 μg/ml.), in a preparation already potentiated by either pheniprazine (0.5 to 10 μg/ml.) or tranylcypromine (0.1 to 5.0 μg/ml.), further augmented the response of the vas deferens to nerve stimulation, and similarly the potentiation induced by pyrogallol was further augmented by the monoamine oxidase inhibitors. Greater potentiation of the response was observed with dexamphetamine (0.1 to 5.0 μg/ml.) than with (−)-amphetamine (0.1 to 5.0 μg/ml.).

Noradrenaline (0.5 μg/ml.) further augmented the increased response induced by previous addition of either monoamine oxidase inhibitors or pyrogallol to the bath. However, dexamphetamine (10 μg/ml.) could not further augment the response in the presence of an existing potentiation induced by tranylcypromine (0.1 μg/ml.).

Fig. 2 demonstrates the effects of interaction of catechol-*o*-methyl transferase and monoamine oxidase inhibitors on the isolated vas deferens preparation. Pyrogallol (2 μg/ml.) elicited a potentiation; when the final bath concentration was increased to 10 μg/ml. the contractions of the vas deferens were reduced. Addition of tranylcypromine (1 μg/ml.) to the bath at this stage was able to potentiate the responses.

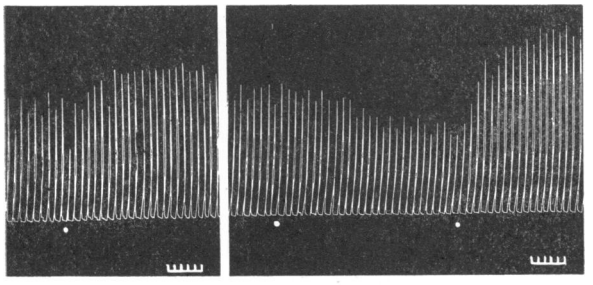


Fig. 2. Effects of pyrogallol (catechol-*o*-methyl transferase inhibitor) and tranylcypromine (monoamine oxidase inhibitor) on the hypogastric nerve-vas deferens preparation. A low dose (2 μg/ml.) of pyrogallol (Pyro) potentiated and a high dose (10 μg/ml.) inhibited the response. Tranylcypromine (Tran), 1 μg/ml., potentiated the response after the inhibition due to pyrogallol. Time scale, 1 min.

Effects of catechol amine metabolites

The methylated metabolites of catechol amines, namely metanephrine (3-methoxyadrenaline) and normetanephrine (3-methoxynoradrenaline) when added to the bath in doses of 10 to 30 μg/ml. augmented the response of the vas deferens to nerve stimulation. Another metabolite of the methylated compounds, 3-methoxy-4-hydroxymandelic acid, was inactive at the same dose. Furthermore, this metabolite was inactive even after previous addition of effective doses of pheniprazine and pyrogallol. The other metabolic product of catechol amines, 3,4-dihydroxymandelic acid (10 to 30 μg/ml.), was also inactive.

Effects of cholinergic and adrenergic blocking agents

The contraction of the isolated vas deferens in response to nerve stimulation could not be inhibited more than 10 to 20% of the initial height by atropine sulphate (0.1 to 2 μg/ml.). However, atropine had no effect on the contractions in response to a stimulus frequency of 60 shocks/sec. The stimulant action of acetylcholine (0.1 μg/ml.) on the nerve-vas deferens preparation was blocked by atropine in the same concentrations.

The responses to physostigmine and tetramethoquin were dependent upon the dose of atropine. A low concentration of atropine (0.1 μg/ml.) blocked the response of 1 to 4 μg/ml. of physostigmine as well as tetramethoquin. An increase in the concentration of the anticholinesterase agents (5 μg/ml.) could overcome the block by atropine. The block with 1 μg/ml. atropine could also be overcome by correspondingly higher doses of physostigmine (15 μg/ml.).

In the present investigation slight or no potentiation of the response to nerve stimulation was seen with 0.5- to 2-μg/ml. concentrations of phenoxybenzamine. With 1 μg/ml. of phenoxybenzamine definite block of the potentiating effect of noradrenaline (1 to 5 μg/ml.) could be demonstrated.

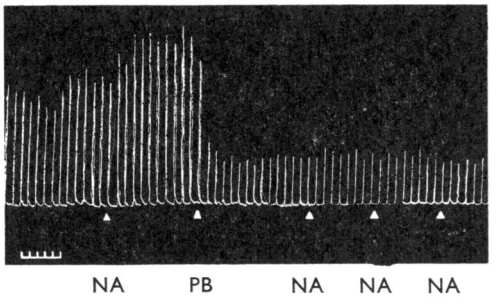


Fig. 3. Potentiating effect of noradrenaline (NA), 1 μg/ml., on the hypogastric nerve vas deferens preparation. Phenoxybenzamine (PB), 1 μg/ml., blocked the effects of subsequent additions of noradrenaline (total, 5 μg/ml.). Time scale, 1 min.

In the experiment illustrated in Fig. 3, noradrenaline (1 μg/ml.) potentiated the response to nerve stimulation. Phenoxybenzamine (1 μg/ml.) blocked the effect of noradrenaline added subsequently. Similarly, the potentiating effects of dexamphetamine and (−)-amphetamine were blocked by phenoxybenzamine (1 μg/ml.). Fig. 4 shows the results of the adrenergic block produced by phenoxybenzamine (1 μg/ml.) on the potentiating effects of monoamine oxidase and catechol-*o*-methyl transferase inhibitors in the isolated vas deferens preparation. Tranylcypromine (1 μg/ml.), pheniprazine (1 μg/ml.) and pyrogallol (1 μg/ml.) potentiated the response of the vas deferens to nerve stimulation; the greatest effect was with tranylcypromine. Phenoxybenzamine (1 μg/ml.) blocked the potentiation induced by the enzyme inhibitors and subsequent addition of these agents was ineffective.

Combined effects of drugs acting on cholinergic and adrenergic mechanisms

In the presence of cholinergically acting agents, physostigmine (0.5 to 1 μg/ml.), tetramethoquin (1 μg/ml.), demecarium bromide (1 μg/ml.) or acetylcholine (0.1 μg/ml.), further

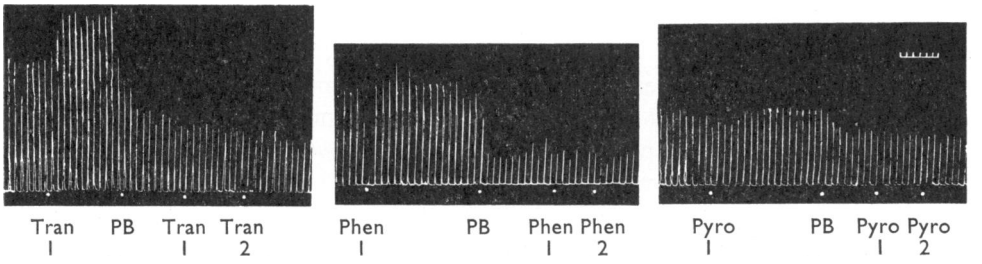


Fig. 4. Blocking action of phenoxybenzamine (PB) on the potentiation induced by monoamine oxidase inhibitors, tranylcypromine (Tran) and pheniprazine (Phen) and the catechol-*o*-methyl transferase inhibitor, pyrogallol (Pyro), in three different experiments. Initially, all the enzyme inhibitors in 1-μg/ml. doses potentiated the responses. Phenoxybenzamine (1 μg/ml.) blocked the response to subsequent addition of 1 and 2 μg/ml. of tranylcypromine, pheniprazine and pyrogallol. Time scale, 1 min.

potentiation could be observed with the adrenergically acting agents, dexamphetamine (0.1 to 10 μg/ml.), (−)-amphetamine (0.5 μg/ml.), pheniprazine (0.5 to 1.0 μg/ml.), tranylcypromine (0.5 μg/ml.) and pyrogallol (1 μg/ml.). Similarly, in the presence of the potentiation induced by adrenergically acting drugs the anticholinesterase agents or acetylcholine could further augment the response of the vas deferens to nerve stimulation. When atropine sulphate (0.1 to 1 μg/ml.) had blocked the effects of the anticholinesterase agents or acetylcholine, the adrenergically acting drugs still potentiated the response of the vas deferens to nerve stimulation.

The effects of cholinergic block (by atropine) on the responses of the isolated vas deferens to physostigmine salicylate (1 μg/ml.), acetylcholine chloride (0.1 μg/ml.) and tranylcypromine (1.1 μg/ml.) are shown in Fig. 5. Atropine (0.1 μg/ml.) blocked the potentiating effects of physostigmine and acetylcholine, whereas the monoamine oxidase inhibitor tranylcypromine potentiated the responses of the vas deferens to nerve stimulation even in the presence of atropine (0.1 μg/ml.).

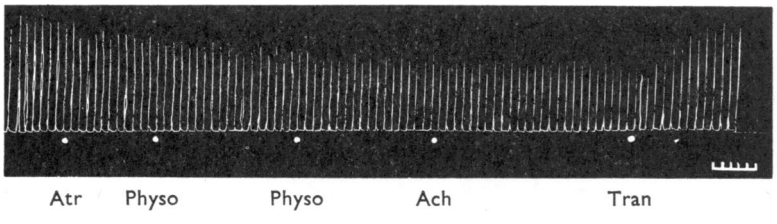


Fig. 5. Atropine (Atr), 0.1 μg/ml., blocked the effects of physostigmine (Physo), 1 μg/ml., and acetylcholine (Ach), 0.1 μg/ml., but tranylcypromine (Tran), 1.1 μg/ml., potentiated the responses. Time marks, 1 min.

Effects in reserpine- or guanethidine-treated animals

In the reserpinized preparations the drugs (−)-amphetamine (1 μg/ml.), dexamphetamine (1 μg/ml.), pheniprazine (1 to 2 μg/ml.), tranylcypromine (0.5 to 1 μg/ml.), pyrogallol (1 to 2 μg/ml.) and phenoxybenzamine (1 μg/ml.) caused no potentiation, whereas they did cause potentiation in normal preparations. Acetylcholine (1 to 5 μg/ml.), physostigmine

(1 to 5 μg/ml.) and tetramethoquin caused definite potentiation which was blocked by atropine.

The potentiation to noradrenaline (5 μg/ml.) was more marked than the potentiation to acetylcholine (5 μg/ml.), and phenoxybenzamine (1 μg/ml.) could block the effects of noradrenaline (Fig. 6). The potentiation induced by physostigmine (5 μg/ml.) could not be blocked by phenoxybenzamine (1 μg/ml.).

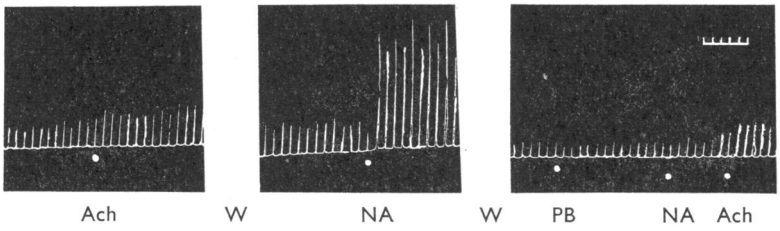


Fig. 6. Effects of acetylcholine (Ach) and noradrenaline (NA) in a reserpinized hypogastric nerve-vas deferens preparation before and after phenoxybenzamine (PB). Acetylcholine (5 μg/ml.) and noradrenaline (5 μg/ml.) each potentiated the responses. After phenoxybenzamine (1 μg/ml.) the noradrenaline response was blocked but the acetylcholine response remained unaltered. Wash at W. Time scale, 1 min.

In the reserpine-treated or guanethidine-treated preparation, results similar to those in the normal preparation were obtained with catechol amine metabolites. Normetanephrine and metanephrine (10 to 30 μg/ml.) increased the contractions whereas 3-methoxy-4-hydroxymandelic acid and 3,4-dihydroxymandelic acid were inactive.

In contrast to the reserpine-treated preparation, the guanethidine-treated preparation showed potentiation of the responses to pheniprazine (1 to 2 μg/ml.), tranylcypromine (0.5 to 1 μg/ml.) and pyrogallol (1 to 2 μg/ml.) similar to that observed in the normal preparations.

In Fig. 7 are shown the effects of physostigmine and tranylcypromine in a normal, a reserpine-treated and a guanethidine-treated preparation. Tranylcypromine (0.5 μg/ml.)

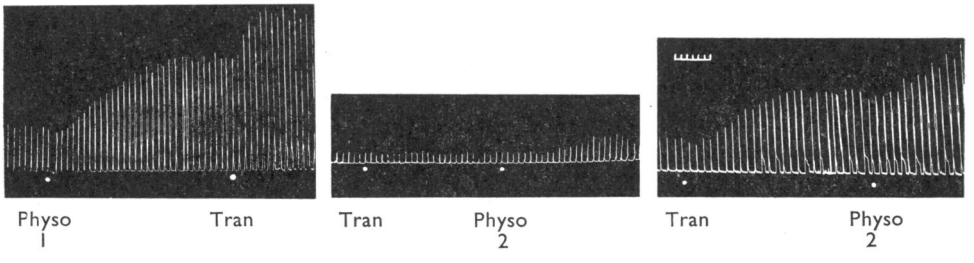
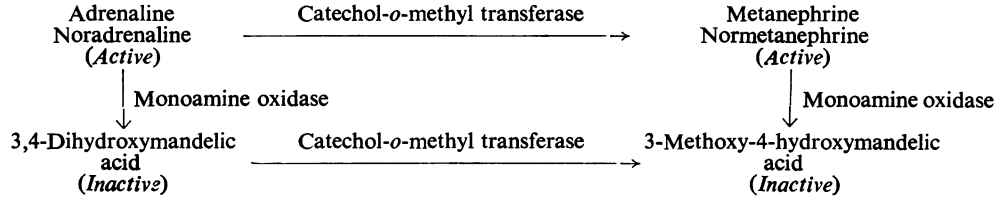


Fig. 7. Effects of physostigmine (Physo) and tranylcypromine (Tran) in normal (left-hand panel), reserpine-treated (middle panel) and guanethidine-treated (right-hand panel) hypogastric nerve-vas deferens preparations. In the normal preparation, physostigmine (1 μg/ml.) potentiated the responses and tranylcypromine (0.5 μg/ml.) further augmented these responses. Tranylcypromine (0.5 μg/ml.) was ineffective in the reserpinized preparation but was effective in the guanethidine-treated preparation. Physostigmine (2 μg/ml.) potentiated the responses in both the treated preparations. Time scale, 1 min.

augmented the potentiation induced by physostigmine (1 μg/ml.) in the normal preparation. Tranylcypromine was inactive in the reserpinized preparation but potentiated the guanethidine-treated preparation. Physostigmine could potentiate in all the three preparations, although in the reserpinized preparation it was much less active.

DISCUSSION

The contractions of the vas deferens to nerve stimulation are potentiated by noradrenaline (Huković, 1961). We have confirmed this finding and have further shown that agents which inhibit monoamine oxidase and catechol-*o*-methyl transferase, responsible for the destruction of catechol amines (Axelrod, 1959), also potentiate the response of the isolated vas deferens to nerve stimulation (Bhargava, Kar & Parmar, 1963). The potentiating effect of monoamine oxidase inhibitors can be further augmented by the catechol-*o*-methyl transferase inhibitor and *vice versa*. The present study indicates that the methylated metabolites are active in high doses whereas the deaminated metabolites are inactive. Thus it seems that monoamine oxidase is the enzyme which is responsible for the production of the inactive metabolic products of the catechol amines as represented below.



The potentiating effects of noradrenaline, the monoamine oxidase inhibitors (pheniprazine, tranylcypromine, dex- and (—)-amphetamines), the catechol-*o*-methyl transferase inhibitor (pyrogallol) and normetanephrine were all blocked by the adrenergic blocking agent phenoxybenzamine (Figs. 3 and 4). The specificity of phenoxybenzamine as a sympathetic receptor blocking agent in the present study is suggested by the effect being mediated by a concentration as low as 1 μg/ml. The potentiating effect of high doses of phenoxybenzamine on the vas deferens preparation appears to be due to mechanisms other than stimulation of sympathetic receptors. Several antiadrenaline drugs including phenoxybenzamine possess anticholinesterase activity and this may account for their stimulant action (Boyd *et al.*, 1960). In the reserpine-treated preparations the enzyme inhibitors which act through the adrenergic mechanism were ineffective whereas noradrenaline showed an enhanced response which could again be blocked by phenoxybenzamine (1 μg/ml.). That the enzyme inhibitors (pheniprazine, tranylcypromine and pyrogallol) potentiated the response of the guanethidine-treated vas deferens preparation may indicate the inability of guanethidine to deplete the stores of catechol amines.

The contractions of the vas deferens to nerve stimulation are potentiated by acetylcholine (Sjöstrand, 1961) and by physostigmine or neostigmine (Boyd *et al.*, 1960; Ohlin & Strömblad, 1963; Burn & Weetman, 1963; Riley & Maanen, 1962). In the present study, anticholinesterases like tetramethoquin and demecarium bromide had actions similar to physostigmine. Atropine blocked the potentiating effects of acetylcholine, physostigmine, tetramethoquin and demecarium bromide on the hypogastric nerve-vas deferens pre-

paration. However, atropine only partially inhibited the normal contractions of vas deferens to nerve stimulation. The effects of drugs acting through the mediation of acetylcholine were unaltered by prior treatment with reserpine and were also blocked by atropine.

It may be concluded that separate muscarinic receptors exist in the vas deferens preparation which are excited by acetylcholine and blocked by atropine. This is further shown by the combined use of agents acting through the mediation of noradrenaline and acetylcholine. When the response of the vas deferens to nerve stimulation was potentiated by acetylcholine or an anticholinesterase, further augmentation of the response could be obtained by noradrenaline, monoamine oxidase or catechol-*o*-methyl transferase inhibitors, and the reverse was also true. Moreover, in the presence of sympathetic block by phenoxybenzamine, physostigmine could potentiate the response and, similarly, in the presence of a muscarinic block by atropine, the adrenergic agents could elicit the potentiation of the responses of vas deferens to nerve stimulation. When both the muscarinic and sympathetic receptors were blocked by atropine and phenoxybenzamine, physostigmine was ineffective. Although reserpinization rendered the agents acting through the mediation of noradrenaline ineffective, noradrenaline and agents acting through a cholinergic mechanism could still potentiate the responses. The potentiation by noradrenaline could be blocked by phenoxybenzamine, whereas the responses induced by physostigmine were unaffected.

Our results indicate the presence of separate sympathetic and muscarinic receptors in the vas deferens. Since both adrenergic and cholinergic transmitter substances cause contraction of the vas deferens and can be specifically blocked by respective blocking agents (phenoxybenzamine or atropine), it seems that both adrenergic and cholinergic fibres exist in the hypogastric nerve. The noradrenaline could arise from postganglionic adrenergic nerve terminals or from the chromaffin cells in the vas deferens. Estimation of noradrenaline content (Sjöstrand, 1962b) and morphological evidence favour the existence of adrenergic nerve terminals in the vas deferens (Falck, 1962).

It thus seems that adrenergic as well as cholinergic mechanisms are concerned in the contractions of the vas deferens to nerve stimulation. Burn & Rand (1960, 1962), Boyd *et al.* (1960, 1961), Chang & Rand (1960) and Burn & Weetman (1963) have repeatedly emphasized the role of acetylcholine in the release of the adrenergic transmitter from the postganglionic adrenergic nerve terminals. Contrary to Dale's principles, these workers assume the presence of acetylcholine and noradrenaline in the same nerve fibre. Such an assumption may be unnecessary when in the vas deferens preparation ganglia have actually been demonstrated histologically (Ohlin & Strömblad, 1963), and with the use of ganglion-blocking agents (Sjöstrand, 1962a, Bentley & Sabine, 1963; Birmingham & Wilson, 1963). Chang & Rand (1960) assumed that a specific inhibition of the synthesis of acetylcholine was concerned in the block by hemicholinium, since acetylcholine is crucial for the release of noradrenaline. However, these workers were stimulating preganglionically and therefore hemicholinium, a postsynaptic blocking agent (Thies & Brooks, 1961), would be expected to block ganglionic transmission.

Burn & Weetman (1963) found that high doses of physostigmine increased the contractions of the vas deferens to nerve stimulation in the presence of atropine and these contractions were attributed to the release of noradrenaline by acetylcholine. We have repeated the experiment and found that more than 5 μg/ml. of physostigmine was required to overcome the block produced by 0.1 μg/ml. of atropine and a correspondingly higher dose

(15 μg/ml.) of physostigmine was necessary to overcome the block produced by 1.0 μg/ml. of atropine. Riley & Maanen (1962), in a study on the guinea-pig isolated vas deferens, indicate a competitive antagonism between atropine and acetylcholine or neostigmine. Furthermore, in the presence of atropine the potentiation observed with physostigmine may be due to a ganglionic (nicotinic) action resulting in the release of noradrenaline by the postganglionic fibres. The latter explanation seems more likely, since in the reserpinized preparation the physostigmine was not effective in overcoming the block produced by atropine. In conclusion, our results support the existence of independent cholinergic and adrenergic mechanisms in the guinea-pig isolated vas deferens preparation.

SUMMARY

1. A pharmacological analysis of the response of the guinea-pig isolated vas deferens to nerve stimulation was made in normal, reserpinized and guanethidine-treated animals.

2. In the normal preparation potentiation of the response was obtained independently with inhibitors of cholinesterase and/or monoamine oxidase and catechol-*o*-methyl transferase. The last two inhibitors mutually augmented their potentiating actions.

3. The methylated metabolites of catechol amines were active, whereas the deaminated metabolites were inactive.

4. The potentiating effect of choline esters and anticholinesterases was further augmented by catechol amines and the inhibitors of their metabolic enzymes, and *vice versa.*

5. The response to drugs acting on sympathetic receptors was blocked by phenoxybenzamine and that to drugs acting on muscarinic receptors by atropine.

6. In the presence of block with phenoxybenzamine the cholinergically acting agents could potentiate and, similarly, in the presence of a cholinergic block with atropine, the adrenergically acting agents could potentiate the response.

7. In the reserpinized preparation the potentiating effect of monoamine oxidase and catechol-*o*-methyl transferase inhibitors could not be demonstrated. Agents acting via the cholinergic system or catechol amines and their active metabolites could, however, cause potentiation.

8. The guanethidine-treated preparations behaved like the untreated normal preparations.

9. The results indicate that cholinergic and adrenergic mechanisms operate independently of each other in this preparation.

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