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THE MECHANISM OF THE PRESSOR RESPONSES TO PHYSOSTIGMINE IN THE RAT AND THEIR MODIFICATION BY MEBUTAMATE AND AMYLOBARBITONE

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Pressor responses to intravenous injections both of physostigmine and of pilocarpine were smaller in rats anaesthetized with either mebutamate or amylobarbitone than in rats anaesthetized with urethane. The response to electrical stimulation of the hypogastric nerve in the isolated hypogastric nerve–vas deferens preparation was diminished by mebutamate and by amylobarbitone, but not by urethane. Similar results were obtained with the cat isolated splenic nerve-spleen preparation. In the rat anaesthetized with urethane, pressor responses to physostigmine were only partially antagonized by hexamethonium but were completely abolished during "depolarizing" ganglionic block by nicotine or tetramethylammonium. It is suggested that, in addition to the central mechanism, there is a peripheral component in the pressor action of physostigmine and in the antihypertensive actions of mebutamate and amylobarbitone.

Intravenous administration of physostigmine causes an appreciable rise in the blood pressure of the rat anaesthetized with urethane. In agreement with the suggestions of Dirnhuber & Cullumbine (1955) concerning the mechanism of the pressor effects of a number of anticholinesterase compounds, Varagić (1955) postulated that hypertension due to physostigmine was mainly due to a central stimulation. This view has been generally accepted by all authors who subsequently dealt with this subject and by those who have used pressor responses to physostigmine to investigate antihypertensive drugs (Hornykiewicz & Kobinger, 1956 ; Medaković & Varagić, 1957 ; Cass & Spriggs, 1961 ; Lešić & Varagić, 1961 ; Della Bella & Gandini, 1962 ; Varagić & Vojvodić, 1962 ; Gokhale, Gulati & Joshi, 1963).

We have used the physostigmine test to study the antihypertensive properties of mebutamate, a drug thought to act centrally (Berger, Douglas, Kletzkin, Ludwig & Margolin, 1961 ; Margolin, Plekss & Fedor, 1963), and in screening related compounds. In accordance with previous reports mebutamate was found to antagonize pressor responses to physostigmine but, to our surprise, gave similar inhibitory results in concurrent experiments with pilocarpine, a ganglion-stimulating drug (Trendelenburg, 1954, 1961 ; Levy & Ahlquist, 1962). This finding, as well as earlier observations by Burn & Weetman (1963) on the potentiation by physostigmine of peripheral adrenergic transmission, led us to suppose that a

peripheral component was involved both in the antihypertensive properties of mebutamate and in the pressor activity of physostigmine.

To verify these hypotheses we carried out the following experiments. First, we compared pressor responses to physostigmine with those to pilocarpine in rats previously treated with urethane, mebutamate and amylobarbitone. The action of a barbiturate was investigated because in previous experiments we had noted that pressor responses to physostigmine in the rat during barbiturate anaesthesia were far smaller than those in animals with urethane (Della Bella & Gandini, 1962). Secondly, we wanted to study the influence of urethane, mebutamate and amylobarbitone on the responses to electrical and chemical stimulation of two sympathetically innervated, *in vitro* preparations, namely the guinea-pig vas deferens and the cat spleen. Thirdly, we have studied the pressor responses to physostigmine in rats anaethetized with urethane, in which a "depolarizing" ganglionic block had been induced by the administration of cumulative high doses of nicotine or tetramethylammonium. These responses are only slightly inhibited by hexamethonium.

METHODS

In vivo *experiments*

Male albino rats of homogeneous strain, weighing 200 to 300 g. were used. They were anaesthetized by intraperitoneal administration of urethane, mebutamate or amylobarbitone given in the doses specified below. Mebutamate and amylobarbitone were administered as suspensions in water with 2% gum arabic. The rats were heparinized, their tracheas cannulated and blood pressures recorded by a cannula inserted in a carotid artery and connected to a mercury manometer. A needle tied into a jugular vein was used for injecting drugs.

In vitro *experiments*

Guinea-pig isolated hypogastric nerve–vas deferens preparation. The method used was that of Huković (1961) as described by Benelli, Della Bella & Gandini (1964). The nerve was stimulated every 2 min by 200 rectangular pulses of 0.5 to 1.5 msec duration at frequencies of 10 or 50 shocks/sec from a constant voltage source at 3.5 to 5 V.

Cat isolated splenic nerve-spleen preparation. The method used was that of Brandon & Rand (1961) as described by Benelli, Della Bella & Gandini (1964). The nerve was stimulated every 3 to 4 min by rectangular pulses of 1 or 2 msec duration at frequencies of 10 or 50 shocks/sec from a constant voltage source at 1.5 to 3.5 V.

Doses given in the text refer to the following: noradrenaline base, mebutamate, atropine sulphate, amylobarbitone, physostigmine sulphate. urethane, pilocarpine hydrochloride, nicotine tartrate. tetramethylammonium bromide, and hexamethonium bromide.

RESULTS

Influence of urethane, mebutamate and amylobarbitone on the pressor responses of the rat to physostigmine and pilocarpine

The following experiments were undertaken to confirm our previous finding that mebutamate is able to inhibit pressor responses both to physostigmine and to pilocarpine. Three groups of sixteen rats were given intraperitoneally 1.2 g/kg of urethane, 300 mg/kg of mebutamate and 200 mg/kg of amylobarbitone respectively. After 40 to 50 min, and thereafter repeatedly at 45 min intervals, eight animals from each group received intravenously 150 μg/kg of physostigmine and eight 300 μg/kg of pilocarpine. Tables 1 and 2, in which the results are

TABLE 1

PRESSOR RESPONSES TO SUCCESSIVE INTRAVENOUS DOSES OF PHYSOSTIGMINE (150 μg/kg) TO THREE GROUPS OF RATS ANAESTHETIZED WITH URETHANE, AMYLOBARBITONE AND MEBUTAMATE RESPECTIVELY

Values are the means and standard errors of eight experiments

Treatment	No. of rats	Pressor responses to physostigmine (mm Hg) I	II	III
Urethane	8	20±1·9	27·9±1·9	28·9±2·3
Amylobarbitone	8	7±1·3	7·6±4·7	10·1±3·9
Mebutamate	8	7·9±1·7	9·6±1·2	12±1·6

TABLE 2

PRESSOR RESPONSES TO SUCCESSIVE INTRAVENOUS DOSES OF PILOCARPINE (300 μg/kg) TO THREE GROUPS OF RATS ANAESTHETIZED WITH URETHANE, AMYLOBARBITONE AND MEBUTAMATE RESPECTIVELY

Values are the means and standard errors of eight experiments

Treatment	No. of rats	Pressor responses to pilocarpine (mm Hg) I	II	III
Urethane	8	18·2±2	17·8±3	16·1±1·6
Amylobarbitone	8	9·8±1	10·5±1·6	8·7±0·9
Mebutamate	8	7±1·3	7±1·5	8±1·6

reported, show clearly that pressor responses to both pressor drugs vary with the previous treatment. The statistical analysis of the differences (Table 3) indicates that optimal pressor responses to either drug were obtained in animals under urethane anaesthesia and that, in comparison with the urethane-treated rats, those receiving either mebutamate or amylobarbitone gave markedly reduced pressor responses. Furthermore, and this observation was most evident in animals with urethane, repeated administration of physostigmine resulted in increasingly higher pressor responses. This was not apparent with pilocarpine.

From Table 4, in which blood pressure values before and after pressor responses are recorded, it appears that: (1) basal pressure values of animals given mebutamate were quite low in comparison with those of rats treated with the other drugs, but got progressively higher after hypertensive treatment; this was more marked with physostigmine; (2) no significant difference may be observed in rats first treated with amylobarbitone and a slight reduction, if anything, is noted after pilocarpine; and (3) in urethane-treated animals basal pressure values following either physostigmine or pilocarpine were lower than the initial ones. In particular it was noted that physostigmine gave more marked responses the lower the basal pressure.

Influence of urethane, mebutamate, amylobarbitone and atropine on the responses of the vas deferens to electrical stimulation, in the absence and in the presence of physostigmine

We wanted to determine whether the reduced pressor responses to pilocarpine and physostigmine observed in rats first treated with mebutamate and amylobarbitone could be ascribed to an inhibitory effect of the drugs at the level of the peripheral sympathetic pathways. To test this hypothesis, experiments were carried out on the

TABLE 3

STATISTICAL ANALYSIS OF THE DIFFERENCES OF PRESSOR RESPONSES TO PHYSOSTIGMINE AND TO PILOCARPINE OF RATS TREATED WITH URETHANE, AMYLOBARBITONE AND MEBUTAMATE

$d\bar{x}$ = difference between two mean pressor values

$\sigma = \sqrt{\frac{S(x-\bar{x})^2}{n}}$

n = number of observations

The *P* values were calculated by Student's one-tailed *t*-test (Fisher & Yates, 1960)

	Responses to physostigmine		
	I	II	III
(a) Differences between urethane- and amylobarbitone-treated rats			
$d\bar{x}$	13	20·25	18·75
n	16	16	16
σ	2·3	5	4·5
P	<0·0005	<0·0005	<0·0005
(b) Differences between urethane- and mebutamate-treated rats			
$d\bar{x}$	12·1	18·25	16·87
n	16	16	16
σ	2·6	2·2	2·9
P	<0·0005	<0·0005	<0·0005

	Responses to pilocarpine		
	I	II	III
(a) Differences between urethane- and amylobarbitone-treated rats			
$d\bar{x}$	8·4	7·4	5·4
n	16	16	16
σ	2·2	3·3	1·83
P	<0·0025	<0·025	<0·01
(b) Differences between urethane- and mebutamate-treated rats			
$d\bar{x}$	11·2	10·9	8·1
n	16	16	16
σ	2·33	3·23	2·26
P	<0·0005	<0·0025	<0·0025

guinea-pig hypogastric nerve–vas deferens preparation, to which a total number of 200 shocks was applied, at a frequency of either 50 or 10 shocks/sec. Results may be summarized as follows.

Urethane. In eleven preparations, responses of the vas deferens to electrical stimulation were not affected at either frequency by urethane concentrations of 250 to 300 μg/ml., even when the drug was left in contact with the preparation for periods of 20 to 30 min. A slight inhibition of the responses to stimulation at

TABLE 4

BASAL BLOOD PRESSURE VALUES OF THREE GROUPS OF RATS ANAESTHETIZED WITH URETHANE, AMYLOBARBITONE AND MEBUTAMATE RESPECTIVELY, BEFORE AND AFTER PRESSOR RESPONSES TO PHYSOSTIGMINE AND PILOCARPINE

Values are the means and standard errors of eight experiments

Pressor agent	Basal pressure (mm Hg) with					
	Urethane		Amylobarbitone		Mebutamate	
	Before	After	Before	After	Before	After
Physostigmine	82±6	55±6	101±11	97±4	60±4	76±2
Pilocarpine	81±7	57±4	90±5	83±7	61±5	65±5

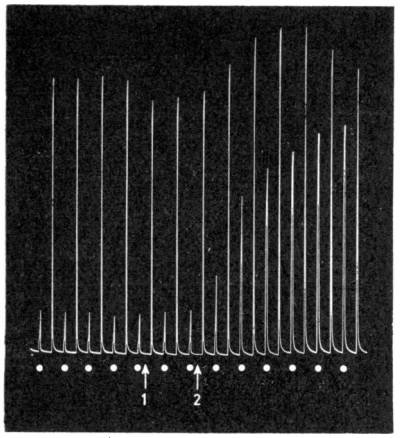

Fig. 1. Contractions of the guinea-pig isolated vas deferens in response to stimulation of the hypogastric nerve. Each stimulation consisted of 200 shocks, applied alternately at 50 and at 10 shocks/sec (at dots) every 2 min. Urethane (500 μg/ml., at 1) depressed only slightly the responses to electrical stimulation and, upon addition of physostigmine to the bath (2.5 μg/ml. at 2), allowed full development of the enhancing effect of physostigmine.

50 shocks/sec was observed for concentrations of 500 to 750 μg/ml. When physostigmine was added to the bath to give a concentration of 2.5 μg/ml., the typical enhancement of responses at low frequency was apparent, in spite of the presence of urethane up to a concentration of 500 μg/ml. (Fig. 1), but this was abolished by higher concentrations (Fig. 2).

Mebutamate and amylobarbitone. Unlike urethane, mebutamate and amylobarbitone concentrations as low as 25 to 50 μg/ml. reduced significantly the

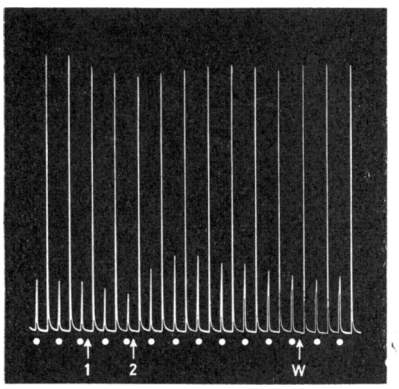

Fig. 2. Contractions of the guinea-pig isolated vas deferens in response to stimulation of the hypogastric nerve. Each stimulation consisted of 200 shocks, applied alternately at 50 and 10 shocks/sec (at dots) every 2 min. At 1, urethane (750 μg/ml.). At 2, physostigmine (2.5 μg/ml.). In the presence of concentrations of urethane higher than those of the experiment illustrated in Fig. 1, the typical enhancing effect of physostigmine was antagonized. At W, thorough washing of the preparation.

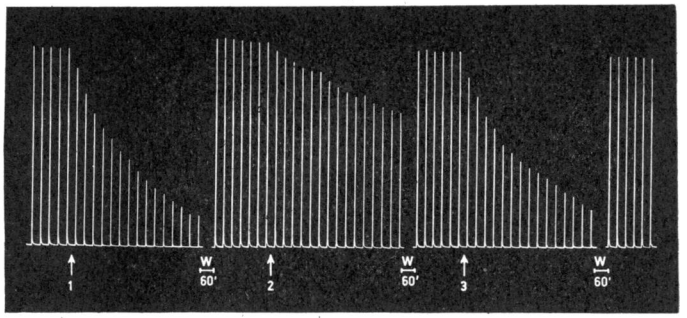

Fig. 3. Contractions of the guinea-pig isolated vas deferens in response to electrical stimulation of the hypogastric nerve. Each stimulation was of 200 shocks, given at 50 shocks/sec, every 2 min. Mebutamate (150 μg/ml., at 1) induced a progressive reduction in the responses of the vas deferens; meprobamate (250 μg/ml., at 2) was far less effective, despite the greater concentration. An inhibitory effect, comparable to that due to mebutamate, appeared upon the addition of amylobarbitone (150 μg/ml., at 3). At W, stimulation and recording were stopped for 60 min and the preparation thoroughly washed.

responses of the vas deferens to electrical stimulation of the hypogastric nerve. The effect was more evident the higher the concentration of the drug and there was a clear dose-effect relationship. From Fig. 3 it may be seen that meprobamate was far less effective and that the inhibitory effect of mebutamate and amylobarbitone developed progressively and was promptly reversible. Physostigmine, added to the bath in the presence of either of these drugs, failed to potentiate the responses of the preparation. A similar inhibitory effect could be observed when mebutamate or amylobarbitone were added after the potentiation due to physostigmine had already developed (Fig. 4). Removal of the drug allowed full recovery of the responses potentiated by physostigmine.

Atropine. In analogous experiments we tested atropine which is known *in vivo* to abolish pressor responses to physostigmine completely. We obtained results somewhat different from those described by Burn & Weetman (1963). Whereas 0.5 to 1 μg/ml. of atropine induced only a slight reduction of the responses to

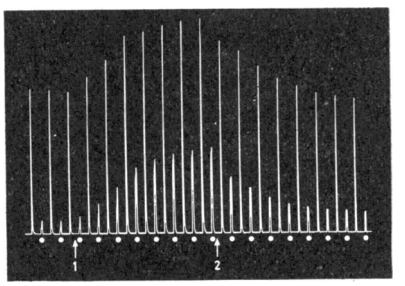

Fig. 4. Contractions of the guinea-pig isolated vas deferens in response to stimulation of the hypogastric nerve. Each stimulation was of 200 shocks, given alternately at 50 and 10 shocks/sec (at dots), every 2 min. Amylobarbitone (50 μg/ml., at 2) abolished the potentiating effect of physostigmine (2.5 μg/ml., at 1) on the responses of the vas deferens at both frequencies.

electrical stimulation at different frequencies, leaving the physostigmine-enhancement almost unimpaired, lower concentrations, such as 0.05 to 0.1 μg/ml., given after the enhancing effect of physostigmine had developed, exerted a powerful inhibitory effect.

As illustrated in Fig. 5, 0.05 μg/ml. of atropine abolished almost completely the responses of the vas deferens to stimulation at low frequency, the effect being immediate and very slowly reversible. Further experiments are now being carried out to elucidate this phenomenon.

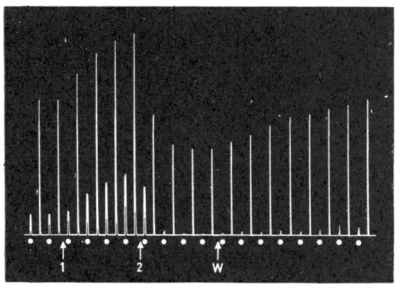

Fig. 5. Contractions of the guinea-pig isolated vas deferens in response to stimulation of the hypogastric nerve. Each stimulation was of 200 shocks, given alternately at 50 and 10 shocks/sec (at dots), every 2 min. At 1, physostigmine (2.5 μg/ml.). At 2, atropine (0.05 μg/ml.). Addition of atropine to the bath reduced strongly the physostigmine-enhanced responses at the higher frequency of stimulation and abolished almost completely those at the lower frequency. The inhibitory effect of atropine appeared to be slowly reversible after thoroughly washing the preparation (at W).

Modifications by urethane, mebutamate and amylobarbitone of the vas deferens responses to noradrenaline

The next experiments were directed to determining whether an antiadrenaline effect or a direct action on the smooth muscle effector cell might be responsible for the inhibitory effect of amylobarbitone and mebutamate on the electrically stimulated hypogastric nerve–vas deferens preparation. Direct stimulation with noradrenaline at a concentration of 0.5 to 1 μg/ml. was used. The responses were not affected by mebutamate (100 μg/ml.), by amylobarbitone (100 μg/ml.) or by urethane (750 μg/ml.).

Influence of urethane, mebutamate and amylobarbitone on the responses of the cat spleen to electrical and chemical stimulation

The results obtained in the experiments on the vas deferens could not allow us to exclude the possibility that a ganglion-blocking component was involved in the inhibitory effect of mebutamate and amylobarbitone; strong evidence has in fact been presented for the existence of ganglionic synapses along the hypogastric nerve (Sjöstrand, 1962; Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Merrillees, Burnstock & Holman, 1963; Ohlin & Strömblad, 1963; Vogt, 1963). For this purpose the cat splenic nerve–spleen preparation was more suitable since

isolated fibres of the splenic nerve are certainly postganglionic in nature and it has been demonstrated that their responses to electrical stimulation are not affected by hexamethonium (Utterback, 1944; Daly & Scott, 1961; Blakeley, Brown & Ferry, 1963).

The results of nine experiments were as follows. Whereas single doses of urethane up to 10 mg injected directly into the splenic artery did not modify at all the responses to electrical stimulation, amylobarbitone and, to a lesser extent, mebutamate were active at doses of 2.5 to 5 mg. The effect was almost immediate, appeared to be slowly reversible (Fig. 6), and there was some evidence of a dose-effect relationship. When direct stimulation with noradrenaline was used, no appreciable modifications of the responses were seen with doses as high as those affecting the electrically stimulated preparation.

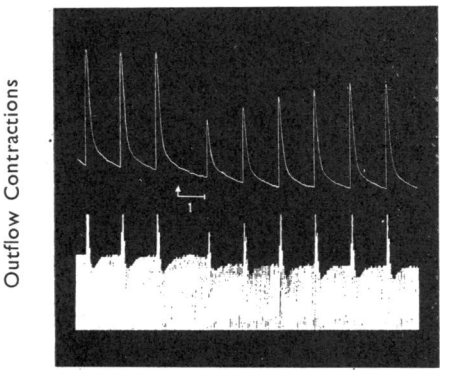

Fig. 6. Cat isolated and perfused spleen preparation. Upper tracing: longitudinal contractions of the spleen in response to electrical stimulation of the splenic nerve (15 shocks/sec for 3 sec every 4 min). Lower tracing: splenic venous outflow. At the arrow and line (1), 5 mg of amylobarbitone in 1 ml. was infused into the splenic artery over a period of 3 min: responses to splenic nerve stimulation were much reduced. The effect appears slowly reversible in the following responses.

Influence of hexamethonium, nicotine and tetramethylammonium on pressor responses of the rat to physostigmine

We wanted next to confirm that the ganglion-blocking drug hexamethonium is only able to modify pressor responses to physostigmine to a small extent, even when given in very high doses. Physostigmine (150 μg/kg, intravenously) was given repeatedly, at 45 min intervals, starting 40 to 50 min after anaesthesia with urethane. Each administration of physostigmine was preceded by hexamethonium intravenously, the first dose being 10 mg/kg and subsequent doses 20 mg/kg. Five experiments confirmed that there is a slight reduction (10 to 25%) in the response after the first dose of hexamethonium but after subsequent doses, in the presence therefore of total amounts of 30 and 50 mg/kg, no further depression occurs (Fig. 7).

Nicotine (5 to 8 mg/kg) was given intravenously in close divided doses until pressor responses to the drug were no longer observable; physostigmine, administered intravenously at this moment, failed to exert its typical effect. As

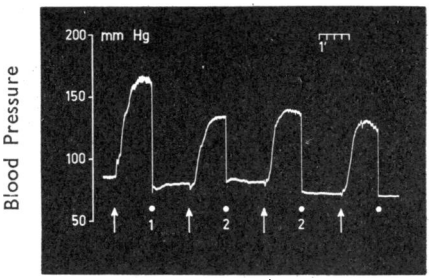

Fig. 7. Rat, 320 g, urethane anaesthesia. Record of the carotid blood pressure. At the arrows: Physostigmine (150 μg/kg, intravenously). At 1, hexamethonium (10 mg/kg, intravenously). At 2, hexamethonium (20 mg/kg, intravenously). A partial inhibition of the pressor responses to physostigmine may be observed after the first administration of hexamethonium. Subsequently higher doses did not reduce the responses further. At dots, the recording was stopped for 45 min.

reported by Varagić (1955), the inhibition by nicotine appeared to be short-lasting (Fig. 8), and physostigmine was again fully effective upon the next administration. A subsequent total dose of 4.5 to 6 mg/kg of nicotine had no inhibitory effect on responses to physostigmine when the latter drug was not administered immediately afterwards.

Like nicotine, tetramethylammonium under the same experimental conditions proved able to abolish the hypertensive responses to physostigmine. The effect developed in the presence of doses lower than those needed for nicotine (2.5 to 5 mg/kg) and seemed more slowly reversible (Fig. 9).

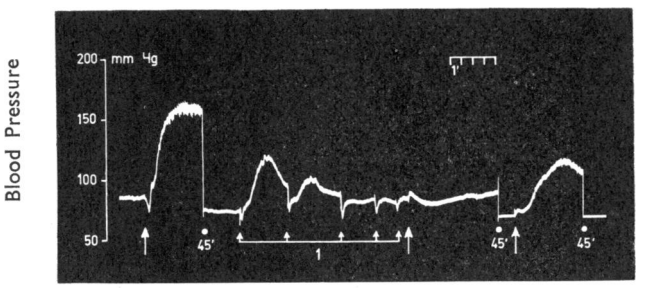

Fig. 8. Rat, 330 g, urethane anaesthesia. Record of the carotid blood pressure. At the arrows: physostigmine (150 μg/kg, intravenously). At the arrow and line (1), nicotine (4 mg/kg, intravenously in divided doses). The panel shows that the pressor response to physostigmine during the phase of " depolarizing " ganglionic block immediately following nicotine administration, failed to develop. The inhibitory effect of nicotine was short-lasting since the next administration of physostigmine evoked a normal hypertensive response. At dots, the recording was stopped for 45 min.

DISCUSSION

The typical hypertensive response to physostigmine in the rat anaesthetized with urethane has been attributed previously to a stimulating effect, mainly central, conveyed to the periphery through established sympathetic pathways. In the light

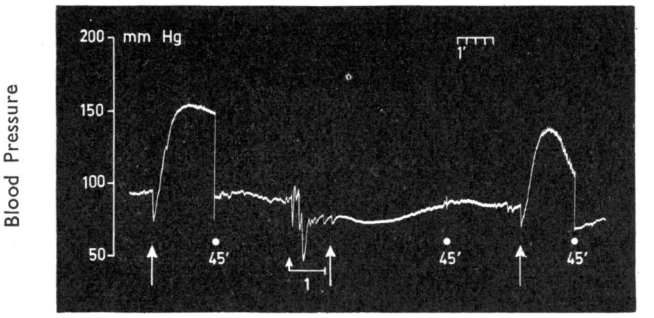

Fig. 9. Rat, 290 g, urethane anaesthesia. Record of the carotid blood pressure. As illustrated in Fig. 8 for nicotine, tetramethylammonium (2.5 mg/kg, intravenously in divided doses at the arrow and line 1) abolished the pressor response to physostigmine (150 μg/kg, intravenously at arrows). Note that recovery of the pressor responses to physostigmine was slower than after nicotine. At dots, the recording was stopped for 45 min.

of this assumption, the reduced pressor responses to physostigmine in the rat anaesthetized with mebutamate could be ascribed to its action on the brain stem and spinal vasomotor centres (Berger *et al.*, 1961 ; Margolin *et al.*, 1963), and with amylobarbitone to depression of hypothalamic centres and peripheral ganglionic synapses (Exley, 1954 ; Goodman & Gilman, 1960).

On the other hand we found that mebutamate and amylobarbitone are able to depress also the hypertensive response to pilocarpine, whose mechanism has been described by Trendelenburg (1954, 1961) as mainly peripheral, acting at the level of the sympathetic postganglionic axons. Furthermore, according to the classification of Levy & Ahlquist (1962), pilocarpine, together with neostigmine, edrophonium and McN-A-343, belongs to category II ganglion stimulants ; these are antagonized by atropine but not by classical ganglion-blocking agents such as hexamethonium. The finding that mebutamate and amylobarbitone antagonize the pressor responses both to physostigmine and to pilocarpine led us therefore to formulate two hypotheses. The first, suggested by the observations on the vas deferens and on the spleen, concerns the possibility that both drugs, besides their well-known central action, interfere with peripheral sympathetic transmission. This is supported by the fact that mebutamate and amylobarbitone reduce the responses of the vas deferens to electrical stimulation of the hypogastric nerve, and abolish completely the potentiating effect of physostigmine on the responses of the same preparation. This last effect of physostigmine has recently been interpreted by Burn & Weetman (1963) as indicating that physostigmine enhances the vas deferens responses through a cholinergic mechanism, thereby promoting a greater output of adrenergic transmitter.

The inhibitory action of amylobarbitone on the responses of the cat spleen to electrical stimulation provides evidence that the drug is able to act on postganglionic fibres directly, since splenic fibres are certainly postganglionic and it has been demonstrated that ganglion-blocking agents do not modify the responses to electrical stimulation. In this respect no problem arises for mebutamate, which is known

to be devoid of any action at the level of peripheral ganglionic synapses (Berger *et al.*, 1961). Similarly, an antiadrenaline action of the drugs can be ruled out since no modification of responses to noradrenaline was observed either on the vas deferens or on the spleen. The pharmacological analysis of the above results led us indirectly to the second hypothesis. This postulates that in the rat anaesthetized with urethane there may be a peripheral mechanism involved in the responses to physostigmine, due to an action on the sympathetic postganglionic axons. This view is consistent with the following findings: (1) the pressor responses to physostigmine, by analogy with those to pilocarpine and similar drugs, are practically unaffected by hexamethonium (only a partial inhibition is seen with very high doses), are completely abolished by atropine, and are still observable in the adrenalectomized animal (Varagić, 1955; Lešić & Varagić, 1961; Gokhale *et al.*, 1963); (2) the pressor response to physostigmine is completely abolished during the "depolarizing" ganglionic block which follows administration of nicotine or tetramethylammonium in high doses, whereas the response is unaffected when physostigmine is given during the later "nondepolarizing" phase of the block. Furthermore the presence of a peripheral component in the pressor action of physostigmine seems to explain more satisfactorily than a purely central mechanism, not only the antagonism to this action shown by bretylium given in doses insufficient to inhibit reflex hypertension induced by clamping the common carotid arteries (Lešić & Varagić, 1961), but also the antagonism by nicotine of the constrictor action of physostigmine on the perfused hind-legs of the rat (Varagić, 1955).

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