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MECHANISM OF SYMPATHOMIMETIC RESPONSES OF ISOLATED GUINEA-PIG ATRIA TO NICOTINE AND DIMETHYLPHENYLPIPERAZINIUM IODIDE

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Trendelenburg (1960) reviewed evidence indicating that not all ganglion stimulating agents are similar to nicotine in their pharmacological properties. The ganglionic stimulating properties of pilocarpine, histamine, 5-hydroxytryptamine, muscarine, etc., in contrast to those of nicotine and dimethylphenylpiperazinium are not antagonized by classical ganglion blocking agents like hexamethonium, but are blocked by cocaine, morphine and methadone. These observations have led to the concept of a second class of ganglion stimulating substances which differ from nicotine in their mode of action (Jones, 1963 ; Jones, Gomez Alonso de Sierra & Trendelenburg, 1963). Thus, drugs that act as sympathetic ganglionic stimulants fall into two categories. Category 1 consists of those agents whose ganglion stimulating properties are blocked by the " classical " ganglion blocking agents like hexamethonium. In this category are included nicotine and dimethylphenylpiperazinium iodide (DMPP). Category 2 consists of those agents whose sympathetic stimulant effects are not antagonized by hexamethonium.

This classification, however, is based on experiments performed, *in vivo*, since, on isolated guinea-pig atria, hexamethonium, in contrast to nicotine, failed to antagonize the cardiostimulant response to dimethylphenylpiperazinium (DMPP) (Bhagat, 1966). Birmingham and Wilson (1965) used the Finkelman preparation of guinea-pigs and rabbit and found that dimethylphenylpiperazinium abolished the inhibition produced by the stimulation of the periarterial sympathetic fibres. This effect was not antagonized by hexamethonium.

The present study provides further evidence that dimethylphenylpiperazinium acts differently on isolated guinea-pig atria and acts more like tyramine than like nicotine.

METHODS

The atria were dissected from the hearts of freshly killed guinea-pigs (300 to 450 g body weight) and suspended in a modified Tyrode solution maintained at 37° C and containing atropine sulphate (0.5 μg/ml.). A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the bathing fluid *via* a sintered glass plate at the bottom of the bath. The Tyrode solution had the following

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composition: NaCl, 0.9% ; KCl, 0.042% ; $CaCl_2$, 0.024% ; $NaHCO_3$, 0.05% ; glucose, 0.2%. The bicarbonate concentration employed maintained the pH at approximately 7.4. The atria were attached to a Grass force displacement transducer and isometric contractile force (resting tension of approximately 0.5 g) and rate of spontaneous beat were recorded by means of a Grass polygraph. The atria were allowed to equilibrate at least 1 hr after being placed in the bath and were washed repeatedly after each addition of drug. Whenever the response to ganglion-stimulants was determined in the presence of any other drug, it was added 15–20 min before the ganglion stimulant and removed with it.

In additional experiments, the effect of ganglion stimulants on the release of (DL)-[^{3}H]-noradrenaline (33.6 μg/millicurie ; purchased from New England Nuclear Corporation) in isolated atria was determined. After incubation with 10 μC/ml. [^{3}H]-noradrenaline for 15 min at 37° C, the atria were washed repeatedly with Tyrode solution removed from the bath, blotted with filter paper and weighed. After exposure to drugs as described in Results the atria were homogenized in 10 ml. ice-cold 0.4 N perchloric acid. The protein-free supernatant solution obtained after centrifugation was then passed over alumina at pH 8.4 and eluted with 0.04 N perchloric acid and assayed for tritiated noradrenaline (Whitby, Axelrod & Weil-Malherbe, 1961). Ethylene diaminetetra-acetic acid (15 μg/ml.) was always present in the bath during incubation with [^{3}H]-noradrenaline to slow the oxidation of the latter.

The following substances were used: dimethylphenylpiperazinium iodide, nicotine, hexamethonium chloride, hemicholinium, atropine sulphate and tyramine hydrochloride. Except for nicotine all concentrations refer to the salt used. Details of the concentrations are given in the appropriate section of the results. Tests of significance were performed according to Student's t test (Snedecor, 1956). $P<0.05$ was regarded as significant.

RESULTS

Responses of the atrial preparation to nicotine and dimethylphenylpiperazinium

Nicotine (10 μg/ml.) or dimethylphenylpiperazinium (10 μg/ml.), in the absence of atropine, consistently exerted a biphasic response; initially a negative response which lasted for 1 to 3 min and subsequently positive chronotropic and inotropic effects which occurred in 6 to 7 min. The negative responses were completely prevented or, once induced, were reversed by atropine sulphate (0.05 to 0.5 μg/ml.). In the presence of atropine (0.5 μg/ml.), maximal positive responses occurred within 20 to 30 sec, after addition of the ganglion stimulants, and were fairly well maintained, but were terminated by 3 to 4 washings with Tyrode solution.

Following the positive response to either of these ganglion stimulants, the preparation was almost refractory to subsequent additions of nicotine or dimethylphenylpiperazinium. The refractoriness could not be attributed to a total lack of responsiveness of the preparation, since addition of noradrenaline (0.005 μg/ml.) still produce a chronotropic and inotropic response. Apparently, the adrenergic receptors were still functional.

Tachyphylaxis developed when the second dose of ganglion stimulant was added within less than 10 min after the first dose had been washed off. However, exact repetition of the positive response to a first dose of ganglion stimulant could be obtained by a second dose of stimulant if the preparation had been washed 3 to 4 times during a 15- to 20-min interval. For this reason care was always taken to wash the preparation 3 to 4 times during 15- to 20-min periods following each addition of the test drug to the bath.

The cardiostimulant response to nicotine did not occur in the presence of hexamethonium (20 μg/ml.) or hemicholinium (10 μg/ml.) but the response to dimethylphenylpiperazinium or tyramine (10 μg/ml.) was not significantly altered. The concentration

of hexamethonium or hemicholinium used in this study had no effect on normal function of the atria. No details of the results are presented here since these effects were described earlier (Lee & Shideman, 1959; Trendelenburg, 1960; Leaders & Long, 1962; Chiang & Leaders, 1965; Barnett & Benforado, 1966; Kotobuki, Iwata, Iwata & Honma, 1966).

Effect of hexamethonium on the release of [^{3}H]-noradrenaline from guinea-pig atria by nicotine, dimethylphenylpiperazinium or tyramine

Several investigators have presented evidence to suggest that cardiostimulatory effects of nicotine or dimethylphenylpiperazinium are mediated through the release of catecholamines (Lee & Shideman, 1959; West, Bhagat & Robinson, 1966). Since hexamethonium blocked the response to nicotine, one possibility is that it might be interfering with the release of catecholamines by nicotine. This possibility was tested in the following experiments. Atria were incubated with 10 μc/ml. [^{3}H]-noradrenaline by 37° C for 15 min. After washing 4 times atria were exposed to 10 μg/ml. nicotine dimethylphenylpiperazinium or tyramine in the presence of 20 μg/ml. hexamethonium added 20 min before the addition of the test drug. Hexamethonium itself did not affect the [^{3}H]-noradrenaline content of the atria. It interfered significantly with the release of [^{3}H]-noradrenaline by nicotine (Table 1) but release by dimethylphenylpiperazinium (Table 1) or tyramine remained unaltered. The mean percentage release by tyramine of [^{3}H]-noradrenaline taken up by guinea-pig atria was 41 (S.E. $\pm$6) and it remained unaltered in the presence of hexamethonium (6 experiments each).

TABLE 1

EFFECT OF HEXAMETHONIUM ON THE RELEASE OF [^{3}H]-NORADRENALINE FROM GUINEA-PIG ATRIA BY NICOTINE OR DIMETHYLPHENYLPIPERAZINIUM

Atria were incubated with 10 μc/ml. [^{3}H]-noradrenaline at 37° C for 15 min. Thereafter, they were repeatedly washed with Tyrode solution and exposed to 10 μg/ml. nicotine or dimethylphenylpiperazinium for 2 min in the presence of hexamethonium (20 μg/ml.) added 20 min before addition of ganglion stimulant. They were washed repeatedly and analysed for [^{3}H]-noradrenaline content. Data are expressed as counts/min/mg.

Treatment	[^{3}H-]noradrenaline Mean$\pm$S.E.	Expts. (no.)
None	498$\pm$19·5	5
Nicotine	225$\pm$5·0[a]	6
Nicotine+hexamethonium	367$\pm$15·0[b]	5
None	504$\pm$16·5	5
DMPP	381$\pm$29·0[c]	6
DMPP+hexamethonium	311$\pm$15·0[d]	5

a. $P<0·001$ (control vs. nicotine treated). b. $P<0·01$ (nicotine treated in presence of hexamethonium). c. $P<0·01$ (control vs. dimethylphenylpiperazinium treated). d. $P>0·05$ (dimethylphenylpiperazinium treated vs. dimethylphenylpiperazinium treated in presence of hexamethonium).

Effect of hemicholinium on the release of [^{3}H]-noradrenaline from guinea-pig atria by nicotine dimethylphenylpiperazinium or tyramine

Hemicholinium prevents the synthesis of acetylcholine by interfering with the transport of choline to the intraneuronal site where the synthesis takes place (MacIntosh, Birks & Sastry, 1950). Since hemicholinium completely abolished both the negative and positive effects of nicotine, blocking at the same time the vagal but not the sympathetic transmission, Leaders & Long (1962) postulated that in both the negative and positive phases

of atrial response to nicotine only the parasympathetic system is involved and that the mobilization of catecholamines from the peripheral stores is probably brought about by the mediation of acetylcholine. However, this possibility is ruled out by the recent observation of Khan, Mantegazza & Piccinini (1965) that the positive chronotropic and inotropic effects of nicotine are still present at temperatures at which the parasympathetic system is completely insensitive both to nicotine and to electrical stimulation. According to Burn & Gibbons (1964), hemicholinium has a peripheral action similar to that of hexamethonium. Since hexamethonium interfered with the release of [^{3}H]-noradrenaline by nicotine experiments were performed similar to those described in the preceding section except that hexamethonium was replaced by hemicholinium (10 μg/ml.). Hemicholinium in the dose employed did not affect the [^{3}H]-noradrenaline content of isolated guinea-pig atria. Results presented in Table 2 indicate that hemicholinium, like hexamethonium, interfered with the release of [^{3}H]-noradrenaline by nicotine, but the release by dimethylphenylpiperazinium remained unaltered. Hemicholinium also failed to alter the release of [^{3}H]-noradrenaline by tyramine. The mean (6 experiments each) percentage released by tyramine was 44 (S.E. $\pm$5) and in the presence of hemicholinium it was 42 (S.E. $\pm$6). The difference between the two means is not significant ($P>0.05$).

TABLE 2

EFFECT OF HEMICHOLINIUM ON THE RELEASE OF ^{3}[H]-NORADRENALINE FROM GUINEA-PIG ATRIA BY NICOTINE OR DIMETHYLPHENYLPIPERAZINIUM

Atria from guinea-pigs were incubated with 10 μc/ml. ^{3}[H]-noradrenaline at 37° C for 15 min. Thereafter, they were repeatedly washed with Tyrode solution and exposed to 10 μg/ml. nicotine or dimethylphenylpiperazinium for 2 min in the presence of hemicholinium (10 μg/ml.) added 20 min before the addition of ganglionic stimulant. They were washed repeatedly and analysed for ^{3}[H]-noradrenaline. Data are expressed as counts/min/mg.

Treatment	[^{3}H] noradrenaline Mean$\pm$S.E.	Expts. (no.)
None	471$\pm$19·5	5
Nicotine	225$\pm$5·0[a]	6
Nicotine+HC_3	342$\pm$16·0[b]	5
None	635$\pm$21·5	5
DMPP	425$\pm$11·5[c]	6
DMPP+HC_3	411$\pm$12·5[d]	6

a. $P<0.001$ (control vs. nicotine treated). b. $P<0.01$ (nicotine treated vs. nicotine treated in presence of hemicholinium). c. $P<0.01$ (control vs. dimethylphenylpiperazinium treated). d. $P<0.05$ (dimethylphenylpiperazinium treated vs. dimethylphenylpiperazinium treated in presence of hemicholinium).

Effect of raising calcium concentration on the release of [^{3}H]-noradrenaline in isolated guinea-pig atria by nicotine, dimethylphenylpiperazinium or tyramine

Burn and Gibbons (1965) presented pharmacological evidence suggesting that the release of noradrenaline by nicotine from sympathetic fibres was dependent on the concentration of calcium ions outside the fibre. It was, therefore, thought of interest to investigate the effect of raising calcium concentration on the release of [^{3}H]-noradrenaline by nicotine, dimethylphenylpiperazinium or tyramine. Atria were incubated with 10 μc/ml. [^{3}H]-noradrenaline at 37° C for 15 min. Thereafter, they were washed repeatedly with Tyrode solution and exposed to 10 μg/ml. nicotine, dimethylphenylpiperazinium or tyramine for 2 min and after washing were analyzed for their [^{3}H]-noradrenaline content. In other series of experiments, the calcium concentration of the Tyrode solution was

raised 6 times from 2.2 mM to 13.2 mM before the atria were exposed to the test drugs. Raising the calcium concentration did not significantly affect ($P>0.05$) the [^{3}H]-noradrenaline content of the atria (Fig. 1) but increased the spontaneous rate by 45 ± 6 (mean $\pm$ standard error of 6 experiments) beats/min. Release of [^{3}H]-noradrenaline by nicotine was significantly enhanced ($P<0.01$) by the rise in calcium concentration. The mean percentage (6 experiments each) of release by tyramine of [^{3}H]-noradrenaline taken up by the guinea-pig atria was 43 (S.E. $= \pm 7$) for 2.2 mM calcium and was 49 (S.E. $= \pm 5$) for 13.2 mM calcium. The difference between the means was not significant ($P>0.05$).

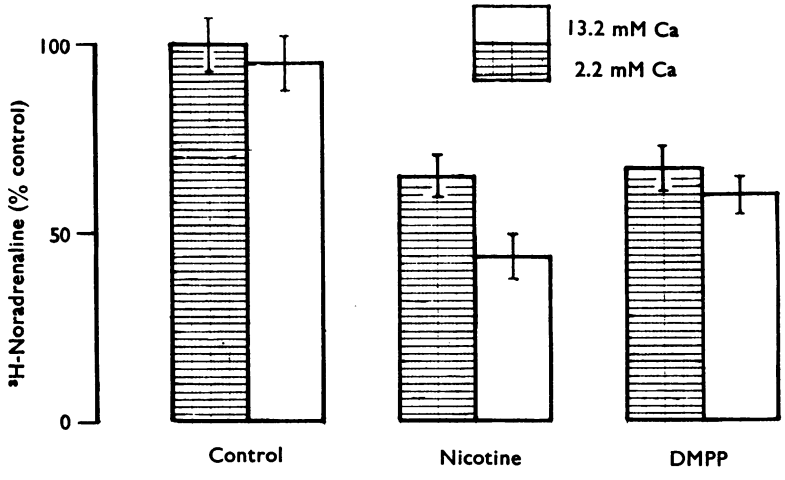

Fig. 1. The effect of increased calcium concentration on the release of [^{3}H]-noradrenaline from isolated guinea-pig atria by nicotine or dimethylphenylpiperazinium. Atria were incubated with 10 μc/ml. [^{3}H]-noradrenaline at 37° C for 15 min. Thereafter, they were washed with Tyrode solution and exposed to 10 μg/ml. nicotine or dimethylphenylpiperazinium for 2 min. After washing 3 or 4 times, atria were analysed for [^{3}H]-noradrenaline content. In other similar experiments the calcium concentration of Tyrode solution was raised 6 times—that is, from 2.2 to 13.2 mM. Each column represents the mean percentage of control atrial [^{3}H]-noradrenaline, and the vertical lines indicate the standard errors of the means of 6 experiments.

DISCUSSION

Nicotine and dimethylphenylpiperazinium, both ganglionic stimulants, had a biphasic effect on isolated guinea-pig atria, initially a negative, and subsequently a positive, chronotropic and inotropic effect. The inhibitory component of the response is believed to be the result of a stimulation of intramural parasympathetic ganglia, but the significance of the secondary positive response is still controversial.

The fact that these ganglionic stimulants fail to exert their cardiostimulatory effects either when the sympathetic receptors have been blocked (Lee & Shideman, 1959; West *et al.*, 1966) or when catecholamine stores have been depleted by reserpine (Burn & Rand, 1958; Lee, McCarty, Zodrow & Shideman, 1960) suggests that the effects are mediated through the release of catecholamines from the post-ganglionic sympathetic nerve endings. In the present study both nicotine and dimethylphenylpiperazinium caused

a significant release of [^{3}H]-noradrenaline from isolated guinea-pig atria, thus providing direct biochemical evidence for this view. However, none of these observations indicates whether ganglionic stimulants liberate catecholamines directly from peripheral stores or indirectly by ganglionic stimulation. Since hexamethonium antagonizes the actions of nicotine, Kottegoda (1953) postulated the existence of sympathetic ganglia in the heart. However, when histological examination of papillary muscle of the cat, on which nicotine exerted a stimulatory effect, failed to reveal the existence of ganglionic cells, Lee & Shideman (1959) postulated that there may exist in the heart certain elements which do not possess the morphological characteristics of ganglia, but which behave pharmacologically as if they were ganglia: nicotine and other ganglion stimulants may act directly on these elements to liberate catecholamines. Failure of hexamethonium to antagonize the cardiostimulatory response to dimethylphenylpiperazinium or its release of [^{3}H]-noradrenaline (Table 2) seems to exclude this possibility. These results, therefore, may be considered as further evidence in favour of the view that the isolated atria of the guinea-pig are not provided with intramural sympathetic ganglia. Thus nicotine may be exerting its cardiostimulatory effects by direct mobilization of catecholamines from some peripheral store and not indirectly through an action on ganglia levels.

Nevertheless, hexamethonium blocks the sympathomimetic effects of nicotine. This could be explained by assuming that hexamethonium has a peripheral antinicotinic activity. Hexamethonium and other ganglionic blocking agents are capable of antagonizing the sympathomimetic effects of nicotine and even of acetylcholine at sites other than subsynaptic membranes, for instance, the excitation of sympathetic postganglionic C fibres (Ferry, 1963; Blakeley, Brown & Ferry, 1963). This, like many other observations, is consistent with the view that acetylcholine or nicotine has an action on the nerve membrane and that hexamethonium blocks the acetylcholine receptors at this site as it does in ganglia. Burn & Gibbons (1964), however, suggested that hexamethonium prevented the sympathomimetic effects of acetylcholine and nicotine, not by blocking the cholinergic receptors, but by preventing drug entry into the nerve fibres.

The nerve impulse in the adrenergic nerve terminals, which is a series of changes of ionic permeability across the cell membrane, permits the entry of calcium ions from the extracellular space. This entry of calcium causes, in some unknown way, the release of noradrenaline. The release of sympathetic transmitter by acetylcholine and nicotine is due primarily to an action on the membrane of the nerve terminals increasing their permeability to ions, the inflow of which is associated with the release of noradrenaline. The present study suggests that the important event is the entry of calcium ions since increasing the calcium ion concentration from 2.2 mM to 13.2 mM increased the release of [^{3}H]-noradrenaline by nicotine. This is consistent with the observations of Douglas & Rubin (1961, 1963) who reported that acetylcholine and nicotine evoked the release of catecholamines from the chromaffin cells of the adrenal medulla by promoting their uptake of calcium. Burn & Gibbons (1965) showed that the rabbit atrial response to acetylcholine in the presence of atropine or hyoscine and to nicotine was dependent on external calcium ion concentration.

While the results presented in this study do not conclusively prove that release by nicotine depends on calcium ion concentration outside the sympathetic fibre, they do lend support to the idea that calcium plays a cardinal role in the process of release

of catecholamines by nicotine from the adrenal medulla (Douglas & Rubin, 1963) and from postganglionic sympathetic fibres (Burn & Gibbons, 1965).

Increasing the calcium ion concentration in the bath increased the release of [^{3}H]-noradrenaline by nicotine but failed to alter the release by dimethylphenylpiperazinium or tyramine. The release of [^{3}H]-noradrenaline by dimethylphenylpiperazinium or by tyramine, therefore, does not appear to depend on the calcium ion concentration.

The results presented and literature reviewed suggest that the mechanism of release of catecholamines by dimethylphenylpiperazinium differs from that for nicotine. Since hexamethonium, hemicholinium or increased calcium ion concentrations failed to affect the release of [^{3}H]-noradrenaline by tyramine or by dimethylphenylpiperazinium, it is suggested that dimethylphenylpiperazinium acts more like tyramine than like nicotine. This is consistent with the view of Lindmar & Muscholl (1961).

According to the classification of ganglion stimulants summarized in the Introduction dimethylphenylpiperazinium and nicotine are grouped together because their ganglionic stimulant properties are antagonized by hexamethonium. It was, therefore, expected that nicotine and dimethylphenylpiperazinium would act similarly on the isolated guinea-pig atria. However, in the present study, the cardiostimulant effects of dimethylphenylpiperazinium were not antagonized by hexamethonium or hemicholinium. This is consistent with the findings of Chiang & Leaders (1965) who used isolated rat atria. Similarly the release of [^{3}H]-noradrenaline from the atria by dimethylphenylpiperazinium or tyramine remained unaltered in the presence of hexamethonium or hemicholinium while that by nicotine was reduced. These findings indicate that results obtained *in vivo* may not be relevant to those obtained *in vitro* and that differences between nicotine and dimethylphenylpiperazinium become more evident *in vitro*.

SUMMARY

1. Ganglionic stimulants can be divided by experiments *in vivo* into a "nicotinic class," which includes nicotine and dimethylphenylpiperazinium, and is antagonized by hexamethonium, and a "non-nicotinic class" which is not blocked by hexamethonium. However, on isolated guinea-pig atria, the stimulatory responses to dimethylphenylpiperazinium, in contrast to those to nicotine, were not antagonized by hexamethonium.

2. Hexamethonium and hemicholinium interfered with the release of [^{3}H]-noradrenaline by nicotine but failed to alter release by dimethylphenylpiperazinium or tyramine.

3. Raising calcium ion concentration to six times normal increased the release of [^{3}H]-noradrenaline by nicotine, while release by dimethylphenylpiperazinium or by tyramine remained unaltered.

4. These results, thus, suggest that the mechanism of action of dimethylphenylpiperazinium is different from that of nicotine. It appears to act on isolated guinea-pig atria more like tyramine than like nicotine.

5. An increased release of [^{3}H]-noradrenaline by nicotine in the presence of an increased calcium ion concentration lends support to the view that calcium plays an important role in the release of catecholamines by nicotine from post-ganglionic sympathetic fibres.

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