EFFECTS OF HEXAMETHONIUM AND OTHER AGENTS ON NORADRENALINE OUTPUT RELEASED BY THE SYMPATHETIC NERVE STIMULATION IN THE ISOLATED PERFUSED RABBIT'S HEART

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The heart has been used as a convenient tool of physiological and pharmacological studies of nerves and muscles. The present experiment was done in rabbit's heart as a possible preparation to be isolated with the sympathetic nerve in a convenient experimental animal. At the beginning of the present experiment, investigation was done as to whether or not there was a relationship between positive mechanical responses to and noradrenaline output released by the sympathetic nerve stimulation. Effects of high concentrations of external Ca<sup>++</sup> and Mg<sup>++</sup>, cocaine and adrenergic β-blockers on noradrenaline output released by the sympathetic nerve stimulation were re-investigated.

The objective of this paper is to discuss the possibility of the existence of intracardiac sympathetic ganglia. Such a hypothesis was based mainly on observations that in heart tissues nicotinic drugs exerted effects like those of the sympathetic nerve stimulation, which could be blocked by ganglionic blocking agents (1–4). The hypothesis appeared open to discussion under evidence (5–7). On the other hand, Juhász-Nagy and Szentiványi (8) and Takenaka et al. (9) have demonstrated the possibility that vasoconstrictor fibers of coronary arteries synapse with ganglion cells in or near the wall of the dog's heart. In the present paper, it was attempted to throw light on the problem by investigation of effects of hexamethonium on mechanical and coronary responses to and noradrenaline output released by the electrical stimulation of the sympathetic nerve in the rabbit's heart. Preliminary reports have already been published (10).

METHODS

About 80 hearts of rabbit, weighing 1.8 to 2.5 kg, of both sexes were used. Animals were anesthetized with pentobarbital sodium (50 mg/kg) injected intraperitoneally. The chest was opened under artificial respiration. Bilateral sympathetic trunks (mainly stellate ganglia) were isolated carefully from the surrounding connective tissues. The aorta was cannulated with a polyethylene tube and perfused with Krebs bicarbonate solution, bubbled...
with 95% O₂ and 5% CO₂, having the following composition (expressed in mM): NaCl 118.4; KCl 4.7; CaCl₂ 2.5; MgCl₂ 7.11; O 1.18; NaHCO₃ 25; KH₂PO₄ 1.2; and glucose 11.1. The heart with bilateral stellate ganglia was isolated and perfused with the perfusion pressure of 60 cm H₂O at the rate of 10 to 15 ml/min at 30°C. The preparation was immersed in external medium in the same composition as perfusion medium (Fig. 1). In some experiments, hearts were perfused with solutions containing excess Ca²⁺ (twofold) or excess Mg²⁺ (fivefold). Changes in tonicity were not compensated. Bilateral stellate ganglia were stimulated for 30 sec with an electronic stimulator MSE-3 (Nihon Kohden) through fluid electrodes (11) at supramaximal voltage with rectangular pulses of 3.5 msec duration at a frequency of 30 cps according to Huković and Muscholl (12). As shown in Fig. 2, each stimulation period was followed by a resting period of 30 sec. This schedule was repeated for 6 min and the standard stimulation procedure was repeated in the same preparation twice at an interval of 30 min. About 15 preparations were suspended in air by means of a perfusion apparatus (Natsume KN-206) for recording, on smoked paper, isotonic changes in spontaneous contractile responses to the nerve stimulation. All experiments were designed to terminate within 30 min after the 1st period of the nerve stimulation.

Venous effluent was collected for 6.5 min during nerve stimulation. Noradrenaline output released in venous effluent was measured fluorometrically by the method of Anton.
and Sayre (13) with a spectrofluorophotometer GF-16 (Shimazu Ltd.). The recovery was about 60%.

Positive chronotropic and contractile responses to the 2nd period of the nerve stimulation were expressed as percentages of those of the 1st procedure. Noradrenaline output released by the 2nd nerve stimulation was expressed as a percentage of that of the 1st procedure. In some experiments, coronary responses to nerve stimulation were determined as an index of changes in volume of coronary flow for 6.5 min during the 1st nerve stimulation. When the significance of data was evaluated Students’ t-test was used. N is the number of estimations.

The solution containing drugs was usually perfused 20 min before and during the 2nd nerve stimulation. Drugs used were cocaine hydrochloride, dichloroisoproterenol hydrochloride, pronethalol hydrochloride, propranolol hydrochloride, and hexamethonium bromide. Atropine sulphate and phenoxybenzamine hydrochloride, which were used in some experiments to investigate coronary responses to the nerve stimulation, were added to Krebs solution at the start. Concentrations of all drugs are expressed in terms of g of the salts/ml in perfusion medium.

RESULTS

Responses of the untreated heart to the sympathetic nerve stimulation

Fig. 2 shows a positive chronotropic and contractile response of the untreated heart to electrical stimulation of bilateral stellate ganglia. In few preparations, initial slight inhibitory responses to the sympathetic nerve stimulation were observed. Percent increases in the 1st positive contractile and chronotropic responses to the 1st period of the nerve stimulation were 59% (n = 6) and 41% (n = 7). Responses to nerve stimulation decreased

Nerve stimulation 10 min

![Graph](image)

Fig. 2. A positive chronotropic and contractile response of an untreated rabbit’s heart to the electrical stimulation of the sympathetic nerve.

Arrows show the beginning of the nerve stimulation, which was performed 6 times, each for 30 sec at an interval of 30 sec, at supramaximal voltage with rectangular pulses of 3.5 msec duration at a frequency of 30 cps. The stimulation was repeated in the same preparation twice at an interval of 30 min. Numbers under the figure show beats/min in maximal response to each stimulation.
Effect of hexamethonium ($5 \times 10^{-4}$) on positive chronotropic responses of the isolated rabbit's heart to the sympathetic nerve stimulation.

The intermittent (6 times) nerve stimulation was repeated twice at an interval of 30 min. Abscissa shows each number of the 1st and 2nd nerve stimulations. In ordinate, each response of the heart to the 2nd nerve stimulation is expressed as a percentage of that to the 1st procedure. Broken line shows a control response. Solid line shows an effect of hexamethonium, which was applied 20 min before the 2nd nerve stimulation. Vertical bars show standard errors. Parentheses indicate number of experiments.

Effect of hexamethonium ($5 \times 10^{-4}$) on positive contractile responses of the isolated rabbit's heart to the sympathetic nerve stimulation.

The intermittent (6 times) nerve stimulation was repeated twice at an interval of 30 min. Abscissa shows each number of the 1st and 2nd nerve stimulations. In ordinate, each response of the heart to the 2nd nerve stimulation is expressed as a percentage of that to the 1st procedure. Broken line shows a control response. Solid line shows an effect of hexamethonium, which was applied 20 min before the 2nd nerve stimulation. Vertical bars show standard errors. Parentheses indicate number of experiments.
gradually. The 6th responses to the 1st period of nerve stimulation were 27\% and 30\%.

After stimulation, responses returned to initial levels within 2 to 3 min. The 2nd period of nerve stimulation 30 min after the 1st produced similar positive chronotropic and slightly reduced contractile responses (Fig. 2). Percent increases in the 1st and 6th positive contractile and chronotropic responses to the 2nd nerve stimulation were, however, 45\% and 38\%, and 25\% and 26\%, respectively. Results are summarized in Figs. 3 and 4. As shown in broken lines of Figs. the 2nd period of the nerve stimulation produced responses not significantly different from those of the 1st.

The 1st nerve stimulation released noradrenaline from 50.0 to 962.4 ng, 158.0 ng on an average of 12 cases. Actual amount of noradrenaline varied in each preparation. However, as shown in Fig. 5, the 2nd nerve stimulation released a relatively constant 34.5 ± 3.5% of the noradrenaline released by the 1st one in each preparation, which was regarded as control in untreated hearts.

Effects of extracellular excess Ca\(^{++}\) and Mg\(^{++}\) on noradrenaline output released by the sympathetic nerve stimulation

It has been generally accepted that Ca\(^{++}\) plays an essential key role in release of various kinds of neurohormones and Mg\(^{++}\) causes inhibitory and antagonistic effects against Ca\(^{++}\).

![Fig. 5. Noradrenaline output released by the electrical stimulation of the sympathetic nerve in untreated rabbit's hearts.](image)

The intermittent nerve stimulation was repeated twice at an interval of 30 min. In ordinate, noradrenaline output released by the 2nd nerve stimulation is expressed as a percentage of that of the 1st procedure. Vertical bar shows a standard error. Parenthesis indicates number of experiments.
Effects of the high concentration of external Ca\(^{2+}\) (twofold) and Mg\(^{2+}\) (fivefold) on noradrenaline output released by the sympathetic nerve stimulation in the isolated rabbit's heart.

The intermittent nerve stimulation was repeated twice at an interval of 30 min. In ordinate, noradrenaline output released by the 2nd nerve stimulation is expressed as a percentage of that of the 1st procedure. The solution containing the high concentration of external Ca\(^{2+}\) or Mg\(^{2+}\) was perfused 20 min before the 2nd nerve stimulation. Vertical bars show standard errors. Parentheses indicate number of experiments.

Atomic photographs of the effects of Ca\(^{2+}\) and Mg\(^{2+}\) on noradrenaline output. The effects of various concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) on noradrenaline output are shown in Figure 6. The ordinate represents the percentage of noradrenaline output released by the 2nd nerve stimulation relative to that of the 1st procedure. The numbers in parentheses indicate the number of experiments. Vertical bars represent standard errors.

For example, in release of acetylcholine in the skeletal muscle (14, 15), adrenaline from the cat's adrenal medulla (16), vasopressin from the neurohypophysis (17,18), and noradrenaline in the cat's spleen (19). Huković and Muscholl (12) have shown that in the rabbit's heart release of noradrenaline depends on the presence of Ca\(^{2+}\). As shown in Fig. 6, perfusion with excess Ca\(^{2+}\) (twofold) Krebs solution raised noradrenaline output caused by the nerve stimulation from 34.5 to 52.8%.

On the other hand, Schümann and Philippu (20) have reported that in isolated perfused suprarenals of cattle, injection of both Ca\(^{2+}\) (25 \(\mu\) moles) and Mg\(^{2+}\) (100 \(\mu\) moles) releases catecholamines to about the same extent and the substitution of Ca\(^{2+}\) by a 4 times higher concentration of Mg\(^{2+}\) restores the acetylcholine-induced release of catecholamines. In preliminary observations of the present experiment, using 3 atrial preparations with stellate ganglia prepared according to the previous paper (11), external Mg\(^{2+}\) (5, 10 and 20 fold) reduced slightly atrial spontaneous rate and force of contraction, but unexpectedly, raised slightly atrial positive chronotropic and contractile responses to the sympathetic nerve stimulation according to concentrations. As shown in Fig. 6, perfusion with excess Mg\(^{2+}\) (fivefold) Krebs solution did not reduce, but raised slightly noradrenaline output from 34.5 to 43.3%. It appears likely that the rabbit's heart may be an exceptional organ concerning the inhibitory effect of Mg\(^{2+}\) on release of noradrenaline caused by the sympathetic nerve stimulation.

Effects of cocaine and adrenergic \(\beta\)-blockers on noradrenaline output released by the sympathetic nerve stimulation

It has been generally accepted that reuptake process plays an essential role in disap-
The intermittent nerve stimulation was repeated twice at an interval of 30 min. In ordinate, noradrenaline output released by the 2nd nerve stimulation is expressed as a percentage of that of the 1st procedure. Drugs were applied 20 min before the 2nd nerve stimulation. Vertical bars show standard errors. Parentheses indicate number of experiments.

Effects of hexamethonium on responses of the heart to the sympathetic nerve stimulation

As shown in Fig. 8, perfusion with a relatively low concentration of hexamethonium (5 x 10^-6) 20 min before the 2nd nerve stimulation reduced significantly (P<0.01, n = 9) noradrenaline output by 48% compared with the corresponding output of control hearts (n=12). In order to rule out a possibility that the drug leaked from perfusion medium to external medium acted directly on the sympathetic ganglia outside the heart, the same dose
FIG. 8. Effects of hexamethonium (5 \times 10^{-6}) on noradrenaline output released by the sympathetic nerve stimulation in the isolated rabbit's heart.

The intermittent nerve stimulation was repeated twice at an interval of 30 min. In ordinate, noradrenaline output released by the 2nd nerve stimulation is expressed as a percentage of that of the 1st procedure. The drug was applied 20 min before the 2nd nerve stimulation, into perfusion medium in the middle column and into external medium in the right column. Vertical bars show standard errors. Parentheses indicate number of experiments.

FIG. 9. Effects of phenoxybenzamine (10^{-6}) and hexamethonium (5 \times 10^{-6}) on a decrease in coronary flow caused by the sympathetic nerve stimulation in the isolated rabbit's heart.

The intermittent nerve stimulation was performed in the presence of atropine (10^{-6}) and propranolol (5 \times 10^{-7}). Ordinate shows percent changes in the volume of coronary flow caused by the nerve stimulation. Vertical bars show standard errors. Parentheses indicate number of experiments.

of the drug was put into external medium 20 min before the 2nd nerve stimulation and did not produce a decrease in noradrenaline output (n = 6).

As shown in solid lines of Figs. 3 and 4, the same dose of hexamethonium did not alter
positive chronotropic and contractile responses to electrical nerve stimulation.

In Fig. 9, the volume of coronary flow for 6.5 min before the nerve stimulation (70 to 90 ml) was regarded as 100%. In every case as shown in this figure, perfusion medium contained propranolol $5 \times 10^{-5}$ and atropine $10^{-5}$, to rule out $\beta$-adrenergic factors, which could cause an increase in coronary flow to be secondary to the increase of cardiac metabolism, and cholinergic factors in changes of coronary flow (8). In control preparations ($n=4$), the sympathetic nerve stimulation produced in the above-mentioned condition a decrease in coronary flow to $87.1 \pm 2.0\%$. As suggested in the demonstration that adrenaline-induced vasoconstriction of coronary arteries was inhibited by $\alpha$-adrenergic blockers in cat’s (27) and dog’s heart (28), the pretreatment with phenoxybenzamine ($10^{-6}$) blocked completely the decrease in coronary flow caused by the electrical nerve stimulation in rabbit’s heart ($n=3$). Perfusion with hexamethonium 20 min before the nerve stimulation blocked significantly ($P<0.05, n=4$) the decrease in coronary flow caused by the sympathetic nerve stimulation. In every experiment shown in Fig. 9, noradrenaline output released by the nerve stimulation was measured and ascertained to be more than 50 ng.

**DISCUSSION**

Huković and Muscholl (12) reported that noradrenaline output decreased equally by 44%, with four subsequent nerve stimulations at intervals of 30 min in the isolated rabbit’s heart. In the present experiment, however, noradrenaline output released by the 2nd nerve stimulation decreased to 34.5% of that of the 1st one. This difference could be derived from different experimental situations, for example, differences of perfusion medium, measurement of noradrenaline, etc. Such a marked decrease in noradrenaline output could be due to difficulties in maintaining the physiological condition of Langendorff’s preparations by perfusion with artificial nutrient solution in addition to the possible general difficulty of synthesis and reuptake of noradrenaline in artificially perfused organs. Experiments of this type should be limited to one hr after the start of perfusion. On the other hand, the 2nd period of the nerve stimulation produced positive chronotropic and contractile responses not significantly different from those of the 1st one. There was no clear parallelism between positive mechanical responses to and noradrenaline output released by the sympathetic nerve stimulation. It seems probable in the present experiment that noradrenaline released by the nerve stimulation adequately reached receptors and even a smaller amount of noradrenaline could produce usual mechanical responses.

Since the early work of Hoffman et al. (1), it has been generally accepted that acetylcholine or the stimulation of the vagus in the presence of atropine and nicotinic drugs exert actions like those of the sympathetic nerve stimulation with liberation of catecholamines and hexamethonium can block these effects in the heart tissues as well as a variety of other organs of various kinds of animals. In the case of heart, a possibility of the presence of intracardiac synaps between cholinergic fibers and adrenergic nerve terminals has been discussed (2-4). Such a hypothesis was refuted by Ferry (5), Cabrera et al. (6), Cooper (7), Bhagat (29) and Brus and Jacobowitz (30). Results obtained in the present experiment, in which electrical
stimuli replacing nicotinic drugs were used as sympathetic nerve impulses, threw light again on the problem of the site and mode of action of hexamethonium. Hukovic and Muscholl (12) have reported no effects of the drug on noradrenaline output released by the sympathetic nerve stimulation, however experimental cases were few. In the present experiment, perfusion with the relatively low concentration of hexamethonium ($5 \times 10^{-4}$) reduced significantly noradrenaline output released by the electrical nerve stimulation. Although hexamethonium did not alter positive chronotropic and contractile responses of the heart to the sympathetic nerve stimulation, the drug did block significantly decrease in coronary flow caused by the sympathetic nerve stimulation.

Results may be explained in several possible ways. Pharmacologically, it appears probable that there could exist rudimentary intracardiac sympathetic ganglia or ganglion-like structures, postganglionic fibers of which might innervate mainly coronary arteries, and the effect of hexamethonium could be derived from a specific ganglionic blocking action on these ganglia. With fluorescent histochemical methods, adrenergic ganglion cells in the myocardium were observed in the frog heart by Falck et al. (31) and demonstrated in the heart of brown and rainbow trout by Gannon and Burnstock (32). Despite extensive research of mammalian hearts, however, no clear adrenergic ganglion cells have been demonstrated, although there do exist intramural adrenergic cells (33). After complete extrinsic cardiac denervation of the canine heart, Napolitano et al. (34) demonstrated intrinsic cardiac nerves, which contained occasional electrondense membrane-limited granules. These appeared to be noradrenaline granules.

As a second possibility, the results could be explained by interactions between cholinergic and adrenergic systems. Mixed cholinergic components in initial inhibitory responses of the isolated rabbit’s heart to the sympathetic nerve stimulation have been often observed (35, 36) and as well as in few cases of the present experiment. There is some possibility that leakage of electrical current from electrodes placed on stellate ganglia stimulates vagal fibers running along the sympathetic nerve, as suggested by Misu and Kirpekar (11) in the cat’s heart. Possibly a small amount of acetylcholine released from postganglionic cholinergic nerve terminals could release secondarily noradrenaline mainly from the sympathetic nerve endings. Hexamethonium can block this type of mode of action of acetylcholine. Recently, Ehinger et al. (37) suggested interesting possibilities that in the rat iris and heart there existed axo-axonal synapses between cholinergic and adrenergic nerve terminals. The main parts of the present experiment, however, were performed in a non-atropinized condition.

Thirdly, if virtually there were no intracardiac sympathetic ganglion-like structures, where would the action of hexamethonium be located? Since hexamethonium has no blocking action on adrenergic axonal conduction, could the drug have a similar action to that of adrenergic neuron blockers only in the fibers innervating coronary arteries?

By way of summary, it seems most probable that there exist rudimentary intracardiac sympathetic ganglia, post-ganglionic fibers of which innervate coronary arteries. The location of these ganglia is as yet unclear.
**SUMMARY**

1. Rabbit's hearts were isolated with bilateral stellate ganglia and perfused with Krebs solution. Preparations were usually immersed in external medium in the same composition as perfusion medium. The rate and force of contraction of the heart increased during stimulation of the sympathetic nerve. Noradrenaline released was measured fluorometrically. Sympathetic stimulation was repeated twice at an interval of 30 min in the same preparation. With the 2nd period of stimulation, positive mechanical responses decreased scarcely, but noradrenaline output decreased to 34.5% of that with the 1st.

2. Effects of agents were studied by perfusion with solutions 20 min before and during the 2nd stimulation, and noradrenaline output was compared with the corresponding output of control hearts. Excess calcium (twofold) raised noradrenaline output, excess magnesium (fivefold) did not reduce it, cocaine, dichloroisoproterenol and pronethalol (5 x 10^-6) raised it and propranolol (5 x 10^-6) did not modify it.

3. Hexamethonium (5 x 10^-6) reduced noradrenaline output significantly. The drug applied in external medium did not reduce it. The drug did not alter positive mechanical responses to the sympathetic nerve stimulation. In hearts pretreated with propranolol and atropine, the nerve stimulation produced a decrease in coronary flow, which was blocked completely by phenoxybenzamine (10^-6). Hexamethonium blocked significantly the decrease in coronary flow caused by the sympathetic nerve stimulation.

4. The fact that there could exist intracardiac sympathetic ganglia in the rabbit's heart, postganglionic fibers of which might innervate coronary arteries has been discussed.

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