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NON-CHOLINERGIC COMPONENT OF RAT SPLANCHNIC NERVES PREDOMINATES AT LOW NEURONAL ACTIVITY AND IS ELIMINATED BY NALOXONE

BY RAVINDRA K. MALHOTRA AND ARUN R. WAKADE

From the Department of Pharmacology, State University of New York, Downstate Medical Center, 450 Clarkson Avenue, Brooklyn, New York 11203, U.S.A.

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SUMMARY

1. Effects of nicotinic (mecamylamine) and muscarinic (atropine) receptor antagonists were investigated on the secretion of catecholamines evoked by stimulation of splanchnic nerve terminals and acetylcholine in the isolated perfused adrenal gland of the rat to determine whether non-cholinergic substances released from nerve terminals participate in the secretion of catecholamines.

2. Increasing the frequency of stimulation from 0·5 to 10 Hz (300 pulses) caused enhanced secretion of catecholamines (26–110 ng/collection period). After blockade of nicotinic and muscarinic receptors with mecamylamine and atropine, the secretion was reduced by 40, 65 and 80% at 0·5, 1 and 10 Hz, respectively. Acetylcholine-evoked secretion of catecholamines, which was roughly equivalent to that produced by stimulation at 10 Hz, was blocked by over 90% by the cholinergic antagonists.

3. Naloxone (3–300 μM) caused a concentration-dependent inhibition of catecholamine secretion evoked by stimulation of splanchnic nerves (1 Hz); acetylcholine-evoked secretion was much less affected by naloxone.

4. The secretion of catecholamines that remained after blockade of cholinergic receptors at different frequencies of stimulation (see 2 above) was almost completely inhibited by inclusion of 30 μM-naloxone in the medium. The inhibitory effect of naloxone was concentration dependent (3–30 μM) and reversible.

5. Splanchnic nerve-evoked secretion of catecholamines was facilitated by 400% in the presence of tetraethylammonium or tetraethylammonium plus mecamylamine and atropine. The facilitatory effect of tetraethylammonium was inversely related to the frequency of stimulation.

6. The residual secretion of catecholamines obtained after blockade of cholinergic receptors was facilitated by increasing concentrations of tetraethylammonium (1–5 mM). 30 μM-naloxone antagonized the facilitatory effects of tetraethylammonium at 1 and 3 mM by 60% and 25%, respectively, but failed at 5 mM-tetraethylammonium; higher concentrations of naloxone (100 μM) were also ineffective.

7. It is concluded that neurally evoked secretion of catecholamines is mediated by acetylcholine and a non-cholinergic substance(s); the contribution of non-cholinergic substance(s) predominates at low neuronal activity, whereas that of acetylcholine is

maximum at high neuronal activity. Blockade of the non-cholinergic component by naloxone suggests that an opioid peptide may be involved in the secretion of catecholamines in the rat adrenal medulla.

INTRODUCTION

The secretion of catecholamines is regulated by the activity of splanchnic nerves innervating the adrenal medullary cells. The prevalent view is that excitation of splanchnic nerve terminals causes release of acetylcholine (Feldberg, Munz & Tsudzimura, 1934), which then activates the cholinergic receptors of the chromaffin cells to evoke the secretion of catecholamines (Dale, 1914; Feldberg *et al.* 1934). The contribution of cholinergic receptors involved in the secretion of catecholamines largely depends on the species of animals used for the investigational purposes. The most widely used chromaffin cells of the bovine adrenal medulla, for example, contain nicotinic receptors that are linked to the secretory apparatus (Wilson & Kirshner, 1976, 1977). On the other hand, in the adrenal chromaffin cells of the cat (Douglas, 1975) and dog (Tsujimoto & Ashikawa, 1975) nicotinic as well as muscarinic receptors are associated with the secretion. Our recent studies on the perfused adrenal gland of the rat have demonstrated that not only nicotinic but muscarinic receptors play a prominent role in the secretion of catecholamines (Wakade & Wakade, 1983). A similar conclusion was reached by Role & Perlman (1983) regarding the participation of nicotinic and muscarinic receptors in the secretion from isolated chromaffin cells of the guinea-pig.

In addition to the above consideration of the cholinergic receptors involved in the secretion of catecholamines, recent studies have shown that a number of polypeptides exist in the adrenal glands of different species and may act as neurotransmitters and/or neuromodulators in this synaptic region. Thus, enkephalins, substance P, vasoactive intestinal polypeptide and somatostatin have been found in the adrenal gland (Schultzberg, Lundberg, Hökfelt, Terenius, Brandt, Elde & Goldstein, 1978; Lundberg, Hamberger, Schultzberg, Hökfelt, Granberg, Efendic, Terenius, Goldstein & Luff, 1979; Linnoila, Diaugustine, Hervanen & Miller, 1980; Livett & Dean, 1980; Viveros, Diliberto, Hazum & Chang, 1980; Bucsies, Soria & Lembeck, 1981; Hökfelt, Lundberg, Schultzberg & Fahrenkrug, 1981; Lemaire, Day, Dumont & Chouinard, 1984; Chaminade, Foutz & Rossier, 1984; Saito, Saito, Ohuchi, Oka, Sano & Hasoi, 1984). However, the physiological roles of these peptides in the secretion of catecholamines remains to be resolved (but see Kumakura, Karoum, Guidotti & Costa, 1980; Dean, Lemaire & Livett, 1982).

The above background obviously raises an important question as to whether, in addition to acetylcholine, some other excitatory substance(s) is released from splanchnic nerves and acts on its receptors to trigger the secretion of adrenal medullary hormones. Therefore, the purpose of the present investigation was to compare the inhibitory effects of nicotinic and muscarinic antagonists on the secretion of catecholamines evoked by either exogenous acetylcholine or stimulation of splanchnic nerves, in the hope that the secretory response to these procedures would be influenced differentially by the antagonists. It was decided to stimulate the nerves at different frequencies, using the same number of pulses, to determine if the

non-cholinergic component, if any, is expressed differently at certain frequencies of stimulation. Finally, among various blocking agents naloxone was selected as an antagonist, with the assumption that any non-cholinergic component might belong to an opioid family of polypeptides, and if released from nerve terminals its action should be blocked by this antagonist of different types of opioid receptors (Jaffe & Martin, 1985). We demonstrate here that more than 50 % of catecholamine secretion is mediated by the action of non-cholinergic substance(s) when splanchnic nerves are activated by low-frequency stimulation and that this response is reduced by naloxone.

METHODS

Perfusion of the adrenal gland

The left adrenal gland of the male rat was perfused as described previously by Wakade (1981). Briefly, rats (300–350 g) were anaesthetized with ether, the left renal vein was cannulated, and the tip of the cannula remained near the junction of renal and adrenal veins. All other blood vessels were ligated. The adrenal gland, along with the tied blood vessels and the cannula, was removed from the rat and placed on a metal plate mounted in a lucite chamber. The metal plate was made up of Ag–AgCl and served as one of the electrodes for stimulation of the gland, and another plate electrode was placed on top of the gland. The chamber was maintained at 37 °C by circulating heated water. The gland was perfused at 0·35 ml/min by means of a Sigma motor pump. The perfusion medium was Krebs bicarbonate solution of the following composition (mM): Na^+, 143·4; K^+, 5·9; Ca^{2+}, 2·5; Mg^{2+}, 1·18; Cl^-, 125·6; SO_4^{2-}, 1·2; HCO_3^-, 25; glucose, 11·7. The solution contained Na_2EDTA (10 μg/ml) to prevent oxidation of catecholamines. The perfusion medium was constantly bubbled with 95 % O_2–5 % CO_2 and the pH was 7·4 ± 0·1 (n = 32). The perfusate escaped from a slit made in the adrenal cortex and was collected in chilled tubes for the analysis of catecholamines.

Stimulation of the adrenal gland

Electrical stimulation of the adrenal gland was achieved by connecting the plate electrodes to a Grass stimulator, Model S88. Stimulation parameters were 300 shocks at different frequencies (1·0 ms duration and 130 mA strength). Agonist-evoked secretion was achieved by injecting various concentrations of acetylcholine into the perfusion stream in a bolus form.

Collection of perfusate

Prior to each stimulation with either electrical stimulation or cholinergic agonist, perfusates were collected to determine the spontaneous secretion of catecholamines. Immediately after the collection of the 'background sample,' collection of the perfusate was continued in another tube, and 15 s later the adrenal gland was stimulated electrically or chemically. The perfusate was collected for various periods of time, depending on the nature of stimulus used. However, the time period for collection of 'background sample' and 'stimulated sample' was identical for a given stimulus. The amounts secreted in the 'background sample' were subtracted from those secreted from the 'stimulated sample' to obtain net secretion of catecholamines, which is shown in all of the Figures.

To study the effects of a test agent on both spontaneous and evoked secretion the adrenal gland was perfused with Krebs solution containing the agent for about 15 min, the perfusate was collected for a specified time period ('background sample'), and then the stimulus was applied in the presence of the test agent and samples were collected for the same period as that for the 'background sample'. Other details are given in the Figure legends.

Measurement of catecholamines

The perfusate was analysed for catecholamine content directly by the fluorometric method of Anton & Sayre (1962), without the intermediate purification on alumina for the reasons described earlier (Wakade, 1981). The content of catecholamines in the perfusate was expressed in terms of adrenaline base. All data are presented as means with standard errors, and differences were compared using Student's t test.

Drugs and their sources

Tetraethylammonium bromide (Eastman Kodak Co., Rochester, NY, U.S.A.), acetylcholine bromide, mecamylamine hydrochloride, atropine sulphate and naloxone hydrochloride (Sigma Chemical Corp., St. Louis, MO, U.S.A.). Drugs were dissolved in distilled water and added to the Krebs solution as required. Acetylcholine was dissolved in saline and injected into the perfusion in a volume of 100 μl. All concentrations are expressed in terms of base.

RESULTS

Inhibition of neurally and acetylcholine-evoked secretion of catecholamines by mecamylamine and atropine

The purpose of these experiments was to compare the inhibitory effects of nicotinic and muscarinic blocking agents on the secretion of catecholamines evoked by

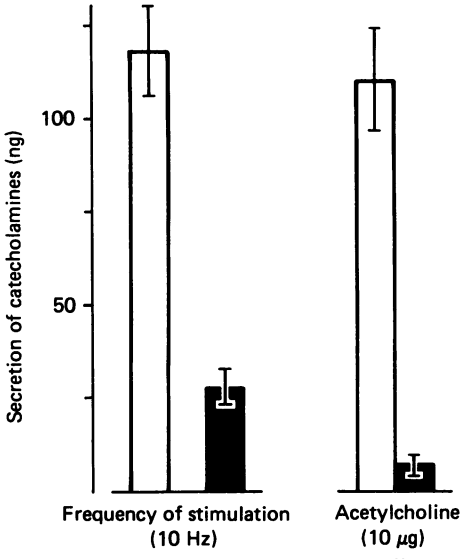

Fig. 1. Effect of mecamylamine plus atropine on neurally and acetylcholine-evoked secretion of catecholamines. Secretion was evoked by electrical stimulation (10 Hz, 300 pulses) and by injecting 10 μg acetylcholine, first in Krebs solution (open columns), and 20 min later in 0·35 mM-mecamylamine plus 0·5 μM-atropine Krebs solution (filled columns). The interval between stimulations was 20–30 min. Each perfusate was collected for 5 min. Each column represents a mean of four experiments. Vertical lines show S.E. of mean.

stimulation of splanchnic nerves and exogenous acetylcholine in the same adrenal gland. Stimulation of splanchnic nerves by delivering 300 pulses at 10 Hz secreted over 100 ng of catecholamines (Fig. 1). After perfusion of the adrenal gland with a combination of mecamylamine and atropine, the evoked secretion of catecholamines was reduced by 78%. Fig. 1 also shows that in the presence of mecamylamine and atropine, acetylcholine-evoked secretion was reduced by 93%. The difference in the degree of inhibition in the case of nerve- and acetylcholine-evoked secretion was statistically significant ($P < 0·01$). A combination of mecamylamine and atropine had no effect on the spontaneous secretion of catecholamines (data not shown).

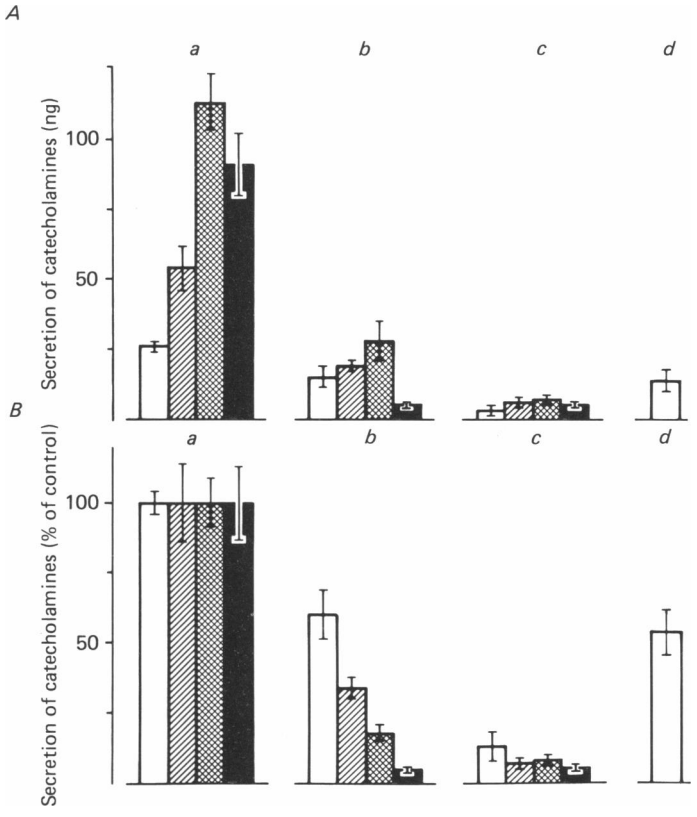

Fig. 2. Effect of cholinergic receptor antagonists and naloxone on catecholamine secretion evoked by different frequencies of stimulation and acetylcholine. *A: a*, 30 min after the beginning of the perfusion with Krebs solution, secretion was evoked in separate groups of glands by delivering 300 pulses at 0·5 Hz (open column), at 1 Hz (hatched column), at 10 Hz (cross-hatched column) and by injecting 10 μg acetylcholine (filled column). *b*, the medium was changed to 0·35 mM-mecamylamine plus 0·5 μM-atropine Krebs solution for 15 min, and then the secretion was evoked as described in *a*. *c*, 30 μM-naloxone was added to the medium used in *b*, and 15 min later secretion was evoked by electrical stimulation and acetylcholine. *d*, finally, the medium was changed to Krebs solution containing 0·35 mM-mecamylamine plus 0·5 μM-atropine for 20 min, and then the secretion was evoked at 0·5 Hz. The interval between each stimulation was 10–15 min. Perfusates were collected for 11 min (0·5 Hz), 6 min (1 Hz), and 5 min (10 Hz and acetylcholine) before and after stimulation. *B*, secretion of catecholamines evoked by each frequency of stimulation and acetylcholine in Krebs solution was taken as 100 % (*a*), and the amounts secreted subsequently by each procedure in different types of media (*b*, *c* and *d*) were represented as a percentage of those in (*a*). Each column is a mean of six experiments. Vertical lines show S.E. of mean.

Effect of mecamylamine and atropine on the secretion of catecholamines evoked at different frequencies of stimulation

Since a major difference existed between the effects of cholinergic antagonists on neurally evoked *vs.* acetylcholine-evoked secretion, it was decided to study the effects of these antagonists on catecholamine secretion evoked at different frequencies of stimulation using the fixed number of pulses. As shown in Fig. 2*Aa*, stimulation of

splanchnic nerves by delivering 300 pulses at 0·5, 1 and 10 Hz resulted in secretion of increasing amounts of catecholamines. The relationship between frequency of stimulation and secretion was consistent with our earlier report (Wakade, 1981). After stimulation with 10 μg acetylcholine, the amounts of catecholamines secreted were roughly comparable to those secreted at 10 Hz stimulation. In the presence of mecamylamine and atropine, the secretion evoked by nerve stimulation and acetylcholine was markedly depressed. However, the inhibitory effect was most pronounced in the case of injected acetylcholine.

As shown in Fig. 2*B*, if the secretion evoked by each frequency of stimulation and acetylcholine in the normal medium was taken as 100% and those amounts secreted in subsequent periods expressed as percentage of those in the control medium, then acetylcholine-evoked secretion was blocked by 95% and neurally evoked secretion was reduced by between 40 and 82%. For example, the secretion evoked by 0·5 Hz was reduced only 40% by mecamylamine and atropine. The degree of inhibition increased as the frequency of stimulation was increased. However, even at 10 Hz, which produces a maximal secretion from this preparation (Wakade, 1981), a secretion of about 20% of control persisted in the presence of cholinergic receptor antagonists. Statistical analysis revealed that the difference in the level of inhibition seen in the case of acetylcholine and different frequencies of stimulation was highly significant ($P < 0·001$).

Effect of naloxone on catecholamine secretion evoked by nerve stimulation and acetylcholine

Results shown in Fig. 2 clearly indicate that a non-cholinergic component was involved in the secretion mediated by stimulation of nerves, which was particularly obvious at lower frequencies of stimulation. Therefore, in an attempt to determine the nature of a non-cholinergic excitatory substance released from splanchnic nerves, the effects of increasing concentrations of the opioid receptor antagonist, naloxone, were investigated on catecholamine secretion evoked by acetylcholine and nerve stimulation (see Introduction). Naloxone exerted a significant inhibitory effect on neurally evoked secretion between 3 and 30 μM (Fig. 3). Acetylcholine-evoked secretion was reduced by only 15% by 30 μM-naloxone, and was not statistically different from the control ($P < 0·5$). A further increase to 300 μM-naloxone led to an additional reduction in catecholamine secretion evoked by both procedures. However, the effect of naloxone was more prominent on the secretion evoked by stimulation of splanchnic nerves. Naloxone did not increase the spontaneous secretion to any significant level, even after perfusion at a concentration of 300 μM (from 7 ± 5 ng to 12 ± 6 ng, $n = 5$).

Effect of naloxone plus mecamylamine and atropine on neurally evoked secretion of catecholamines

Since 30 μM-naloxone selectively antagonized the secretion evoked by nerve stimulation, it was decided to test the effects of this concentration of naloxone on the secretion of catecholamines evoked by various frequencies of stimulation in the presence of mecamylamine and atropine. The results of such experiments are included in Fig. 2. Secretion of catecholamines remaining after blockade of nicotinic and

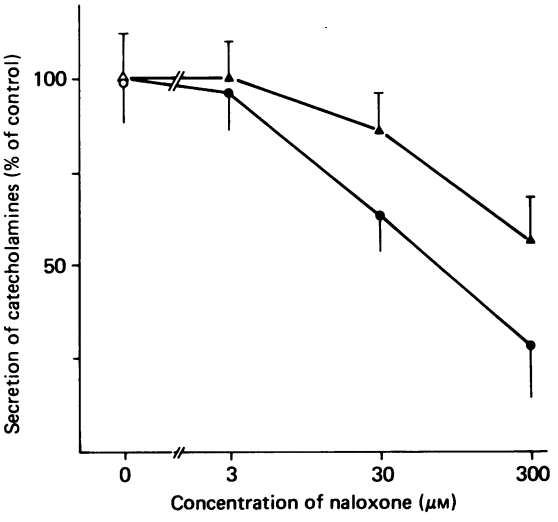

Fig. 3. Inhibition of stimulation-evoked secretion of catecholamines by naloxone. Secretion was evoked first in Krebs solution by stimulation of splanchnic nerves by delivering 300 pulses at 1 Hz (○) or by injecting 10 μg acetylcholine (△) into the perfusion stream. The perfusion medium was then changed over to increasing concentrations of naloxone, as shown. Then the secretion was evoked by electrical stimulation (●) and acetylcholine (▲). Each concentration of naloxone remained in contact with the adrenal gland for 15 min, and then in its presence samples were collected before and after stimulation. Samples were collected for 6 min. The interval between stimulations was 10–15 min. Secretion of catecholamines was expressed as a percentage of the net secretion in the Krebs solution evoked by electrical stimulation (62 ± 8 ng/6 min) and acetylcholine (80 ± 12 ng/6 min). Each point is a mean of six experiments. Vertical lines show S.E. of mean.

muscarinic receptors was practically abolished by 30 μM-naloxone. The effect of naloxone was evident at all the frequencies of nerve stimulation, and was reversible. This is the first incidence where neurally evoked secretion of catecholamines was reduced by as much as 90% in the presence of cholinergic receptor antagonists and opioid receptor antagonist.

To determine the relative potency of naloxone as an inhibitor of the non-cholinergic component of the splanchnic nerves, effects of increasing concentrations of naloxone were tested on the residual secretion of catecholamines evoked at 0·5 Hz in the presence of mecamylamine and atropine. These results are shown in Fig. 4. Secretion of catecholamines evoked at 0·5 Hz was reduced by about 30% in the presence of a combination of mecamylamine plus atropine. Increasing concentrations of naloxone, from 3 to 30 μM, inhibited the secretion in a concentration-dependent manner. Thus, 30 μM-naloxone reduced the non-cholinergic component of the secretion by almost 80%. The inhibitory effect of naloxone was reversible.

Effect of tetraethylammonium on neurally evoked secretion of catecholamines in the absence and presence of mecamylamine and atropine

Tetraethylammonium has been known to markedly facilitate the stimulation-evoked release of transmitter from sympathetic nerve terminals (Thoenen, Haefely

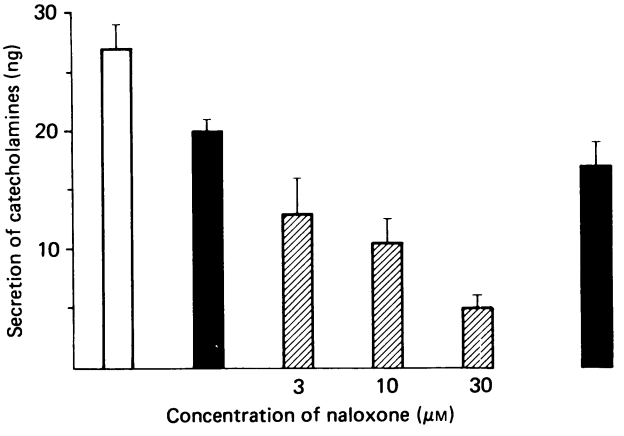

Fig. 4. Inhibition by naloxone of catecholamine secretion mediated by non-cholinergic component of splanchnic nerves. Secretion was evoked by stimulation of splanchnic nerves by delivering 300 pulses at 0·5 Hz, first in Krebs solution (open column), and 15 min later in Krebs solution containing 0·35 mM-mecamylamine and 0·5 μM-atropine (filled column). To this medium, increasing concentrations of naloxone were added, as shown (hatched columns), and finally, the secretion of catecholamines was evoked 20 min after wash-out of naloxone by Krebs solution containing mecamylamine and atropine (filled column). Each sample was collected in the presence of these antagonists for 11 min. Each column is a mean of three experiments. Vertical lines show S.E. of mean.

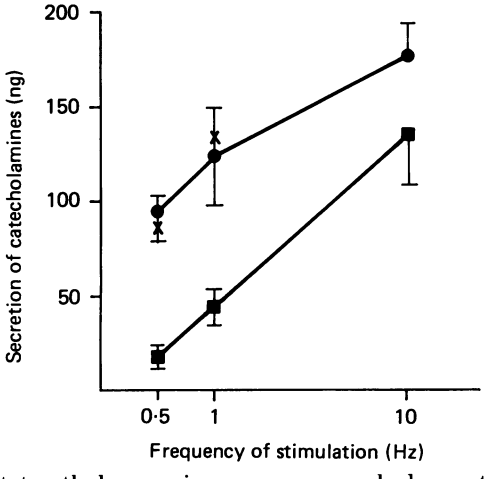

Fig. 5. Effect of tetraethylammonium on nerve-evoked secretion of catecholamines. 30 min after perfusion with Krebs solution the adrenal gland was transmurally stimulated by passing 300 pulses at the frequencies shown. Perfusates were collected for 6 min in the case of 1 and 10 Hz stimulation, and for 11 min in the case of 0·5 Hz stimulation. Interval between each stimulation period was 10 min. After obtaining secretion under control conditions, the adrenal gland was perfused with 5 mM-tetraethylammonium Krebs solution for 15 min, and in its presence the samples were collected before and after stimulation (●). In other adrenal glands, the identical protocol, as described above, was repeated, except that tetraethylammonium Krebs solution also contained 0·35 mM-mecamylamine and 0·5 μM-atropine (X). All the control values ($n = 10$) in both groups of adrenal glands have been pooled (■). Other points represent a mean of five observations. Vertical lines show S.E. of mean.

& Staehelin, 1967; Wakade & Wakade, 1982). It is suggested that excess amounts of calcium ions are made available to the secretory process during the extended duration of action potential resulting from inactivation of outward potassium current by tetraethylammonium (Kirpekar, Wakade & Prat, 1976). It was of interest to see if tetraethylammonium could facilitate the release of transmitters from splanchnic nerves and thereby enhance the secretion of catecholamines upon electrical stimulation.

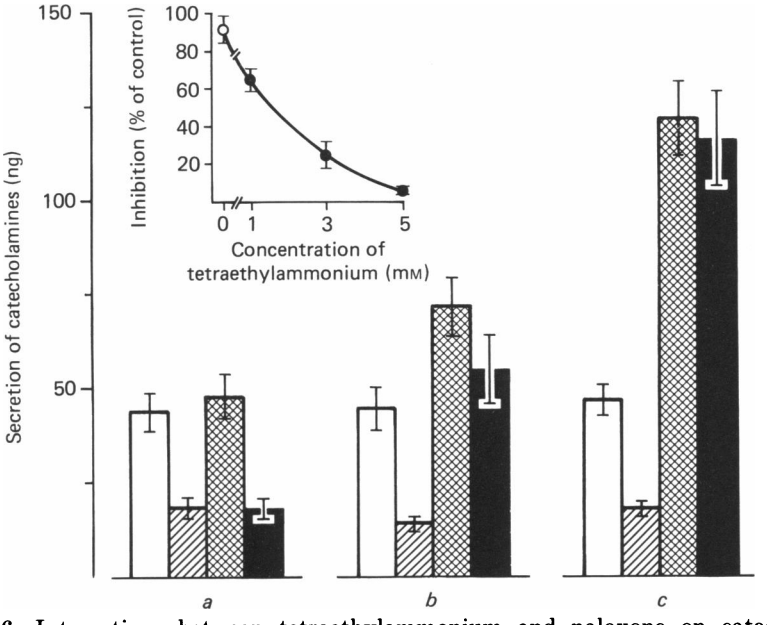

Fig. 6. Interactions between tetraethylammonium and naloxone on catecholamine secretion evoked by stimulation of splanchnic nerves in the presence of mecamylamine and atropine. *a*, secretion was evoked by delivering 300 pulses at 1 Hz in different types of media: Krebs solution (open column), 0·35 mM-mecamylamine plus 0·5 μM-atropine Krebs solution (hatched column), 0·35 mM-mecamylamine, 0·5 μM-atropine Krebs solution plus 1 mM-tetraethylammonium (cross-hatched column), and 0·35 mM-mecamylamine, 0·5 μM-atropine Krebs solution plus 30 μM-naloxone (filled column). Identical protocol was carried out in other adrenal glands, except that the concentration of tetraethylammonium was 3 mM (*b*) and 5 mM (*c*). Each type of medium remained in contact for at least 15 min and then samples were collected for 6 min in the presence of these agents. Each bar is a mean of three or four experiments. Vertical lines show S.E. of mean. Inset shows the relationship between concentration of tetraethylammonium and percentage inhibition of catecholamine secretion by naloxone. Data were obtained from Figs. 2 and 6.

As shown in Fig. 5, under normal conditions the secretion of catecholamines increased with an increase in the frequency of stimulation. In the presence of 5 mM-tetraethylammonium, the secretion evoked at 0·5 Hz increased from a control value of 18 ng to 94 ng. The facilitatory effect of tetraethylammonium was also noticeable at 1 and 10 Hz, but to a lesser extent (3- and 1·2-fold increases, respectively). Facilitation of secretion could be a result of the action of excess quantities of acetylcholine and non-cholinergic substance(s) on the chromaffin cell. Therefore, mecamylamine and atropine were included in subsequent experiments.

Fig. 5 shows that secretion of catecholamines evoked at 0·5 and 1 Hz in the presence of tetraethylammonium, mecamylamine and atropine was still comparable to that obtained after treatment with tetraethylammonium alone.

Effect of naloxone on tetraethylammonium-induced facilitation of catecholamine secretion

These results are shown in Fig. 6. As shown earlier, catecholamine secretion evoked at 1 Hz was reduced by about 60% by mecamylamine and atropine. However, the residual secretion was markedly potentiated and restored towards the normal level by addition of 1 mM-tetraethylammonium. Inclusion of naloxone into the perfusion

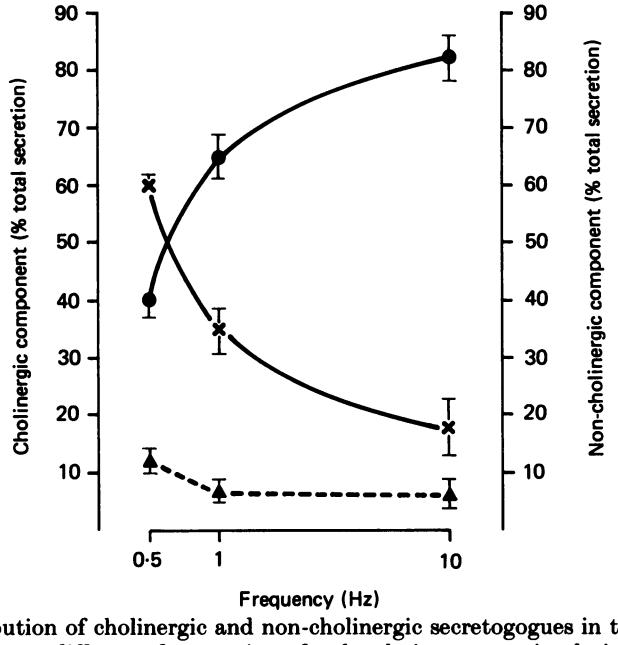

Fig. 7. Contribution of cholinergic and non-cholinergic secretogogues in the secretion of catecholamines at different frequencies of splanchnic nerve stimulation. Cholinergic component (●); non-cholinergic component (×); residual component (▲) after elimination of cholinergic and non-cholinergic components by mecamylamine, atropine and naloxone. Data obtained from Fig. 2.

medium caused over 60% inhibition of catecholamine secretion (Fig. 6*a*). If the residual secretion of catecholamines was further potentiated by addition of 3 mM-tetraethylammonium, naloxone caused only about 20% inhibition of the secretion (Fig. 6*b*), and in 5 mM-tetraethylammonium medium naloxone was ineffective (Fig. 6*c*). The inset of Fig. 6 shows the relationship between increasing concentrations of tetraethylammonium and the degree of inhibition of catecholamine secretion by naloxone. Naloxone is most effective (90% inhibition) in eliminating catecholamine secretion that remains after blockade of cholinergic receptors. However, the effect of naloxone becomes less and less as the residual component is enhanced by increasing concentrations of tetraethylammonium.

DISCUSSION

If acetylcholine was the only neurotransmitter released from splanchnic nerves, one would expect almost a complete inhibition of the secretion of catecholamines after blockade of nicotinic and muscarinic receptors. However, it was consistently found that neurally evoked secretion of catecholamines was not as effectively reduced by a combination of mecamylamine and atropine as that evoked by injected acetylcholine. Results shown in Figs. 1 and 2 indicate that some substance other than acetylcholine must be involved in the secretion of catecholamines in the rat adrenal medulla. The contribution of the non-cholinergic substance(s) towards evoking the secretion was most prominent when nerves were stimulated at low frequencies. On the other hand, the contribution of the cholinergic component was most obvious at high frequencies of stimulation. The involvement of cholinergic and non-cholinergic neurotransmitters in the secretion of catecholamines at different levels of neuronal activity is schematically shown in Fig. 7. If more than one type of neurotransmitter exists in splanchnic neurones (see below), then the release of each type seems to be controlled by the rate of impulse traffic. The physiological rate of firing of autonomic nerve fibres is around 1 Hz (Hillarp, 1960). If such a measure applies to the splanchnic nerves of the rat, it would seem that at 1 Hz there is a 65% cholinergic and 35% non-cholinergic contribution towards the secretion of catecholamines. The proportion of both components varies significantly at stimulation frequencies both above and below the physiological rate.

The present observations obviously raise a question of considerable interest regarding the storage and release of different transmitters in presynaptic neurones. We have considered two possibilities. One possibility is that each type of transmitter substance is stored in its own vesicles, and these vesicles have a different threshold for calcium ions to participate in the exocytosis. Thus, at low frequency the number of calcium ions entering splanchnic neurones per unit time would be low, and only a certain population of vesicles with high affinity for calcium ions would be released. At high rates of stimulation, other vesicles requiring higher levels of ionized calcium would enter the exocytotic process. There is some evidence that the magnitude of the entry of calcium ions is related to the frequency of stimulation (Kirpekar, Prat & Wakade, 1975). Another possibility is that presynaptic nerve fibres may contain a heterogeneous population of neurones (Hökfelt *et al.* 1981) which have different types of neurotransmitters. These neurones may have different thresholds for firing and conduction of impulses, which may account for variable participation of storage vesicles containing different transmitters at different frequencies of stimulation.

Of considerable importance was the observation that the secretion of catecholamines which remained after blockade of cholinergic receptors was reduced by the opioid receptor antagonist, naloxone (Fig. 7). Naloxone is classified as an antagonist of different types of opioid receptors and blocks the action of morphine and several other related agonists, including enkephalins (Lord, Waterfield, Hughes & Kosterlitz, 1977; Sawynok, Pinsky & LaBella, 1979; Jaffe & Martin, 1985). High-affinity opioid receptor-binding sites have been found in the adrenal medulla (Chavkin, Cox & Goldstein, 1979; Kumakura *et al.* 1980; Dumont & Lemaire, 1984). These reports raise a possibility that enkephalins may be a possible neurotransmitter of splanchnic

nerves. However, we have found that methionine-enkephalin was an extremely weak agent in evoking the secretion of catecholamines from the rat adrenal medulla (Dixon, Semafuko, Chen, Chandra & Wakade, 1985).

The secretion of catecholamines remaining after blockade of cholinergic receptors was facilitated in a concentration-dependent manner by tetraethylammonium through its secondary effect on calcium influx (see Results). Facilitation of secretion observed in the presence of tetraethylammonium, mecamylamine and atropine might be due to the action of the non-cholinergic substance released from nerve terminals in greater amounts. The effect of unknown substances was substantially antagonized by naloxone in 1 and 3 mM-tetraethylammonium; however, if the secretion was enhanced over 6-fold by 5 mM-tetraethylammonium, even high concentrations of naloxone could not antagonize the secretion. We do not know whether naloxone fails to compete with higher concentrations of unknown transmitters, or if the non-cholinergic component is made up of more than one type of transmitter for which we need another type of antagonist. Still another possibility is that tetraethylammonium has effects on both splanchnic nerve fibres and chromaffin cells, and that naloxone can only interfere with tetraethylammonium-induced facilitation which involves non-cholinergic transmitters but not chromaffin cell-based facilitation.

In conclusion, we have presented evidence that in addition to acetylcholine – a classical neurotransmitter – other substances released from splanchnic nerves also have a prominent stimulatory effect on the chromaffin cells, and these secretogogues assume an important physiological function when conduction traffic is low along the splanchnic nerves.

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