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VASOACTIVE INTESTINAL POLYPEPTIDE STIMULATES THE SECRETION OF CATECHOLAMINES FROM THE RAT ADRENAL GLAND

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SUMMARY

1. Our previous studies have indicated that splanchnic nerves release a substance(s), other than acetylcholine, that induces the secretion of catecholamines from the rat adrenal medulla. To identify the nature of the non-cholingeric substance, the effects of met-enkephalin and vasoactive intestinal polypeptide (VIP) were investigated in the perfused adrenal gland of the rat.

2. The secretion of catecholamines increased from a basal level of 8 ng to a maximum value of 18 ng during perfusion with 100 μM-met-enkephalin. The secretion evoked by 10 μg acetylcholine increased from 118 to 143 ng in the presence of 10 μM-met-enkephalin. Higher concentrations of met-enkephalin (100 μM) had no additional effect. Secretion of catecholamines evoked by stimulation of splanchnic nerves (10 Hz for 30 s) was even less (8%) affected by met-enkephalin.

3. 0·3 μM-VIP caused a significant increase in the secretion of catecholamines, and the effect increased with an increase in the concentration of VIP. About 115 ng of catecholamines were secreted during 15 min perfusion with 3 μM-VIP.

4. VIP-evoked secretion was not affected by antagonists of nicotinic and muscarinic receptors, nor by chronic splanchnicotomy. However, removal of calcium ions from, and inclusion of 1 mM-EGTA in, the perfusion medium completely inhibited the secretion evoked by VIP.

5. VIP-evoked secretion was reduced (20–75%) in a concentration-dependent manner by 3–30 μM-naloxone.

6. It is suggested that VIP may be the non-cholinergic excitatory substance present in the splanchnic nerves and released along with acetylcholine during simulation of the nerves to evoke secretion of catecholamine from the rat chromaffin cells.

INTRODUCTION

Most recently, it was demonstrated that the stimulatory effect of exogenous acetylcholine on the adrenal medulla of the rat was fully blocked by nicotinic and muscarinic receptor antagonists, but the secretion of catecholamines evoked by stimulation of splanchic nerves persisted to a significant degree even in the presence of cholinergic antagonists (Malhotra & Wakade, 1986). Therefore, it was suggested that splanchnic nerves may release an excitatory transmitter in addition to acetyl-

choline. The effect of non-cholinergic transmitter was especially prominent when splanchnic nerves were stimulated at low (0·5 Hz) rather than high frequencies (10 Hz). Furthermore, splanchnic nerve-evoked secretion of catecholamines was almost completely blocked at all frequencies of stimulation if naloxone was included in the perfusion medium along with cholinergic receptor antagonists (Malhotra & Wakade, 1987).

Different types of peptides such as enkephalins and vasoactive intestinal polypeptide (VIP) exist in the splanchnic nerves of the adrenal gland (Schulzberg, Lundberg, Hökfelt, Terenius, Brandt, Elde & Goldstein, 1978; DiGiulio, Nang, Fratta & Costa, 1979; Hökfelt, Lundberg, Schultzberg & Fahrenkrug, 1981). However, their physiological role in the secretion of medullary hormones has not been established. Therefore, we decided to study the effects of enkephalin and VIP on the secretion of catecholamines in an attempt to determine the nature of the non-cholinergic component of the splanchnic nerves involved in the secretion of catecholamines. Another reason for testing the effects of enkephalin was that naloxone – an opioid receptor antagonist – effectively reduced the secretion induced by stimulation of splanchnic nerves. Now we demonstrate that VIP is a powerful secretogogue and that its effect can be blocked by naloxone.

METHODS

Perfusion of the adrenal gland

Retrograde perfusion of the left adrenal gland of male rats (300–400 g) was carried out using Krebs–bicarbonate solution equilibrated with 95 % O_2 + 5 % CO_2; the pH was 7·4 (Wakade, 1981*a*). The solution contained disodium EDTA (30 μM) to prevent oxidation of catecholamines. Perfusate (0·35 ml/min) escaped from a slit made in the adrenal cortex and was collected in chilled tubes. Different test agents were added to the perfusion medium to investigate their effects on catecholamine secretion (Wakade & Wakade, 1984). The other details are given in the Results section. Catecholamine content (about 70 % adrenaline and 30 % noradrenaline) in the perfusate (0·2 ml) was analysed fluorometrically by the method of Anton & Sayre (1962) without using alumina to purify and concentrate the sample. For other details – stimulation, sample collection, etc. – see Wakade (1981*a*) and Malhotra & Wakade, 1987. Splanchnic nerves innervating the left adrenal gland were severed 10–15 days prior to use (splanchnicotomy). The operated animals were obtained from Taconic Farms (Germantown, NY, U.S.A.). The success of denervation was tested by transmural stimulation of the adrenal gland. As reported earlier (Wakade, 1981*b*), denervated adrenal glands did not secrete catecholamines after stimulation at 10 Hz (300 pulses). Only those glands that failed to respond to electrical stimulation were used for the present study.

The content of catecholamines in the perfusate was calculated in terms of adrenaline base and presented as means with standard errors, and differences were compared using Student's *t* test.

Drugs used

Met-enkephalin (Boehringer Mannheim Biochem, Indianapolis, IN, U.S.A.); acetylcholine bromide, vasoactive intestinal polypeptide, porcine sequence (lot 26F-00181), naloxone hydrochloride, atropine sulphate (Sigma Chemical Co., St Louis, MO, U.S.A.); tetraethylammonium bromide (Eastman Kodak Co., Rochester, NY, U.S.A.).

RESULTS

Ability of met-enkephalin to evoke or modulate catecholamine secretion

Perfusion of the adrenal gland with increasing concentrations of met-enkephalin (1–100 μM) enhanced the secretion from a control value of 8 ± 3 ng to a maximum value of 18 ± 4 ng/5 min collection period (Fig. 1). Furthermore, it was found that

the secretion evoked by 10 μg acetylcholine increased from a control value of 118 ± 16 ng/5 min to 143 ± 15 ng/5 min in 10 μM-met-enkephalin–Krebs solution. 100 μM-met-enkephalin caused no additional facilitation. The effect of met-enkephalin was even less impressive ($8 \pm 5\%$) on the splanchnic-nerve-evoked secretion of

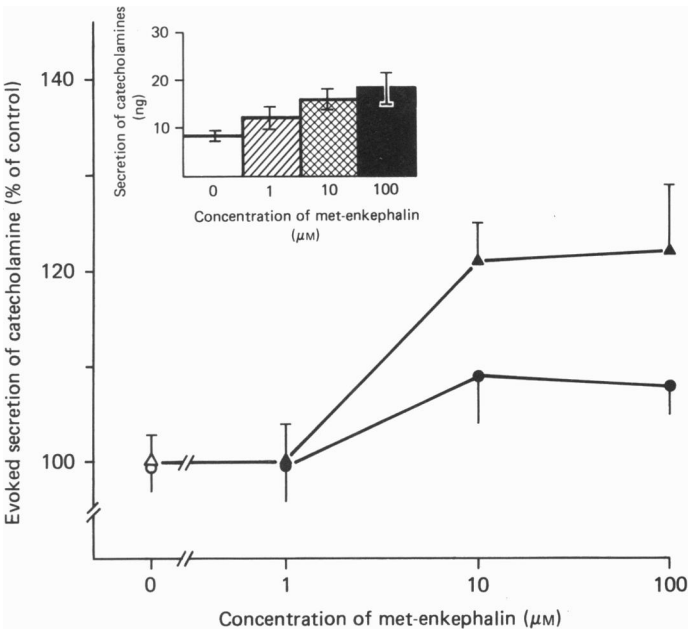

Fig. 1. Effects of met-enkephalin on catecholamine secretion. Secretion was evoked first in Krebs solution either by injecting 10 μg acetylcholine (△) or transmural stimulation at 10 Hz for 30 s (○), and then in increasing concentrations of methionine (met-enkephalin) as shown (▲ and ●). Each concentration remained in contact with the adrenal gland for 15 min and then in its presence the samples were collected before and after stimulation. The interval between stimulation by acetylcholine and electrical pulses was about 30 min. Amounts of catecholamines secreted spontaneously during the non-stimulation period have been subtracted from those during the stimulation period to obtain net secretion, which is expressed as 100% (118 ± 16 ng by acetylcholine and 152 ± 11 ng by 10 Hz). Each sample was collected for 5 min. Each symbol represents a mean of four experiments. The inset shows the effect of increasing concentrations of met-enkephalin on the secretion of catecholamines. After determining the amounts secreted spontaneously in Krebs solution (open column), increasing concentrations of met-enkephalin were added to the perfusion medium as shown, and the amounts secreted during 5 min exposure to each concentration of met-enkephalin were determined. Each column is a mean of four experiments. Vertical lines show S.E. of mean.

catecholamines, and was statistically not significant ($P > 0{\cdot}1$) at any concentration of met-enkephalin. Almost identical results were obtained with 100 μM-met-enkephalinamide (not shown).

Stimulation of catecholamine secretion by VIP

Fig. 2 shows that introduction of 0·3 μM-VIP in the perfusion medium produced a modest increase in the secretion of catecholamines. A gradual increase to 3 μM-VIP caused a concentration-dependent increase in the secretion. There was no desensit-

ization of secretion during continued perfusion with low or high concentrations of VIP. Wash-out of the polypeptide caused rapid decline in the secretion toward the basal level.

Need for calcium in VIP-evoked secretion of catecholamines

Since VIP proved to be a good stimulatory agent in the present test preparation, the next question was whether calcium was essential for the secretion. As shown in

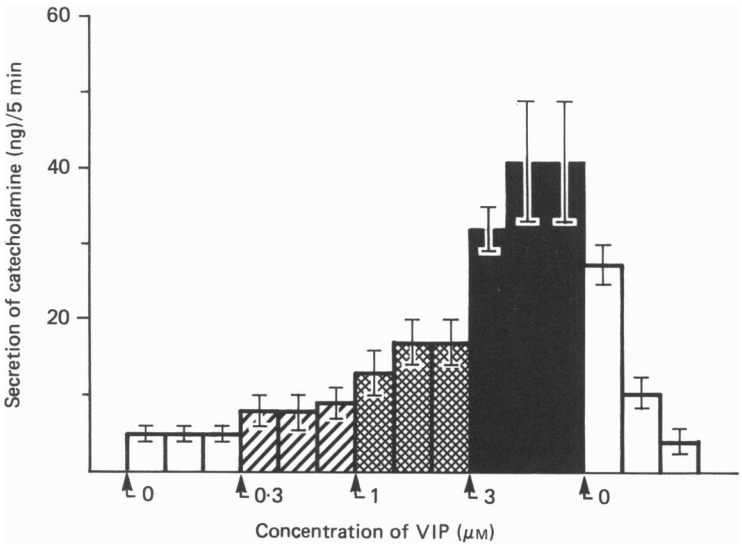

Fig. 2. Secretion of catecholamines induced by VIP. 30 min after the perfusion of the adrenal gland with Krebs solution, samples were collected to determine the spontaneous secretion of catecholamines (open columns). The perfusion medium was then switched to Krebs solution containing 0·3, 1 and 3 μM-VIP. Each concentration of VIP remained in contact with the adrenal gland for 15 min. After testing the effects of 3 μM-VIP, the medium was changed over to Krebs solution. The entire protocol was carried out in the same adrenal gland; the collection period for each sample was 5 min. Each column represents a mean of four experiments. Vertical lines show S.E. of mean.

Fig. 3, after perfusion of the adrenal gland with calcium-free Krebs solution (plus 1 mM-EGTA) for 15 min, 3 μM-VIP was unable to evoke the secretion of catecholamines. Perfusion of the same adrenal gland with 2·5 mM-calcium-containing medium restored the stimulatory effects of VIP.

Direct action of VIP on the chromaffin cells

VIP-evoked secretion could be a result of (1) a direct stimulation of chromaffin cells by activation of VIP receptors and (2) an indirect stimulation of chromaffin cells by acetylcholine or other stimulatory agent released by the primary action of VIP on splanchnic nerves. To distinguish between these possibilities, two series of experiments were carried out. In one, the nicotinic and muscarinic receptors of chromaffin cells were blocked by tetraethylammonium and atropine, respectively, and then VIP was tested for its stimulatory effect. As shown in Fig. 4*A*, the presence of nicotinic

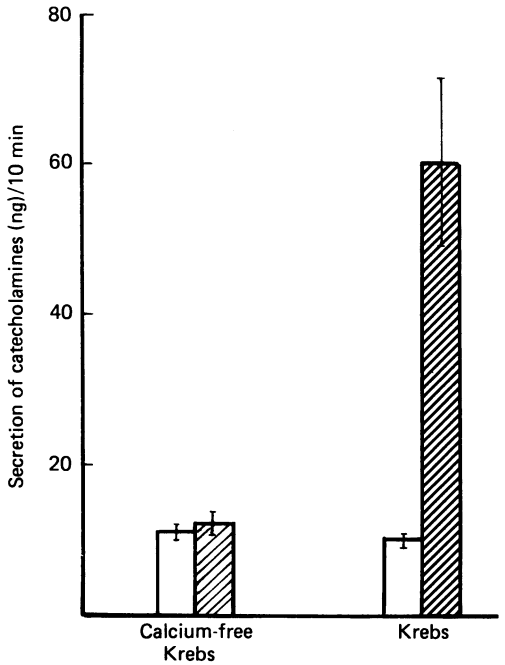

Fig. 3. Role of calcium in VIP-evoked secretion of catecholamines. The adrenal gland was perfused with calcium-free and 1 mM-EGTA–Krebs solution for 15 min. One 10 min sample was collected to determine the spontaneous secretion of catecholamines (open column), and then 3 μM-VIP was introduced in this medium for 10 min (hatched column). The perfusion medium was finally changed to Krebs solution for 15 min, and again, samples were collected before and after VIP to estimate catecholamine secretion. Each column represents a mean of three observations. Vertical lines show S.E. of mean.

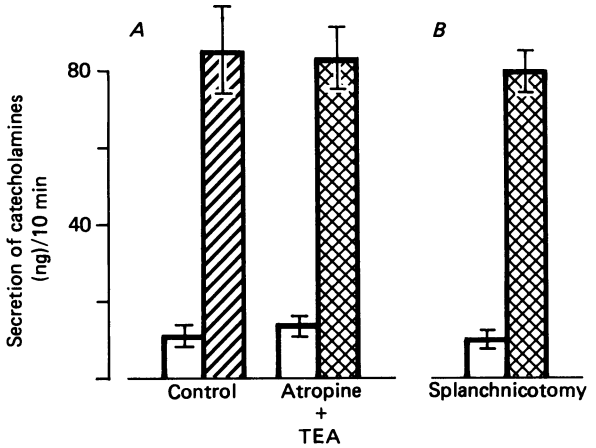

Fig. 4. Effects of blockade of cholinergic receptors and splanchnicotomy on VIP-evoked secretion of catecholamines. *A*, 30 min after perfusion of the adrenal gland with Krebs solution, samples were collected to determine the spontaneous secretion (open column) and 3 μM-VIP-evoked secretion (hatched column). The perfusion medium was then switched to Krebs solution containing 0·5 μM-atropine and 1 mM-tetraethylammonium (TEA) for 15 min, and then samples were collected in this medium as described above. *B*, the left adrenal gland was chronically denervated for 6–8 days prior to use and perfused with Krebs solution to determine the secretion before (open column) and after (hatched column) 3 μM-VIP. Each sample was collected for 10 min. Each column is a mean of four observations. Vertical lines show S.E. of mean.

and muscarinic blockers did not affect the secretion of catecholamines evoked by VIP. In another series, presynaptic splanchnic nerves were allowed to degenerate prior to the use of such denervated adrenal glands for the study. As shown in Fig. 4*B*, the stimulatory effect of VIP on the secretion of catecholamines was not reduced by degeneration of splanchnic nerve terminals.

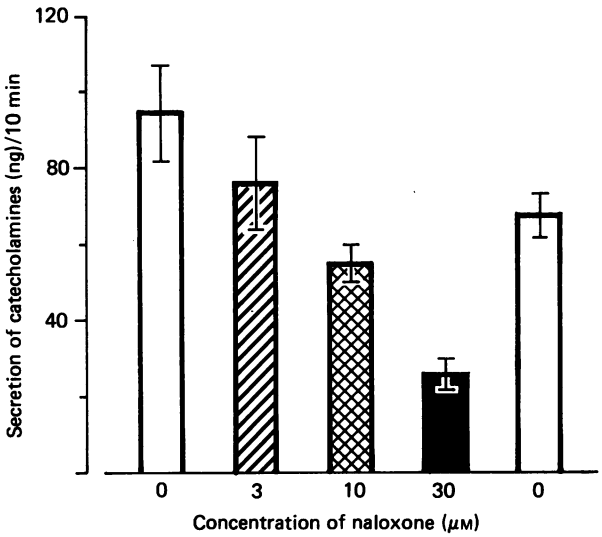

Fig. 5. Effect of naloxone on VIP-evoked secretion of catecholamines. The secretion was evoked in Krebs solution by perfusion with 3 μM-VIP, and then the medium was changed over to increasing concentrations of naloxone, as shown. Each concentration of naloxone remained in contact with the gland for 15 min, and then the samples were collected before and after VIP. Finally, the secretion was evoked in Krebs solution 30 min after wash-out of 30 μM-naloxone. The amounts of catecholamine secreted (6·4–12·2 ng/10 min) immediately prior to stimulation with VIP have been subtracted from each stimulated sample to obtain net secretion of catecholamines. Each sample was collected for 10 min. Each column is a mean of four experiments. Vertical lines show S.E. of mean.

Antagonism between VIP and naloxone

The effect of naloxone on the secretion of catecholamines evoked by 3 μM-VIP was investigated to see if some endogenous substance was involved and if the opioid receptor antagonist had any effect on VIP-evoked secretion of catecholamines in the adrenal gland. Perfusion with increasing concentrations of naloxone (3–30 μM) produced a concentration-dependent reduction in the secretion of catecholamines (Fig. 5). A significant reduction ($21 \pm 8\%$, $P < 0{\cdot}05$) occurred at 3 μM, reached nearly a 50% level at 10 μM, and was $80 \pm 7\%$ at 30 μM. In control experiments, repeated stimulation of the adrenal medulla with 3 μM-VIP at 15–20 min intervals in Krebs solution produced a modest decline in the secretion in four subsequent stimulation periods ($S_1 = 100 \pm 9\%$, $S_2 = 93 \pm 11\%$, $S_3 = 88 \pm 7\%$, and $S_4 = 79 \pm 12\%$, $n = 5$). Spontaneous secretion of catecholamines was not altered by naloxone (not shown).

The interaction between VIP and naloxone was further examined by using different concentrations of VIP before and after treatment with 20 μM-naloxone. As shown in Fig. 6, the concentration–secretion curve was shifted to the right in a parallel manner

in the presence of 20 μM-naloxone. In three control experiments the identical protocol was repeated, except naloxone was omitted from the Krebs solution to determine the reproducibility of the secretory response to the second challenge of VIP. The secretion of catecholamines evoked by 1 and 3 μM-VIP in the second period was 73 ± 11 % and 89 ± 9 %, respectively, of that obtained in the first period.

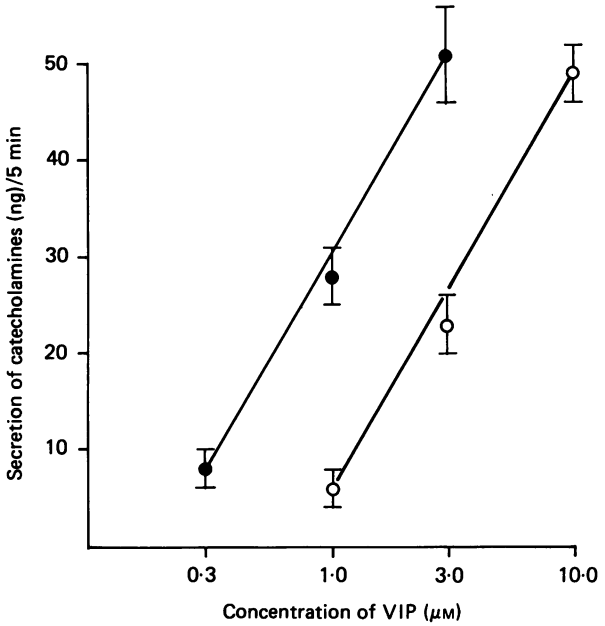

Fig. 6. The nature of antagonism between VIP and naloxone. The secretion of catecholamine was evoked, first in Krebs solution by increasing concentrations of VIP, as shown (●), without washes inbetween. After testing the last concentration of VIP, the medium was changed over to 20 μM-naloxone, and after 15 min increasing concentrations of VIP were introduced, as described above, along with naloxone (○). Each sample was collected for 5 min. The amounts of catecholamines secreted just prior to stimulation with VIP have been subtracted from each stimulated sample to obtain net secretion of catecholamines. Each circle represents a mean of four experiments. Vertical lines show S.E. of mean.

DISCUSSION

The existence, release and presence of ligand sites for opioid peptides in the adrenal medulla have led to a wide speculation that these (and other) peptides may have a physiological role in the modulation of neurohumoral secretion. In support of such a proposal, it was shown that met^5-enkephalin, leu^5-enkephalin, β-endorphin, dynorphin, met^5-enkephalin-Arg^6-Phe^7, etc., inhibited nicotine- and acetylcholine-induced secretion of catecholamines from cultured bovine chromaffin cells (Kumakura, Karoum, Guidotti & Costa, 1980; Lemaire, Livett, Tseng, Mercier & Lamaire, 1981; Dean, Lemaire & Livett, 1982; Saiani & Guidotti, 1982; Livett & Boksa, 1984). However, concentrations of peptides required to demonstrate the modulatory action ranged between micromolar and millimolar concentrations. Furthermore, the compounds did not exhibit stereospecificity and the actions of opioid peptides were poorly reversed by the opioid receptor antagonist, naloxone. Most recently, Marley,

Mitchelhill & Livett (1986) examined several endogenous opioid peptides, all having a sequence of either met^5-enkephalin or leu^5-enkephalin, for their ability to modulate nicotine-evoked secretion of catecholamines from bovine adrenal medullary cells. Since none of the peptides had a prominent action on the secretory response, they concluded that either opioid receptors of the adrenal medulla may be uniquely different from opioid receptors at other peripheral sites, or opioid peptides may not play a significant role in controlling the secretion of adrenal medullary hormones.

In the present investigation we found that very high concentrations of met-enkephalin (100 μM) were required to induce even a modest secretion of a few nanograms of catecholamines. It was also shown that met-enkephalin was a weak agent in modulating the secretion of catecholamines evoked by either stimulation of splanchnic nerves or exogenous acetylcholine. More recently, we have tested the effects of 100 μM-etorphine, which was reported to be more potent than morphine and met-enkephalin in the isolated bovine chromaffin cells (Saiani & Guidotti, 1982), and found it to be a totally ineffective inducer of secretion of catecholamines ($2{\cdot}5 \pm 1{\cdot}7$ ng/5 min *vs.* $3{\cdot}1 \pm 2{\cdot}1$ ng/5 min, $n = 7$). In view of these facts, it is unlikely that met-enkephalin plays the role of a neurotransmitter or neuromodulator in the rat adrenal medulla. It is to be noted that most of the information in the past about the involvement of opioid receptors in the secretion of catecholamines has been derived from the experiments performed on the dissociated bovine chromaffin cells maintained in culture, whereas the present data are obtained from an intact adrenal medulla perfused with a physiological salt solution.

In contrast to the effects of met-enkephalin, VIP was a very potent evoker of the secretion of catecholamines in a calcium-dependent manner. Of particular importance was the fact that VIP exerted its stimulatory effect in the presence of nicotinic and muscarinic receptor antagonists. This observation suggests that the polypeptide acts on receptors that are distinct from the cholinergic receptors, and that the effect is not mediated indirectly by stimulation of presynaptic cholinergic nerve terminals to liberate acetylcholine. A possibility of release of some other excitatory substance by VIP from splanchnic nerve terminals was also eliminated by demonstrating the unaltered response to VIP in chronically denervated adrenal medulla. Therefore, it is clear that the stimulatory effect of VIP on chromaffin cells does not depend on the intact innervation of the adrenal medulla or cholinergic receptors.

The distribution of VIP is widespread (Fahrenkrug, 1979). VIP and acetylcholine coexist in central and peripheral neurones (Said & Rosenberg, 1976; Giachetti, Said, Reynolds & Koniges, 1977; Anggard, Lundberg, Hökfelt, Nilsson, Fahrenkrug & Said, 1979; Lundberg, Anggard, Emson, Fahrenkrug & Hökfelt, 1981; Kobayashi, Kyoshima, Olschowka & Jacobowitz, 1983). Most importantly, VIP-containing neurones have been demonstrated in the rat adrenal medullar (Hökfelt *et al.* 1981). In consideration of this information, our previous report (Malhotra & Wakade, 1987) and the current findings, we would like to propose that VIP may be released along with acetylcholine as a co-transmitter from splanchnic nerves to evoke the secretion of catecholamines from the chromaffin cells. Earlier, Ip, Ho & Zigmond (1982) suggested that VIP and secretin are the likely candidates for the non-cholinergic transmission in the superior cervical ganglia of the rat. Additional experiments to determine VIP content, release, and blockade of its stimulatory actions on chromaffin

cells by specific VIP antagonists and VIP antisera are in progress to establish VIP's role as a neurotransmitter in the rat adrenal gland.

One of the unexpected findings in the present study was that the stimulatory effects of VIP on catecholamine secretion were blocked in a concentration-dependent fashion by naloxone, an opioid-receptor antagonist. How does naloxone block the effect of VIP? One possibility is that VIP releases another agent from the adrenal gland which activates naloxone-sensitive receptors of the chromaffin cells to induce the secretion of catecholamines. Since the secretory response remains unaltered after denervation, the most likely site of action for VIP to release an intermediate substance would be the chromaffin cells. Another possibility is that the high concentrations of naloxone used in the present study can directly affect the function of VIP receptor in a non-specific manner to suppress the secretion. High concentrations of naloxone are known to interfere with the function of non-opioid receptors (Dingledine, Iversen & Breuker, 1978; Dean *et al.* 1982; Malhotra & Wakade, 1987).

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