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THE PEPTIDE VIP IS A NEUROTRANSMITTER IN RAT ADRENAL MEDULLA: PHYSIOLOGICAL ROLE IN CONTROLLING CATECHOLAMINE SECRETION

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SUMMARY

1. The perfused adrenal gland of the rat was used to establish the identity of a non-cholinergic substance involved in splanchnic nerve-mediated secretion of catecholamines.

2. The perfused adrenal medulla was rich in vasoactive intestinal polypeptide (VIP) content (28 pmol g^{-1} of wet tissue). VIP-immunoreactive nerve fibres were present in the adrenal medulla and the adrenal cortex.

3. Field stimulation (10 Hz for 15 min plus 1 Hz for 15 min) caused a large increase in the output of VIP in the perfusate over the spontaneous release of VIP. Secretion of catecholamines was also greatly elevated by field stimulation. Field stimulation-evoked output of VIP and catecholamines was abolished after chronic denervation of the adrenal glands.

4. Infusion of acetylcholine (ACh) did not increase the output of VIP but caused a robust secretion of catecholamines.

5. The VIP output declined when the stimulation frequency was increased ($8{\cdot}6 \times 10^{-3}$ fmol $pulse^{-1}$ at 1 Hz and $4{\cdot}0 \times 10^{-3}$ fmol $pulse^{-1}$ at 10 Hz).

6. In contrast, the output of ^{3}H-acetylcholine (^{3}H-ACh, expressed as a fraction of tissue ^{3}H-ACh content) increased from $7{\cdot}0 \times 10^{-2}$ $pulse^{-1}$ at 1 Hz to $16{\cdot}3 \times 10^{-2}$ $pulse^{-1}$ at 10 Hz.

7. Secretion of catecholamines evoked by low-frequency stimulation (1 Hz) was reduced by 40% in the presence of cholinergic receptor antagonists (atropine plus hexamethonium). Inclusion of a VIP receptor antagonist ([Ac-Tyr^{1}, D-Phe^{2}]-GRF 1-29 amide) caused about 75% inhibition.

8. The VIP receptor antagonist inhibited VIP-evoked secretion of catecholamines without affecting ACh-evoked secretion.

9. In conclusion, VIP satisfies all the essential criteria to assume the role of a neurotransmitter in the rat adrenal medulla. The contribution of VIP to the secretion of adrenal medullary hormones is more prominent at low rates of neuronal activity whereas ACh is the major contributor at higher activity.

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INTRODUCTION

Perfused adrenal gland of the rat has become a very useful preparation to study the mechanism of catecholamine secretion by stimulation of splanchnic nerves that innervate the chromaffin cell (Wakade, 1981*a*). A significant finding reported in recent years was that splanchnic nerve-mediated secretion was not as effectively blocked by cholinergic receptor antagonists as that evoked by exogenous acetylcholine (ACh). Furthermore, the secretion evoked by a low-frequency stimulation was only partially depressed by cholinergic receptor blocking agents but that evoked at high frequency was much more sensitive to these antagonists (Malhotra & Wakade, 1987*a*). Extension of this work showed that the secretion of catecholamines was fairly well maintained for six hours of continuous stimulation of splanchnic nerves at a low frequency and was unaffected by cholinergic receptor antagonists (Wakade, 1988). These findings provided clear indication that secretion of catecholamines from the rat adrenal gland must be under the control of multiple presynaptic neurotransmitters.

To identify these, we have tested several neuropeptides and other putative neurotransmitter substances that have been reported by others to be localized in splanchnic nerves of the adrenal gland (Marley & Livett, 1985). Enkephalin analogues proved disappointing because of their very weak effects on catecholamine secretion. In contrast, vasoactive intestinal polypeptide (VIP) exerted a significant stimulatory effect on the rat adrenal medulla (Malhotra & Wakade, 1987*b*). These results provided the first clue that VIP may be a potential candidate for the non-cholinergic transmitter in the rat adrenal gland. In the rat superior cervical ganglia Ip, Perlman & Zigmond (1983) and Ip & Zigmond (1984) found that postsynaptic events triggered by stimulation of presynaptic nerves were only partially blocked by cholinergic receptor antagonists. Secretin or VIP was considered to be a likely non-cholinergic neurotransmitter in the superior cervical ganglia. Neuronal cell bodies and terminals containing VIP-like immunoreactivity were detected by Hokfelt, Lundberg, Schultzberg & Fahrenkrug (1981) and Holzwarth (1984).

In the present investigation we have followed these leads and now show that VIP-like immunoreactive nerve fibres are present in the perfused adrenal medulla and most importantly, VIP is released in the perfusate after splanchnic nerve stimulation. These results, taken together with our previous work, satisfy essentially all the criteria for VIP to assume a role of a neurotransmitter in the rat adrenal gland. Results of the present investigation were published in an abstract form (Malhotra, Blank, Wakade & Wakade, 1989; Wakade, 1990).

METHODS

Retrograde perfusion of adrenal gland

Male rats (300–400 g) were anaesthetized by ether inhalation and maintained on the anaesthetic throughout the operation. The left adrenal gland was surgically prepared for retrograde perfusion at 0·35 ml min^{-1} with Krebs bicarbonate solution, as described previously by Wakade (1981*a*). The composition of the solution was as follows (mM): NaCl, 119; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4$, 1·2; glucose, 11; and $NaHCO_3$, 25. In addition, the solution contained 0·027 mM-Na_2EDTA to prevent oxidation of catecholamines. The solutions were constantly bubbled with 95% O_2 plus 5% CO_2 to maintain the pH at 7·4. The perfusate escaped from a slit made in the adrenal capsule and was collected in chilled tubes for the analysis of catecholamines.

Stimulation of the adrenal gland

The adrenal gland was stimulated electrically by connecting plate electrodes to a Grass stimulator (Model S88). Stimulation parameters were 1·0 ms duration and 100 mA strength, with varying number of pulses at different frequencies. Field stimulation selectively activates splanchnic nerves but not the chromaffin cells (Wakade, 1981*a*, *b*). Agonist-evoked secretion was achieved by adding the agent to the perfusion medium or injecting directly into the perfusion stream.

Collection of perfusate

Prior to electrical stimulation or introduction of a stimulatory agent, perfusates were collected for 5 min 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. Total collection time was 5 min. The amounts of catecholamines secreted in the 'background sample' were subtracted from those in the 'stimulated sample' to obtain the net secretion of catecholamines.

Analysis of catecholamine content

The perfusate was analysed for catecholamine content by the fluorometric method of Anton & Sayre (1962) without the intermediate purification on alumina for the reasons described earlier (Wakade, 1981*a*). Catecholamine content in the perfusate was expressed in terms of adrenaline base.

Analysis of choline acetyltransferase and proteins

Rat adrenal medullae were extracted in 0·5 ml of ice-cold Tris-acetate buffer, pH 6·0, with 0·1% Triton X-100 and homogenized. The homogenate was centrifuged at 10000 *g* for 20 min at 4 °C and the supernatant was used for the estimation of proteins and choline acetyltransferase (ChAT) activity (Fonnum, 1976). The enzyme activity was expressed as picomoles of ACh formed per minute per milligram of protein. Proteins were estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

Analysis of VIP

Normal or chronically denervated adrenal gland was perfused, as described above, and perfusates were collected under different experimental conditions. For example, perfusates were collected in the absence of stimulation, after field stimulation (10 Hz for 15 min followed by 1 Hz for 15 min) or after perfusion with 2 μM-ACh–Krebs solution. The duration of collection of each perfusate was 30 min. Perfusates were collected in tubes containing aprotinin (2000 KIU (ml perfusate)$^{-1}$). An aliquot (0·2 ml) of the perfusate was immediately taken for catecholamine estimation and the remaining perfusate was lyophilized and subsequently reconstituted in phosphate buffer for estimation of VIP concentration by radioimmunoassay (Mitchell & Bloom, 1978). Briefly, the antiserum (kindly donated by Professor S. R. Bloom) was raised in a New Zealand White rabbit against natural porcine VIP. Radio-iodinated VIP was prepared by chloramine T oxidation of synthetic porcine VIP and purified by ion exchange chromatography. The assay was able to detect 0·5 fmol tube^{-1} with 95% confidence.

To measure the tissue VIP content, the medulla was separated from the adrenal cortex and placed in boiling 0·5 M-acetic acid (100 μl). Aliquots of the extract were then assayed in duplicate for VIP, as described above.

Immunohistochemistry of the adrenal gland

The adrenal glands were perfused with 4% formalin in 0·1 M-phosphate buffer and fixed overnight in the same fixative. After buffer wash, tissue was immersed in 30% sucrose overnight, embedded in 30% gelatin and sectioned with a cryostat into 20–40 μm sections. Sections were mounted on subbed slides. Tissue was dehydrated through graded concentrations of alcohol and incubated for 20 min in 100% methanol in 0·1% H_2O_2 to quench endogenous peroxidase. Sections were then rehydrated with Tris saline and incubated for 1 h at room temperature in Tris saline containing bovine serum albumin (BSA). Tissue was incubated in rabbit anti-VIP serum (Amersham) at a 1:500 dilution in Tris saline–BSA overnight at 4 °C. Slides were then washed in

Tris saline and treated with 1:100 goat anti-rabbit IgG in Tris saline–BSA for 1 h at room temperature. After another Tris saline wash, tissue was incubated in 1:250 rabbit peroxidase antiperoxidase for 1 h. Reaction product was visualized following treatment with 0·05% diaminobenzidine in the presence of 0·015% H_2O_2. After repeated Tris saline washes, sections were dehydrated and mounted with Depex.

Output of ^{3}H-ACh

A complete and more detailed account of the method used to study the output of ^{3}H-ACh from the rat adrenal gland is in preparation. Briefly, the adrenal glands were perfused with Krebs solution containing ^{3}H-choline chloride (50 μCi (5 ml)$^{-1}$, specific activity 88·7 Ci mmol^{-1}, New England Nuclear Corporation). The solution was recirculated to economize on the radioactive material. During a 45 min recirculation period the gland was electrically stimulated (3 Hz for 5 min) five times at 5 min intervals to improve the loading of the tissue with ^{3}H-choline. The perfusion medium was then switched to unlabelled choline chloride (10 μg ml^{-1})–Krebs solution for 30 min, followed by regular Krebs solution for 10 min. Perfusates were collected every 5 min and when the effluent radioactivity levelled off the gland was stimulated at 10 Hz for 30 s and the perfusate was collected for 5 min. After a 10 min rest the collection of sample was continued for 6 min; the gland was stimulated at 1 Hz for 5 min and the perfusate was collected for a further 6 min. This process was repeated in the same gland.

The perfusates were then treated with choline oxidase to separate ^{3}H-ACh from ^{3}H-choline, as described by Cooper (1988). The radioactivity (^{3}H-ACh) was counted in a liquid scintillation counter and expressed as a fraction of the total tissue ^{3}H-ACh content at the time of release.

To measure the ^{3}H-ACh content of the adrenal medulla the tissue was separated from the cortex at the end of the experiment and homogenized in 0·05 N-perchloric acid (0·5 ml). The homogenate was added to 1·5 ml Krebs solution, centrifuged and the supernatant was subsequently treated with choline oxidase for 30 min (see above) to measure ^{3}H-ACh.

Statistics

All the data were presented as means with standard errors and differences were compared using Student's *t* test.

Drugs and their sources

The following drugs were used in these experiments: atropine sulphate, hexamethonium bromide and acetylcholine chloride (Sigma Chemical Corp, St Louis, MO, USA); VIP and VIP antagonist [Ac-Tyr^1, D-Phe^2]-GRF 1-29 amide (Penninsula Laboratories, Belmont, CA, USA).

RESULTS

Content and localization of VIP in adrenal medulla

The average content of VIP of the left perfused adrenal medulla was $28·5 \pm 7·0$ pmol (g wet tissue)$^{-1}$ ($n = 7$). Immunohistofluorescence studies were carried out to localize the site of VIP storage in the adrenal gland. The results are shown in Fig. 1. VIP-immunoreactive nerve fibres were visible over a diffuse background fluorescence of medullary tissue. Most fluorescent fibres were visible around the chromaffin cells. Occasionally we observed VIP-immunoreactive fibres in the cortical region of the adrenal gland. However, unlike previous reports (Hokfelt *et al.* 1981; Holzwarth, 1984) we did not find VIP-like immunoreactive neuronal cell bodies in our sections. Although not shown, cat adrenal medulla also contained a rich neuronal network of VIP-like immunoreactive substance.

We determined the activity of a cholinergic marker enzyme, choline acetyltransferase. The average activity was 182 ± 28 pmol ACh (g protein)$^{-1}$ min^{-1} ($n = 5$). Three days after ablation of splanchnic nerve fibres (Wakade, 1981*b*) the enzyme activity was reduced by 98 ± 7% ($n = 4$).

Output of VIP and catecholamines from normal adrenals

Perfusates of adrenal glands collected under different experimental conditions were first analysed for catecholamines, then lyophilized and subsequently reconstituted in phosphate buffer for measurement of VIP. The results are shown in Fig. 2. The amount of VIP found in the perfusates of unstimulated adrenal glands was near the detection limit of the assay. Field stimulation (10 Hz for 15 min plus 1 Hz for 15 min) caused a significant increase in the output of VIP compared to the output from unstimulated glands ($P < 0{\cdot}001$) (Fig. 2*A*). In each experiment the stimulation-evoked release was at least 2–3 times greater than that observed in a non-stimulated sample collected from the same adrenal preparation. As expected, field stimulation produced a massive secretion of catecholamines (Fig. 2*B*).

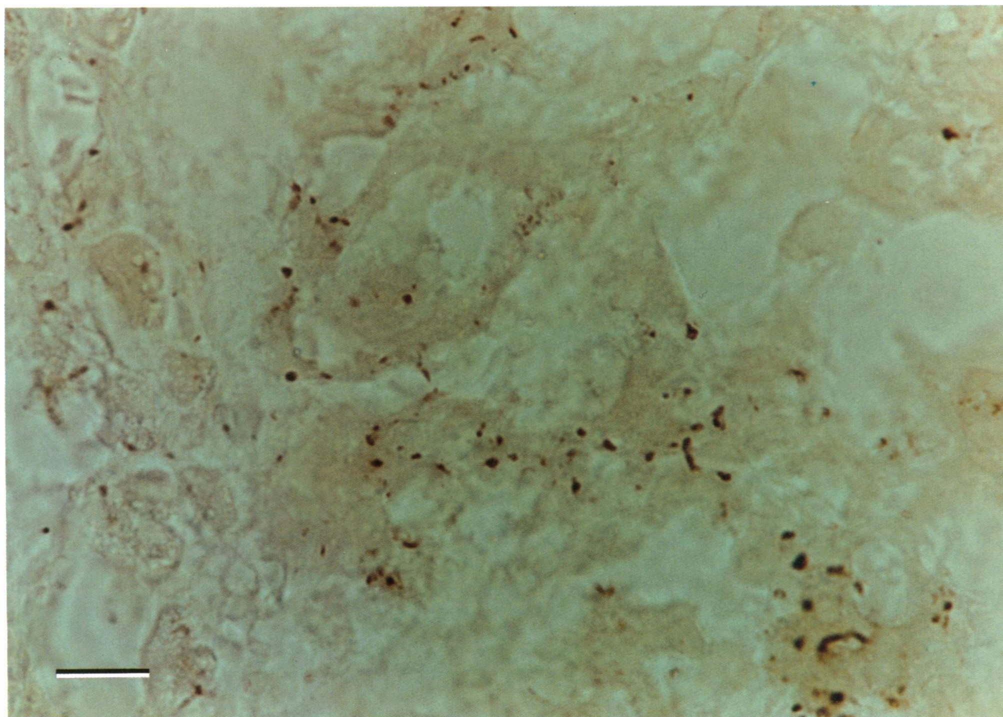

Fig. 1. Photomicrograph showing VIP-like immunoreactive nerve fibres. Several nerve fibres containing dense brown spots are visible over a diffuse background in the adrenal medullary region. A few immunoreactive fibres are also visible in the extreme left portion of the photograph, which represents the adrenal cortical region. Scale bar is 50 μm.

Figure 2*A* and *B* also shows that stimulation of the adrenal gland by 2 μM-ACh Krebs solution for 30 min did not increase the output of VIP but the secretion of catecholamines was greatly enhanced. In fact, catecholamine secretion by ACh was almost identical to that evoked by field stimulation.

Output of VIP and catecholamines from denervated adrenals

Our previous work has repeatedly shown that the parameters used for field stimulation preferentially excite splanchnic nerve terminals (or sympathetic nerve terminals) but not the chromaffin cells of the adrenal gland (Wakade, 1981*a*, *b*;

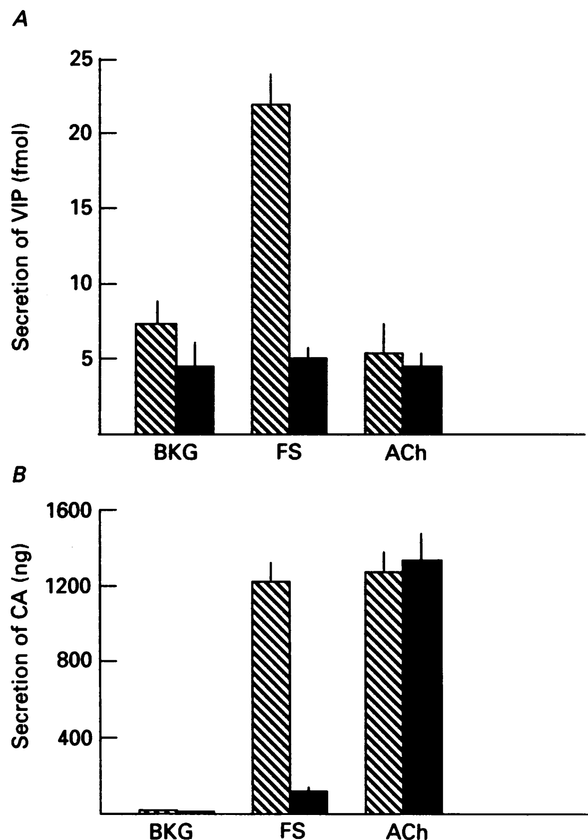

Fig. 2. Enhanced output of VIP by field stimulation. The perfusates were collected in the absence of stimulation (background, BKG), 30 min of field stimulation (FS) (10 Hz for 15 min followed by 1 Hz for 15 min) and 30 min of perfusion with 2 μM-ACh–Krebs solution (ACh) from normal (hatched columns) and chronically denervated (filled columns) adrenal glands. Perfusates were assayed for VIP (*A*) and catecholamine (*B*; CA) contents, as described in Methods. Each column represents a mean of five values and vertical lines show S.E.M.

Wakade, Malhotra, Wakade & Dixon, 1986). To assess the origin of VIP release by field stimulation we continued the release experiments in adrenal glands whose splanchnic neuronal supply was ablated 3 days prior to the experiments. As shown in Fig. 2, a field stimulation did not enhance the output of VIP over that seen in unstimulated adrenal perfusate. There was also a marked reduction in the secretion of catecholamines by field stimulation from denervated adrenals. However, direct

stimulation of chromaffin cells by exogenous ACh produced normal amounts of catecholamine secretion from the denervated adrenal glands (Fig. 2*B*).

Relationship between stimulation frequency and output of VIP and ^{3}H-ACh

A relationship between frequency of stimulation and the output of VIP and ^{3}H-ACh was investigated in two groups of adrenal glands. The results are shown in Fig.

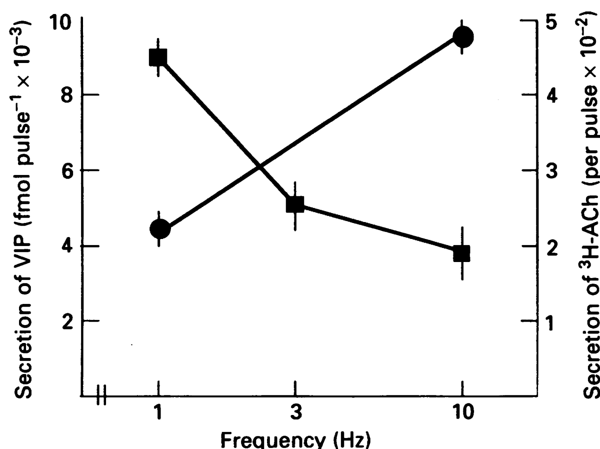

Fig. 3. Frequency of stimulation and output of VIP and ^{3}H-ACh. In one group, the perfused adrenals were stimulated by delivering 3600 shocks at 1 Hz and 7200 shocks at 3 Hz and 10 Hz at 15 min intervals. The process was repeated by reversing the order of frequencies (10, 3 and 1 Hz) in the same adrenals. Perfusates collected during each frequency (1 h for 1 Hz, 40 min for 3 Hz and 12 min for 10 Hz) were pooled, lyophilized and subsequently analysed for VIP. The output of VIP was expressed as femtomoles per pulse (■). In the other group, the adrenal glands were labelled with ^{3}H-choline and subsequently used to measure the output of ^{3}H-ACh. 300 pulses were delivered at 1 Hz and 10 Hz at 15 min intervals. Perfusates were collected for 6 min. Prior to stimulation, background perfusates were also collected for 6 min. All samples were processed for ^{3}H-ACh content and the amounts released in the background samples were subtracted from those in corresponding stimulated samples to obtain the net output of ^{3}H-ACh at each pulse (●) expressed as a fraction of the total tissue ^{3}H-ACh content at time of release. Each point is a mean of five observations. Vertical lines show S.E.M.

3. VIP output decreased as the frequency of stimulation increased from 1 Hz to 10 Hz. Although different number of pulses were given at 1, 3 and 10 Hz for practical reasons, the release was expressed as femtomoles per pulse at each frequency. It should be stressed here that stimulation frequencies were delivered in ascending and descending order in each gland and the amounts released by each frequency were averaged.

The output of ^{3}H-ACh increased when the stimulation frequency was increased from 1 Hz to 10 Hz. Three hundred pulses were delivered at both frequencies. Here again the stimulations were applied in ascending and descending order and values in each experiment were averaged for each frequency.

Effects of VIP antagonist on VIP and ACh-evoked catecholamine secretion

Recently we have demonstrated that VIP-mediated hydrolysis of polyphosphoinositides was partially antagonized by a VIP antagonist (Malhotra, Wakade & Wakade, 1988). Therefore, it was of interest to see if the same antagonist could

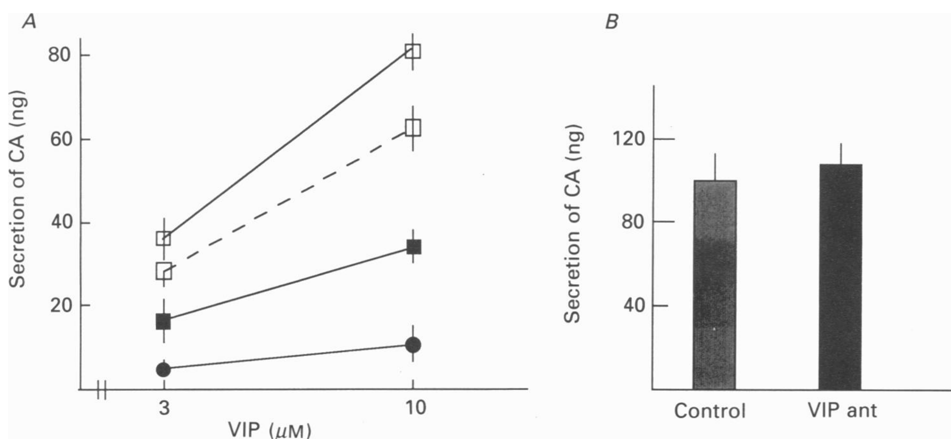

Fig. 4. Effects of VIP antagonist on VIP-evoked and ACh-evoked secretion of catecholamines. *A*, the secretion was evoked by injecting two concentrations of VIP, as shown, first in Krebs solution (□——□), in the presence of 3 μM (■) or 10 μM-VIP antagonist (●) and 30 min after wash-out of the VIP antagonist (□----□). *B*, the effect of VIP antagonist (10 μM) on catecholamine secretion evoked by ACh (2 μM). The gland was perfused with the antagonist for 15 min prior to stimulation with VIP or ACh. Each perfusate was collected for 15 min. Each value is a mean of six experiments. Vertical lines represent S.E.M.

interfere with the stimulatory effects of VIP on catecholamine secretion. The stimulatory effect of VIP was depressed in a concentration-dependent manner by the VIP antagonist (Fig. 4*A*). Ten micromolar VIP antagonist caused over 80% inhibition of catecholamine secretion. The inhibitory effect was partially reversible after wash-out of the VIP antagonist. Figure 4*B* also shows that ACh-evoked secretion of catecholamines was unaffected by 10 μM-VIP antagonist, demonstrating the selectivity of the VIP antagonist, at least in the concentrations used in our study. The VIP antagonist did not enhance the spontaneous secretion of catecholamines (not shown).

Effect of VIP antagonist on secretion of catecholamines evoked by field stimulation

One of the consistent findings we noted was that secretion of catecholamines, evoked at low frequency (0·5–1 Hz), was less susceptible to the blocking actions of cholinergic receptor antagonists than that at higher frequencies (10 Hz) (Malhotra & Wakade, 1987*a*; Wakade, 1988). If VIP is the non-cholinergic transmitter responsible for mediation of secretion of catecholamines then one would anticipate a

reduction in the secretion by VIP antagonist at low-frequency stimulation. Results shown in Fig. 5 confirm such a prediction. A combination of muscarinic and nicotinic receptor antagonists (atropine and hexamethonium) produced only a partial (about 40%) blockade of catecholamine secretion by 1 Hz stimulation. After inclusion of

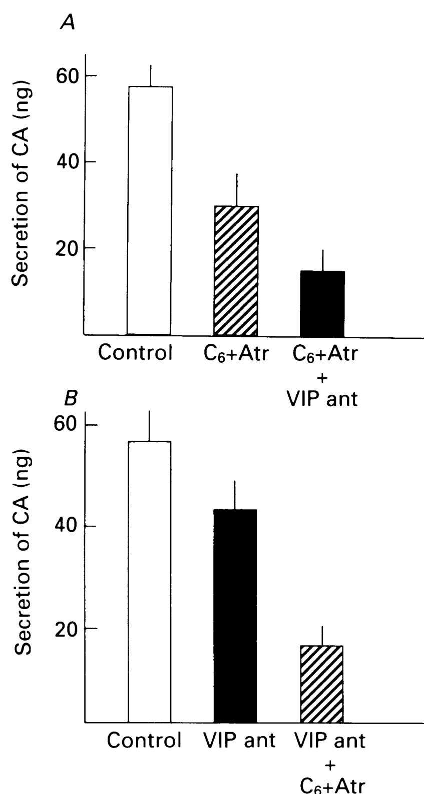

Fig. 5. Effect of VIP antagonist on catecholamine secretion evoked by field stimulation. *A*, the secretion was evoked by stimulation of splanchnic nerves at 1 Hz for 5 min in Krebs solution (Control), hexamethonium (50 μM, C_6) plus atropine (0·5 μM, Atr), and C_6+Atr and VIP antagonist (10 μM, VIP ant). *B*, in the other group, the secretion was evoked by stimulation at 1 Hz for 5 min in Krebs solution (Control), VIP antagonist, and VIP antagonist plus C_6+Atr. Each treatment was for 15 min prior to collection of samples. Perfusates were collected for 6 min. Each column is a mean of four observations. Vertical lines show S.E.M.

VIP-antagonist to this medium the stimulation-evoked secretion was reduced by almost 75% (Fig. 5*A*). In another series we reversed the order of antagonists added to the perfusion medium. As shown in Fig. 5*B*, neurally-evoked secretion (1 Hz) was reduced by about 30% ($P < 0·01$) by the VIP antagonist alone. Greater reduction of secretion (75%) was observed when atropine and hexamethonium were included together with the VIP antagonist. It is to be noted that a combination of cholinergic receptor antagonists caused 95% inhibition of catecholamine secretion induced by exogenous ACh (Malhotra & Wakade, 1987*a*).

DISCUSSION

The most noteworthy observation of the present investigation was the detection of significant amounts of VIP in the perfusate and its dramatic increase upon stimulation of splanchnic nerves of the isolated rat adrenal gland. We believe this finding, along with the previous work performed on similar preparations, provides the crucial piece of evidence for VIP as a neurotransmitter in the adrenal medullary synapse. The five major criteria satisfied by VIP are as follows.

VIP-like immunoreactive nerve fibres have been demonstrated in the adrenal medullary synapse (Hokfelt *et al.* 1981; Holzwarth, 1984; Maubert, Tramu, Croise, Beauwillain & Dupouy, 1990; Yoshikawa, Saito, Sano, Ohuchi, Ishimura, Morita, Saito & Oka, 1990). In this study we have provided additional support for the existence of VIP-containing nerve fibres and have quantified the amount of VIP-like immunoreactive material in the rat adrenal medulla.

Secondly, VIP-like immunoreactive material was detected in the perfusate and the output increased upon excitation of the neuronal endings in the adrenal medulla. This piece of direct evidence constitutes the major strength for the earlier contention that VIP could serve as a neurotransmitter in the rat adrenal medulla (Malhotra & Wakade, 1987*b*; Malhotra, Wakade & Wakade, 1989). Bloom, Edwards & Jones (1988) have also detected significant amounts of VIP in the adrenal effluents of the conscious calf after stimulation of splanchnic nerve fibres. Most recently Yoshikawa *et al.* (1990) found VIP-like immunoreactive material in the perfusate after infusion of excess K^+ in the bovine adrenal gland.

Thirdly, VIP exerts a direct stimulatory effect on the chromaffin cells of the rat adrenal medulla to evoke the secretion of catecholamines (Malhotra & Wakade, 1987*b*; Malhotra *et al.* 1989). Morphological evidence shows that VIP-mediated secretion occurs by exocytosis (Carmichael, Brooks, Malhotra, Wakade & Wakade, 1989). The stimulatory effect of VIP is not restricted to the rat adrenal medulla but chromaffin cells of several other species also respond to VIP by secreting catecholamines (Cheung & Holzwarth, 1986; Misbahuddin, Oka, Nakanishi & Morita, 1988). In addition, VIP alters other parameters of the chromaffin cells such as an increase in biosynthesis of enkephalins by activation of ProEnk A gene expression (Wan & Livett, 1989) and phosphorylation and activation of tyrosine hydroxylase (Haycock & Wakade, 1991).

Further analysis of the effects of VIP on chromaffin cells has given new insights in to the molecular mechanism of action of the peptide. VIP turns on two separate signalling pathways in the rat adrenal medulla: stimulation of the phosphoinositide–phospholipase C pathway and activation of the adenylate cyclase–cyclic AMP pathway (Malhotra *et al.* 1988, 1989). It is hypothesized that the direct stimulatory action of VIP is mediated by the hydrolysis of the phosphoinositide pathway, which increases the intracellular concentration of Ca^{2+} through the liberation of inositol 1, 4, 5-trisphosphate to initiate the exocytosis. A rise in cellular cyclic AMP contributes to the facilitatory action of VIP on catecholamine secretion through some unknown mechanism but probably not by further enhancing the cytosolic concentration of Ca^{2+}.

Fourthly, the stimulatory effect of exogenous VIP on the secretion of catechol-

amines is mimicked by stimulating the splanchnic nerves. Undoubtedly, ACh is the main stimulatory transmitter known to be released from the splanchnic nerve endings. However, the effect of nerve stimulation could not be abolished by atropine and hexamethonium. The residual secretion, which was termed as non-cholinergic, accounted for as much as 60% under certain conditions of nerve stimulation (Malhotra & Wakade, 1987*a*). The non-cholinergic portion of the secretion was not due to inadequate blockade of cholinergic receptors because exogenous ACh (10 μg) secretion was reduced by 95% by atropine and hexamethonium.

Fifthly, stimulatory effects of VIP and nerve stimulation are antagonized by VIP antagonist. We were successful in demonstrating the antagonistic action of the VIP antagonist on catecholamine secretion evoked by administered VIP and nerve stimulation. Secretion by VIP was reduced by more than 80% and the non-cholinergic component of nerve-mediated secretion was reduced from 40 to 75% when VIP antagonist was added together with cholinergic receptor antagonists. This pharmacological evidence is consistent with the idea that neurally mediated secretion has a VIP-ergic component.

All the above five criteria are the hallmarks of any substance that is to be designated as a neurotransmitter in the peripheral and central nervous system. Accordingly, VIP can be regarded as a presynaptic neurotransmitter released upon activation of splanchnic neurones and partly responsible for the secretion of adrenomedullary hormones. There is also substantial evidence that VIP may be a neurotransmitter in the preganglionic nerve fibres of the rat superior cervical ganglia (Ip & Zigmond, 1984; Ip *et al.* 1988; Baldwin, Sasek & Zigmond, 1991). The presence of ACh with VIP, as suggested for other synaptic regions (Lundberg, Anggard, Fahrenkrug, Hokfelt & Mutt, 1980; Kobayashi, Kyoshima, Olschowka & Jacobowitz, 1983; Eckstein & Baughman, 1984) also applies to the rat adrenal medulla. Additional work is needed to understand the precise localization of VIP in presynatic sites as well as the synthesis, storage and the physiological disposition of the peptide in different synaptic regions.

There is a widespread belief that neuropeptides are released during high rates of stimulation parameters (Fahrenkrug, Galbo, Holst & Mukadell, 1978; Goyal, Rattan & Said, 1980; Matsuzaki, Hamasaki & Said, 1980; Lundberg, Anggard & Fahrenkrug, 1982*a*; Lundberg, Anggard, Fahrenkrug, Lundgren & Holmstedt, 1982*b*). It is not that the output of peptide per pulse necessarily rises, but that repetitive stimulation is necessary to get a sufficient total output to trigger a postsynaptic response (Jan, Jan & Kuffler, 1979, 1980; Lundberg *et al.* 1982*b*). In the present study, we have found that the output of VIP actually declined per pulse as the stimulation frequency was increased. In the same preparation we also demonstrated that the output of ^{3}H-ACh increased per pulse with increasing stimulation frequency. Bloom *et al.* (1988) also found that the output of VIP at 4 Hz was slightly greater than that obtained at 40 Hz in the calf adrenals. The non-cholinergic component was most prominent at lower frequencies than at higher frequencies in the rat adrenals (Malhotra & Wakade, 1987*a*). These findings suggest that the release of VIP is, in relation to that of ACh, more prominent at lower neuronal activity.

Some of the above literature on the question of release of VIP at different frequencies is confusing partly because of the variations in experimental protocols

used by different investigators and also in the expression of the published data. In fact, recalculation and expression of the data is consistent with the current proposal that the output of VIP per pulse is greater at lower frequencies. There is also a possibility that the frequency–release relationship may vary from one organ to the other. Additional consideration is that neuronal traffic may occur in bursts rather than at fixed rates of frequencies (Hagbarth, Hallin, Hongell, Torebjork & Wallin, 1972; Hallin & Torebjork, 1974) and it would be more meaningful to study the release of substances after brief bursts of activity, as reported by Andersson, Bloom, Edwards & Järhult (1982) and Edwards & Jones (1989).

REFERENCES

ANDERSSON, P-O., BLOOM, S. R., EDWARDS, A. V. & JARHULT, J. (1982). Effects of stimulation of the chorda tympani in bursts on submaxillary responses in the cat. *Journal of Physiology* **322**, 469–483.

ANTON, A. H. & SAYRE, D. F. (1962). A study of factors affecting the alumina oxide–trihydroxy indole procedure for the analysis of catecholamines. *Journal of Pharmacology and Experimental Therapeutics* **138**, 360–375.

BALDWIN, C., SASEK, C. A. & ZIGMOND, R. D. (1991). Evidence that some preganglionic sympathetic neurons in the rat contain vasoactive intestinal peptide – or peptide histidine isoleucine amide-like immunoreactivities. *Neuroscience* **40**, 175–184.

BLOOM, S. R., EDWARDS, A. V. & JONES, C. T. (1988). The adrenal contribution to the neuroendocrine responses to splanchnic nerve stimulation in conscious calves. *Journal of Physiology* **397**, 513–526.

CARMICHAEL, S. W., BROOKS, J. C., MALHOTRA, R. K., WAKADE, T. D. & WAKADE, A. R. (1989). Ultrastructural demonstration of exocytosis in the intact rat adrenal medulla. *Journal of Electron Microscopy Technique* **12**, 316–322.

CHEUNG, C. Y. & HOLZWARTH, M. A. (1986). Fetal adrenal VIP distribution and effects on medullary catecholamine secretion. *Peptides* **7**, 413–418.

COOPER, J. R. (1988). A simple method to determine released acetylcholine in the presence of choline. *Life Sciences* **45**, 2041–2042.

DALE, H. H. (1914). The action of certain esters of choline and their relation to muscarine. *Journal of Pharmacology and Experimental Therapeutics* **6**, 147–190.

ECKSTEIN, F. & BAUGHMAN, R. W. (1984). Two types of cholinergic innervation in cortex, one co-localized with vasoactive intestinal polypeptide. *Nature* **309**, 153–155.

EDWARDS, A. V. & JONES, C. T. (1989). Adrenal responses to splanchnic nerve stimulation in conscious calves given naloxone. *Journal of Physiology* **418**, 339–351.

FAHRENKRUG, J., GALBO, H., HOLST, J. J. & MUCKADELL, O. B. (1978). Influence of the autonomic nervous system on the release of vasoactive intestinal polypeptide from the porcine gastrointestinal tract. *Journal of Physiology* **280**, 405–422.

FONNUM, F. (1976). A rapid radiomechanical method for the determination of choline acetyltransferase. *Journal of Neurochemistry* **24**, 407–409.

GOYAL, R. K., RATTAN, S. & SAID, S. I. (1980). VIP as a possible neurotransmitter of non-cholinergic non-adrenergic inhibitory neurons. *Nature* **288**, 378–380.

HAGBARTH, K.-E., HALLIN, R. G., HONGELL, A., TOREBJORK, H. E. & WALLIN, B. G. (1972). General characteristics of sympathetic activity in human skin nerves. *Acta Physiologica Scandinavica* **84**, 164–176.

HALLIN, R. G. & TOREBJORK, H. E. (1974). Single unit sympathetic activity in human skin nerves during rest and various manoeuvres. *Acta Physiologica Scandinavica* **92**, 303–317.

HAYCOCK, J. & WAKADE, A. R. (1991). Frequency-dependent activation and multiple-site phosphorylation of tyrosine hydroxylase in perfused rat adrenal gland. *Journal of Neurochemistry* (in the Press).

HOKFELT, T., LUNDBERG, J. M., SCHULTZBERG, M. & FAHRENKRUG, J. (1981). Immunohistochemical evidence for a local VIP-energic neuron system in the adrenal gland of the rat. *Acta Physiologica Scandinavica* **113**, 575–576.

HOLZWARTH, M. A. (1984). The distribution of vasoactive intestinal peptide in the rat adrenal cortex and medulla. *Journal of the Autonomic Nervous System* **11**, 269–283.

IP, N. Y., PERLMAN, R. L. & ZIGMOND, R. E. (1983). Acute transsynaptic regulation of tyrosine 3-monooxygenase activity in the rat superior cervical ganglion: Evidence for both cholinergic and noncholinergic mechanisms. *Proceedings of the National Academy of Sciences of the USA* **80**, 2081–2085.

IP, N. Y. & ZIGMOND, R. E. (1984). Pattern of preganglionic nerve activity can determine the type of neurotransmitter regulating a postsynaptic event. *Nature* **311**, 472–474.

JAN, Y. N., JAN, L. Y. & KUFFLER, S. W. (1979). A peptide as a possible transmitter in sympathetic ganglia of the frog. *Proceedings of the National Academy of Sciences of the USA* **76**, 1501–1505.

JAN, Y. N., JAN, L. Y. & KUFFLER, S. W. (1980). Further evidence for peptidergic transmission in sympathetic ganglia. *Proceedings of the National Academy of Sciences of the USA* **77**, 5008–5012.

KOBAYASHI, I., KYOSHIMA, K., OLSCHOWKA, J. A. & JACOBOWITZ, D. M. (1983). Vasoactive intestinal polypeptide immunoreactive and cholinergic nerves in the whole mount preparation of the major cerebral arteries of the rat. *Histochemistry* **79**, 377–381.

LOWRY, D. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

LUNDBERG, J. M., ANGGARD, A. & FAHRENKRUG, J. (1982*a*). VIP as a mediator of hexamethonium-sensitive, atropine-resistant vasodilation in the cat tongue. *Acta Physiologica Scandinavica* **116**, 387–392.

LUNDBERG, J. M., ANGGARD, A., FAHRENKRUG, J., HOKFELT, T. & MUTT, V. (1980). Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: Functional significance of coexisting transmitters for vasodilation and secretion. *Proceedings of the National Academy of Sciences of the USA* **77**, 1951–1980.

LUNDBERG, J. M., ANGGARD, A., FAHRENKRUG, J., LUNDGREN, J. & HOLMSTEDT, B. (1982*b*). Corelease of VIP and acetylcholine in relation to blood flow and salivary secretion in cat submandibular gland. *Acta Physiologica Scandinavica* **115**, 525–528.

MALHOTRA, R. K., BLANK, M., WAKADE, T. D. & WAKADE, A. R. (1989). Vasoactive intestinal polypeptide serves as another neurotransmitter in the rat adrenal medulla. *Regulatory Peptide* **26** (2), 168.

MALHOTRA, R. K. & WAKADE, A. R. (1987*a*). Non-cholinergic component of rat splanchnic nerves predominates at low neuronal activity and is eliminated by naloxone. *Journal of Physiology* **383**, 639–652.

MALHOTRA, R. K. & WAKADE, A. R. (1987*b*). Vasoactive intestinal polypeptide stimulates the secretion of catecholamines from the rat adrenal gland. *Journal of Physiology* **388**, 285–294.

MALHOTRA, R. K., WAKADE, T. D., & WAKADE, A. R. (1988). Vasoactive intestinal polypeptide and muscarine mobilize intracellular Ca^{2+} through breakdown of phosphoinositides to induce catecholamine secretion. *Journal of Biological Chemistry* **263**, 2123–2126.

MALHOTRA, R. K., WAKADE, T. D. & WAKADE, A. R. (1989). Cross-communication between acetylcholine and VIP in controlling catecholamine secretion by affecting cAMP, inositol trisphosphate, protein kinase C, and calcium in rat adrenal medulla. *Journal of Neuroscience* **9**, 4150–4157.

MARLEY, P. D. & LIVETT, B. (1985). Neuropeptides in autonomic nervous system. In *CRC Critical Reviews in Clinical Neurobiology*, vol. 1, ed. Ross, A., pp. 201–283. CRC Press, Inc., Boca Raton, FL, USA.

MATSUZAKI, Y., HAMASAKI, Y. & SAID, S. I. (1980). Vasoactive intestinal peptide: A possible transmitter of noradrenergic relaxation of guinea pig airways. *Science* **210**, 1252–1253.

MAUBERT, E., TRAMU, G., CROISE, D., BEAUWILLAIN, C. J. & DUPOUY, J. P. (1990). Co-localization of vasoactive intestinal polypeptide in the nerve fibers of the rat adrenal gland. *Neuroscience Letters* **113**, 121–126.

MISBAHUDDIN, M., OKA, M., NAKANISHI, A. & MORITA, K. (1988). Stimulatory effect of vasoactive intestinal polypeptide on catecholamine secretion from isolated guinea pig adrenal chromaffin cells. *Neuroscience Letters* **92**, 202–206.

MITCHELL, S. J. & BLOOM, S. R. (1978). Measurement of fasting and postprandial VIP in man. *Gut* **19**, 1043–1048.

WAKADE, A. R. (1981*a*). Studies on secretion of catecholamines evoked by acetylcholine or transmural stimulation of the rat adrenal gland. *Journal of Physiology* **313**, 463–480.

WAKADE, A. R. (1981*b*). Facilitation of secretion of catecholamines from rat and guinea-pig adrenal glands in potassium-free medium or after ouabain. *Journal of Physiology* **313**, 481–498.

WAKADE, A. R. (1988). Noncholinergic transmitter(s) maintains secretion of catecholamines from rat adrenal medulla for several hours of continuous stimulation of splanchnic neurons. *Journal of Neurochemistry* **50**, 1302–1308.

WAKADE, A. R. (1991). Contribution of acetylcholine and vasoactive intestinal polypeptide in controlling catecholamine secretion. *Journal of the Autonomic Nervous System* **33**, 176.

WAKADE, A. R., MALHOTRA, R. K., WAKADE, T. D. & DIXON, W. R. (1986). Simultaneous secretion of catecholamines from the adrenal medulla and of [^{3}H] norepinephrine from sympathetic nerves from a single test preparation: Different effects of agents on the secretion. *Neuroscience* **18**, 877–888.

WAN, D. C. & LIVETT, B. (1989). Vasoactive intestinal peptide stimulates proenkephalin A mRNA expression in bovine adrenal chromaffin cells. *Neuroscience Letters* **101**, 218–222.

YOSHIKAWA, M., SAITO, H., SANO, T., OHUCHI, T., ISHIMURA, Y., MORITA, K., SAITO, S. & OKA, M. (1990). Localization and release of immunoreactive vasoactive intestinal polypeptide in bovine adrenal medulla. *Neuroscience Letters* **111**, 75–79.