STUDIES OF VASOACTIVE INTESTINAL POLYPEPTIDE (VIP)

IN THE MAMMALIAN GENITOURINARY SYSTEM

MARION ANNE BLANK

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

ROYAL POSTGRADUATE MEDICAL SCHOOL

UNIVERSITY OF LONDON

ABSTRACT

The aim of these studies was to examine the role of VIP in the genitourinary system. By means of a specific and highly sensitive radioimmunoassay for VIP, its quantitative distribution in the normal mammalian genitourinary system was established. Physiological studies were undertaken to investigate possible roles for VIP in this system. In addition, the pharmacological action of VIP and related peptides on genitourinary smooth muscle preparations was investigated. Finally, studies were made of the changes in distribution of VIP in certain pathological conditions of the genitourinary system.

To determine the origin of the VIP-containing nerves found in the bladder and genitalia, denervation studies were carried out. Following pelvic and hypogastric nerve section, there was no decrease in VIP content in these tissues suggesting that the predominant source of VIP is from local ganglia. However, there was a minor source of VIP-innervation of genitourinary tissues which appeared likely to be sensory. A VIP-containing system was discovered in the lumbosacral spinal cord in man and the cat: dorsal rhizotomy, dorsal root ganglionectomy and cord hemisection in the cat demonstrated its probable sensory origin.

As vasodilatation may be an important mechanism contributing to penile erection, and VIP is known to be a potent vasodilator, studies were made of the role of VIP in mammalian penile erection. VIP was found to be released into the dorsal penile vein during tactile stimulation of the penis.

A comparison of the pharmacological effect of VIP and related peptides was made on rabbit genitourinary smooth muscle preparations in an attempt to establish further that VIP is the natural effector in the non-adrenergic non-cholinergic relaxation and in doing so, further characterise the VIP-family of receptors in these preparations.

It was concluded that VIP-containing nerve fibres in the mammalian genitourinary tract are predominantly of local origin, although VIP also selectively marks pelvic nerve afferent fibres in the sacral spinal cord. VIP is likely to be an important non-adrenergic non-cholinergic vasodilator and smooth muscle relaxant in the genitourinary tract, and may be of significance in certain pathological conditions, including the unstable bladder and erectile impotence in diabetes.

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CHAPTER 1

INTRODUCTION

Vasoactive intestinal polypeptide (VIP) was first isolated from porcine duodenum (Said & Mutt 1970a, 1970b, 1972) and for some years considered to be a gut hormone (see Grossman 1974). It was the discovery in 1976 that VIP was widely present in the mammalian central and peripheral nervous system (Bryant et al 1976; Said & Rosenberg 1976) which led to its study in a number of peripheral organs, including the genitourinary tract. The first studies of VIP in the genitourinary system, based mainly on immunohistochemical techniques, reported that VIP-containing fibres were found in all regions investigated, and distributed within smooth muscle, around blood vessels and beneath the epithelium (Larsson et al 1977a, 1977b; Alm et al 1977). Subsequent investigations demonstrated the biological activity of VIP within the genitourinary tract. In the female, VIP inhibited contractions of smooth muscle of the uterus, cervix and Fallopian tube (Clark et al 1981; Ottesen 1981; Walles et al 1980; Carter et al 1981) and increased myometrial blood flow in vivo (Carter et al 1981; Ottesen & Fahrenkrug 1981). In the male, VIP inhibited contractions of genital tract smooth muscle in vitro (Larsen et al 1981; Sjöstrand et al 1981; Willis et al 1981). In the urinary tract, too, reports indicated that VIP affected smooth muscle contractility (Levin & Wein 1981; Erspamer et al 1981; Johns 1979).

However, many questions regarding the role of VIP in the mammalian genitourinary system remained unanswered, and led to the anatomical, physiological, pharmacological and pathological studies of VIP within the genitourinary system which are presented in this thesis.

The first and essential requirement was to establish a sensitive, accurate and reliable method of measuring VIP in biological tissues and fluids. A radioimmunoassay was chosen as it is specific, highly sensitive (capable of measuring femtomole levels) and permits simultaneous processing of many samples which may be of very small volume. The efficiency of extraction and stability of VIP in extracts was assessed before its distribution in the genitourinary system was reliably determined.

Anatomical studies were undertaken to determine the origin of VIP-containing fibres in the genitourinary system of the cat and rat. These included a comprehensive study of the origin and distribution of VIP containing fibres in the spinal cord of the cat.

A physiological study was made of the role of VIP in mammalian penile erection, since VIP was known to be a potent vasodilator, and vasodilatation may be an important mechanism contributing to penile erection.

The pharmacological action of VIP and related peptides was studied on genitourinary smooth muscle preparations (rabbit urethra and anococcygeus muscle) to further establish VIP as a natural nonadrenergic non-cholinergic effector, and to characterise the receptors of the VIP family of peptides in these preparations.

Finally, studies were made of the changes in distribution of VIP in certain pathological conditions of the genitourinary system. To determine whether VIPergic innervation is altered in human impotence, biopsies taken from patients with diabetic impotence were compared with penises obtained at gender reassignment operations. Also, in view of the relaxant effect of VIP on bladder smooth muscle, bladder biopsies from patients with an unstable bladder were studied.

Introduction to VIP

VIP was originally discovered in porcine lung extracts on account of its potent vasodilatory activity in anaesthetized dog hind limb (Said & Mutt 1969). Using the same bioassay, it was later discovered that chromatographic fractions from porcine duodenum obtained during the purification of secretin also contained a vasodilator peptide. This led to the isolation of VIP (Said & Mutt 1970a, 1970b, 1972). The presence of a vasodepressor substance in intestinal extacts had actually been noted by Bayliss & Starling almost 70 years earlier, during their experiments leading to the discovery of secretin (Bayliss & Starling 1902).

Following its discovery, isolation and characterisation (Mutt & Said 1974), porcine VIP was synthesized (Bodanszky et al 1973, 1974). It is a highly basic 28 amino acid peptide (molecular weight 3326 Da), closely resembling the structures of secretin and glucagon. Gastric inhibitory peptide shares a less extensive structural homology but is still considered a member of the VIP family of peptides (see Table 1:1). VIP shares a very high degree of sequence homology with the

recently discovered peptide containing NH_2-terminal histidine and COOH-terminal isoleucine (PHI), also isolated from porcine intestine (Tatemoto & Mutt 1980).

The presence of macromolecular forms of VIP was initially reported in certain pancreatic islet and neurogenic tumours producing the peptide (Gozes et al 1983; Yamaguchi et al 1980). Subsequently, Obata and co-workers reported that human neuroblastoma cell lines synthesized VIP from a precursor molecule (Pro-VIP) with a molecular weight of 17,500 (Obata et al 1981).

More recently, the same group of investigators identified the primary translation product of the messenger RNA encoding VIP (Pre-Pro-VIP) with a molecular weight of 20,000 (Itoh et al 1983) (Figure 1:1). These investigators also disclosed that PHM (a peptide nearly identical to PHI and having COOH-terminal methionine instead of isoleucine) was co-synthesized from the same precursor and confirmed that the sequence of human VIP was identical to that of the porcine peptide.

A consequence of the formation of peptides by the cleavage of long precursor forms is that various intermediate cleavage forms may be present in tissues and/or the circulating blood. In the case of VIP, no other form other than the octacosapeptide has yet been isolated, but there is no reason to believe that none occurs. Indeed, there are two reports of a smaller molecular weight form of VIP-immunoreactivity in non-malignant tissue (Dimaline & Dockray 1978; Bryant et al 1976) and a larger form in the plasma and tumour extracts of patients with VIP-secreting tumours (Bloom & Polak

1979). Only the 28 amino acid form of VIP has been reported to occur in tissues of the genitourinary system.

Distribution of VIP

Although originally suggested to occur in endocrine-like cells of the gastrointestinal tract (Polak et al 1974), VIP is now known to have a widespread distribution, occurring in neurones in the central and peripheral nervous systems. The latter include the digestive and respiratory tracts as well as the genitourinary tract, the heart, skin, ear, exocrine glands, the thyroid and adrenal glands and certain autonomic nerve fibres and ganglia (for review see Said 1982, 1984). In all these regions, the VIP nerve terminals are intimately associated with smooth muscle cells, close to mucosal surfaces, glandular epithelia and around blood vessels. This distribution pattern accords well with the main effects of VIP, which include relaxation of smooth muscle, vasodilatation and enhancement of secretion. Other actions of VIP, including interactions with hormones and neurotransmitters, have also been reported (see Said 1984).

Mechanisms of Action

Neurotransmitters induce their effects by interacting with specific receptors, which in turn are linked to effector mechanisms. Receptors for VIP have been extensively investigated (see Amiranoff & Rosselin 1982; Robberecht et al 1982) and characterized into subtypes, depending on their affinity for natural peptides and synthetic analogues (Christophe et al 1983). How the binding of VIP

to its receptor is coupled to its actions is not clear, but cyclic AMP seems to be involved as second mesenger - a stimulation of adenylate cyclase in isolated plasma membranes and/or a rise of the cellular cyclic AMP level can be detected (Amiranoff & Rosselin 1982). Cyclic AMP, resulting from the stimulation of the receptor by VIP, phosphorylates and thereby activates protein kinases and these in turn bring about the cellular response.

Neurotransmitter Co-Existence and VIP

VIP co-exists with acetylcholine in some cholinergic neurones supplying exocrine glands (Lundberg 1981) and in some intracortical neurones of the rat (Eckenstein & Baughman 1984). The finding that VIP also co-exists with PHI in normal tissues and tumours (see Yanaihara et al 1983) is not unexpected, as both peptides are derived from a common precursor (Itoh et al 1983).

The functional significance of co-existence of the neurotransmitter acetycholine and the putative neurotransmitter VIP (and possibly PHI) is still uncertain,although a tentative model has been proposed for the interaction of VIP and acetylcholine released from neurones supplying blood vessels and exocrine glands (Lundberg et al 1982). The presence of PHI in neurones containing VIP will further add to the complexity of this model, particularly as PHI shares many of the biological activities of VIP, although in most systems PHI has a somewhat lower potency (Bataille et al 1980; Brennan et al 1982; Dimaline & Dockray 1980; Suzuki et al 1984; Szecowka et al 1980; Lundberg & Tatemoto 1982).

The presence of VIP in cholinergic neurones of the genitourinary

system remains predominantly, as yet, unreported. However, preliminary studies by Dail et al (1983) demonstrate that many penile neurones are strongly positive for acetylcholinesterase, and that some of these neurones stain also for VIP. Further studies, using more definitive markers to determine neurotransmitter type, will be necessary to answer the question whether VIP and acetylcholine co-exist in neurones of the mammalian genitourinary system.

TABLE 1:1 The amino acid sequences of the members of the VIP family of peptides.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Porcine VIP	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln	Met	Ala	Val	Lys	Lys	Tyr
Chicken VIP	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Ser	Arg	Phe	Arg	Lys	Gln	Met	Ala	Val	Lys	Lys	Tyr
PHI	His	Ala	Asp	Gly	Val	Phe	Thr	Ser	Asp	Phe	Ser	Arg	Leu	Leu	Gly	Gln	Leu	Ser	Ala	Lys	Lys	Tyr
Secretin	His	Ser	Asp	Gly	Thr	Phe	Thr	Ser	Glu	Leu	Ser	Arg	Leu	Arg	Asp	Ser	Ala	Arg	Leu	Gln	Arg	Leu
Glucagon	His	Ser	Gln	Gly	Thr	Phe	Thr	Ser	Asp	Tyr	Ser	Lys	Tyr	Leu	Asp	Ser	Arg	Arg	Ala	Gln	Asp	Phe
GIP	Tyr	Ala	Glu	Gly	Thr	Phe	Ile	Ser	Asp	Tyr	Ser	Ile	Ala	Met	Asp	Lys	Ile	Arg	Gln	Gln	Asp	Phe

	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43
Porcine VIP	Leu	Asn	Ser	Ile	Leu	Asn	NH_2														
Chicken VIP	Leu	Asn	Ser	Val	Leu	Thr	NH_2														
PHI	Leu	Glu	Ser	Leu	Ile	NH_2															
Secretin	Leu	Gln	Gly	Leu	Val	NH_2															
Glucagon	Val	Gln	Trp	Leu	Met	Asn	Thr														
GIP	Val	Asn	Trp	Leu	Leu	Ala	Gln	Gln	Lys	Gly	Lys	Lys	Ser	Asp	Trp	Lys	His	Asn	Ile	Thr	Gln

Abbreviations:
GIP = gastric inhibitory polypeptide; PHI = porcine histidine isoleucine amide-containing peptide; VIP = vasoactive intestinal polypeptide

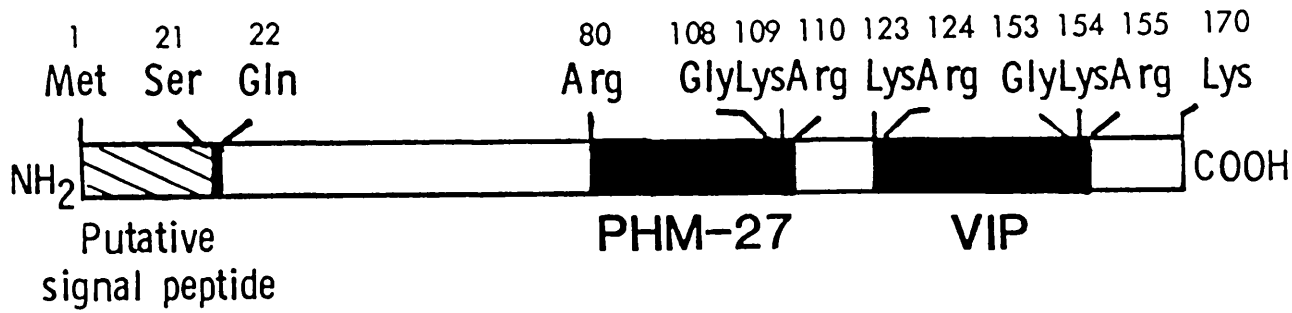


FIGURE 1:1 Schematic representation of the structure of pre-pro VIP/PHM-27. VIP (125-152) and PHM (81-107) are represented by the closed bars. The amino acid residues at the post-translational processing sites are shown.

CHAPTER 2

MEASUREMENT OF VIP

In this chapter, the following aspects of radioimmunoassay of VIP are addressed: (A) Introduction to the principles of radioimmunoassay; (B) Description of the radioimmunoassay of VIP used in the studies presented in this thesis; (C) Methods of extraction of VIP from tissue; (D) Chromatographic analysis of VIP-like immunoreactivity in tissue extracts and (E) Measurement of VIP in plasma.

In section (A), the principles of the method used to arrive at all quantitative determinations of VIP are outlined. In (B), the particular features of the VIP assay used in these studies are described. This assay has undergone modifications since its initial development (Mitchell & Bloom 1978) and these modifications are high-lighted. Studies involving the measurement of VIP-like immunoreactivity in tissues necessitated a systematic investigation of the stability of the peptide in tissue extracts. In section (C), a number of sources of error have been identified and an attempt made to eliminate them. A number of studies in the literature fail to analyze the nature of VIP-like immunoreactivity presented (eg the release of VIP in mast cells (Cutz et al 1978)). Section (D) describes how the characterization of VIP-like immunoreactivity in tissue extracts measured by this assay is carried out. Section (E) outlines the differences between measurement of VIP-like

immunoreactivity in tissue extracts and plasma samples.

(A) INTRODUCTION TO PRINCIPLES OF RADIOIMMUNOASSAY

In radioimmunoassay (RIA), a fixed concentration of radioactively labelled antigen (Ag*) is incubated with a constant dilution of antiserum (Ab) such that the concentration of antigen binding sites on the antiserum is limiting. If unlabelled antigen (Ag) is added to this system there is competition between labelled and unlabelled antigen for the limited and constant number of binding sites on the antiserum, and thus the amount of labelled antigen bound to the antiserum will decrease as the concentration of unlabelled antigen increases.

$$Ag\ Ab \rightleftharpoons Ag + Ab + Ag^* \rightleftharpoons Ag^*\ Ab$$

The amount of radiolabelled antigen bound to the antiserum can be measured after separating antiserum-bound from free antigen and counting one or other, or both fractions. A calibration or standard curve is set up with increasing concentrations of standard unlabelled antigen, and from this curve the amount of antigen in unknown samples can be calculated. Thus the four basic requirements for an RIA system are (1) an antiserum to the substance to be measured; (2) a radioactively labelled form of the substance; (3) a method for separation of antiserum-bound and unbound radiolabelled antigen; (4) a standard preparation of antigen, to allow construction of a standard curve.

(1) Antiserum Production

The sensitivity and specificity of an RIA will depend above all on the properties of the antiserum. Antiserum to a peptide is prepared by injecting the pure peptide or a conjugate of the peptide, in an adjuvant into a suitable animal. Small peptides (ie haptens) have to be coupled to an immunogenic protein (carrier, eg bovine serum albumin (BSA), keyhole limpet haemocyanin) in order to elicit an immune response.

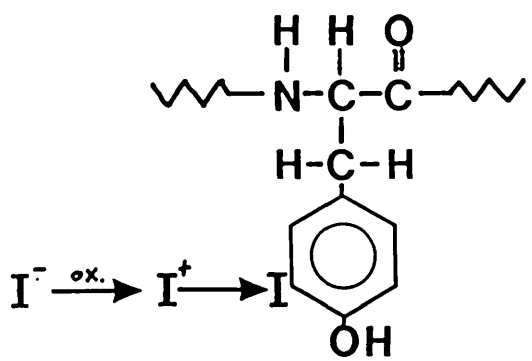
The dilution of antiserum to be used for a particular RIA is determined from an antiserum dilution curve constructed by incubating a fixed concentration of labelled antigen with increasing dilutions of antiserum.

(2) Production of Radiolabelled Antigen

Since the tracer is intended simply to provide a measure of the total antigen in the bound and free phases of the system, it is obvious that its property of combination with the antiserum must be as similar as possible with that of the unlabelled antigen.

Isotopes of iodine are almost universally employed at present. ^{125}I is used in preference to ^{131}I because of its more convenient half-life (60 days as opposed to 8 days), its greater isotopic abundance in commercially available preparations, and the better counting efficiency of the gamma radiation emitted by the isotope in conventional gamma scintillation counters (^{131}I is too hard and requires very large crystals to ensure adequate counting efficiency).

A variety of chemical methods exists for the introduction of iodine into peptides. The most widely adopted procedure involves the use of the oxidising agent chloramine T (Hunter & Greenwood 1962). The reaction involves oxidation of $^{125}I^-$ to free ^{125}I and generation of the cation $^{125}I^+$, which is then incorporated into the aromatic ring of a tyrosyl residue in the peptide by electrophilic substitution.



The reaction is terminated by the addition of a reducing agent, usually sodium metabisulphite, which reduces the unreacted chloramine T. Peptides such as VIP are susceptible to oxidation, mainly due to the presence of methionine residues. If the methionine residue is part of the antigenic site a significant loss of immunoreactivity may occur. Similarly, iodination of an antigenically important tyrosine residue may affect immunoreactivity resulting in loss of potential assay sensitivity. For these reasons, alternative methods of iodination may need to be considered, eg enzymatic iodination using lactoperoxidase (Marchalonis 1969) or the use of iodine monochloride (McFarlane 1958). These two alternative methods also involve oxidation, but are less harsh than chloramine T. Conjugation labelling in which

iodine is first attached to a suitable reagent and then the complex is conjugated to the peptide (Bolton & Hunter 1973) is another iodination procedure. By careful choice of reaction conditions, peptide oxidation may be avoided.

When an oxidative iodination procedure is complete, the reaction mixture contains the following: unlabelled antigen (damaged and undamaged); labelled antigen (damaged and undamaged); free iodide and salts including the oxidising and reducing agent. Since the tracer used in the assay should consist of undamaged labelled antigen, purification of the reaction mixture is essential.

Many variations in methodology exist for peptide label purification. Gel permeation chromatography was the most widely adopted technique. Here, the iodinated peptide is separated from the other reactants mainly on the basis of molecular size.

Ion exchange chromatography provides a more discriminating and reproducible system separating molecules mainly on the basis of charge differences. Provided conditions are correctly chosen, the technique is capable of separating monoiodinated, diiodinated and non-iodinated hormone. The phenolic hydroxyl group of tyrosine has a pK (ie the pH at which it is 50% ionized) of about 10.0. After tyrosine monoiodination its pK falls to about 8.5 and after diiodination to 7.0. Therefore at about pH 9.0, non-iodinated tyrosines are mainly non-ionized, while iodinated tyrosines are mainly ionized and carry a negative charge. At pH 8.5 monoiodinated tyrosines are only half ionized, while diiodotyrosines are almost fully charged. These charge differences result in different degrees

of adhesion to charged ion exchange materials (eg CM-C25 or DEAE Sephadex). In practice, buffer molarity must be carefully selected to give partial retardation of the peptide during chromatography and sufficient time allowed for successful separation. Isocratic elution from ion exchange columns has the disadvantage of being very sensitive to external factors such as temperature, degree of column equilibration and other buffer constituents etc. Therefore a very shallow gradient is often utilised in order to purify labelled peptides during ion exchange chromatography. The ion exchange purification technique allows the use of 'trace iodination' in which a considerable excess of peptide is used in the iodination reaction. Under these conditions, the ratio of oxidant to peptide is lower, minimizing oxidation damage and preventing the over-iodination which results in formation of diiodotyrosine, with subsequent loss of immunoreactivity. Such 'trace' iodination critically depends on obtaining the correct conditions for a very discriminating purification system so that the radiolabelled monoiodo-peptide can be fully separated from the great excess of non-radioactive peptide in order to obtain a preparation of high specific activity.

Following purification, two properties of the labelled antigen must be established: the specific activity (ie radioactivity per mole of ligand) and its reactivity toward the antiserum.

Calculation of the specific activity of the new labelled antigen by self-displacement, while only approximate, is the method of choice (Roulston 1979). Increasing amounts of labelled antigen are incubated with a constant amount of antiserum under similar

conditions to those employed routinely in the assay; the amount of tracer present in the tubes is then determined by interpolating from the standard curve the percentage bound for each label increment; the amount of non-radioactive antigen displaced by each increment is equated to the amount of radioactive antigen in the tubes.

Fractions of the radiolabelled antigen, showing similar maximal immunoreactivity and specific activity should be pooled, diluted and aliquoted for storage at -20°C. The diluent should contain a high concentration of protein, such as BSA, so as to reduce the adsorption to tube walls and protect the labelled peptide from irradiation damage. Peptides which are sensitive to proteolytic enzymes and oxidation may be more stable if stored with a protease inhibitor and cysteine. Correctly stored, the monoiodo-peptide preparations are usually found to remain stable for several months after purification, and only the predicted slow loss of specific activity due to radioactive decay occurs.

(3) Separation of Bound and Free Antigen

An effective method must be used for complete and rapid separation of antiserum-bound antigen from free antigen so that the distribution of radioactivity between the two phases can be accurately measured. The methods used for separation are based on chemical or immunological differences between the free and the antiserum-bound antigen. The differences utilized for separation include charge, size, solubility, surface configuration (immunological determinants), and the adsorption to solid material such as cellulose, glass powder, silica, talc and active charcoal.

Activated charcoal, coated with dextran or albumin, is the most widely used adsorbent - it is cheap, easy to dispense and produces reproducible results. It is thought that the free antigen enters the pores of the charcoal particles and is adsorbed, whilst the antigen-antiserum complex is excluded. Following centrifugation, the liquid phase can be separated from the charcoal by simple aspiration. The amount of radioactive antigen bound to the antiserum can then be measured by counting the liquid phase (supernatant) or charcoal pellet, or both.

Coating the charcoal particles is thought to reduce the adsorption of the antigen-antiserum complex.

The concentration of charcoal required must be tested for an individual assay.

(4) The Standard Curve

The accuracy of an assay relies on comparisons of the unknown sample with the standards, which contain a pre-determined amount of the material to be measured. Ideally, the assay incubation solutions containing standard and unknown antigen should be identical in every respect. Also it is necessary to ensure assay consistency by establishing a stable standard preparation and using quality control samples to monitor interassay variation.

With the appropriate amounts of antiserum and radiolabel, the concentrations of standard are chosen to achieve a practical range of detection which may or may not provide maximal sensitivity in

terms of the minimum detectable concentration.

(B) RADIOIMMUNOASSAY FOR VIP

(1) Antiserum Production

The antiserum (V9) used in the RIA of VIP in the studies presented in this thesis was raised as described by Mitchell and Bloom (1978).

VIP (3 µmol) was conjugated to BSA (1 µmol) by carbodiimide condensation and administered emulsified in Freund's adjuvant to rabbits. After primary immunization and two boosts, nearly all animals developed detectable antisera to VIP. However, only two rabbits (V9 and V25) produced antisera of high affinity and specificity.

V9 is able, under the conditions described below, to detect a minimum concentration of VIP of 0.3 fmol/assay tube with 95% confidence. It is used at a final dilution of 1:320,000.

Synthetic VIP fragments 1-6, 1-8, 1-22, 7-28, 11-28 and 18-28 and pure chicken VIP were used to assess the binding characteristics of V9. It was found to react fully only with whole VIP:-

VIP Type	VIP (mol per assay tube) which caused 50% displacement of ^{125}I-Porcine VIP from V9 at normal working dilution and 4 days incubation
1- 6	greater than 5×10^{-9}
1-18	6×10^{-10}
1-22	1×10^{-11}
7-28	2×10^{-13}
11-28	9×10^{-13}
18-28	6×10^{-8}
Chicken	greater than 5×10^{-10}
Porcine	5×10^{-14}

With V9 there was no displacement on addition of 5 nmol/assay tube of glucagon, secretin, gastrin, motilin, cholecystokinin, gastric inhibitory peptide, substance P or PHI.

(2) Preparation of Radiolabelled VIP

In the assay described by Mitchell & Bloom (1978) ^{125}I-VIP was prepared using the method of lactoperoxidase oxidation.

In the studies presented in this thesis, chloramine T oxidation was the iodination method of choice to produce consistently pure ^{125}I-VIP of high specific activity, antigenicity equal to that of natural porcine VIP and a 'shelf-life' of at least three months.

VIP contains two tyrosine residues and incorporation of iodine is readily achieved. However, the methionine at position 17 is

susceptible to oxidation producing the highly-charged methionine sulphoxide. Using a method of 'trace iodination' reduces the risk of obtaining oxidised radiolabelled VIP.

Protocol for Iodination of VIP

1) Weigh 6.0 nmol (20 µg) porcine VIP.
2) Dissolve in 50 µl phosphate buffer (0.3 M) pH 7.4.
3) Add 0.4 nmol $Na^{125}I$ (1 mCi 10 µl) (IMS 30 Radiochemical Centre, Amersham, UK)
4) Add 26 nmol (7.3 µg) Chloramine T (BDH) in 20 µl (0.3M) phosphate buffer, pH 7.4.
5) React for 10 seconds.
6) Add 138 nmol (26 µg) sodium metabisulphite (BDH) in 100 µl (0.3M) phosphate buffer, pH 7.4 and mix.
7) Dilute reaction mixture in 1 ml column buffer (see below) containing 1% potassium iodide. The potassium iodide serves as a carrier for the ^{125}I, preventing significant quantities of the radioisotope adsorbing to surfaces during the purification procedures.

Purification of ^{125}I-VIP

The iodination product was purified by cation exchange chromatography on a carboxy-methyl (CM25) Sephadex column (60 x 1.5 cm) equilibrated in borate buffer (0.05M), pH 8.8, containing 1% BSA. After loading the reaction mixture, the column was eluted with a linear salt gradient of 0-0.2M sodium chloride in a total volume of 400 ml of the column buffer.

Two ml fractions of eluate were collected and counted by a gamma-scintillation counter (see Figure 2:1). Fractions across the peaks of radioactivity were selected and tested for immunoreactivity. Aliquots of the fractions under test (from Figure 2:1, fractions 76, 113, 118, 121 and 126) were diluted in assay buffer (see Assay Technique Section) so as to obtain approximately 400 counts per 10 seconds in 100 µl. In a final volume of 0.8 ml of the assay buffer contained in duplicate sets of tubes, the diluted fractions were tested as follows:-

Condition 1	100 µl diluted fraction without	antiserum V9
2	50 µl diluted fraction with	antiserum V9
3	200 µl diluted fraction with	antiserum V9
4	100 µl diluted fraction with	antiserum V9
5	100 µl diluted fraction with	antiserum V9 and 4 fmol VIP
6	100 µl diluted fraction with an excess of	antiserum

Following 3 days incubation at 4°C, separation of free and bound VIP was carried out and the percentage of ^{125}I-VIP bound to the antiserum (%B) assessed. The specific activities of the selected fractions of the new radiolabel were estimated by the method of self-displacement (see Introduction).

Fraction Number	76	113	118	121	126
	%B	%B	%B	%B	%B
Condition 1	4	5	5	2	5
2	52	57	64	65	66
3	38	43	48	49	45
4	47	53	58	59	57
5	36	42	44	46	46
6	75	79	83	84	87
Estimated Specific Activity (Bq/fmol)	68	52	70	66	42

The first major peak of radioactivity (a) (see Figure 2:1) was unreacted iodide which, because of its negative charge, was excluded from the negatively charged gel column and eluted in the void volume.

The small peak of radioactivity (b) (Fraction 76) was found to contain immunoreactivity. It demonstrated a high specific activity and, therefore, was probably a diiodinated product eluting ahead of the monoiodinated product because of its less positive charge. Despite its initially greater specific activity, this diiodinated product rapidly deteriorated and there was a loss of immunoreactivity due to the effects of 'decay catastrophe'.

The specific activities of fractions in peak c were found to be lower in the fractions of the trailing edge than in the leading edge and peak fractions. Thus, the stock of radiolabelled VIP was

prepared by pooling the peak fraction and those of the leading edge (in Figure 2:1, fractions 115-124). The pooled fractions were diluted in a solution of 0.5M acetic acid containing 5% lactose, 2.5% BSA, 6mM cysteine hydrochloride and 1600 KIU/ml aprotinin (Trasylol, Bayer). This was then aliquoted and stored at -20°C.

By this method, a radiolabelled preparation of VIP was regularly obtained with a specific activity of 60-70 Bq/fmol. The maximum specific activity for pure monoiodinated peptide is 81 Bq/fmol. Therefore the ^{125}I-VIP product of this iodination appeared to be about 80% pure - it probably contained some uniodinated peptide.

The immunoreactivity of the radiolabelled VIP was monitored regularly and showed no significant decline over several months. Although peptide fragments generated by 'decay catastrophe' would have accumulated they would be likely to have little immunoreactivity and, being non-radioactive, would not influence the specific activity of the preparation. Thus any decrease in specific activity would be largely due to non-radiolabelled peptide of the initial product providing proportionally more of the total immunoreactivity, while that of the radiolabelled VIP was declining.

(3) Separation of Bound and Free VIP

Reliable separation of antiserum-bound from free radiolabelled VIP was obtained with dextran-coated charcoal. The quantity of charcoal required was selected by testing increasing amounts from 1.0 to 40.0 mg/tube (see Adrian 1982). Figure 2:2 shows the effect on different assay parameters of adding increasing amounts of charcoal to the

standard incubation mixture for separating bound and free fractions. Ten mg charcoal (Norit GSX Hopkins and Williams Ltd) and 1 mg clinical grade dextran (Sigma) per assay tube was the routinely chosen amount but the assay exhibited a wide margin of tolerance to the quantity of charcoal added.

Counting of the charcoal pellet and supernatant was carried out in multiple-well gamma counters (Nuclear Enterprises 1600). These machines were linked via a microprocessor and counted 96 tubes simultaneously. The microprocessor calculated the percentage bound (%B) as:

$$\frac{\text{Counts in the Supernatant (B)}}{\text{Counts in the Supernatant (B) + Counts in Charcoal Pellet}} \times 100$$

The counting time was adjusted to give a mean count of 3,000-4,000 counts per tube. The magnitude of the counting error is dependent upon the number of disintegrations recorded. The more events recorded, the lower the error. However, it is not worth reducing the counting error much below the pipetting error.

(4) Preparation of Standard VIP

VIP is unstable because of its susceptibility to proteolysis, oxidation, deamidation and adsorption to active surfaces. For these reasons, stock solutions of VIP were prepared according to the following procedure in order to ensure both accuracy and precision by minimizing losses both during handling and long-term storage.

VIP was weighed accurately on an electrostatic microbalance (Cahn Electrobalance, model M10) and then dissolved in an aqueous solution containing the following:-

50 ml	0.5M acetic acid
40 µmol/l	BSA (to prevent binding of VIP to surfaces)
6 mmol/l	L-cysteine hydrochloride (to prevent oxidation damage to VIP)
140 mmol/l	Lactose (to produce a large soluble pellet to aid dissolving)
10 mmol/l	Citric acid (to maintain a low pH for better stability during freeze-drying)
8×10^5 KIU/l	Aprotinin (Trasylol) (to inhibit proteolytic enzymes)

Aliquots of the solution containing 1 pmol VIP were then lyophilized and stored in vacuo at -20°C.

In order to assess the accuracy of preparation of the VIP standards and to compare RIA results of the VIP assay described here and those conducted by other researchers in the field using locally raised antisera, aliquots of 1 pmol VIP standards, prepared as described above, were sent to three other laboratories. The results were as follows:-

	Concentration of VIP determined in 1 pmol standard aliquot of VIP
Prof S Said (Oklahoma, USA)	0.9 pmol
Prof G Dockray (Liverpool, UK)	0.7 pmol
Prof J Fahrenkrug (Copenhagen, Denmark)	1.6 pmol

These results indicated a consistency between values obtained from three separate VIP assays, each utilizing different antisera, which is not often observed (Chard 1982). It would also appear that a satisfactory accuracy has been obtained during the preparation of VIP standards for use in the assay described in this chapter.

For preparing an assay standard curve, a vial containing 1 pmol of freeze-dried standard was reconstituted in 2.5 ml of assay buffer. From this solution (0.4 pmol/ml) duplicate aliquots were taken by micropipette for each of the following volumes: 2,3,5,7,10,15,20,25, 50 and 100 µl and added to the appropriate assay tubes. See Figure 2:4 for diagram of a mean VIP standard curve obtained from 6 replicate series of assay tubes.

Assay Technique

The VIP assay was designed to be sensitive over a practical concentration range (1-40 fmol/tube). The sensitivity of an assay is the smallest difference in concentration which can be reliably detected. It is dependent on the slope of the standard curve (which

is dependent upon antiserum/antigen affinity), and the error of a given value on the standard curve. Sensitivity is proportional to the slope divided by the error.

Duplicate 2 ml polystyrene test tubes were set up, each with a total assay volume of 800 µl, according to the format shown in Table 2:1. Buffer used in the assay was EDTA phosphate buffer (0.05M), pH 7.4, containing 10 mmol/l EDTA, 0.05 mmol/l (0.3%) BSA and 0.05% sodium azide (bacteriostat). ^{125}I-VIP was prepared in the assay buffer and 100 µl added to give a concentration of 1 fmol ^{125}I-VIP per assay tube.

Antiserum V9 was also prepared in the assay buffer and added in a volume of 100 µl to produce a final dilution of 1:320,000.

Usually, 10 µl aliquots of tissue extracts or of diluted (in 0.5M acetic acid) extracts were assayed and to account for any effect that this quantity of acetic acid might have on binding of antigen to antiserum, an equal volume of acetic acid was added to all other assay tubes.

The various quality controls listed in Table 2:1 were included for the following reasons:-

Blank These tubes contained all the reagents except antiserum to evaluate the non-specific binding of ^{125}I-VIP.

1/2 x Label These tubes contained half the amount of label added to the rest of the tubes in the assay. This was useful for assessing whether greater sensitivity could have been achieved in an assay by

adding less label.

2 x Label Tubes that contained twice the amount of label added to the other assay tubes which is useful for assessing the sensitivity of the assay with respect to the amount of label added. These tubes could also be used for calculating the specific activity of ^{125}I-VIP.

Zero Tubes containing ^{125}I-VIP and antiserum, but no standard or samples, placed at regular intervals throughout the assay in order to detect and assess the degree of assay drift, e.g. due to reagent deterioration.

Excess Antiserum These tubes contained excess antiserum and ^{125}I-VIP. In order not to waste antiserum of high affinity (V9), another antiserum, V57, was used at a final concentration of 1:80. This low affinity antiserum was inadequate for sensitive RIA, but the degree of binding of ^{125}I-VIP in the presence of excess V57 was used as a measure of the immunological integrity of ^{125}I-VIP and in conjunction with the 'blank' value to assess the quality of ^{125}I-VIP.

The assays were incubated for 5-7 days at 4°C (a balance between maximum sensitivity and minimal proteolytic degradation of VIP) after which time separation of antiserum-bound from free VIP was performed.

Quality Control

Constant evaluation and monitoring of the VIP assay was undertaken during the studies presented in this thesis. In addition to the inclusion of the tubes referred to in Table 2:1, certain additional tubes containing tissue extract quality control samples were added

to each assay.

Tissue extract quality control samples were prepared by boiling 10g of specimens of pig brain, colon and liver in approximately 100 ml of 0.5M acetic acid. Following 10 minutes boiling, each solution was allowed to cool and 0.2 ml aliquots of each were then lyophilized and stored in vacuo at -20°C until required for assay. For assay, an aliquot of each of the pig brain, colon and liver extracts were reconstituted in 200 µl of 0.5M acetic acid and duplicate 10 µl aliquots were taken and added to assay tubes at the beginning and end of each assay. The pools of tissues were chosen in such a way that their concentrations of VIP represented high (colon), medium (brain) and low to undetectable (liver) values in the assay. This provided an on-going check of precision at different parts of the standard curve.

The inter-assay variation was calculated from the quality control values obtained across a series of 36 assays (see Figure 2:3). The values for inter-assay variation (in terms of percentage coefficient of variation) for the colon and brain extracts were 21.0% and 19.5% respectively, where coefficient of variation (CV) = $\frac{SD}{\bar{x}} \times 100\%$ where SD = standard deviation of quality control values and $\bar{x}$ = mean of quality control values.

An estimate of the precision of an assay can be obtained from replicate standard curves. Figure 2:4 shows a mean standard curve obtained from 6 replicate series of assay tubes in a single assay

containing standard concentrations of VIP. Also shown are the approximate 95% confidence limits (ie ± 2 standard deviations) of the percentage bound value at each standard concentration. It can be seen that the variance of the %B value changed with standard VIP concentration. Thus the precision, and hence sensitivity, of the assay varied along the standard curve. By dividing the standard deviation of each %B-value by the slope of the curve at each point, the relative error, or intra-assay variation in estimated concentrations of VIP was determined across the concentration range (ie a precision profile was constructed) (See Figure 2:5). The coefficient of variation of VIP concentration was seen to vary with dose and this is a common feature of RIAs (Rodbard 1974). The precision was relatively low at lower VIP concentrations but above 4 fmol per assay tube there was an approximate plateau of maximum precision followed by a progressive deterioration towards the extremes of standard concentrations. This level of precision (7-12%) was acceptable and was exhibited over an appropriate range of VIP concentrations for the studies in this thesis in which this assay was employed.

The intra-assay variation was also tested by assaying five replicates of pig colon quality control tissue in a single assay. Expressed as a coefficient of variation, the intra-assay variation was calculated to be 7% which is consistent with the value obtained from the plateau region of the precision profile.

(C) TISSUE EXTRACTION

Introduction

It is clear from the discrepancies in the literature that the determination of peptide quantities in tissue is problematic (Fahrenkrug 1979). The problems have, however, been less well recognised than those that occur with measurement in plasma.

Regulatory peptides are contained in the storage granules of neurones and endocrine cells. These structures are readily disrupted, eg by physical trauma (sonication or homogenisation), by freezing or boiling, or by extremes of pH or osmotic pressures. Of these procedures, mechanical homogenisation is least preferred as it appears to expose peptide binding sites, resulting in variable loss of peptide in the soluble fraction (Bryant & Bloom 1982). After solubilisation the peptides must be protected from degradation by enzymes, for example released from the disrupted lysosomes. Their extraction must, therefore, also involve protein (enzyme) disruption, eg by use of organic solvents, extremes of pH, protein-precipitating agents or changes of ionic strength caused by the addition of large amounts of salts. Unfortunately these agents may, in turn, also affect the antibody binding in the subsequent RIA. Therefore, protein-disrupting agents have to be removed again. Occasionally they can be diluted out but often the concentration of regulatory peptides present in a tissue is too low for this to be possible. For this reason, boiling is preferred (Mutt 1978).

The commonest current technique for extraction makes use of the fact

that small peptides are usually stable at 100°C, whereas large proteins are not. Thus if a tissue is maintained at 100°C for 10 minutes, all enzymic activity and membranes are destroyed, allowing small peptides to diffuse out undamaged. This technique is efficient and quick. Some peptides have proven more soluble at neutral pH (for example the acidic small molecular forms of cholecystokinin and gastrin), whereas others (the majority, including VIP) are more soluble at low pH. It was, therefore, necessary to employ acid in order to extract VIP from tissues.

Pre-weighed polypropylene tubes (Sarstedt) were filled with 0.5M acetic acid in a volume approximately ten times the mass of tissue and placed in a vigorously boiling covered water bath with their caps on loosely. The weighed tissue was added once the acid in the tubes had reached a temperature of at least 96°C, and was left at this temperature for 10-15 minutes. Large pieces of tissue were always cut up to yield pieces no bigger than 100 mg in size, prior to adding to the boiling acid. The tubes were then removed, allowed to cool, dried on the outside and weighed. By knowing the weight of the empty tube, the tissue weight and the weight of the tube plus contents following boiling, it was possible to determine accurately the volume of acid in which the tissue was extracted. The extracts were then firmly capped and stored at -20°C until time of assay for VIP.

Regulatory peptides must, in order to regulate activity, be susceptible to rapid destruction and are, therefore, usually labile by nature. Tissue damage causes release of lysosomal proteolytic enzymes. Post-mortem stability of neuropeptides in unextracted

tissues is well documented (Cooper et al 1981; Emson et al 1980; Rossor et al 1981; McGregor & Bloom 1983) and these studies emphasize the need for rapid harvesting of tissues from undamaged organs and if immediate extraction is not undertaken, tissue samples should be stored in liquid nitrogen. When the samples are eventually extracted, it is essential that thawing should not occur prior to addition to the boiling acid.

The stability of peptides after extraction is variable and may also depend on conditions of storage. Some peptides are very susceptible to oxidative damage or other forms of slowly progressive chemical change, such as deamidation. These changes have not been well studied and methods for preventing them are ill-documented. In order to investigate these possibilities it was thought important to undertake a study of the stability of VIP in various tissue acid extracts during different types of storage for differing periods of time. In addition, the effect of repeated freezing and thawing on the VIP concentration of the acid extracts was investigated.

Method

The colon, stomach, duodenum, brain, liver, heart and kidneys were removed from 2 male, age-matched rats (Albino Wistar), killed by an overdose of ether (rats (1) and (2)). Brains were divided into fore- and hind halves. Each tissue was then extracted in 0.5M acetic acid, as described above. Exogenous natural porcine VIP was added to the extracts of liver, heart and kidneys to produce a concentration of 1 pmol of exogenous VIP/ml extract. Tissue extracts from each of rats (1) and (2) were divided into 9 aliquots

and treated as follows:-

Condition (1) Aliquots were assayed for VIP immediately following extraction.

Condition (3) Aliquots were frozen rapidly on dry ice and then placed in boiling water to produce rapid thawing. This freezing and thawing process was carried out ten times prior to assay for VIP.

Condition (4) Aliquots were stored at 4°C for 24 hrs.

Condition (5) Aliquots were stored at -20°C for 24 hrs and following thawing, were assayed for VIP in the same assay as those subjected to condition (4).

Condition (6) Aliquots were stored at 4°C for seven days.

Condition (7) Aliquots were stored at -20°C for seven days.

Condition (8) Aliquots were frozen following extraction and then allowed to thaw. They were left at room temperature (24°C) for eight hours before being re-frozen. This process was repeated over 5 successive days.

Condition (9) Aliquots were frozen following extraction and then allowed to thaw and left at room temperature (24°C) for a period of 2 hrs before being re-frozen. This process was repeated on five occasions.

Aliquots subjected to conditions (6), (7), (8) and (9) were then assayed for VIP in a single assay.

Condition 10 Aliquots were frozen at -20°C for three months prior to assay for VIP.

Results

The results of the extract stability study are illustrated in Figures 2:6 a-h. In each case the concentration of VIP measured in each aliquot of tissue extract (pmol/ml) from rats (1) and (2) was plotted for each condition. Aliquots subjected to repeated freezing and thawing (ie conditions (3), (8) and (9)) were grouped together, as were aliquots stored at 4°C (ie conditions (4) and (6)) and those stored at -20°C (conditions (5), (7) and (10)).

In the case of the colon, similar concentrations of VIP were measured in all conditions except where the aliquot had been subjected to freezing and thawing over eight hours on five successive occasions (condition (8)). Under these conditions an approximately 50% loss of VIP was incurred. The rat stomach showed a similar pattern - condition (8) resulted in a marked reduction in VIP. In the duodenum, fore- brain and hind brain, consistent concentrations of VIP were measured for all conditions.

In the case of tissues where exogenous VIP was added to the acid extract, relatively constant VIP concentrations were detected, except in the case of the kidney and liver where freezing and thawing for periods of two and eight hours (conditions (8) and (9)) resulted in marked losses of VIP. In the kidney, conditions (6) and (7) also resulted in reduced concentrations of VIP but this reduction was seen neither in the heart nor the liver aliquots, under identical conditions.

Discussion

The results of this study have demonstrated that VIP, whether endogenous or exogenous, in acid extracts of various tissues is remarkably stable under various storage conditions. Of those investigated here, only 'careless' freezing and thawing of the aliquot (ie thawing and then standing at room temperature for eight hours), with the exception of the duodenum, consistently resulted in a reduced VIP content.

In all the tissues examined, storage at -20°C for a period of three months did not result in any significant change in VIP concentration when compared to those aliquots assayed immediately following tissue extraction. Likewise short-term storage at 4°C appeared to have no detrimental effect on VIP concentration.

The results of this study indicate that VIP in acidic tissue extracts is stable for long time periods if stored frozen and providing repeated thawing to room temperature for prolonged periods is avoided. This also applied to exogenously added VIP except in the case of thawed extracts of liver and kidney where there was a significant loss of immunoreactivity, possibly due to adsorption to membrane structures, following freezing and thawing.

(D) GEL PERMEATION CHROMATOGRAPHY

The antiserum to VIP (V9) used in the studies presented in this thesis required whole molecule VIP in order to obtain maximal

binding. However, V9 was shown to cross-react with carboxy-terminal fragments (Mitchell & Bloom 1978). Therefore any polypeptide containing the same sequence of amino acids as those constituting the antigenic site of VIP to which V9 combines may be readily detected by the antiserum. Such potential cross-reacting substances could include larger putative precursor forms of VIP (Itoh et al 1983) or subfragments of the peptide molecule (particularly carboxy-terminal fragments) which may occur naturally or have been generated prior to or during extraction. The possible existence of such molecular forms of VIP-like immunoreactivity within the tissues of the mammalian genitourinary system presented in this thesis was investigated using gel permeation chromatography.

Tissue extracts (0.3-0.6 ml), prepared as described earlier, were loaded on to an equilibrated 60 x 1.5 cm column of Sephadex G-50 superfine gel. They were eluted at 4°C at a flow rate of 3 ml/hr with 0.05M phosphate buffer containing 0.2M sodium chloride, 1% BSA and 0.05M EDTA. A high salt concentration was used to prevent electrostatic interaction of VIP with negatively charged residues which exist within the Sephadex matrix. Adsorption losses due to hydrophobic interactions between the peptide and the column matrix were reduced by the BSA. Fractions of 0.5 ml were collected and assayed for VIP-like immunoreactivity. Each column was pre-calibrated with dextran blue and sodium ^{125}I as molecular size markers for the void volume and total volume respectively, cytochrome C and pure porcine VIP. With each chromatographic run dextran blue, cytochrome C and $Na^{125}I$ were included with the unknown sample as internal markers.

A typical elution profile of immunoreactive VIP is shown in Figure 2:7.

From the gel permeation chromatographic analyses, it appeared that in all the tissues investigated a single form of immunoreactivity was detected, which did not differ significantly in molecular size from pure porcine VIP, ie the immunoreactivity in the unknown samples and the pure porcine VIP, both eluted with a mean Kav of 0.54 where Kav = (Ve-Vo)/(Vt-Vo) (Andrews 1970).

Ve = observed elution volume

Vo = column void volume (ie elution volume of a large, completely excluded molecule such as dextran blue).

Vt = total bed volume (ie elution volume of small, totally included molecules such as $Na^{125}I$).

It can be concluded that subfragments or large precursor forms containing any part of the VIP molecule were not present to any significant extent in the genitourinary tissue extracts investigated. When a radioimmunoassay result is quoted in this thesis, VIP should be understood to refer to VIP-like immunoreactivity.

(E) MEASUREMENT OF VIP IN PLASMA

Studies presented in this thesis have involved predominantly the technique of RIA of tissue extracts. However, in Chapter 7, VIP was measured in plasma samples.

Assay of Plasma Samples

Blood (0.5-1 ml) for VIP estimation was taken into heparinized tubes, containing 2,000 KIU aprotinin (Trasylol) per ml blood to retard proteolysis. The blood was immediately centrifuged and the plasma stored at -20°C until assay for VIP.

The methodology for plasma assay was similar to that described earlier for tissue extracts (see Table 2:1). The volume of plasma added was 100 µl.in each case, therefore, the buffer volume had to be reduced in order to maintain a total volume of 800 µl. To account for any effect that this quantity of plasma might have on binding of antigen to antiserum, an equal volume of 'representative' plasma was added to all other tubes, ie blank, zero, standard, 1/2 x and 2 x label and excess antiserum tubes. For example, in an assay to determine the VIP concentration in penile plasma samples, an equal amount of plasma taken from a peripheral vein of the animal (eg caudal or limb vein) was added to the other tubes to minimize any residual error caused by non-specific plasma effects.
All other assay parameters involved in the measurement of plasma VIP were identical to those used in the measurement of VIP in tissue extracts.

TABLE 2:1 Assay Format

	Buffer Volume (µl)	Volume of Label Added (µl)	Volume of Antiserum Added (µl)
Blank	700	100	-
1/2 x Label	650	50	100
2 x Label	500	200	100
Zero	600	100	100
Excess Antiserum	600	100	100
Standards from			
0.4 pmol/ml			
2,3,5,7,10,15,20,25 µl	600	100	100
50 µl	550	100	100
100 µl	500	100	100
Samples	600	100	100

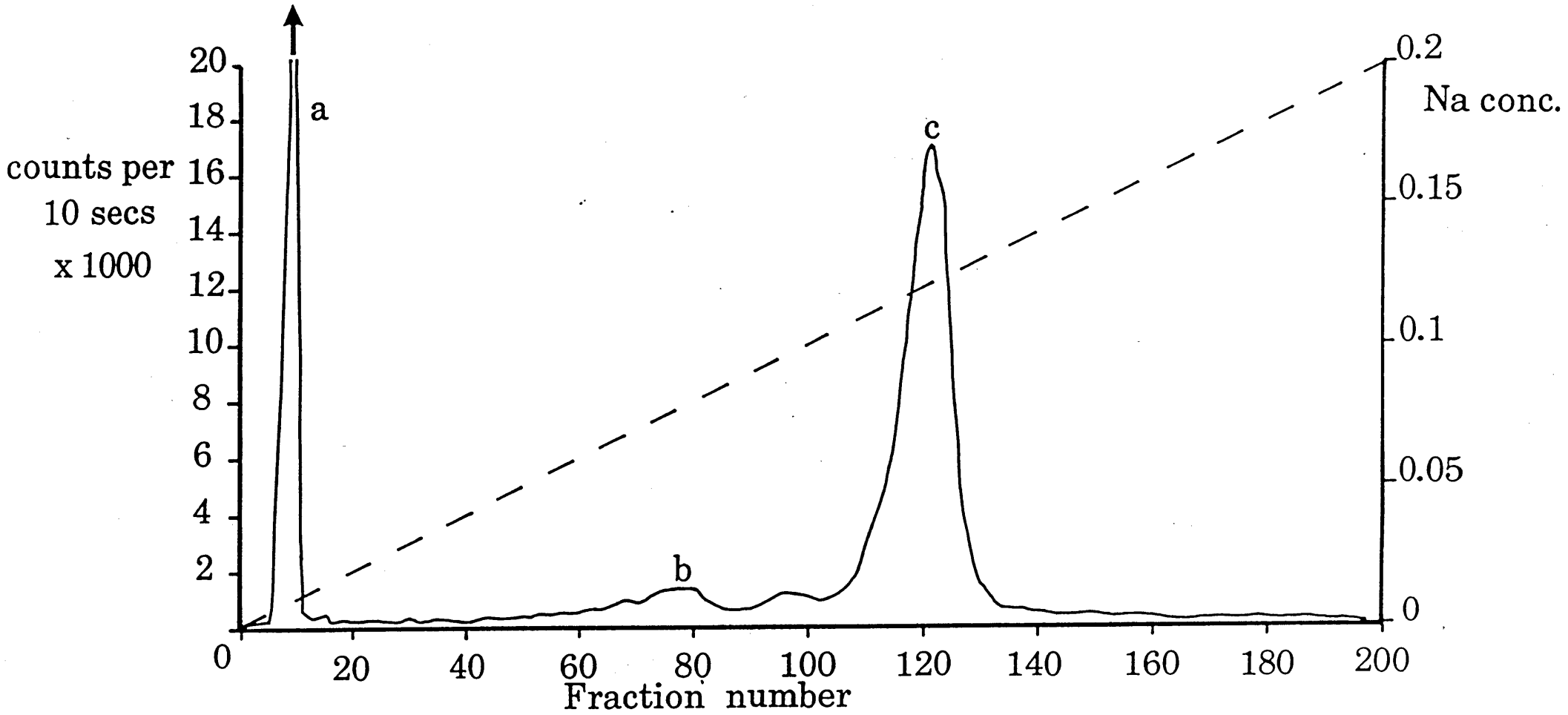


FIGURE 2:1 Profile of radioactivity in the eluate from CM25 Sephadex column following the chromatographic separation of the VIP iodination mixture. The column was eluted with a linear gradient (dashed line) of sodium (Na) chloride in buffer, pH 8.8. Letters a, b and c indicate the peaks of radioactivity.

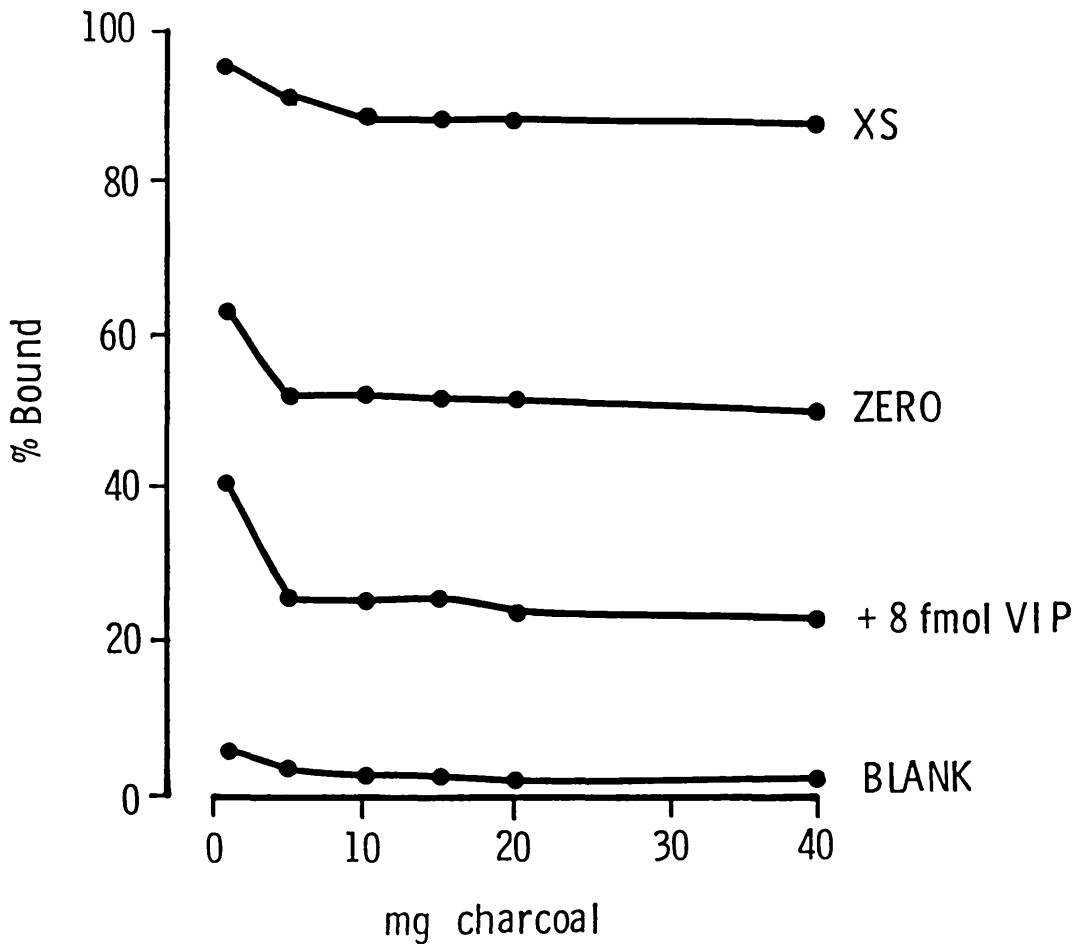


FIGURE 2:2 Effect of increasing amounts of charcoal on the non-specific binding of labelled VIP (blank) and binding in the presence of antiserum only (zero), antiserum plus 8 fmol per tube of cold VIP and excess antiserum (XS).

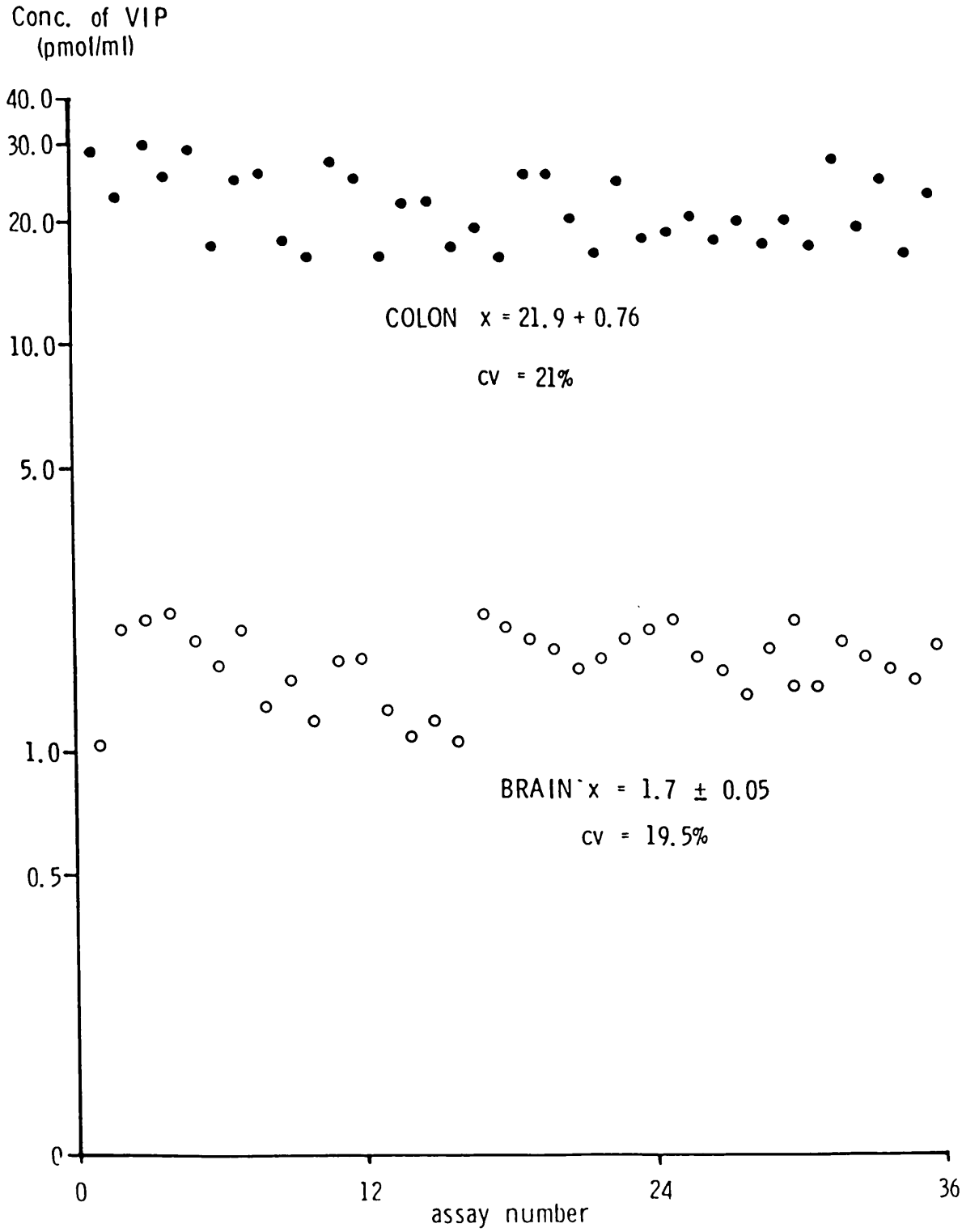


FIGURE 2:3 Values of VIP concentration of quality control extracts of porcine brain (O) and colon (●) obtained from a series of 36 assays. Values are plotted on a log scale and for each quality control the calculated mean (X) ± SEM and the coefficient of variation (CV) (the inter-assay variation) is indicated.

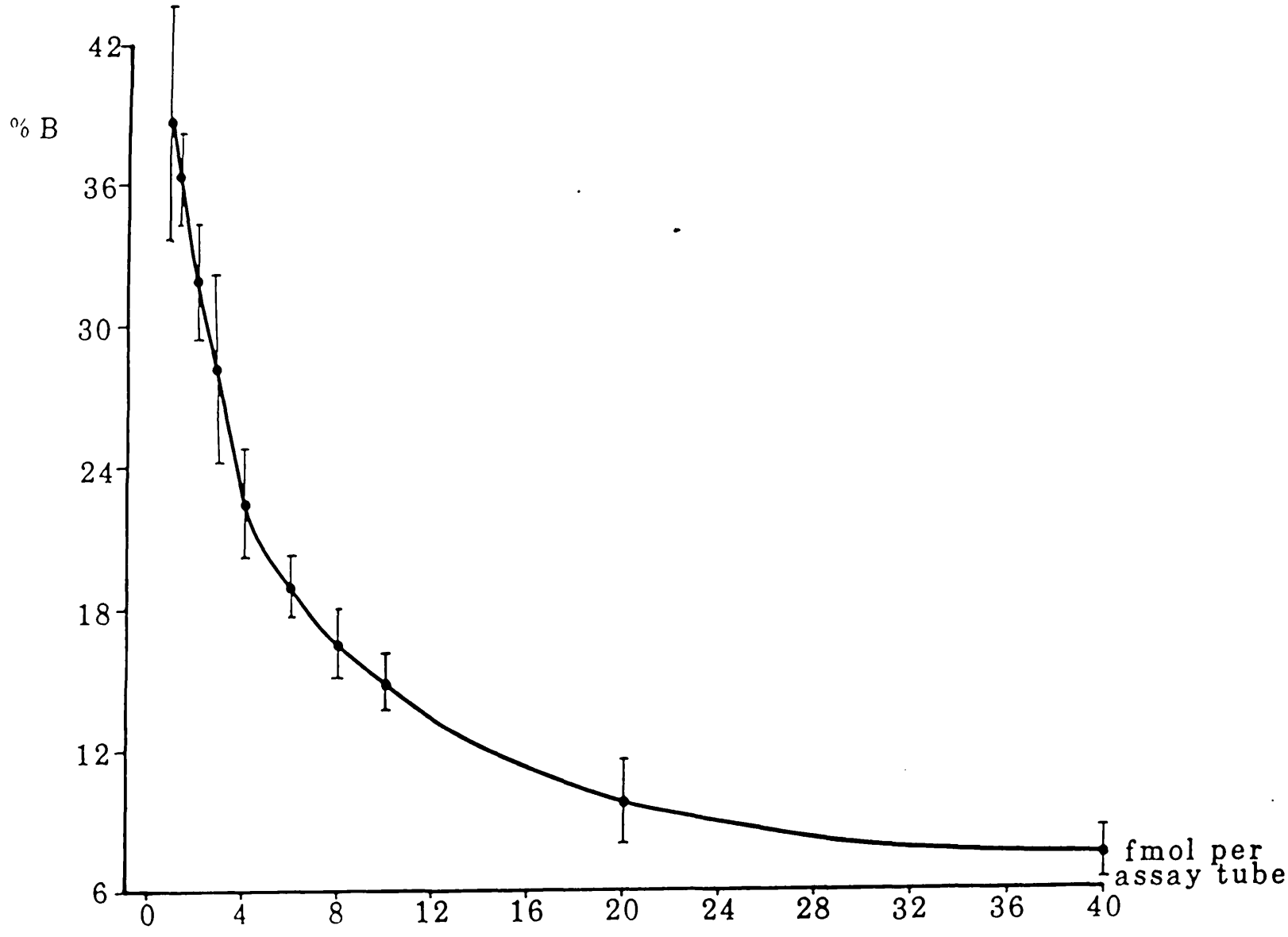


FIGURE 2:4 Mean VIP standard curve obtained from six replicate series of assay tubes containing standard concentrations of VIP. The bars indicate 2 standard deviations on either side of the mean percentage bound (%B) value at each VIP concentration.

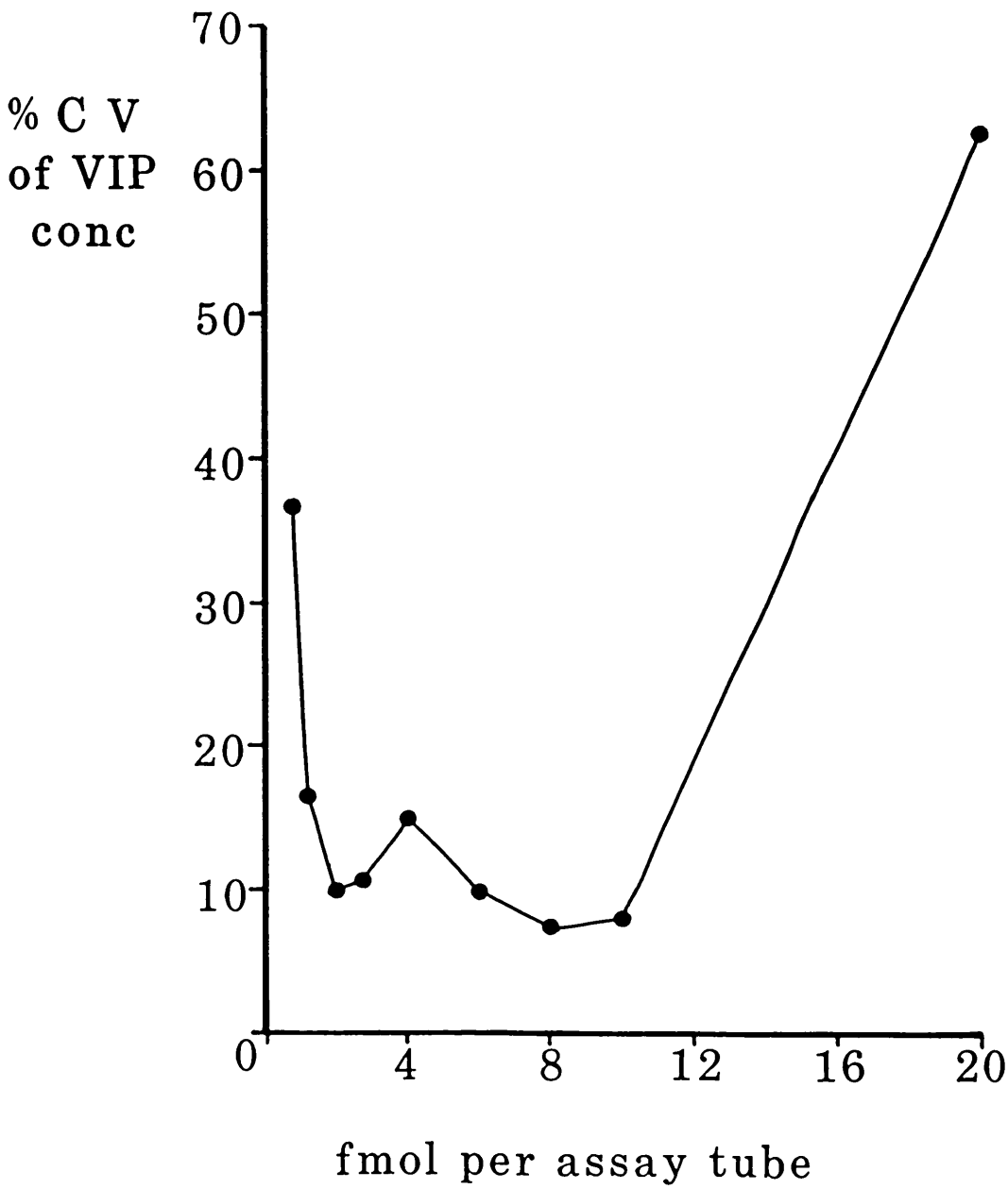


FIGURE 2:5 Precision profile of VIP assay is illustrated here as the percentage coefficient of variation (%CV) at each of nine points on the standard curve.

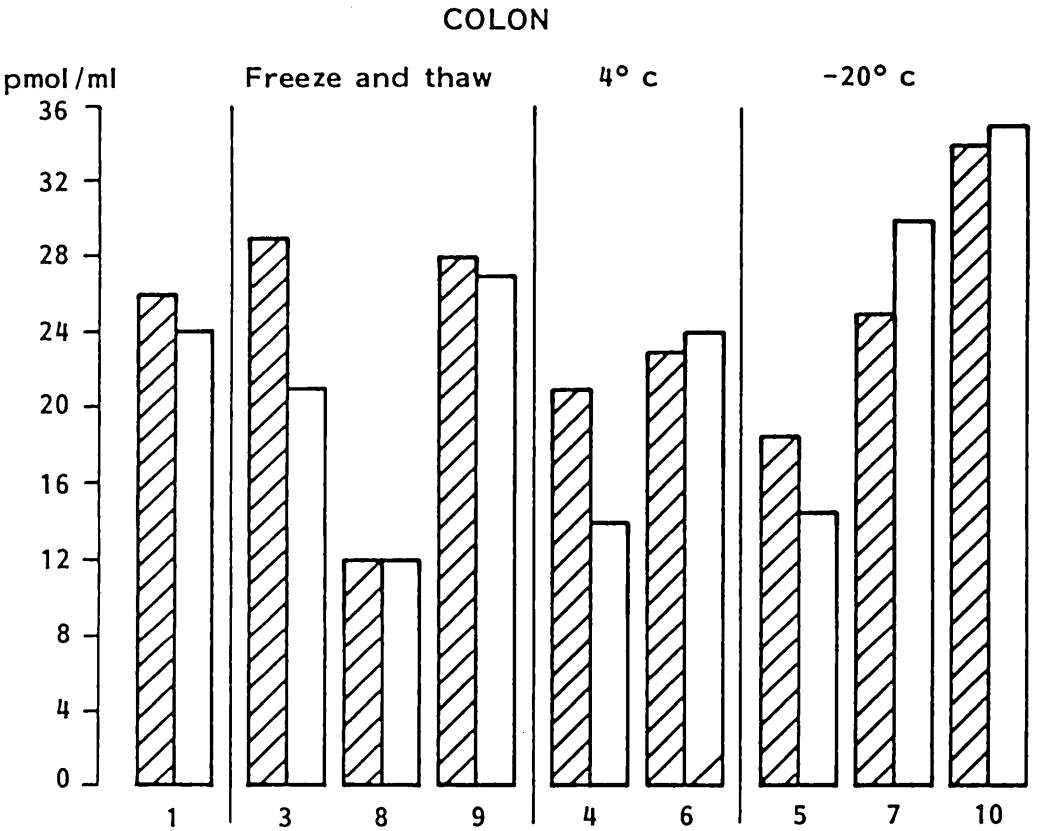


FIGURE 2:6(a) Extract stability study.

Concentrations of VIP (pmol/ml) in acid extracts are plotted for various storage conditions.

Rat 1

Rat 2

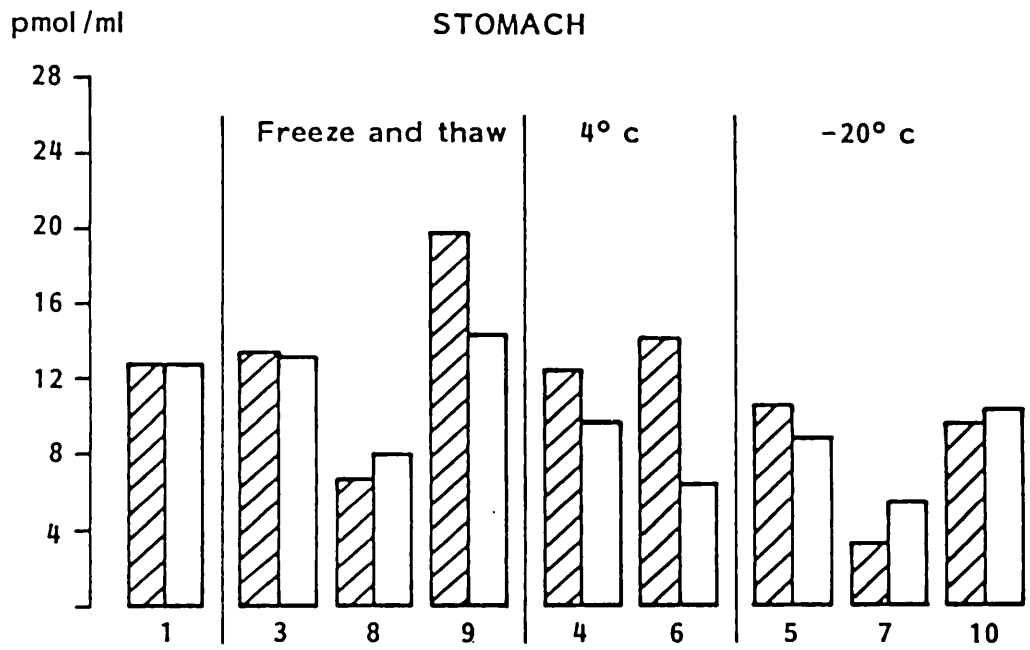

pmol/ml
STOMACH
28
24
20
16
12
8
4
Freeze and thaw
4° c
-20° c
1
3
8
9
4
6
5
7
10

FIGURE 2:6(b)

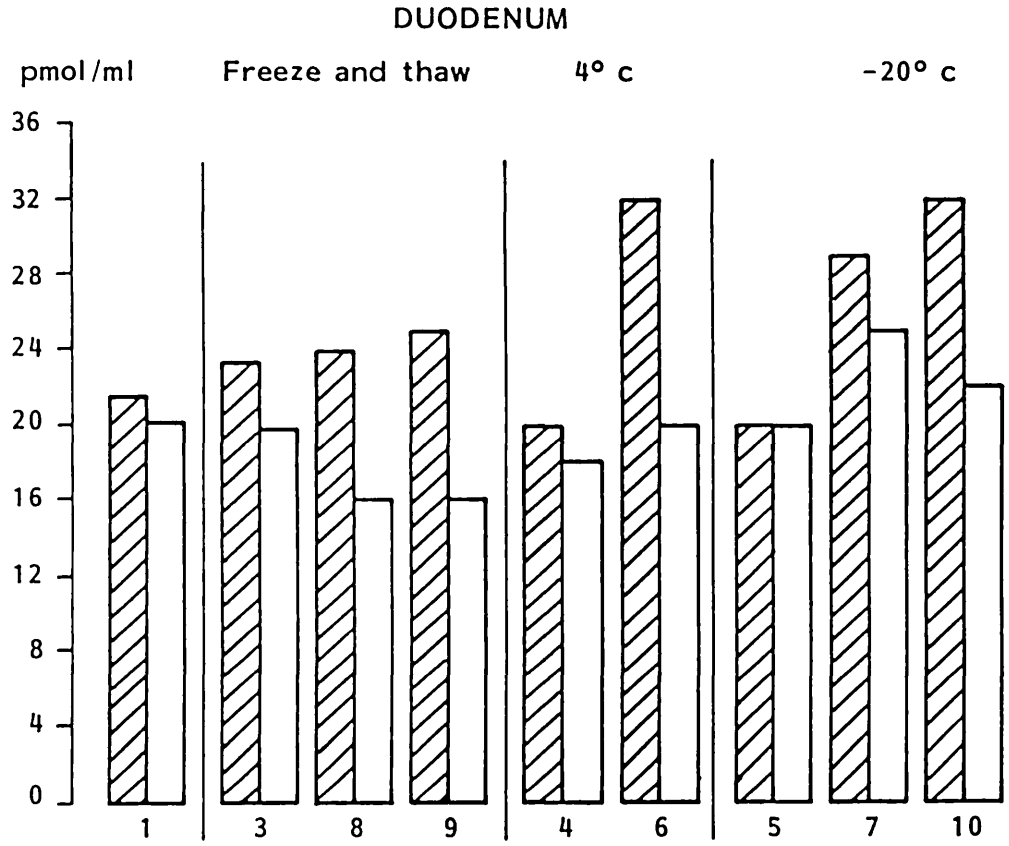

DUODENUM
pmol/ml
Freeze and thaw
4° c
-20° c
36
32
28
24
20
16
12
8
4
0
1
3
8
9
4
6
5
7
10

FIGURE 2:6(c)

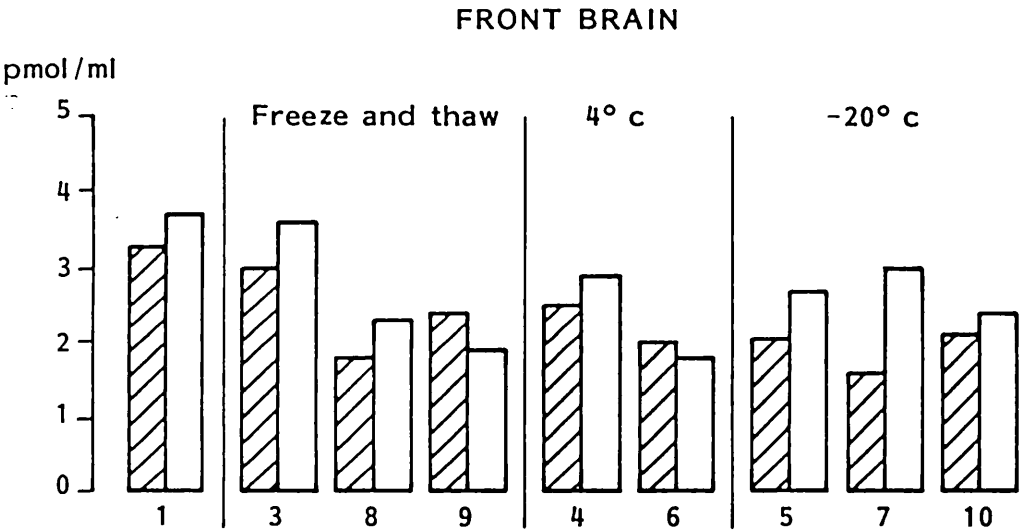

FRONT BRAIN
pmol / ml
5
4
3
2
1
0
Freeze and thaw
4° c
-20° c
1
3
8
9
4
6
5
7
10

FIGURE 2:6(d)

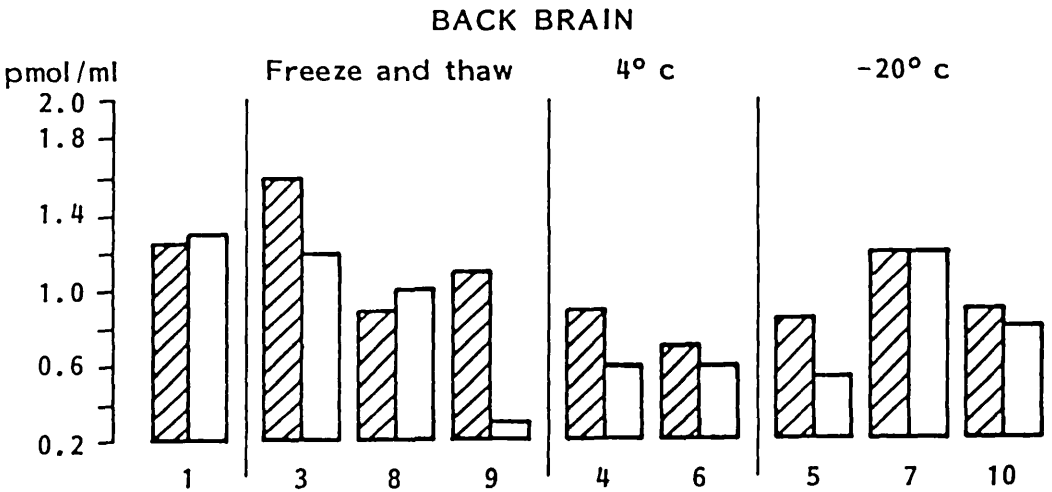

BACK BRAIN
pmol/ml
Freeze and thaw
4° c
-20° c
2.0
1.8
1.4
1.0
0.6
0.2
1
3
8
9
4
6
5
7
10

FIGURE 2:6(e)

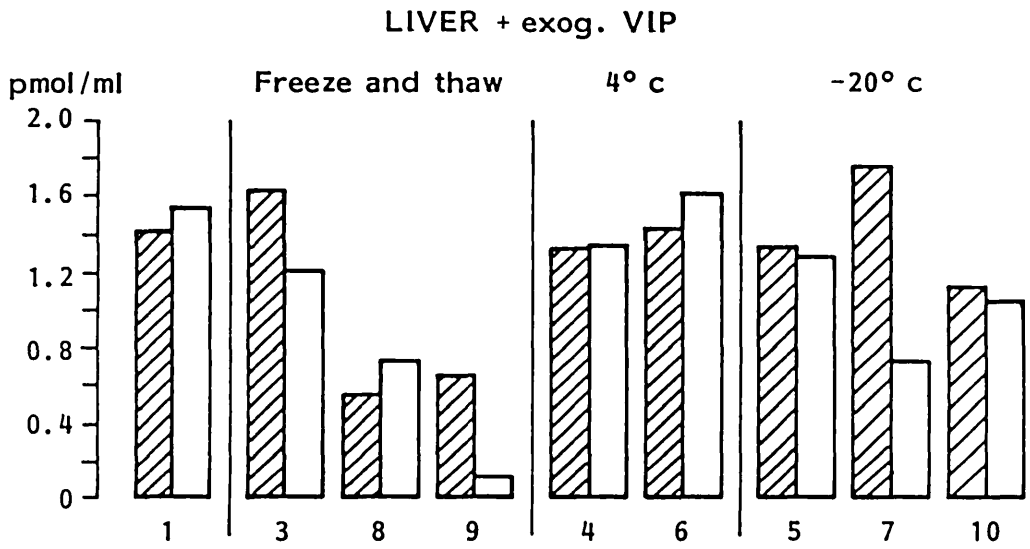

LIVER + exog. VIP
pmol/ml
Freeze and thaw
4° c
-20° c
2.0
1.6
1.2
0.8
0.4
0
1
3
8
9
4
6
5
7
10

FIGURE 2:6(f)

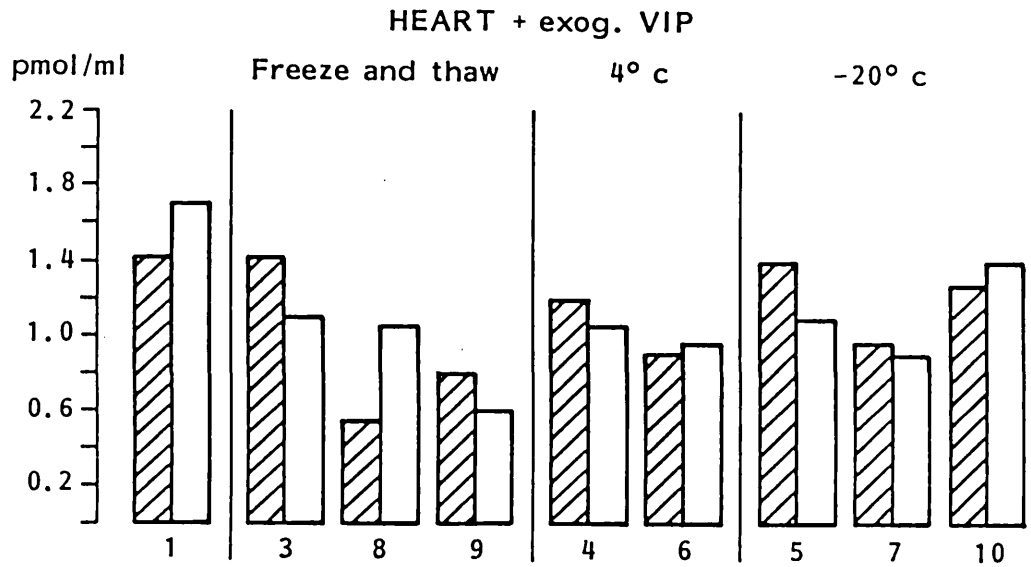

HEART + exog. VIP
pmol/ml
Freeze and thaw
4° c
-20° c
2.2
1.8
1.4
1.0
0.6
0.2
1
3
8
9
4
6
5
7
10

FIGURE 2:6(g)

KIDNEY + exog. VIP

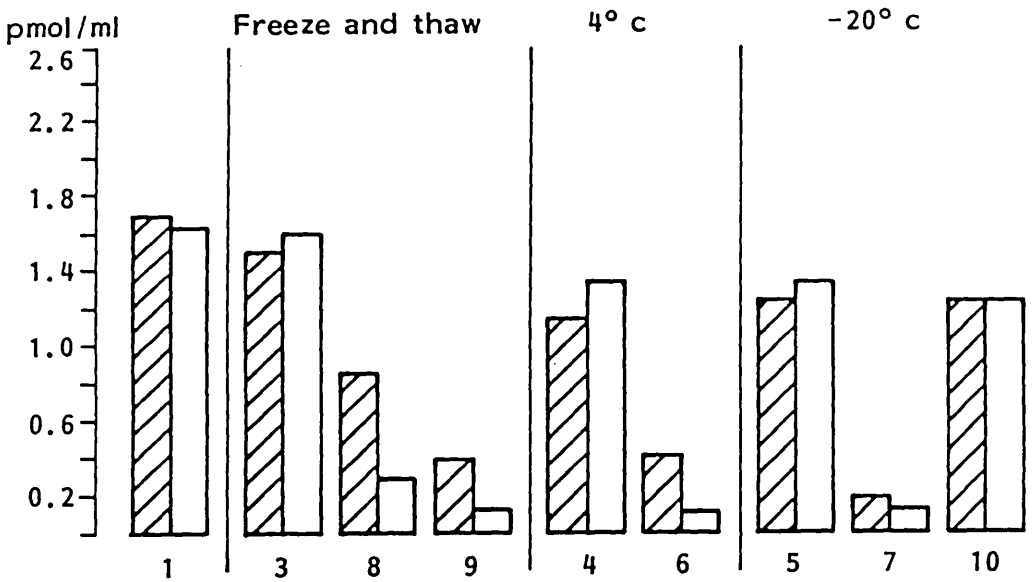


FIGURE 2:6(h)

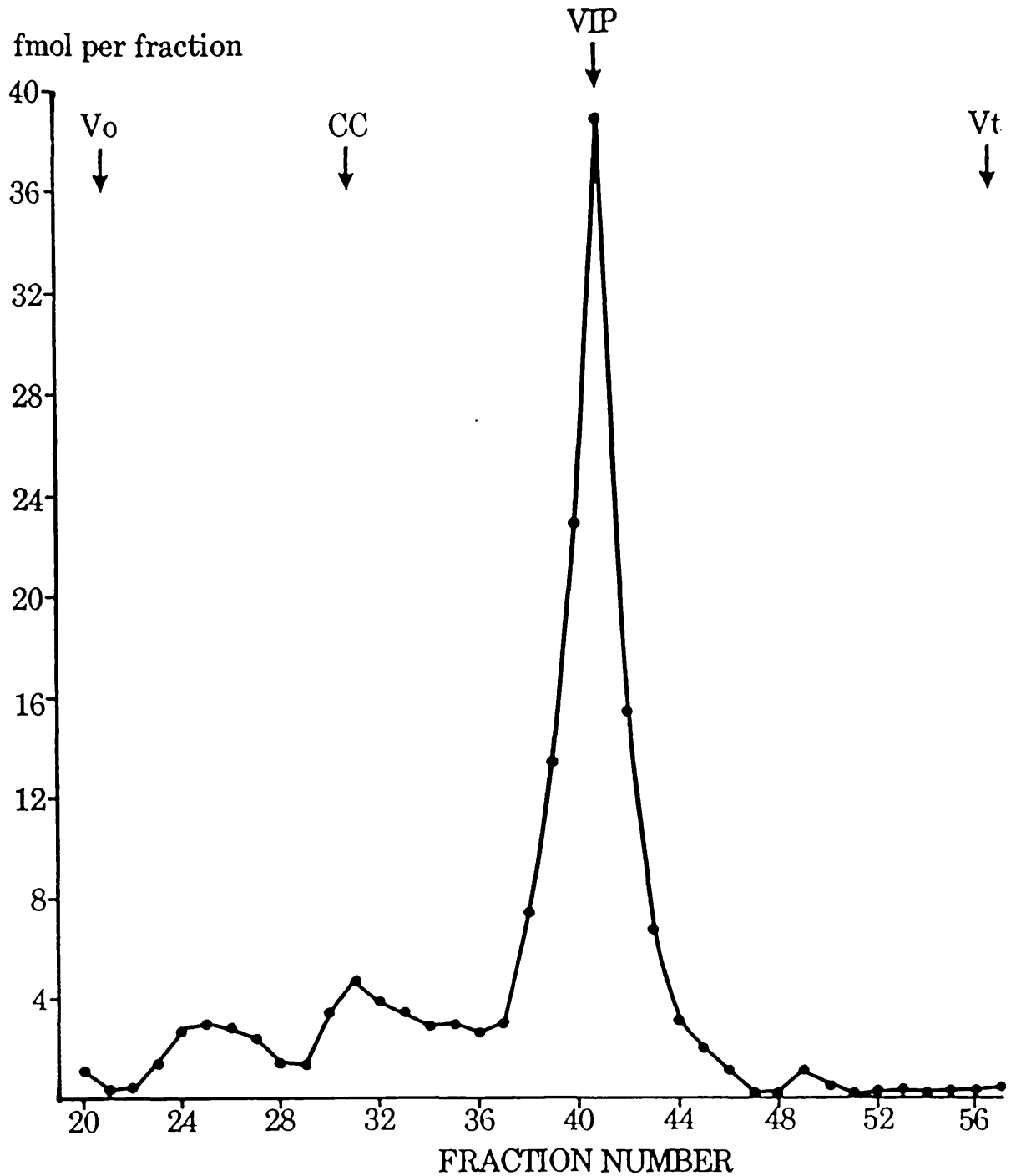


FIGURE 2:7 G-50 gel permeation chromatographic profile of VIP-immunoreactivity extracted from human urinary bladder (dome). Molecular size markers are Vo: dextran blue; CC: cytochrome C; Vt: Na ^{125}I.

VIP denotes the position of pure porcine VIP. Recovery from column = 93%.

CHAPTER 3

DISTRIBUTION OF VIP IN THE GENITALIA

The discovery that VIP was localized within neural tissue (Bryant et al 1976; Larsson et al 1976; Said & Rosenberg 1976) resulted in numerous investigations of the distribution of VIP-containing nerve fibres in peripheral organs, including the genitalia.

Female Genitalia

VIP has been found to be one of the most abundant and widely distributed peptides in the female genital tract. The first demonstrations of VIP in this organ were predominantly immunohistochemical (Larsson et al 1977a; Alm et al 1977,1980a,1980b; Bishop et al 1979; Ström et al 1981). In all species studied VIP-containing fibres were seen to innervate epithelial cells, blood vessels and smooth muscle cells. As VIP was generally considered to exist exclusively in nerve fibres, the relationship of VIP-containing innervation to the parasympathetic and sympathetic systems within the female genital tract was of interest.

The mammalian female genital organs are known to be well supplied by parasympathetic and sympathetic fibres. Adrenergic and cholinergic nerve fibres have been demonstrated in these organs, with the adrenergic fibres predominating (see Bell 1974). In both rat and

man there is an increasing density of adrenergic innervation from the body of the uterus to the cervix, but no gradation of innervation density has been reported in other species studied. In addition to the adrenergic innervation, acetylcholinesterase-positive nerves have been reported to be sparsely distributed in the uterine musculature of the rabbit, guinea-pig and cat, and more densely in that of rat. In man, acetylcholinesterase-positive fibres show an overall distribution similar to that of the adrenergic fibres, being rare in the uterine body and numerous in the cervix and isthmus of the Fallopian tube. Adrenergic and cholinergic cell bodies have been described in the paracervical ganglia, with the cholinergic ganglionic cells predominating.

The presence in the genital tract of an ultrastructural autonomic profile quite distinct from the cholinergic and adrenergic types was first reported by Hervonen in 1973. In 1977 Sporrong et al, on the basis of the characteristics of the synaptic vesicles in the cat uterus, described three types of axon varicosities - adrenergic, cholinergic and a small number having only large-sized vesicles with a moderately electron-dense content. They suggested these varicosities may belong to a system usually designated "p-terminals" (Baumgarten et al 1970), and proposed to contain polypeptides. Since that time at least six different polypeptides, including VIP, have been shown to be present in the innervation of the female genital tract.

More recent studies on the distribution of VIP in the female genital tract have included quantitative data as well as immunohistochemical data (Lynch et al 1980; Ottesen 1983; Ottesen et al 1982b; Helm

et al 1981; Goodnough et al 1979). VIP is particularly abundant in the vagina, cervix and clitoris, present in lower concentrations in the uterine body and Fallopian tube and rare or absent in the ovary. The highest density is observed around the natural sphincters, ie the internal and external cervical os and the isthmic part of the Fallopian tube.

However, there are discrepancies in the reported human quantitative data (see Discussion, this chapter). A study was, therefore, first undertaken to identify the sources of error and, by taking these into account, arrive at an accurate regional distribution of VIP in the normal human female genital tract. In addition, a detailed study of the regional distribution of VIP in rat, mouse and guinea-pig female genitalia was made for the first time.

Male Genital Tract

The human, rat, cat and monkey male genital tracts are supplied by sympathetic and parasympathetic nerve fibres (see McConnell et al 1982). Using light and electron microscopic histochemical methods, numerous adrenergic fibres have been demonstrated throughout the muscular layers, in the connective tissue and in the adventitia of most blood vessels. In the vas deferens and penis, acetyl-cholinesterase - positive nerve fibres are encountered less frequently at the light microscopic level than adrenergic fibres, and few typical cholinergic varicosities are seen in these organs by electron microscopy (see McConnell et al 1982). In addition, another type of nerve fibre was shown to be present in large numbers in each of the organs studied; it was proposed that this was

peptidergic.

In 1977 Alm et al first described the immunocytochemical distribution of VIP in male cat genitalia. VIP-containing fibres were found in particular abundance in the epididymis, prostate and vas deferens. Ganglia located close to or within the wall of the upper urethra were reported to contain numerous immunoreactive nerve cell bodies, and therefore suggested to be the origin of the fibres containing VIP that innervate these regions. Later in the same year, Larsson et al (1977b) mapped the distribution of VIP in the cat genital tract by immunocytochemistry and radioimmunoassay. The epididymis, prostate and seminal vesicle contained the greatest concentrations of VIP, but the penis also contained significant amounts. The fibres were seen to occur in association with the arterial supply and smooth musculature of these organs. In the human prostate and seminal vesicle, VIP-containing nerves were shown by immunocytochemistry to be mainly associated with the epithelium. Only a few VIP nerves were detected in the smooth muscle and connective tissue layer surrounding the epithelium.

The quantitative distribution of VIP in human male genitalia was reported in 1981 (Polak et al; Larsen et al). Polak et al carried out a more extensive study and found VIP in considerable quantity in all areas of the genital tract. VIPergic nerves were most densely concentrated in the penis around the pudendal arteries and in the erectile tissue of the corpus cavernosum. Local VIP-containing ganglia were seen in the penis, especially near blood vessels of the corpus cavernosum. In their study Larsen et al compared the VIP concentrations in cat and human genitourinary tracts. They found

levels in the cat to be generally higher than those in man, but in both species the vas deferens and prostate contained the highest concentrations. Considerable amounts of VIP were reported in the rabbit male genital tract by Willis et al (1981) and a comparison of the human and rabbit male genital tract was made (Willis et al 1983).

In the rat, the penile blood vessels and intrinsic smooth muscle of the penis were shown by immunocytochemistry to receive a VIPergic nerve supply. Most of the VIP innervation was found in the proximal portion of the rat penis (Dail et al 1983). Moreover, the major pelvic ganglion was shown to be a major source of VIP fibres to erectile tissue. Local VIP neurones were not found in the cavernous bodies.

In a previous study, Dail et al (1975) showed that cholinergic neurones tended to cluster toward the pelvic nerve end of the major pelvic ganglion, overlapping to a great extent the area occupied by VIPergic neurones to the penis. Preliminary staining studies showed that many penile VIP neurones were strongly positive for acetylcholinesterase suggesting that VIP and acetylcholine may coexist in penile neurones. This was the first, and is the only, report suggesting that VIP is co-stored with a classical transmitter in neurones of male or female genitalia, although their co-storage in other organshad been reported previously (see Lundberg et al 1982; Eckenstein & Baughman 1984).

A study was undertaken to examine species variations in the distribution of VIP in the male genital tract and to report for the

first time the quantitative distribution of VIP in guinea-pig and rat male genitalia. These studies were necessary prior to investigating the role of VIP in the physiology and pathology of the male genital tract (Chapter 7).

Materials and Methods

Human female genitalia specimens were obtained from six patients at Hammersmith Hospital undergoing hysterectomy for either fibroids or uterine prolapse. The age of the patients ranged from 39 to 49 years (mean age 42 years) and all were pre-menopausal. The phase of the menstrual cycle was documented. Immediately following resection, the tissues were divided into the following regions:- cervix, uterine body (subdivided into myometrium and endometrium), Fallopian tubes and upper portion of the vagina. Only regions of the organs exhibiting grossly and histologically normal appearance were included in this study. Each region was then divided, one half processed for immunocytochemistry and the other weighed and immediately extracted in boiling 0.5M acetic acid as described in Chapter 2. Tissue extracts were stored at -20°C until time of assay.

Adult female mice (n = 5), rats (n = 5) and guinea-pigs (n = 5) were killed with an overdose of ether. The genital tracts were removed and, following the removal of any fat and connective tissue, were dissected into vagina, cervix, uterus, Fallopian tube and ovary.

Adult male rats (n = 5) and guinea-pigs (n = 10) were also killed with an overdose of ether. Their genital tracts were removed, cleared of fat and connective tissue, and dissected into the following regions:- testis, penis, epididymis, prostate gland, vas deferens and seminal vesicle.

In the case of both male and female rat and guinea-pig genitalia and female mouse genitalia, an equal number of animals were killed and processed for immunocytochemistry.

For radioimmunoassay, the tissues were immediately weighed following dissection and extracted in boiling 0.5M acetic acid as described in Chapter 2. Extracts were stored at -20°C until time of assay for VIP.

RESULTS

Female Genitalia

The regional concentration of VIP in human female genitalia is shown in Figure 3:1. VIP was present in all regions, with the highest levels in the vagina and the lowest in the endometrium and Fallopian tube.

The regional concentrations of VIP in the female genitalia of mouse, rat and guinea-pig are tabulated (Table 3:1). The mouse was found to contain a higher concentration of VIP than the other two species, the cervix having the highest concentration. The guinea-pig demonstrated the lowest concentrations of VIP and the rat showed intermediate levels. The mouse ovary was not investigated but low concentrations of VIP were detected in the rat ovary and a higher level seen in the guinea-pig.

Male Genitalia

The distribution of VIP in the rat and guinea-pig male genitalia is shown in Table 3:2. The highest concentrations detected were in the vas deferens of both species but the prostate gland, seminal vesicle and penis demonstrated substantial levels. In general, the guinea-pig male genitalia contained less VIP than the rat and both species contained considerably less than human genitalia.

DISCUSSION

Female Genitalia

VIP has been quantified by radioimmunoassay in human, mouse, rat and guinea-pig female genitalia. Of the four species, the mouse contained the highest concentration. The guinea-pig exhibited very low concentrations of VIP in the female genitalia, which is in accordance with other studies. In the parallel immunocytochemical studies, VIP was localized exclusively to nerve fibres which innervated blood vessels, smooth muscle cells and seromucous

glands. It was noteworthy that towards the utero-tubal junction, VIP fibres were more frequent than in the rest of the uterus. The VIP-containing fibres in the Fallopian tube were mainly distributed in the muscular coat of the tube and were particularly concentrated in the circular muscle layer. It was noticeable that in the cervicovaginal junction of the rat, in particular, clusters of ganglion cells were found, and about fifty percent of these cells were immunoreactive to VIP antisera. Significantly, in all the samples studied there were no apparent differences in the frequency and distribution patterns of the VIP-immunoreactivity in different phases of the menstrual cycle.

There are discrepancies between the results obtained on the human genitalia and results reported by other investigators (Lynch et al 1980; Helm et al 1981; Goodnough et al 1979). It has been suggested that the discrepancies are due to differences between VIP assays used (Ottesen 1983). It is probable, however, that a major source of the differences is the use of non-optimal extraction procedures by the different investigators, including time delay between dissection of tissue and extraction (see Chapter 2). The levels of VIP measured in the rat genitalia here compare favourably with the results of Ottesen et al (1982b), although only two regions were investigated in their study.

The role of VIP-containing fibres in the genital tract has not been firmly established, but on the basis of their quantitative and histological distribution, functional roles may be hypothesized.

In the vagina and cervix, the significantly higher concentrations of VIP indicate it may be responsible in part for the increase in blood flow and mucus production that occurs during sexual activity (Wagner & Levin 1980). In fact, VIP concentrations have been found to increase in the female peripheral circulation during sexual arousal (Ottesen et al 1982b). In addition, the effect of local intra-arterial infusion of VIP on the myometrium of rabbit and goat was to increase local blood flow (Carter et al 1981; Ottesen & Fahrenkrug 1981). In the vagina of non-pregnant women, an increase in blood flow was seen during infusion of VIP (Ottesen et al 1983a).

The particularly dense innervation of the utero-tubal junction seen by immunocytochemistry in these studies is significant. Here the VIP fibres are associated mainly with the non-vascular smooth muscle layer. Both local nerve stimulation (Helm et al 1982; Murcott & Carpenter 1977) and infusion of VIP in vivo (Fredericks & Lundquist 1983) markedly inhibit contractile activity in this region. Pharmacologically, Walles et (1980) and Owman et al (1983) showed VIP to produce a dose-dependent reduction in spontaneous motor activity of human tubal isthmus muscle strips. These actions of VIP on the smooth muscle of the Fallopian tube may relate to the observation of Lindblom et al (1979) that in the human, adrenergic

nerves and nerves liberating an unidentified transmitter substance control contractility of this region. They proposed a concept of a specific sphincter-like function of the ampullary-isthmic junction of the human oviduct, controlling ovum transport. In rabbits, at least, oedema has been shown to occur in the utero-tubal junction (Mastroianni 1977), thereby delaying the passage of ova. As VIP is a powerful vasodilator and oedema potentiator (Williams 1982), it may be hypothesized that the VIP innervation of the utero-tubal junction in female genitalia affects retention times of ova within the Fallopian tube during fertilization, in normal and pathological states.

Studies on the action of VIP on smooth muscle from other regions of the female genital tract (uterine body, cervix and vagina) show a dose-dependent inhibitory effect (Stjernquist & Owman 1984; Ottesen 1981; Ottesen et al 1982b,1983a,1983b). The high concentrations of VIP in these regions make a study of its role in pregnancy and parturition of considerable interest (Goodnough et al 1979; Ottesen et al 1982b)

Male Genitalia

In this investigation, VIP in the male genitourinary tract of rat and guinea-pig has been quantified for the first time. The levels are considerably lower than those found in man and cat, but, in the parallel immunocytochemical studies, in all species the terminals of

the VIP-containing nerve fibres were seen to innervate vascular and non-vascular smooth muscle as well as epithelial cells. In the erectile tissue of the penis, VIP- containing varicose nerves were found in association with arteries and arterioles. In the prostate gland and seminal vesicle, VIP nerve fibres were also seen in the connective tissue and following the folds projecting into the lumen of the glands. The implication of these findings is that VIP may play a neurotransmitter/modulator role in genitourinary blood flow, motility and secretion.

A number of studies of the effect of VIP on smooth muscle specimens from the vas deferens and corpus cavernosum in vitro support such a role. VIP was observed to display a dose-related relaxatory effect in both regions (Larsen et al 1981; Willis et al 1981; Sjöstrand et al 1981).

The direct effect of VIP on penile blood flow has been demonstrated. Bolus injection of the peptide into human corpus cavernosum induced various degrees of tumescence or full erection. A concomitant decrease in the local cavernous elimination of ^{133}Xe was demonstrated. This reduction in penile blood flow was thought to account partly for the increased concentration of VIP detected in cavernosal blood from the erect penis (Ottesen et al 1984). A full account describing the possible role of VIP in mammalian penile erection is presented in Chapter 7.

The high level of VIP seen in the rat and guinea-pig epididymis, seminal vesicle and prostate, and the immunocytochemical localization of VIP to fibres supplying epithelial cells would suggest a role for VIP in the local regulation of secretion from these organs, as in other secretory epithelium (Lindkaer Jensen et al 1978; Ahrén et al 1980).

In conclusion, these studies have demonstrated the wide distribution of VIP in the genital tract. In some species, higher concentrations of VIP were seen when compared to those reported previously which, it is proposed, is due to a reduction in a number of sources of error in the processing of tissues. These observations lead to proposals of the role of VIP-containing fibres, a number of which are supported by physiological studies.

	Mouse (n = 5)	Rat (n = 5)	Guinea-Pig (n = 5)
Cervix	((89.1 ± 17.2	12.4 ± 1.9	1.7 ± 0.6
Vagina	(	12.4 ± 2.2	1.8 ± 0.3
Uterus	57.4 ± 14.8	9.1 ± 1.9	1.5 ± 0.5
Fallopian Tube	4.3 ± 4.0	4.6 ± 0.8	1.9 ± 0.9
Ovary	Not Done	0.6 ± 0.2	2.3 ± 1.1

TABLE 3:1 Mean concentrations (pmol/g) (± standard error of the mean) of VIP in the female genitalia of mouse, rat and guinea-pig.

	Rat	Guinea-Pig
	(n = 5)	(n = 10)
Testis	< 0.3	1.75 ± 0.2
Penis	4.6 ± 0.2	2.7 ± 0.1
Epididymis	4.1 ± 0.4	3.9 ± 0.6
Prostate Gland	2.0 ± 0.2	5.3 ± 0.5
Vas deferens	16.4 ± 2.4	6.3 ± 0.5
Seminal Vesicle	4.8 ± 0.4	4.7 ± 0.5

TABLE 3:2 Mean concentrations (pmol/g) (± standard error of the mean) of VIP in the male genitalia of rat and guinea-pig.

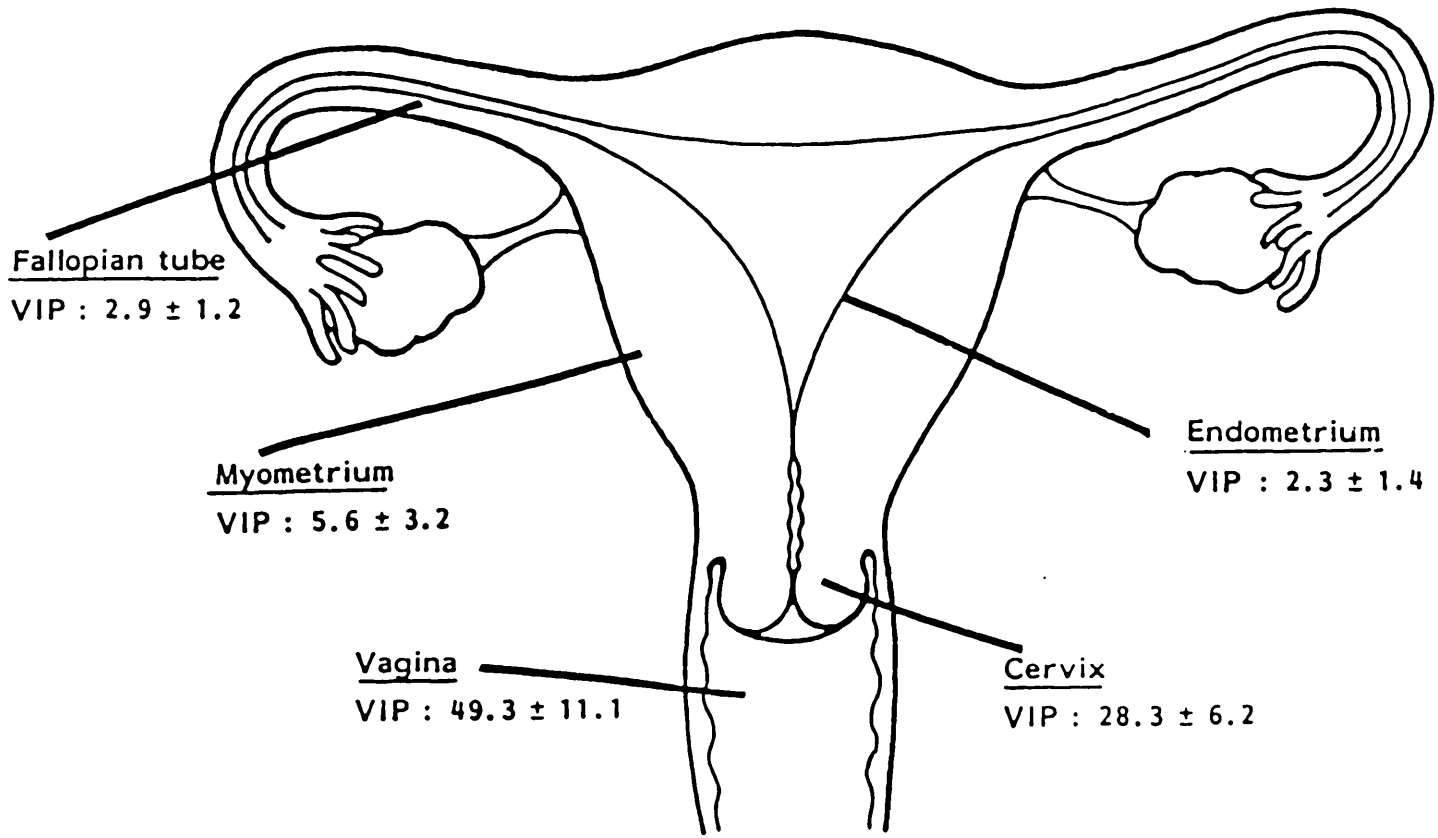


FIGURE 3:1 Regional concentration of VIP (pmol/g) in human female genitalia.

CHAPTER 4

DISTRIBUTION OF VIP IN THE URINARY TRACT

INTRODUCTION

The mammalian urinary tract is richly innervated by parasympathetic and sympathetic fibres within the pelvic and hypogastric nerves and entering the organ following the course of the blood vessels (see El-Badawi & Schenk 1966; McConnell et al 1982; Ek et al 1977).

Adrenergic and acetylcholinesterase-positive fibres are present in all mammalian bladder regions, the proximal urethra and the ureter. In general, the greatest number of fibres of either type are seen in the trigone, with fewer fibres in the anterior base and fewest in the bladder body. Although the basic pattern of innervation is similar in all species, certain variations exist in the relative abundance and arrangement of epithelial and subepithelial fibres. In all species studied, the muscularis has a uniform cholinergic innervation but a variable adrenergic innervation. In the cat, many adrenergic fibres innervate the bladder base, but the rat shows a smaller degree of adrenergic innervation. Dog, monkey and man demonstrate intermediate levels. In the ureter there is a progressive increase in the density of the nervous network away from the kidney in all species, with the terminal ureter being richly innervated (Schulman et al 1972).

In the ureteral musculature, the nerve fibres are related to small

bundles of muscle so that the nerve-muscle ratio is low. In the bladder, the innervation is related to individual muscle cells, producing a higher nerve-muscle ratio.

The anatomical arrangement of the sympathetic and parasympathetic pathways to the urinary tract must be considered before a study of the origin of peptide-containing fibres is made.

The secondary ganglia of the sympathetic system mainly lie in the inferior mesenteric plexus and those of the parasympathetic mainly in the pelvic plexus, although sympathetic 'short' neurones also exist in close vicinity of the bladder (Sjöstrand 1965). After the hypogastric and pelvic nerves meet to form the pelvic plexus, sympathetic and parasympathetic postganglionic nerves are randomly mixed.

Cholinergic and adrenergic ganglion cells also exist in all layers of the bladder wall, with particular abundance in the bladder base and around the ureteral orifices. They are extremely sparse or absent in the middle and upper ureter (El-Badawi & Schenk 1968). Some investigators report an absence of intramural ganglion cells in the rat urinary bladder although clusters of cells are frequently found outside the bladder wall in the connective tissue surrounding the base of the bladder (Alm & Elmer 1975). Where both types of ganglion cells are present, evidence exists for an interaction between the cholinergic and adrenergic elements in the bladder wall (El-Badawi & Schenk 1966,1968). This pattern of the autonomic innervation explains why it is impossible to denervate the urinary tract by simple section of the extrinsic nerves.

The presence of another population of nerve fibres in the bladder, which did not fit the morphological criteria for either cholinergic or adrenergic fibres, were observed by McConnell et al (1982). They were hypothesized to be peptidergic.

The occurrence of VIP in the urinary tract was first reported by Larsson et al in 1977. By immunocytochemistry it was found to be intimately associated with blood vessels and smooth muscle and also to form a subepithelial nerve plexus. Radioimmunoassay of ureter, bladder and urethra of the cat revealed the highest concentration of VIP in the ureter. A more extensive immunocytochemical study conducted by Alm et al (1977) demonstrated numerous nerves containing VIP in the trigone, ureterovesical junction and upper urethra of the cat and also ganglia staining for VIP located close to or within the wall of the trigone and upper urethra. Ganglia containing VIP-immunoreactive nerve cell bodies were reported in the distal ureter, ureterovesical junction, proximal urethra and trigone in the guinea-pig and cat (Wharton et al 1981; Alm et al 1980b).

In the studies reported here, a systematic investigation was made of the quantitative distribution of VIP in the rat, cat, rabbit and human urinary tract. Further experiments were performed to determine the origin of VIP-containing fibres in the rat bladder, including selective chemical and surgical denervation procedures.

Methods

Comparative Distribution in Rat, Cat, Rabbit and Human

The bladder, distal ureter and upper urethra were removed from five

rats (three female, two male) and five rabbits (male) which had been killed by cervical dislocation. Following the removal of fat and connective tissue, the bladders were dissected into dome and trigone. Four cats (two female, two male) were killed by intraperitoneal injection of sodium pentobarbitone and the bladder, urethra and distal ureter were removed. The bladders were dissected into the following regions: dome, trigone, lateral wall and bladder neck.

Human urinary bladders were obtained at cystectomy (n = 7; whole thickness of bladder) and per urethra bladder biopsy (n = 8; mucosa, submucosa and about one-third muscle layer) from patients with functionally normal bladders, suffering from either bladder carcinoma or urinary sphincter dysfunction.

In the case of rat and rabbit tissue, an equal number of animals were processed separately for immunocytochemistry. Cat and human tissue was divided, one half was processed for radioimmunoassay and the other for immunocytochemistry.

Cat, rabbit and rat urinary tract tissues were extracted in 0.5M acetic acid immediately following dissection (see Chapter 2). Human tissue was stored in liquid nitrogen following surgery and then all the samples were extracted simultaneously (as described in Chapter 2) prior to assay for VIP.

Selective Surgical Denervations (carried out by Dr J F B Morrison, Dept Physiology, Leeds University)

Adult female rats (n = 24) were operated on under sodium pentobarbitone general anaesthesia. The denervation of the urinary bladder was carried out in three ways and each was performed on six rats. In one group the sympathetic pathways were sectioned bilaterally at the points before they join the pelvic plexus. In the second group the parasympathetic pathway was sectioned unilaterally at a point just proximal to the pelvic nerve ganglion. This method, however, inevitably left certain parasympathetic ganglia and the sympathetic ganglia intact distal to the sectioning point. In a third group of rats both sympathetic and parasympathetic pathways were sectioned at the above points. Sham-operations were performed on the fourth group of six rats, to serve as controls. The design of the operation is shown in Figure 4:1.

The rats were killed by cervical dislocation eight days after the operations. The bladders were removed and extracted in boiling 0.5M acetic acid, prior to assay for VIP. The bladders from the pelvic and combined pelvic and hypogastric denervated groups were divided into left and right halves before extraction.

Neonatal Capsaicin Treatment

Separate groups of rats of the same litter were injected on day 2 after birth with capsaicin (50 mg/kg body weight) (n = 6) or vehicle solution (10% ethanol, 10% Tween 80 in isotonic saline). The animals were killed at three months of age by cervical dislocation

and the bladders were removed, divided into dome and trigone and extracted.

RESULTS

VIP was detected in all regions of the rat, cat, rabbit and human urinary tract (see Table 4:1). The cat demonstrated the highest levels and the rat the lowest, with human and rabbit having intermediate levels. In the cat, rat and rabbit, the urethra was the region demonstrating the highest concentration of VIP. In all species, the concentration of VIP was greater in the trigone than in the dome of the bladder. In the cat, where the bladder neck was investigated, the concentration of VIP was very similar to the concentration in the trigone.

The concentration of VIP in the rat bladders was increased after all three types of surgical denervation when compared to the sham-operated groups, but to different extents (see Figure 4:2). After unilateral parasympathetic denervation, the concentration of VIP was markedly increased in both the left and right halves of the bladder ($p < 0.05$, Student's t test). The bilateral hypogastric denervation caused a slight, although non-significant increase in the concentration of VIP. Sectioning of both nerve types led to a considerable, although non-significant, increase in the concentration of VIP in both halves of the bladder, but the increase was less marked than that caused by parasympathetic denervation alone.

Neonatally administered capsaicin had no effect on the concentration of VIP in the trigone and dome of the rats when compared to their vehicle-treated littermates (see Figure 4:3).

DISCUSSION

The quantitative distribution of urinary tract VIP has been demonstrated and compared in 4 mammalian species: rat, rabbit, cat and human. Variations between species have been demonstrated. The cat was found to contain the highest overall concentration of VIP and the rat the lowest. The parallel immunocytochemical studies localised the VIP to nerve fibres beneath the transitional epithelium, innervating the non-vascular smooth muscle and surrounding most of the blood vessels. A rich VIP-immunoreactive nerve supply was also found in the lamina propria, where, apart from a few blood vessels, no apparent muscular or glandular structures were present. This could indicate a possible sensory function for VIP. Scattered ganglia in the trigone, and occasionally in the dome of the cat, rabbit and human were found to be immunoreactive to VIP antisera. A smaller number were observed in the rat. These ganglia, small in size and few in number, were located in the muscle layer and the adventitia. In addition, scattered VIP-containing ganglion cells were seen around the distal ureter and ureter-bladder junction.

The high concentration of VIP in the detrusor muscle of the mammalian urinary bladder may indicate a role for this peptide in the control of bladder contractility. The action of VIP on urinary tract smooth muscle is conflicting (see Chapter 6), although most reports favour an inhibitory effect of the peptide (Larsen et al 1981; Klarskov et al 1984; Andersson et al 1983; Hills et al 1984; Levin & Wein 1981). The occurrence of VIP-containing nerve fibres around blood vessels would suggest another role in the urinary tract - the control of local blood flow.

It was found in this study that the concentration of VIP in the rat bladder was increased following the interruption of hypogastric and/or pelvic pathways. Previous experiments have shown that a decrease of acetylcholinesterase-positive nerves in the rat bladder occurred in less than one week of pelvic denervation but from two weeks onwards an outgrowth of the same nerve type was found (Alm & Jörgen 1981). Therefore, in this study, the time length chosen to investigate the changes after surgery was eight days. The implications of this increase are not immediately obvious.

The absence of any depletion of the VIP content after denervation suggests that its major origin is from local ganglia. The increase in the VIP content after the various denervations may represent a loss of drive of local cell bodies, or a compensatory re-innervation

phenomenon, ie replacement of lost fibres by outgrowth from the neighbouring intact nerve fibres. That neonatally administered capsaicin, which has been shown to cause selective degeneration of primary sensory neurones (Jancso et al 1977), had no effect on the concentration of VIP in the rat bladder when compared to control bladders, would provide further support for this local origin of VIP in the rat bladder. Capsaicin was effective at degenerating the primary sensory neurones of the bladder samples as indicated by the significant depletion (93%) in these tissues of substance P, a known marker of primary sensory neurones (Hökfelt et al 1975), when compared to controls. However, a minority of the VIP-containing fibres in the bladder may be afferents of pelvic nerve origin, evidence for which is presented in Chapter 5.

The increase of VIP in the bladder following pelvic nerve section alone was greater than when the hypogastric nerves were also sectioned. This suggests that part of the former increase was dependent on the integrity of the hypogastric nerves. It may indicate that the VIP-containing neuronal cell bodies in the pelvic and visceral plexuses, or inside the bladder wall, are influenced by sympathetic transmission. It has been reported that the sympathetic efferents facilitate or inhibit transmission through some pelvic non-adrenergic ganglion cells located proximal to the bladders of cat and rat (Craggs & Stephenson 1981; De Groat & Saum 1976; De Sy et al 1974; Hamberger & Norberg 1965). It is also possible that certain VIP nerve fibres originate from ganglia located higher in the sympathetic pathway, eg hypogastric plexus or sympathetic chain. This would be in keeping with the finding that large numbers of VIP-immunoreactive ganglia were present in the hypogastric plexus

of the guinea-pig (Hökfelt et al 1978).

VIP in the Unstable Human Bladder

Introduction

Bladder contractions should not occur during the filling phase of the micturition reflex. If present, the bladder is regarded as 'unstable' - a condition first described by Bates et al (1970) and defined in 1979 by Turner-Warwick as the presence of contractile activity that elevates bladder pressure above 15 cm H_2O. Between the ages of about ten and thirty, probably some 10-15% males and females have unstable bladder function; the incidence increases with age (Turner-Warwick & Whiteside 1982).

The quantitative distribution and localization of VIP in the bladder, as described earlier in this chapter, and its reported inhibitory activity on urinary tract smooth muscle (see Chapter 6), resulted in an investigation of the concentration of VIP in biopsy material taken from patients suffering from an unstable bladder.

Methods

Biopsies were taken by transurethral resection from the posterolateral wall of the bladder in 20 patients suffering from urodynamically proven idiopathic detrusor instability, and from 20 control patients undergoing endoscopic surgery for carcinoma or other problems (at Institute of Urology, London). There were 12 women and 8 men in the unstable group with an age range of 26 to 66

years (mean 49 yrs) and 8 women and 12 men in the control group with an age range of 20 to 68 years (mean 51 yrs). Normality in the control group was assessed by lack of symptoms, a normal voided volume chart, a normal flow rate and absence of residual urine.

Following removal each biopsy was divided and one half was extracted for radioimmunoassay of VIP (see Chapter 2) and the other was processed for immunocytochemistry and conventional haematoxylin and eosin histology.

Results

The concentration of VIP detected by radioimmunoassay was markedly decreased in the unstable bladder biopsies when compared to control biopsies ($p < 0.01$, Student's t test) (see Figure 4:4).

No significant changes were observed in the haematoxylin and eosin preparations.

Discussion

The results of this study reveal that there was a dramatic decrease of VIP in the unstable bladder in comparison with the controls. By immunocytochemistry, a marked decrease was seen in the density of distribution and the brightness of VIP-immunoreactive nerves in all of the unstable bladders in comparison with the controls. This decrease was much more marked in the muscle layer than in the lamina propria and around the blood vessels.

The aetiology of detrusor instability is unknown. The normal human detrusor contains relaxation-mediating beta-adrenoceptors, whereas contraction-mediating gamma-adrenoceptors seem to have little functional importance (Andersson & Sjögren 1982). Theoretically, therefore, besides an increased contribution of the non-adrenergic, non-cholinergic contraction component which is known to exist (Cowan & Daniel 1983), the unstable bladder may be associated with a decreased beta-receptor function, an increased gamma-receptor function, or an increased muscarinic receptor function. In addition, the contribution of the non-adrenergic, non-cholinergic relaxation component may be reduced in this condition. It is possible that in the bladder VIP acts to modify the background activity of the detrusor muscle, keeping it relaxed and maintaining normal bladder compliance until a voiding detrusor contraction is voluntarily initiated. There is evidence that VIP exerts a relaxant effect on human bladder smooth muscle (Sjögren et al 1985; Klarskov et al 1984; Andersson et al 1983). It may be hypothesized that a deficiency of VIP might be expected to lead to the unrelaxed and irritable detrusor of the human bladder.

TABLE 4:1 Mean concentrations (pmol/g) (± standard error of the mean) of VIP in the urinary tract of rat, cat and rabbit. Also listed are some values obtained from human bladder and ureter for which mean concentrations were only obtained from the bladder.

	RAT	CAT	RABBIT	HUMAN
	n = 5	n = 4	n = 5	n = 7
Dome	1.2 ± 0.3	9.2 ± 3.4	2.5 ± 1.5	37.0 ± 12.0+ 19.4 ± 5.9*
Trigone	11.3 ± 4.9	45.0 ± 16.0	5.5 ± 1.4	31.7 ± 8.3*
Lateral Wall	NOT DONE	6.3 ± 1.2	NOT DONE	NOT DONE
Bladder Neck	NOT DONE	38.0 ± 6.4	NOT DONE	NOT DONE
Distal Ureter	3.9 ± 1.8	38.0 ± 7.1	29.1 ± 3.6	40.6 ; 50.0
Urethra	22.5 ± 5.9	169.0 ± 19.6	80.4 ± 22.2	NOT DONE

+ = Surgical (whole thickness of bladder)

* = Per urethra biopsy (mucosa, submucosa, and about one-third muscle layer)

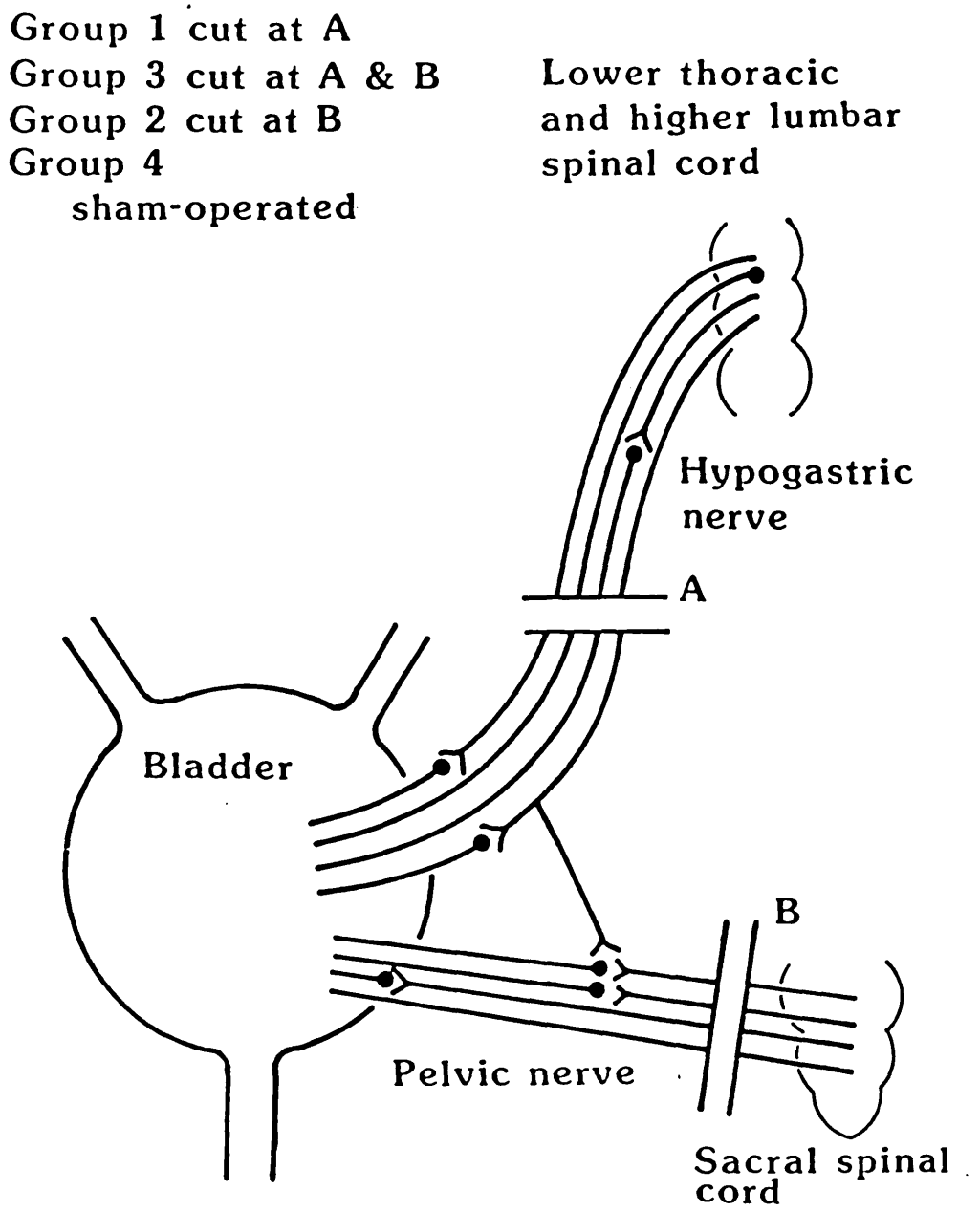


FIGURE 4:1 Design of surgical sectioning of nerve supply to the rat bladder.

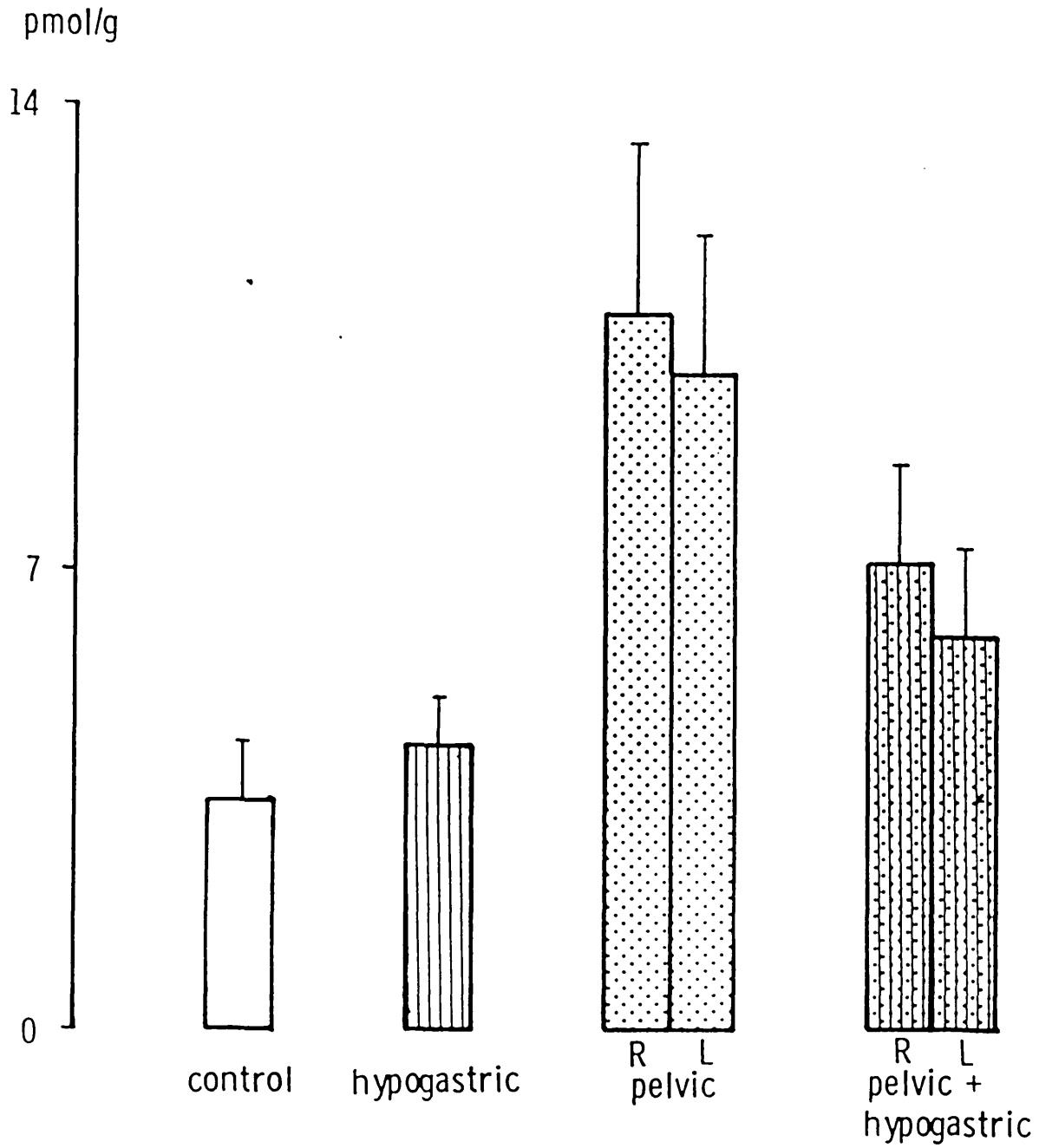


FIGURE 4:2 VIP (pmol/g) in rat bladder after surgery. (Mean ± SEM)

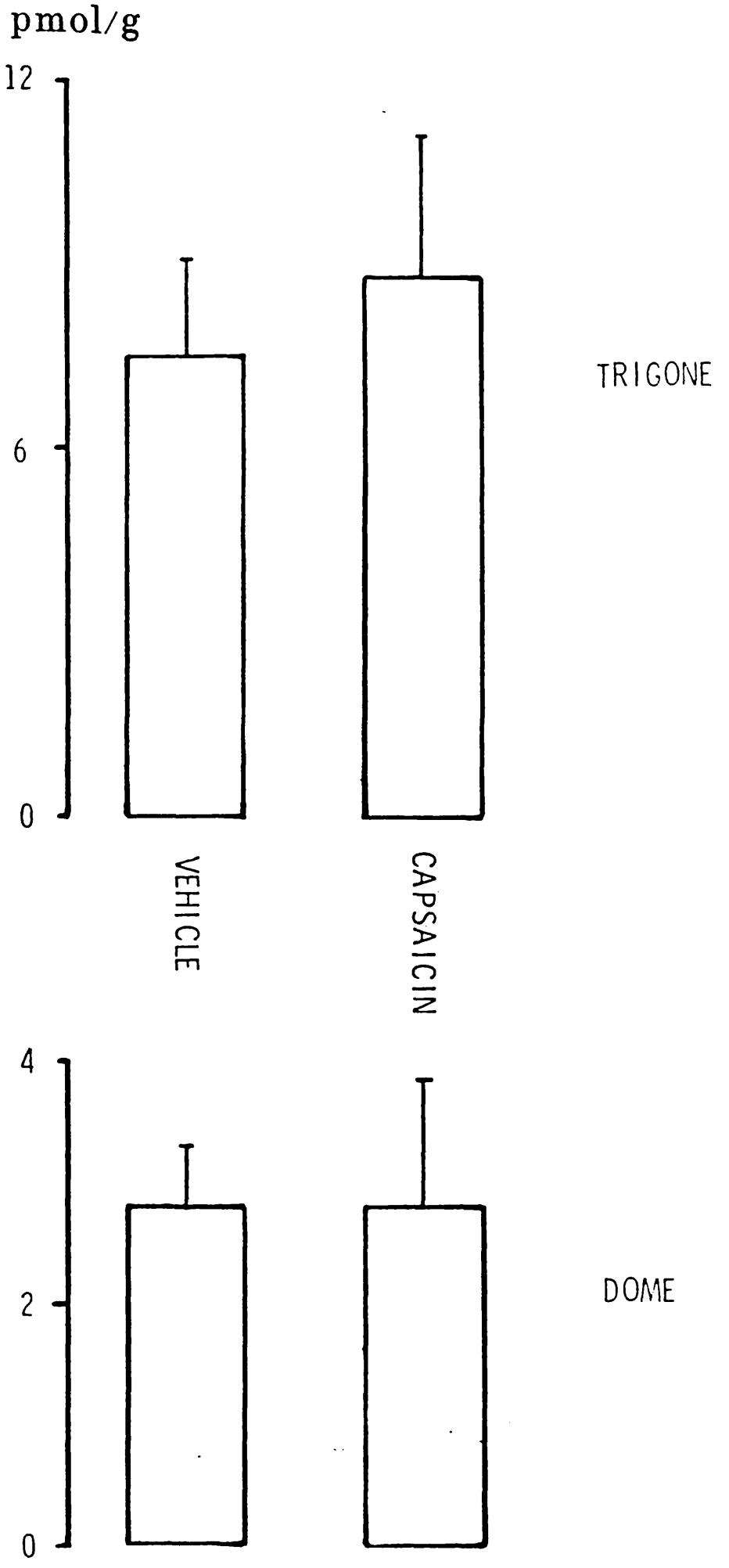


FIGURE 4:3 Effect of capsaicin on the VIP concentration (pmol/g) in the rat trigone and dome. (Mean ± SEM)

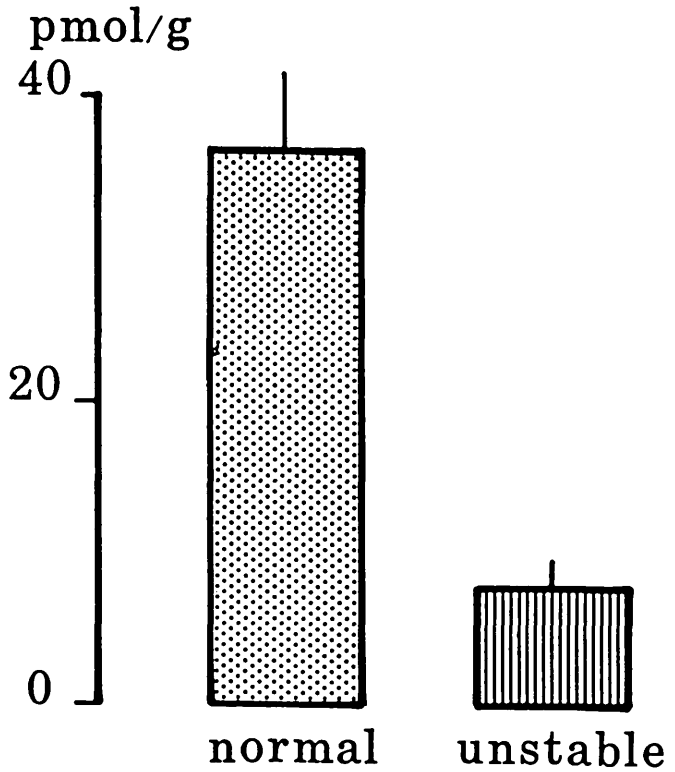


FIGURE 4:4 VIP (pmol/g) in the normal and unstable human bladder. (Mean ± SEM)

CHAPTER 5

THE ORIGIN AND DISTRIBUTION OF VIP IN MAMMALIAN SPINAL CORD

A rich VIP-containing innervation of the mammalian genitourinary tract has been described (see chapters 3 and 4) and evidence presented that the majority of VIP-containing fibres in the bladder were of local origin. In the studies described in this chapter, an investigation was made of the possibility that some of the VIP fibres in the genitourinary tract were sensory or of spinal cord origin.

Previous studies, using radioimmunoassay and immunocytochemistry, have documented the presence of VIP in mammalian spinal cord (Fahrenkrug & Schaffalitzky de Muckadell 1978; Emson 1979; Lorén et al 1979; Hökfelt et al 1980; Fuxe et al 1977; Gibson et al 1981; Jancsó et al 1981; Yaksh et al 1982). Although VIP had been demonstrated in vagal afferent neurones in the nodose ganglion (Lundberg et al 1978) and in afferent projections from the intestine to prevertebral ganglia (Dalsgaard et al 1983) no study had been made of VIP in neural pathways involved in genitourinary reflexes, and in particular within the sacral spinal cord.

A systematic investigation was therefore first carried out of the regional distribution of VIP along the entire length of the normal spinal cord of man (adult and neonate), cat and rat. The results of this study led to a series of experiments to demonstrate the

presence of VIP in afferent fibres travelling within the pelvic nerve of the cat. Unilateral lumbosacral dorsal rhizotomies were performed to interrupt the entry of afferent fibres into the lumbosacral spinal cord. By analogy with the depletion of substance P seen in sensory fibres in the rat spinal cord within ten days of sciatic nerve transection (Jessell et al 1979), unilateral pelvic nerve transection was undertaken to see if a similar phenomenon occurred in VIP-containing afferent fibres.

Finally, the thoracic spinal cord was hemisected in a series of cats to exclude the possibility that some of the VIP fibres in the sacral cord were in descending pathways of supraspinal origin which are known to control urinary reflexes (see De Groat 1975; Nathan & Smith 1958).

Methods

Human Spinal Cord

Human spinal cord specimens were collected from 8 adults (six males, two females; mean age 60 years, range 48 to 83 years) and neonate/foetal cases (three male, one female; mean gestational age 43 weeks, range 26 to 52 weeks) with no evidence of nervous system pathology. All cadavers were stored at 4°C within 2 hours of death and the spinal cords removed within 24 hours (by the pathologist on duty, Hammersmith Hospital). With the cadaver prone, the spinal musculature was removed allowing the laminae to be sawn through with an electric saw. The caudal dura, with the spinal cord in situ, was raised while the spinal roots were progressively cut. The meninges were dissected away. For radioimmunoassay, transverse slices (0.5

cm thick) from cervical, thoracic, lumbar and sacral regions were divided into ventral and dorsal regions by section through the central canal, and stored in liquid nitrogen until extraction and assay for VIP (see Chapter 2). (The human cord dissections were carried out by Dr P Anand, Hammersmith Hospital). For immunocytochemistry, transverse spinal cord slices from the four regions were taken.

Rat Spinal Cord

Four adult albino Wistar rats were killed with an overdose of sodium pentobarbitone (Sagatal). The spinal cords were removed immediately after a full length laminectomy procedure (by S Facer, Hammersmith Hospital). The dura and roots were dissected away and transverse slices of whole cord were taken from cervical, thoracic, lumbar and sacral regions and processed as described in Chapter 2 prior to assay for VIP.

Cat Spinal Cord

Three sets of anatomical studies were performed on cat spinal cord: (1) unilateral lumbosacral dorsal rhizotomy; (2) unilateral pelvic nerve transection; (3) lower thoracic cord hemisection. Surgical procedures were carried out by Dr J F B Morrison, Leeds University.

In the first experiment four adult mongrel cats were subjected to unilateral lumbosacral rhizotomy. They were anaesthetized with sodium pentobarbitone (Sagatal), and a lumbosacral laminectomy was performed. The dura was then split on one side and the lumbosacral

dorsal roots, L2 to S4, exposed. These roots were transected and the region packed with gelfoam (Upjohn). The muscle and skin layers were sutured. The cats were killed four weeks post-operatively by an overdose of sodium pentobarbitone. The spinal cord was dissected as described for human cord and the samples prepared for radioimmunoassay and immunocytochemistry.

In the second experiment a unilateral pelvic nerve transection was performed. Four adult mongrel cats were anaesthetized as above and the pelvic nerve branches adjacent to the urinary bladder exposed. These were then transected unilaterally and the skin sutured. Four weeks later the cats were sacrificed with an overdose of sodium pentobarbitone, and the spinal cord removed and dissected as described above. On this occasion, however, the dissected regions were not frozen but extracted fresh. Furthermore, in the sacral region, the S2 segment was dissected out for assay for VIP.

In the third experiment, four adult mongrel cats were anaesthetized as above, and an upper laminectomy performed and the dura cut to expose the thoracic spinal cord. With a scalpel blade, a hemisection was made in the T9-T11 region. The cord was packed with gelfoam and muscle and skin layers sutured. The animals were sacrificed two weeks later and the spinal cords were removed, dissected, and processed for assay for VIP as described above.

In these studies of cat spinal cord, separate but adjacent transverse slices of spinal cord were taken for immunocytochemical analysis.

Results

Human Spinal Cord

The regional concentrations of VIP in adult and perinatal human spinal cord are shown in Table 5:1. The concentration of VIP was extremely low in the cervical and thoracic regions, whereas the lumbar and especially sacral regions contained much higher concentrations; the dorsal levels significantly exceeded ventral concentrations ($p < 0.05$ Student's paired t-test). Perinatal cord showed a very similar pattern.

Immunocytochemical studies were in accord with the above results. There were few VIP-containing fibres in cervical and thoracic regions, and were seen only as small bundles in Lissauer's tract and the most dorsal portion of the substantia gelatinosa. VIP-containing fibres were more widespread in the dorsolateral lumbar cord and became abundant in the sacral cord, in the dorsolateral grey-white matter border and between the intermediolateral column and the central canal.

Rat Spinal Cord

The regional concentrations of VIP in rat spinal cord are shown in Table 5:2. The sacral spinal cord did not show markedly greater concentrations of VIP when compared to cervical cord.

Immunocytochemical studies showed sparse VIP innervation of the laminae I and II at all levels, and the distinctive pattern in the lumbosacral segments, although present, was not marked.

Cat Spinal Cord

The results of the unilateral lumbosacral rhizotomy experiment are shown in Table 5:3a. Contralateral to the rhizotomies, the pattern of regional VIP distribution was remarkably similar to that described above for human spinal cord, both by radioimmunoassay and immunocytochemistry. Ipsilateral to the rhizotomies, there was a marked reduction of extractable VIP in the dorsal lumbar and particularly sacral spinal cord. No change was observed in the other regions. By immunocytochemistry, a striking decrease was seen in the number of VIP-immunoreactive fibres from the ipsilateral dorsal horn of sacral segments, in laminae I and II, Lissauer's tract, and the area dorsal to the central canal. A few fibres remained, however, in the regions of the dorsal horn and intermediolateral column.

A similar result to the above was obtained ipsilateral to the transection of pelvic nerve branches supplying the bladder (see Table 5:3b). By immunocytochemistry, there was a dramatic loss of fibres in the distinctive pattern seen in the dorsal sacral region.

In contrast, thoracic cord hemisection in the cat failed to produce a change by either radioimmunoassay (Table 5:3c) or immunocytochemistry in the VIP distribution in the sacral spinal cord.

Finally, in accord with the low concentration of VIP seen in ventral cord, and lack of change there following the various surgical procedures, no VIP-staining was seen in motoneurones or parasympathetic preganglionic cell bodies in any of the three

species studied.

Discussion

A comparison of the regional distribution of VIP in the spinal cord of the three species studied shows a remarkable VIP-containing system concentrated in the lumbosacral region, particularly in man and cat. In general, a number of neuropeptides show an increase in caudal regions; this has been attributed to the caudal increase in the ratio of grey to white matter, the former being the area in which peptides are predominantly found. However, the pattern of VIP is still exceptional: whereas for other peptides, such as substance P and somatostatin, there is at most a five-fold increase in dorsal sacral concentration when compared to cervical levels, in the case of VIP there is a fifty-fold higher concentration in dorsal sacral cord when compared to dorsal cervical cord. Although this increase is not as marked in perinatal human cord, the same pattern is clearly seen. A recent immunocytochemical study of the human foetal and newborn infant spinal cord has confirmed this finding (Charnay et al 1985). The cat spinal cord provided a model for human cord, and hence the further anatomical studies discussed below (see Figure 5:1). The regional distribution in rat cord shows that species differences exist, but immunocytochemistry shows that the same VIP-containing system is present in the rat cord, albeit in sparse form. Other immunocytochemical studies have demonstrated the same finding (Basbaum & Glazer 1983; Sasek et al 1984).

The distinctive distribution of VIP in the human and cat lumbosacral spinal cord originally suggested that VIP-containing fibres could

originate from primary afferent fibres, spinal interneurones or descending supraspinal tracts. It was thought that at least some of the VIP fibres were primary afferents from pelvic visceral organs. There was a striking similarity between the immunocytochemical localization of VIP in cat and human lumbosacral cord and the central termination of fibres from the pelvic nerve of the cat, as labelled by retrograde horseradish peroxidase tracing (Morgan et al 1981). The pelvic nerve terminals in cat spinal cord were mostly collaterals of axons in Lissauer's tract that crossed laterally and medially around the dorsal horn, and were located in close proximity to preganglionic cell bodies and dendrites. These pelvic nerve afferents shared other features with the distribution of VIP-immunoreactive terminals - there was a periodic or metameric collection of afferents in the sacral cord, and they terminated rostrally and caudally beyond the limits of the sacral parasympathetic neurones.

The dramatic loss of this distinctive set of fibres and terminals, in conjunction with extractable VIP, after transection of ipsilateral dorsal lumbosacral roots and vesical branches of the pelvic nerve, supported the view that VIP in sacral spinal cord was present in some pelvic afferent fibres. The mechanisms underlying the depletion of some neuropeptides from sensory afferents following peripheral axotomy remain uncertain, but this depletion has helped to establish VIP as a selective marker of pelvic nerve afferents in sacral spinal cord.

Using immunocytochemistry alone, two other groups have confirmed that the VIP distribution in cat sacral cord corresponds to the

above description (Honda et al 1983; De Groat et al 1983). A more recent publication, also using immunocytochemistry alone, reports the presence of VIP immunoreactivity in thoracic segments of the cat spinal cord. However, the density of staining in this region was much lower than that observed in the sacral region (Kuo et al 1985).

The two other groups have also confirmed that the VIP fibres are mainly pelvic afferents. Honda et al (1983) have shown a similar ipsilateral decrease of VIP fibres in sacral cord by dorsal rhizotomy or ganglionectomy. In addition, they have demonstrated in cat sacral cord the presence of VIP-like immunoreactivity in dense core vesicles within axonal enlargements containing both large dense core and smaller clear round vesicles; synaptic connections were infrequently observed but, when encountered, were of the simple axodendritic type. The other group (De Groat et al 1983; Kawatani et al 1983) has used dye-tracing experiments combined with immunocytochemistry to demonstrate that VIP is located in some visceral afferent perikarya in the sacral dorsal root ganglia and also in terminals in the sacral autonomic nucleus.

Both these groups also agree with our results that not all the VIP-immunoreactivity is removed by deafferentation; a few fibres are preserved, especially in the region of the central canal. There is evidence in monkeys that visceral afferents do not synapse directly with autonomic efferent neurones (Petras & Cummings 1972), although they may do so in the sacral cord of the cat (Mawe et al 1984). In any case, visceral reflexes are mediated via interneurones, and involve supraspinal pathways (see De Groat 1975). Loss of supraspinal influences on micturition reflexes may be observed after

lesions that affect white matter pathways cephalic to the human lumbosacral cord (Nathan & Smith 1958). The location of the residual VIP in our studies was compatible with a presence in either interneurones, descending pathways or collaterals of ascending pathways. In one study which examined only the lumbar region of cat cord, cervical hemisection failed to produce a decrease in dorsal lumbar VIP levels (Yaksh et al 1982). However, as the VIP-containing system was present mainly in sacral cord, and the possibility existed that descending fibres crossed below the level of the cervical cord section, we undertook a study of lower thoracic hemisection in cat sacral cord. The lack of change suggested that the residual VIP after deafferentation was not likely to be in descending or ascending pathways, and was therefore probably in interneurones. Although this has not yet been examined in the cat, VIP-containing cell bodies have been observed in substantia gelatinosa and lamina X in the lumbar spinal cord of the rat after colchicine treatment, and in the dorsolateral funiculus of rat lumbar cord following rostral cord transection (Fuji et al 1983). Sasek et al (1984) have reported that spinal cord transection in the rat failed to affect staining of VIP-like immunoreactive fibres in the sacral region. The level at which the transection was made was not stated.

The peripheral distribution, within the pelvic organs, of the VIP-containing afferents terminating in the sacral cord is at present unknown. As they form only a small minority of VIP-containing fibres within pelvic organs (the majority being of local origin), selective tracer studies may be necessary to answer this question. Further studies are of course also necessary to establish the role

of VIP within pelvic nerve afferents. However, in this chapter, it has been demonstrated that VIP is a highly selective marker of some pelvic nerve afferents in sacral spinal cord.

TABLE 5:1 Regional concentration of VIP (mean ± SEM) (pmol/g) in foetal and neonate human spinal cord, in comparison with adult spinal cord.

	Adult	Neonate
Cervical Dorsal	0.64 ± 0.19	1.2 ± 0.3
Cervical Ventral	0.63 ± 0.06	0.8 ± 0.2
Thoracic Dorsal	0.90 ± 0.28	1.0 ± 0.3
Thoracic Ventral	0.86 ± 0.18	0.7 ± 0.3
Lumbar Dorsal	10.75 ± 2.6	4.8 ± 0.5
Lumbar Ventral	0.82 ± 0.2	1.2 ± 0.4
Sacral Dorsal	30.0 ± 6.2	16.5 ± 4.4
Sacral Ventral	7.7 ± 1.9	5.5 ± 2.6

TABLE 5:2 Regional concentration of VIP (mean ± SEM) (pmol/g) in rat spinal cord.

Cervical	Thoracic	Lumbar	Sacral
3.0 ± 0.5	1.9 ± 0.2	2.4 ± 0.4	4.1 ± 0.6

TABLE 5:3 Concentration of VIP (mean ± SEM) (pmol/g) in cat spinal cord following surgical procedures.

(a) Dorsal lumbosacral rhizotomy (n = 4)

	Ipsilateral	Contralateral
Cervical Dorsal	0.21 ± 0.06	0.26 ± 0.09
Cervical Ventral	0.32 ± 0.08	0.47 ± 0.18
Thoracic Dorsal	0.16 ± 0.03	0.23 ± 0.06
Thoracic Ventral	0.15 ± 0.03	0.38 ± 0.21
Lumbar Dorsal	0.42 ± 0.19	2.58 ± 1.90
Lumbar Ventral	1.02 ± 0.66	0.65 ± 0.32
Sacral Dorsal	1.32 ± 0.08*	12.91 ± 6.4
Sacral Ventral	0.32 ± 0.09	0.29 ± 0.07

* $p < 0.05$ Student's paired t-test.

(b) Pelvic nerve transection (n = 4)

	Ipsilateral	Contralateral
Sacral Dorsal (S2)	22.4 ± 6.3*	70.9 ± 5.0

* $p < 0.01$ Student's paired t-test.

(c) Thoracic cord hemisection (n = 4)

	Ipsilateral	Contralateral
Sacral Dorsal	29.8 ± 9.9	38.9 ± 12.4

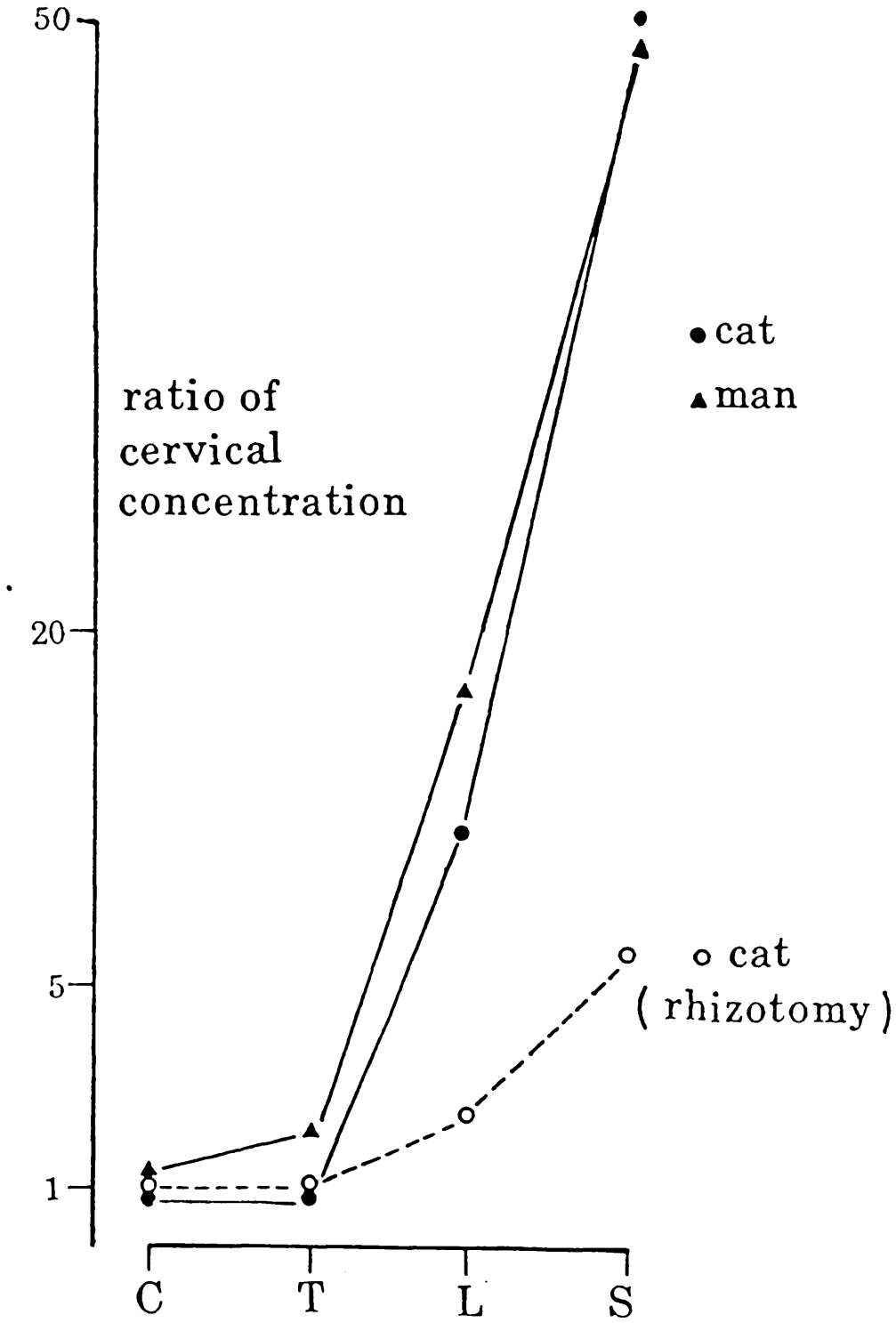


FIGURE 5:1 VIP in the dorsal cord regions of normal man and cat as a ratio of the dorsal cervical concentrations plotted on a log scale. After unilateral lumbosacral rhizotomies in the cat, there is a marked decrease in lumbar and sacral concentrations of VIP.
C = cervical; T = thoracic; L = lumbar; S = sacral.

CHAPTER 6

PHARMACOLOGY OF VIP AND RELATED PEPTIDES

ON GENITOURINARY SMOOTH MUSCLE

INTRODUCTION

As discussed in Chapter 3, several pharmacological studies with VIP have provided evidence, which, in addition to its presence in the innervation of genitourinary smooth muscle, indicate that it may be an inhibitory neurotransmitter which mediates non-adrenergic, non-cholinergic relaxation in this region.

VIP is a member of a family of peptides which share a varying degree of structural homology (see Table 6:1). Of the members so far identified, peptide HI (PHI, peptide with N-terminal Histidine and COOH-terminal Isoleucine) is the most similar to VIP in amino acid sequence. It was isolated from porcine duodenum by Tatemoto and Mutt (1981), is derived from the same precursor gene as VIP (Christofides et al 1982; Itoh et al 1983) and generally marks the same population of neurones (Lundberg et al 1984). PHI shares many of the biological activities of VIP, although in most systems PHI has a somewhat lower potency (Bataille et al 1980; Brennan et al 1982; Dimaline & Dockray 1980; Suzuki et al 1984; Szecowka et al 1980).

It was considered important to compare the action of VIP and PHI and other related peptides (glucagon and secretin) on preparations of

genitourinary smooth muscle in order to establish whether VIP is the natural non-adrenergic non-cholinergic inhibitor in this region. In doing so, it would further characterize receptors for VIP-like peptides in these preparations. The concentrations of these VIP-related peptides in the tissue preparations were measured by radioimmunoassay.

The action of Gila monster venom on these smooth muscle preparations was also investigated as recent evidence suggests that it contains a peptide with structural similarities to members of the VIP family (Robberecht et al 1984).

In 1982, venom from the Gila monster (family Helodermatidae) was shown to increase enzyme secretion in dispersed acini from guinea-pig pancreas to the same extent as VIP, secretin and PHI. Like VIP, secretin and PHI, the venom caused an increase in cellular cyclic AMP and inhibited the binding of ^{125}I-VIP to its receptors on pancreatic acini (Raufman et al 1982). This was the first report to suggest that Gila monster venom contained a peptide with the ability to stimulate pancreatic enzyme secretion by interacting with VIP receptors on pancreatic acinar cells and thereby activating adenylate cyclase and increasing cellular AMP. Gila monster venom has also been shown to increase amylase release from rat pancreatic acini to a greater extent than VIP and secretin (Dehaye et al 1984). In binding studies it was shown to completely inhibit the binding of ^{125}I-VIP to human and rat intestinal epithelial cell membranes and stimulate adenylate cyclase (Amiranoff et al 1983). Adenylate cyclase activity in rat heart, brain and pancreas and human heart has been reported to be stimulated by Gila monster venom

as efficiently as by secretin and VIP (Robberecht et al 1984).

The possibility that this peptide present in Gila monster venom is VIP or a similar peptide was investigated using radioimmunoassay and gel permeation chromatography.

In the present study the effects of PHI, glucagon, secretin and Gila monster venom were compared with VIP on the rabbit urethra and rabbit anococcygeus muscle. The influence of VIP on urinary tract smooth muscle is well documented, although conflicting. It has been reported to exert a concentration-dependent relaxation of pig trigone, detrusor, bladder neck and urethra, rabbit urethra and bladder, cat ureter, bladder and urethra and human detrusor and urethra (Larsen et al 1981; Klarskov et al 1984; Andersson et al 1983; Hills et al 1984; Sjögren et al 1985; Levin & Wein 1981) but a contraction of guinea-pig urinary bladder (Johns 1979; MacKenzie & Burnstock 1984). Erspamer et al (1981) found VIP (and glucagon and secretin) to be inactive on the longitudinal smooth muscle of the human urinary bladder and it is also reported to be inactive in the rat bladder (Choo & Mitchelson 1980) and cat urethra (Abdel-Hakim et al 1983). PHI has been shown to be without any direct excitatory action on guinea-pig detrusor muscle, although to attenuate excitatory responses elicited by VIP when pre-incubated in the preparation in very high concentrations(MacKenzie & Burnstock 1984).

The pharmacology of the anococcygeus muscle has been thoroughly investigated, particularly by Gillespie (1972, 1980), but for many years there was substantial controversy surrounding the nature of

the neurotransmitter released by the inhibitory nerves of the muscle. The first evidence of VIP being an inhibitory transmitter here was provided by Gibson and Wedmore (1981). They found that VIP produced relaxations of the isolated mouse anococcygeus muscle in which tone had been raised by carbachol. In 1984 Gibson et al demonstrated that of a number of neuropeptides tested, only VIP caused powerful relaxations of the mouse anococcygeus muscle. Recently, Hunter et al (1984) provided further evidence in support of VIP being an inhibitory transmitter in the anococcygeus muscle. They demonstrated high concentrations of VIP in the anococcygeus muscle of rat, cat and rabbit, and on applying exogenous VIP to isolated preparations produced dose-related relaxations of tone in all three species. The pharmacological effects of VIP-related peptides, however, have not previously been examined on this tissue.

MATERIALS AND METHODS

Bioassay

Male rabbits (Froxfield Californians, 2.0-2.5 kg) were killed by cervical dislocation and the urinary bladder with the urethra attached and the anococcygeus muscles were removed. The dissection of the anococcygeus muscles was carried out as described by Creed et al (1977). All visible connective tissue and fat were removed. Following separation from the bladder at a point just below the neck, the urethra was split open longitudinally. Transverse strips of the upper urethra were then cut and tied at each end to produce two preparations from each urethra of approximately 1.5 cm in length and 4 mm wide, unstretched.

The anococcygeus muscle and urethra preparations were set up under 0.5g tension in 2 ml organ baths containing Tyrode solution (mM:NaCl, 136.8; KCl 2.8; $NaHCO_3$ 11.9; NaH_2PO_4 0.3; $CaCl_2$ 1.8; $MgCl_2$ 2.1; glucose 5.5). Organ bath temperature was maintained at 37°C and the Tyrode solution was gassed continuously with 95% O_2/5% CO_2. Changes in tension were measured isotonically and displayed on a W+W Electronic Scientific Instruments 4 channel recorder. Each preparation was allowed to equilibrate for at least 30 minutes prior to the start of the experiment. The organ baths were silanized (5% dimethyl-chlorosilane in toluene) to prevent the peptides adhering to the glass.

In order to measure the relaxant effects of the peptides and Gila monster venom, the tone of the urethra and anococcygeus muscle had to be raised. The agonist used to contract the urethra was noradrenaline, and histamine was found to be most effective at contracting the anococcygeus muscle. The agonists were used at sub-maximal concentrations (3×10^{-6}M). (Drug concentrations used in this chapter represent the total concentrations in the bath). The tissue contact time for the agonists was 3 minutes in the case of the urethra and 4 minutes in the anococcygeus muscle.

In both tissue preparations, the method of physiological antagonism was used to investigate the effects of VIP, PHI, glucagon, secretin and Gila monster venom on noradrenaline- or histamine-induced contractions. Having first obtained identical and reproducible control stimulations of the urethra and anococcygeus muscle, the peptide/venom under investigation was added to the bath and allowed an initial contact time of 2 minutes before further addition of the

agonist. The height of the tissue contraction in the presence of the peptide/venom was then compared with the height of the previous control stimulations. From this, the percentage inhibition of the noradrenaline- or histamine-induced contractions produced by the peptide/venom was calculated. The following dose of the peptide/venom was not added until reproducible control contractions of the muscle had once more been obtained (approximately 30 minutes). In the case of each peptide and the Gila monster venom, studies were carried out on a minimum of 5 separate preparations of urethra (or anococcygeus muscle).

Drugs

Drugs used were: noradrenaline (Arterenol, Sigma); histamine diphosphate (Sigma); atropine sulphate (Sigma); indomethacin (Sigma); papaverine hydrochloride (Sigma); bovine serum albumin (Sigma); VIP (porcine, Bachem); PHI (porcine, Bachem); glucagon (bovine/porcine mixture, Sigma); secretin (porcine, Sigma); Gila monster venom (Heloderma Horridum, batch V-8375, Sigma). Constituents of Tyrode solution were obtained from BDH Chemicals.

VIP, PHI, glucagon and secretin were dissolved in 0.01M acetic acid containing 1% BSA. Aliquots of the solutions were lyophilized and stored at -20°C until use. Aliquots were reconstituted in Tyrode solution for addition to the organ bath. Any remaining at the end of the experiment was discarded. Peptides were not re-frozen.

Gila monster venom was dissolved in distilled water and then aliquoted for lyophilization.

Volumes of pharmacological agents added to the organ baths did not exceed 60 µl.

Because of the possibility of loss of peptide during these experiments (eg by adherence to glass organ baths) aliquots of peptide prior to addition to the bath and aliquots of bathing fluid at the end of each dose cycle, were removed and their respective peptide concentrations measured by radioimmunoassay, so that any necessary corrections could be made to the dose-response curves. Results given in the chapter are presented as the mean ± SEM, where n is the number of preparations.

Radioimmunoassays

The concentrations of VIP-, PHI-, secretin- and glucagon-like immunoreactivities in the urethra and anococcygeus muscles of four rabbits were measured by radioimmunoassay. The tissues were excised in a manner identical to that used for bioassay and extracted in boiling 0.5M acetic acid as described in Chapter 2. VIP-like immunoreactivity was measured as described in Chapter 2 and PHI-like immunoreactivity was measured using an antiserum directed to the mid-C-terminal region of the molecule (Christofides et al 1983). Secretin was measured by the method of Greenberg (1982) and glucagon was measured by the method of Christofides (1982).

The concentrations of the peptides in the aliquots added to the organ baths and in the bathing fluid were measured using the same radioimmunoassays.

Gila monster venom was assayed for VIP-like immunoreactivity in the following dilutions: undiluted; 1:50; 1:500; 1:2,500 and 1:20,000. In addition, the effect of boiling on the VIP-like immunoreactive content of Gila monster venom was assessed.

Gel Permeation Chromatography of Gila Monster Venom

A lyophilized aliquot containing 0.4 mg Gila monster venom was reconstituted in 0.7 ml normal saline and applied to a Sephadex G-50 superfine column. Equilibration and calibration of the column was carried out as described in Chapter 2. Fractions (approx 0.7 ml) were collected at a flow rate of 12 ml/hr. Aliquots of each fraction were assayed for VIP-like immunoreactivity. Following reconstitution in 0.7 ml saline, another aliquot of Gila monster venom was boiled for 10 minutes and chromatographed as above.

The above was then repeated on a column of Sephadex G-100 superfine.

RESULTS

Rabbit Urethra

The rabbit urethra lacked spontaneous tone and activity. On addition of noradrenaline (3×10^{-6}M) the resulting contraction was only briefly sustained.

VIP and PHI dose-dependently (10^{-9}-2×10^{-7}M) inhibited the contraction of the urethra induced by noradrenaline (3×10^{-6}M) (Figure 6:1). A maximum inhibition of 73 ± 5.5% was produced by VIP

and one of 51.5 ± 13% produced by PHI. The relative potency of VIP:PHI was 1:0.3 at 25% inhibition of the noradrenaline-induced contraction.

After repeated high doses of VIP and PHI the urethra exhibited tachyphylaxis to both peptides. VIP and PHI no longer produced the inhibition that could be seen prior to desensitization. Having obtained dose-response curves of the urethra to VIP and PHI, repeated high doses ($3x10^{-7}M$) of VIP alone, leading to desensitization of the tissue would also result in a lack of response to PHI in low concentrations and a reduced response when added in high concentrations (see Figure 6:2). This cross-tachyphylaxis of the urethra to VIP and PHI was also obtained when the tissue was desensitized by high doses of PHI alone.

During desensitization, when high concentrations of VIP or PHI failed to inhibit contractions of the urethra, addition of the smooth muscle relaxant papaverine (1 µM) to the contracted urethra, would result in a rapid, total relaxation.

Gila monster venom also showed a dose-related (0.1-100 µg/ml) inhibition of the noradrenaline-induced contraction of the rabbit urethra (Figure 6:3). A maximum inhibition of 86 ± 2% was obtained with 100 µg/ml Gila monster venom. The dose response curves to VIP and Gila monster venom were superimposable (Figure 6:4). Having obtained a dose-response curve to Gila monster venom and then desensitized the urethra to VIP and PHI, addition of a further high dose of Gila monster venom (eg 10 µg/ml) occasionally resulted in a smaller inhibition than that obtained prior to the desensitization.

When this cross-tachyphylaxis occurred, addition of a low dose of Gila monster venom (eg 0.5 µg/ml) had no effect on the contraction. The cross-tachyphylaxis obtained between Gila monster venom and/or PHI was considerably less marked than that obtained between VIP and PHI.

Glucagon (10^{-9}-$3x10^{-6}$M) and secretin (10^{-9}-$3x10^{-6}$M) had no effect on either resting tone or noradrenaline-induced contractions of the rabbit urethra.

The presence of the prostaglandin synthetase inhibitor, indomethacin (10^{-6}M) and atropine (10^{-6}M) in the organ bath did not alter the response of the rabbit urethra to VIP, PHI, glucagon, secretin or Gila monster venom.

Rabbit Anococcygeus Muscle

The rabbit anococcygeus muscle exhibited spontaneous tone and activity both in the basal and contracted states. Contractions produced by histamine ($3x10^{-6}$M) were not sustained.

VIP and PHI reversibly, reproducibly and dose-dependently (10^{-9}-$6x10^{-7}$M) inhibited the contraction of the anococcygeus muscle induced by histamine but had a negligible effect on the spontaneous activity (Figures 6:5 and 6:6). A maximum inhibition of 73.2 ± 21.5% was obtained by VIP and a lesser inhibition of 30 ± 16.5% by PHI. The relative potency of VIP:PHI was 1:0.1 at 25% inhibition.

Gila monster venom also caused a dose-dependent inhibition of the

histamine-contracted anococcygeus muscle (0.001-10 μg/ml) producing a dose-response curve which was parallel to that produced by VIP (Figure 6:7). The maximum inhibition produced by the Gila monster venom was 89.0 ± 5.2%.

Glucagon (10^{-9}-$3x10^{-6}$M) and secretin (10^{-9}-$3x10^{-6}$M) had no effect on either resting tone or histamine-induced contractions of the rabbit anococcygeus muscle.

As in the urethral preparation, the presence of indomethacin (10^{-6}M) or atropine (10^{-6}M) did not alter the response of the rabbit anococcygeus muscle to VIP, PHI, secretin, glucagon or Gila monster venom.

Radioimmunoassay of Urethral and Anococcygeus Muscle Extracts

The concentrations of VIP- and PHI-like immunoreactivity in acetic acid extracts of rabbit anococcygeus and urethra were as follows:-

	VIP	Mid-C-Term PHI
Urethra	80.4 ± 22.2	23.0 ± 5.5
Anococcygeus Muscle	86.6 + 13.5	62.0 ± 18.0

Results are expressed as pmol/g tissue (n = 4). The antiserum to PHI did not exhibit any significant crossreactivity with VIP. Glucagon and secretin were undetectable.

Radioimmunoassay of Gila Monster Venom

	Concentration of VIP-Like Immunoreactivity
Unboiled Gila monster venom	0.9 pmol/mg venom
Boiled Gila monster venom	0.8 pmol/mg venom

Gel Permeation Chromatography of Gila Monster Venom

The elution profile on G-50 Sephadex of VIP-like immunoreactivity in Gila monster venom is shown in Figure 6:8. The elution profile of boiled Gila monster venom was found to be identical. The majority of the VIP-like immunoreactivity was eluted in the void volume, although a small peak of immunoreactivity occurred in fraction 30 (Kav = 0.3). No immunoreactivity was detectable in the position of natural porcine VIP. Recovery of VIP-like immunoreactivity from G-50 column = 70%.

The elution profile on Sephadex G-100 of VIP-like immunoreactivity is shown in Figure 6:9. As with G-50 chromatography, the majority of the immunoreactivity eluted in the void volume. No immunoreactivity was detectable in the position of natural porcine VIP. Recovery of VIP-like immunoreactivity from G-100 column = 62.5%.

DISCUSSION

These studies have demonstrated that of the members of the

VIP-family of peptides tested on smooth muscle preparations of rabbit urethra and anococcygeus muscle, only VIP and PHI exerted any measurable biological activity. Both peptides produced a dose-dependent inhibition of induced contractions. VIP was three to ten times more potent than PHI which is in accordance with studies carried out by other investigators on other tissue organ preparations (Bataille et al 1980; Brennan et al 1982; Dimaline & Dockray 1980; Suzuki et al 1984; Szecowka et al 1980). VIP and PHI were both shown to be present in high concentrations in the anococcygeus muscle and upper urethra. The observation that VIP and PHI were present in these tissues and that both showed biological activity would suggest that PHI is an additional candidate, together with VIP, as mediator of relaxation of rabbit urethra and anococcygeus muscle. As indomethacin and atropine did not affect the responses of VIP and PHI, it can be concluded that the relaxation is not mediated via a cholinergic pathway, or prostaglandin synthesis.

The cross-tachyphylaxis between VIP and PHI demonstrated in the rabbit urethra would suggest that their activity is mediated via a common receptor or a common second messenger. It is reported that VIP, PHI and secretin interact with the same or similar plasma membrane receptors (Robberecht et al 1982); based on their affinity for natural peptides and on their ability to recognize synthetic analogues, the VIP/PHI/secretin receptors can be sub-divided into 'VIP/PHI preferring' and 'secretin-preferring' (Christophe et al 1983). These receptors do not recognise glucagon. As secretin had no effect on these preparations of rabbit urethra and anococcygeus muscle, it is possible that only the 'VIP/PHI preferring' receptors

are present in these regions and that the activity produced by VIP and PHI is mediated via this receptor category. As glucagon had no effect on the rabbit urethra and anococcygeus muscle preparations, it could be assumed that receptors specific for glucagon are absent in these regions of genitourinary smooth muscle. The use of specific antagonists is required in order to fully characterize the receptors.

Gila monster venom demonstrated dose-dependent inhibitions of rabbit urethra and anococcygeus muscle. In both preparations the dose-response curves to VIP and Gila monster venom were parallel but the Gila monster venom exhibited greater potency in the anococcygeus muscle than in the urethra relative to the VIP response. VIP and Gila monster venom appear, therefore, to cause relaxation via the same receptor in each tissue, although there is a difference in relative affinity. However, the cross-tachyphylaxis between Gila monster venom and VIP and/or PHI was not complete. The possibility that Gila monster venom may contain other relaxant substances which are acting via different receptors as well as the 'VIP receptor' should not be overlooked.

It was not possible to properly characterize the VIP-like immunoreactivity which was detected in the Gila monster venom. It was predominantly of large molecular weight (greater than 150,000), eluting in the void volume on G-100. It may reflect non-specific interference with the assay by other constituents of Gila monster venom rather than being a large molecular weight form of VIP. That it may be due to enzymic degradation of the VIP radiolabel is unlikely since boiling had no effect on the immunoreactivity.

The primary structure of a VIP-secretin-like peptide isolated from Gila monster venom has now been elucidated (Hoshino et al 1984). It was found to be a 35 amino acid peptide with a high degree of sequence similarities to VIP, PHI and secretin over the entire N-terminal 1-27 sequence. In particular, the amino acid residues in positions 3, 6 and 7 were found to be common to 9 peptides of the family. This is thought to correspond to the peptide described in the introduction which interacts with VIP or secretin receptors in membrane preparations and activates adenylate cyclase (and is designated 'helodermin'). Parker et al (1984) also reported the amino acid sequences of 2 closely related peptides from Gila monster venom:- helospectin I is a 38-residue peptide and helospectin II is a 37-residue peptide identical to helospectin I except that it lacks serine 38. Comparison of the 28 residues of VIP with residues 1-28 of helospectin reveals that identical amino acids occur in 15 positions. Helospectin 1-37 and helodermin share a structural homology of 83%. None of these peptides would seem to account for all the VIP-like immunoreactivity detected in Gila monster venom by the assay since this is mainly of larger molecular weight. However, the relatively small amount of immunoreactivity eluting later on the G-50 column (at Kav 0.3) may be one of these peptides. It remains to be determined whether helodermin /the helospectins are responsible for the pharmacological activity of Gila monster venom on preparations of rabbit urethra and anococcygeus muscle demonstrated in this chapter.

TABLE 6:1 Amino acid sequences of VIP, PHI, secretin and glucagon

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Porcine VIP	His-	Ser-	Asp-	Ala-	Val-	Phe-	Thr-	Asp-	Asn-	Tyr-	Thr-	Arg-	Leu-	Arg-
Porcine PHI	His-	Ala-	Asp-	Gly-	Val-	Phe-	Thr-	Ser-	Asp-	Phe-	Ser-	Arg-	Leu-	Leu-
Secretin	His-	Ser-	Asp-	Gly-	Thr-	Phe-	Thr-	Ser-	Glu-	Leu-	Ser-	Arg-	Leu-	Arg-
Glucagon	His-	Ser-	Gln-	Gly-	Thr-	Phe-	Thr-	Ser-	Asp-	Tyr-	Ser-	Lys-	Tyr-	Leu-

	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Porcine VIP	Lys-	Gln-	Met-	Ala-	Val-	Lys-	Lys-	Tyr-	Leu-	Asn-	Ser-	Ile-	Leu-	Asn-NH_2
Porcine PHI	Gly-	Gln-	Leu-	Ser-	Ala-	Lys-	Lys-	Tyr-	Leu-	Glu-	Ser-	Leu-	Ile-	NH_2
Secretin	Asp-	Ser-	Ala-	Arg-	Leu-	Gln-	Arg-	Leu-	Leu-	Gln-	Gly-	Leu-	Val-	NH_2
Glucagon	Asp-	Ser-	Arg-	Arg-	Ala-	Gln-	Asp-	Phe-	Val-	Gln-	Trp-	Leu-	Met-	Asn-Thr

Abbreviations

PHI = Peptide-containing N-terminal histidine and C-terminal isoleucine

VIP = Vasoactive intestinal polypeptide

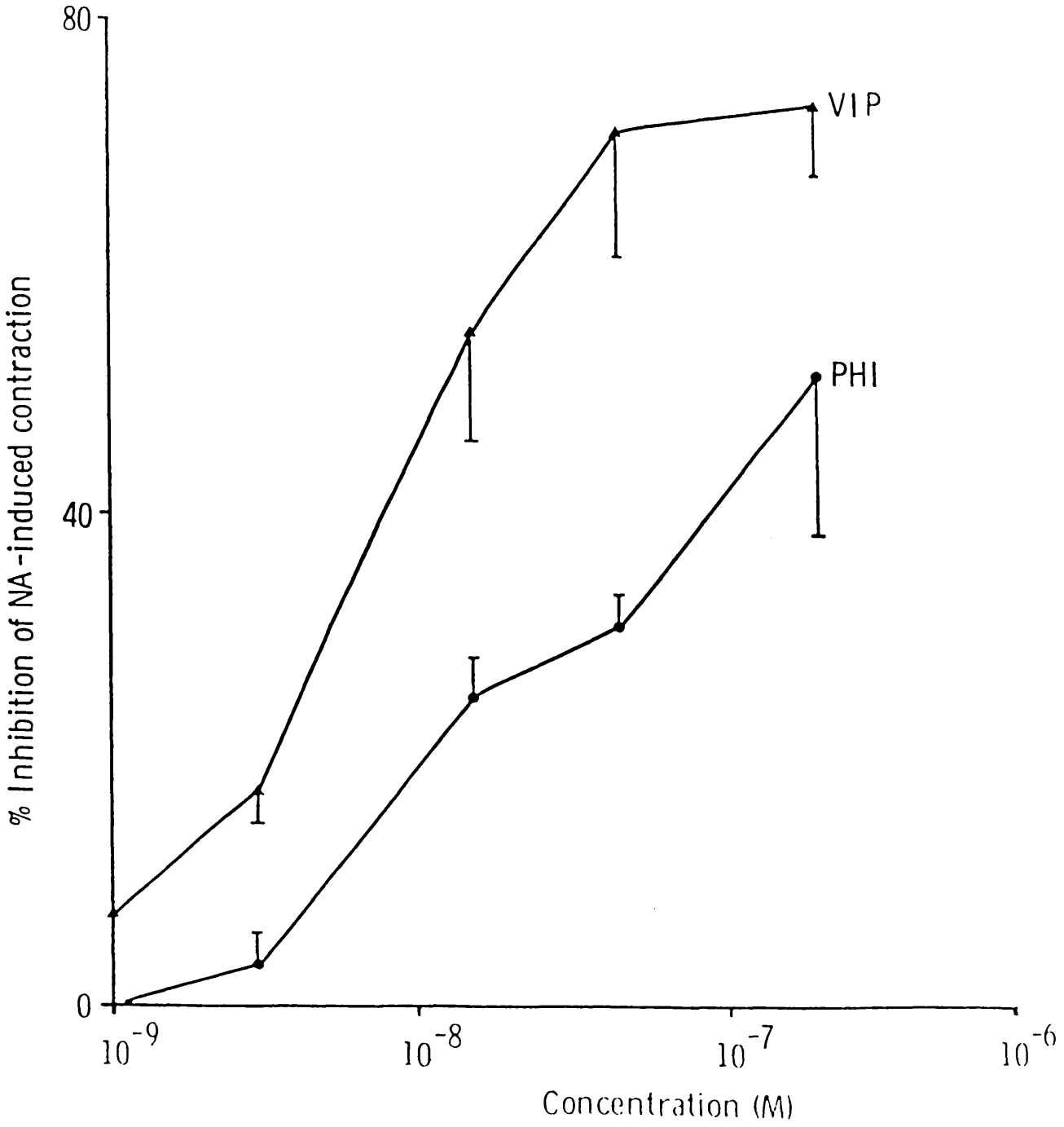


FIGURE 6:1 Dose response curve of rabbit urethra to VIP and PHI (n = 5).

Concentrations were determined by radioimmunoassay and are plotted on a log scale.

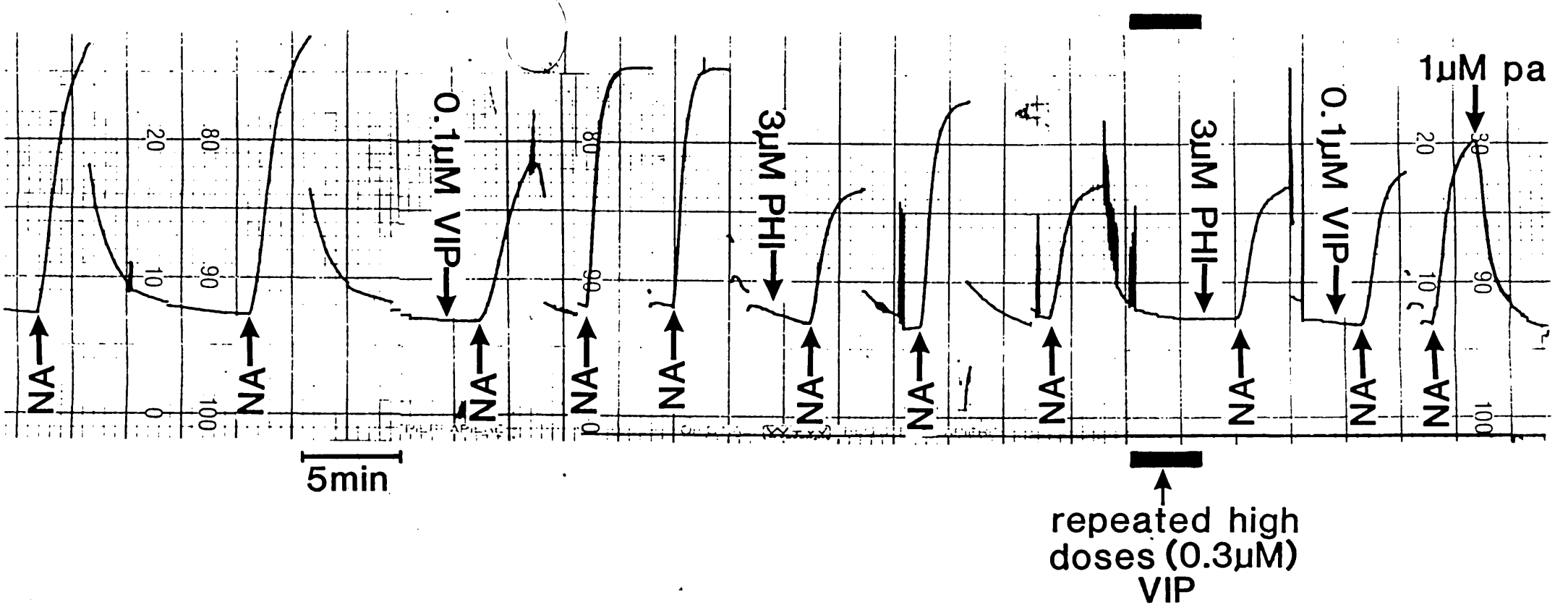


FIGURE 6:2 Demonstration of cross-tachyphylaxis of rabbit urethra to VIP (0.1 µM) and PHI (3 µM) following repeated high doses (0.3 µM) of VIP. Inhibition of noradrenaline (NA)-induced contractions by VIP and PHI are visible prior to the addition of repeated high doses of VIP. Papaverine (1 µM) (pap) produced total inhibition of NA-induced contraction.

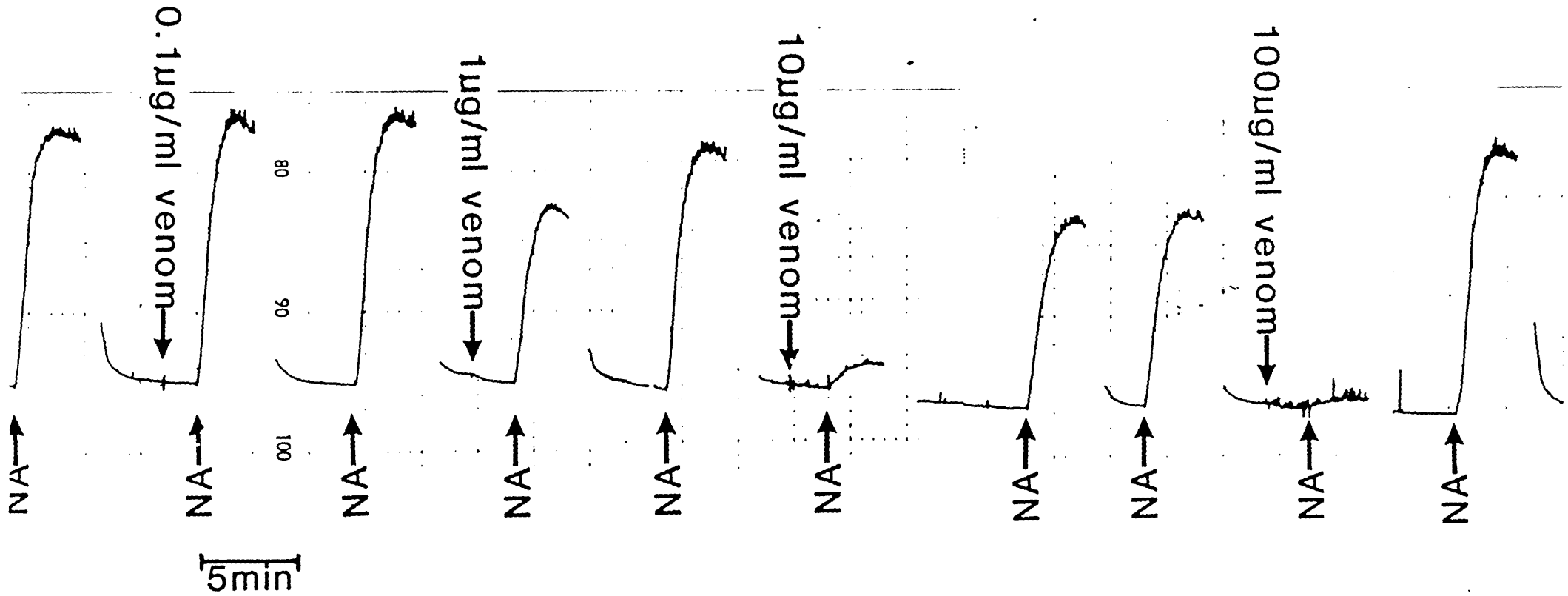


FIGURE 6:3 Gila monster venom modulation of noradrenaline (3 µM) (NA)-induced contractions of rabbit urethra.

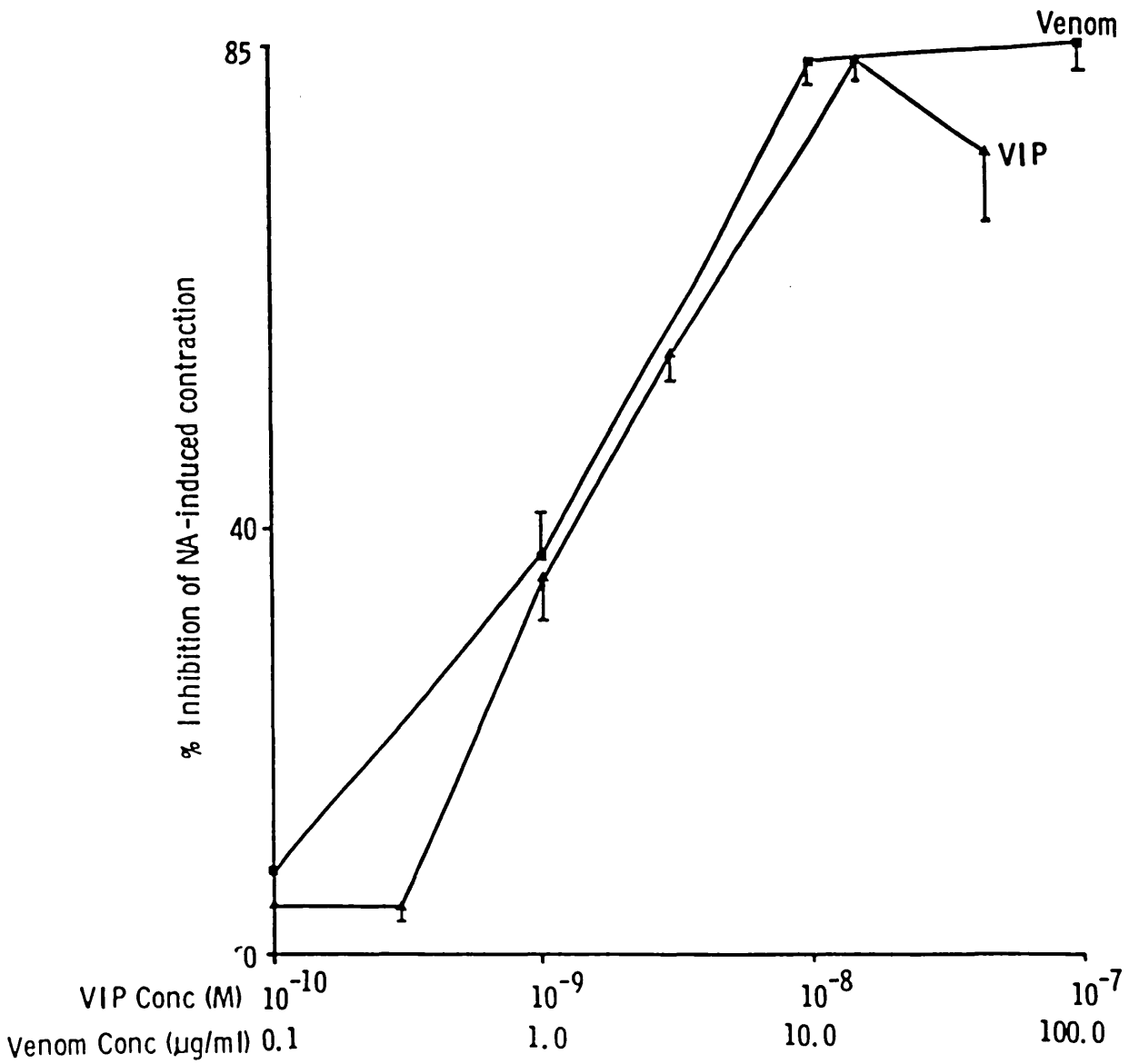


FIGURE 6:4 Dose response curve of rabbit urethra to Gila monster venom and VIP (n = 8).

VIP concentrations were determined by radioimmunoassay.

Concentrations are plotted on a log scale.

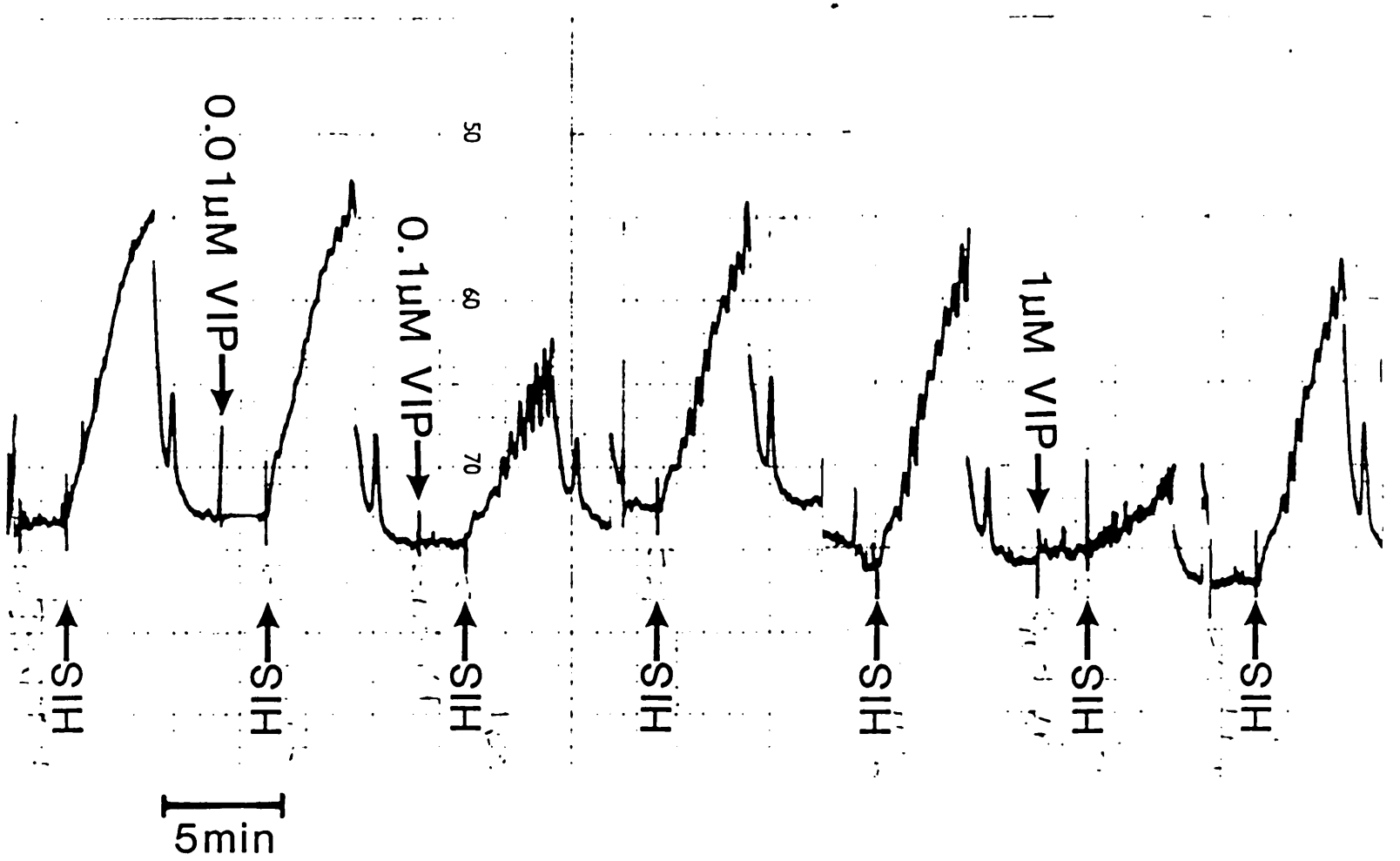


FIGURE 6:5 VIP modulation of histamine (3 µM) (HIS)-induced contractions of rabbit anococcygeus muscle.

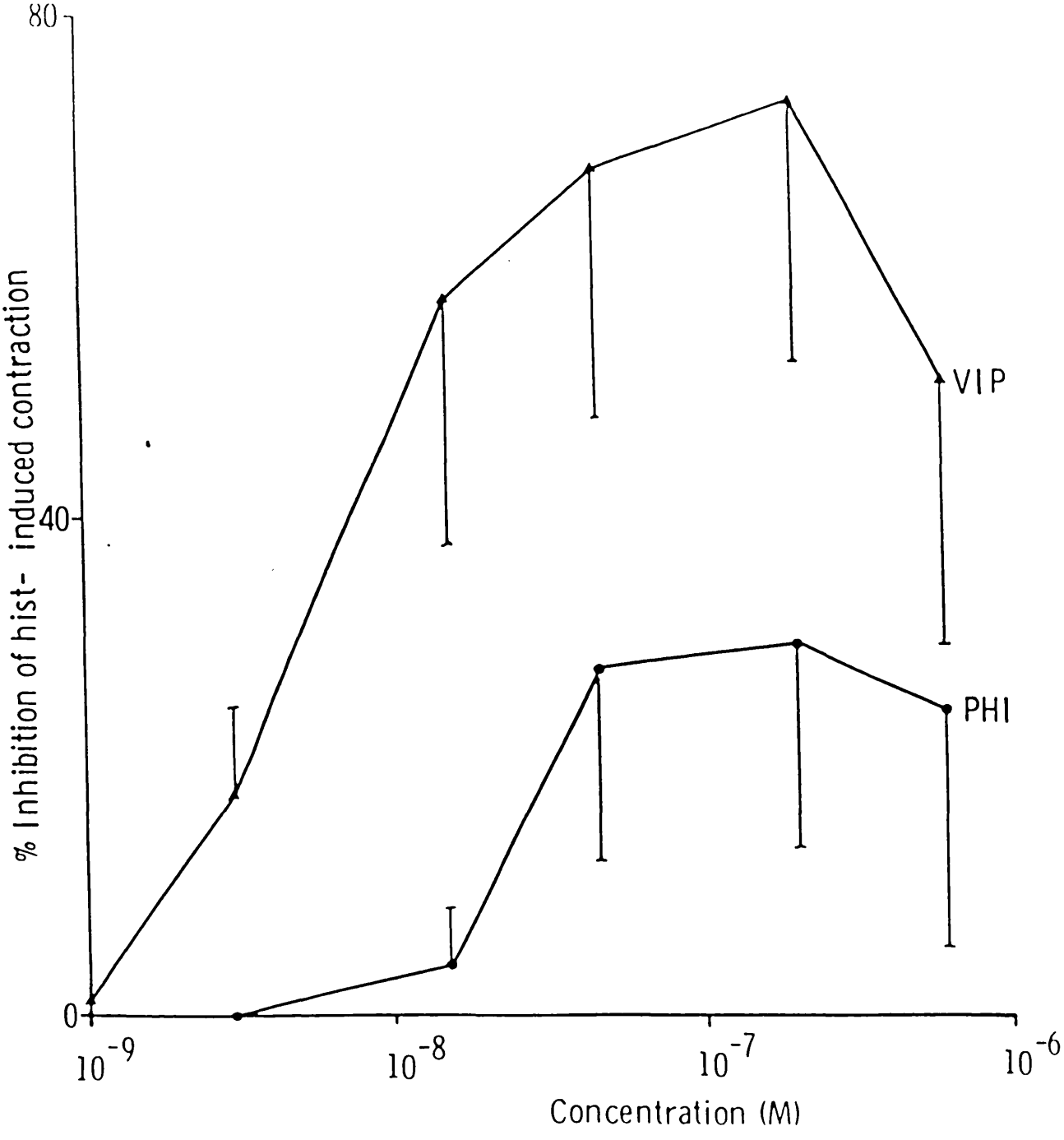


FIGURE 6:6 Dose response curve of rabbit anococcygeus muscle to VIP and PHI (n = 5).
Concentrations were determined by radioimmunoassay and are plotted on a log scale.

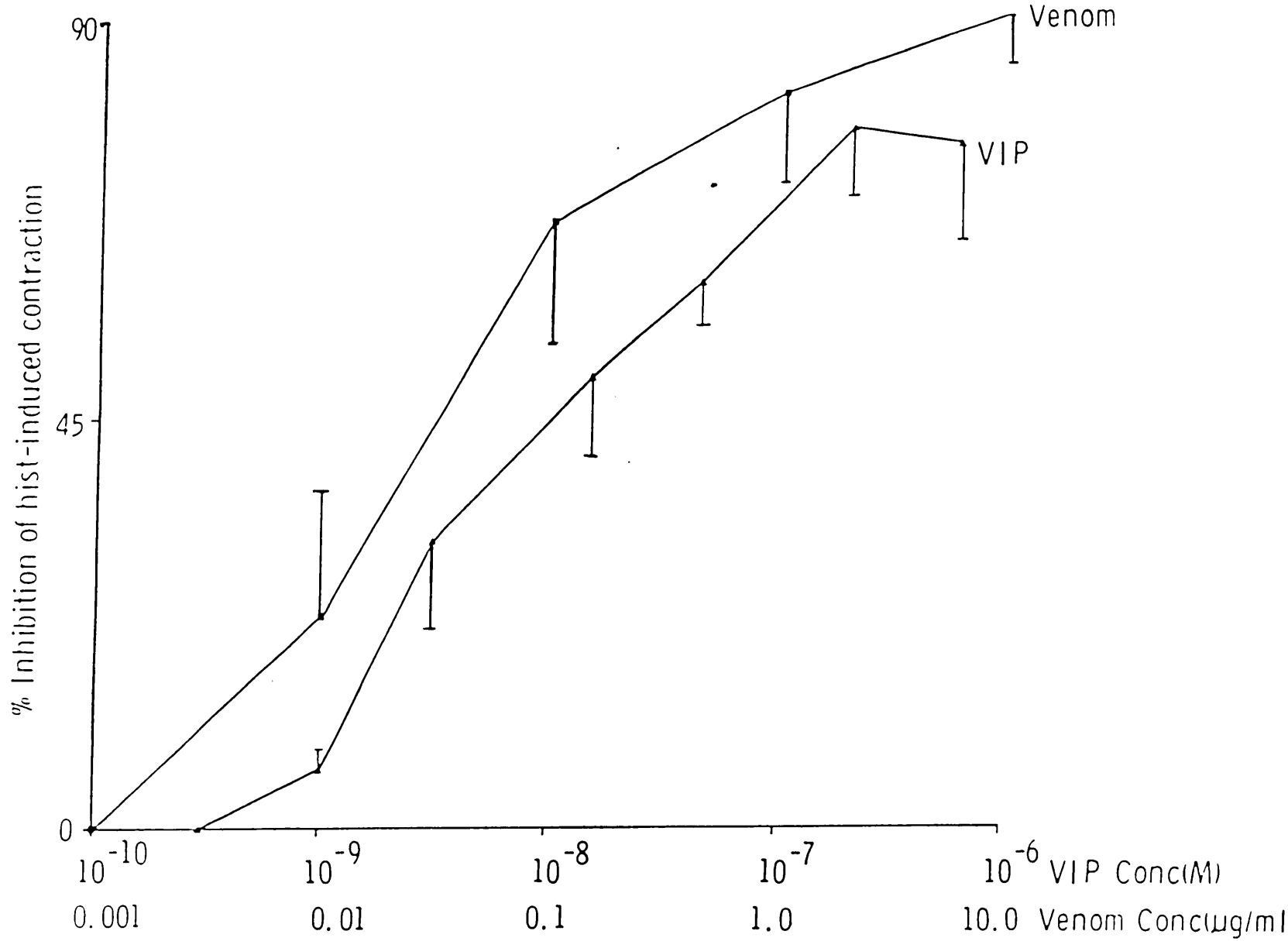


FIGURE 6:7 Dose-response curve of rabbit anococcygeus muscle to VIP and Gila monster venom (n = 5). VIP concentrations were determined by radioimmunoassay. Concentrations are plotted on a log scale.

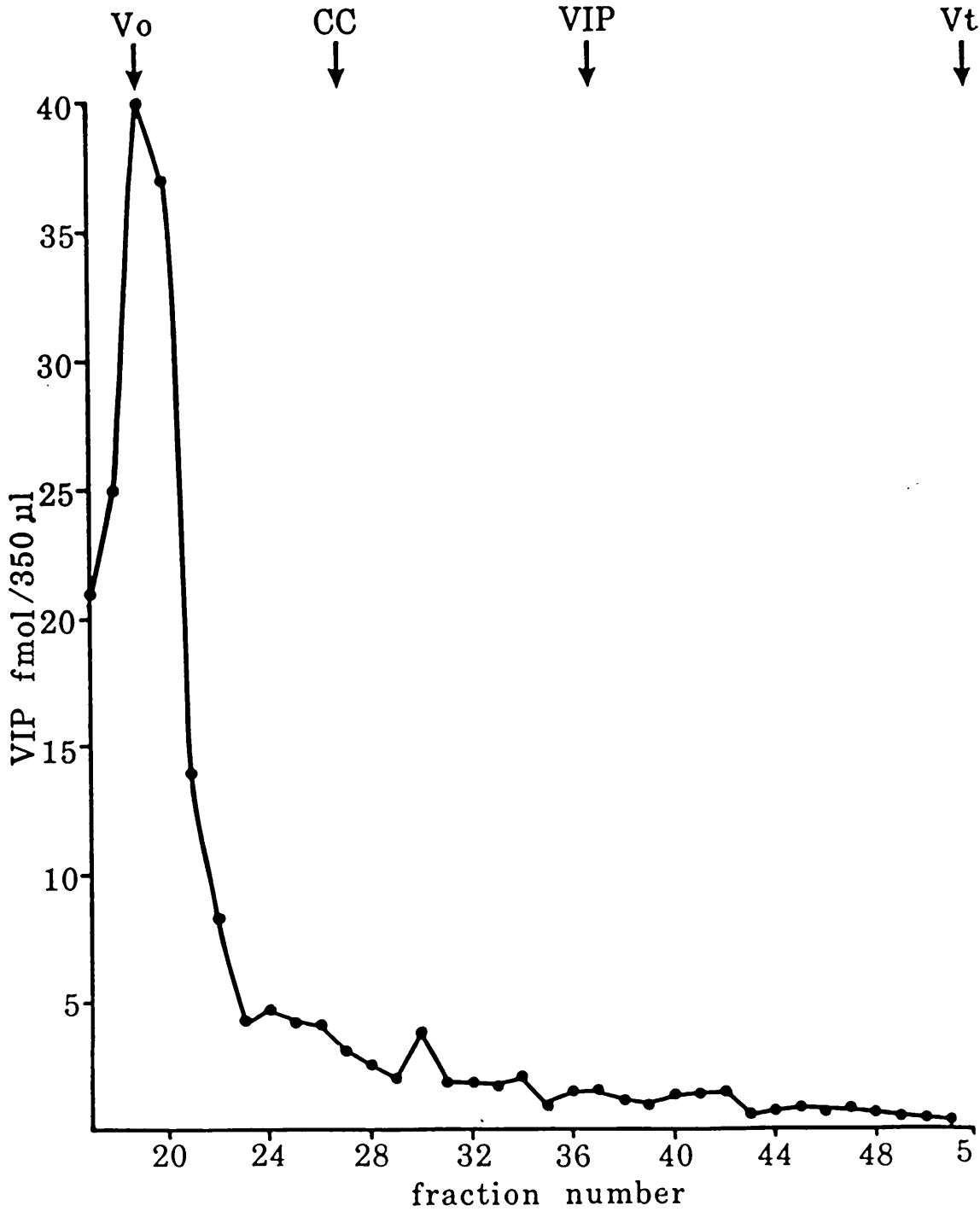


FIGURE 6:8 Sephadex G-50 gel permeation profile of VIP-like immunoreactivity in Gila monster venom.

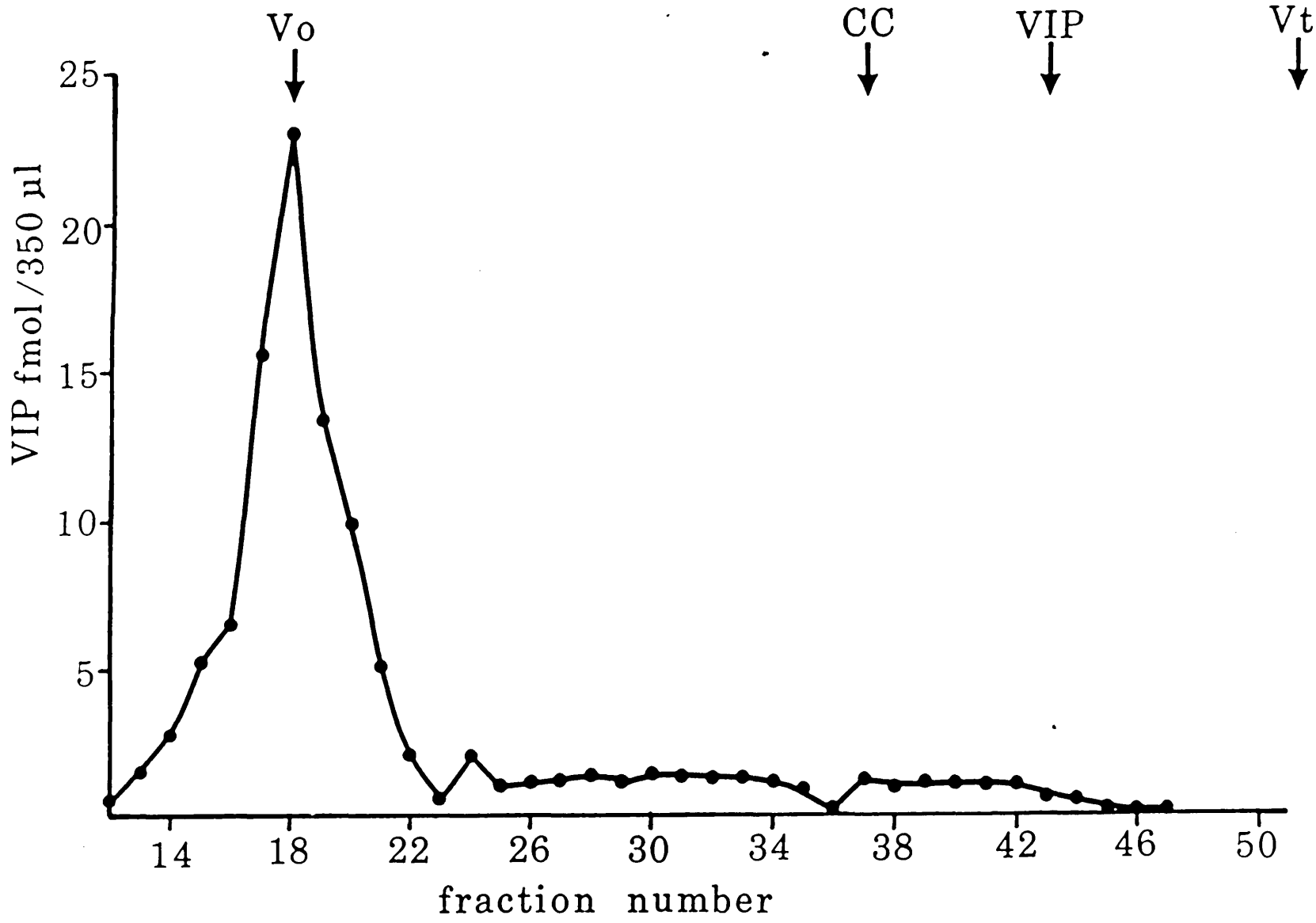
Vo = column void volume.

Vt = total fluid bed volume of column.

CC = elution position of horse heart cytochrome C.

VIP = position of pure porcine VIP.

FIGURE 6:9 Sephadex G-100 gel permeation profile of VIP-like immunoreactivity in Gila monster venom.
Vo = column void volume.
Vt = total fluid bed volume of column.
CC = elution position of horse heart cytochrome C.
VIP = position of pure porcine VIP.



CHAPTER 7

THE PHYSIOLOGICAL ROLE OF VIP IN MAMMALIAN PENILE ERECTION AND ITS PATHOLOGICAL ROLE IN IMPOTENCE

The physiology of mammalian penile erection is highly complex and several theories have been suggested as to the possible mechanisms. These include increased arterial inflow to the penis, altered shunting of blood within the organ, and restricted venous penile outflow.

Although the first proposed mechanism of erection was based on restricted venous outflow, there is little support now for this view. Varolius (1573) claimed that mechanical obstruction of venous drainage from the penis, caused by reflex contraction of the ischiocavernosus and bulbocavernosus muscles, induced penile erection. Later investigators concluded that obstruction of venous outflow by mechanical means or by active venous constriction plays little or no role in the erectile process (Dorr & Brody 1967). However, there is evidence that some venous compromise may be necessary for the induction or maintenance of erection (Newman & Northup 1981).

The best accepted mechanism of erection involves increased arterial inflow combined with altered shunting of blood in the penis. Semans and Langworthy (1938) observed that mechanical constriction of the

aorta during erection in cats caused detumescence, suggesting the key involvement of arterial inflow in erection. Dorr and Brody (1967) perfused the dorsal arteries of the canine penis, measured dorsal venous outflow, and concluded that erection is produced by a large decrease in penile arterial resistance which leads to an increase in blood flow. The magnitude of increased flow is sufficient to fill the erectile tissue.

Conti based his theory of erection (1952) on the process of shunting within the penis and maintained that there are valve-like structures called 'polsters' containing smooth muscle located at the anastomoses. He assumed erection was effected by active relaxation of the polsters within the arteries leading to the cavernous spaces, coincident with contraction of the polsters within the veins draining the penis. The morphological basis of these theories is still controversial, particularly the nature of the shunts and valve-like structures associated with them. Anastomoses were discovered in the canine penis by Müller (1835) and in the human by Hoyer (1877). They are present between the arterioles and the vascular spaces in the erectile tissue. Distension of the penis may be effected by the opening of these anastomoses. Recently, Wagner et al (1982) described the presence of another type of shunt vessel in the penis - a connection of the deep arteries in the corpus cavernosum with vessels in the corpus spongiosum. Constriction of these shunt arteries would divert blood through the helicine arteries into the spaces of the corpora cavernosa, producing tumescence and erection. The existence and nature of the valve-like structures associated with the anastomoses is even more controversial. In 1900, Von Ebner described cushions and ridges in

the penile arteries and this finding was confirmed by Kiss (1921). Newman and Tchertkoff (1980) could not find cushions in the penis of the newborn human, and postulated that they acted in adults solely as passive resistances to blood flow in the penis and did not represent a significant anatomic structure for erection. Benson et al (1980) were also unable to find evidence of muscular columns in the arteries of the corpus cavernosum, but found a few such structures in small veins or cavernous spaces of the corpus spongiosum of penile tissue obtained from humans undergoing penectomy for gender reassignment. Leeson and Leeson (1965) were also unable to find polsters in rat corpus cavernosum. It is of interest that Ruzbarsky and Michel (1977) have reported a fibrous substitution of the longitudinal smooth muscle of Ebner pads only in men older than 38 years; it may be that dysfunction of these cushions is associated with impotence in aged men.

In addition to the mechanisms mentioned above, it appears likely that the dynamics of cavernous filling during penile erection are also influenced by the contractile state of the smooth muscles in the walls of the cavernous spaces. These muscles relax as the erectile tissue becomes engorged (Klinge & Sjöstrand 1977).

The nature of the neural regulation of penile erection has been the subject of a long-standing dispute in the literature. The following four aspects are dealt with here; the parasympathetic and sympathetic control, the effect of supraspinal influences and the nature of the neurotransmitters involved.

In 1863, Eckhard reported that electrical stimulation of the

parasympathetic branches of nerves from the sacral spinal cord produced penile erection in dogs. A few years later Langley and Andersen (1895) studied the nerve supply to cat and rabbit male genitalia and demonstrated that electrical stimulation of parasympathetic nerves from the sacral roots produced an increased blood flow, and that this was the only pathway for vasodilation. This assumption was maintained by Semans and Langworthy (1938): stimulating the first sacral root in the cat elicited a slight protrusion of the penis, while stimulating the third sacral root resulted in vasodilation without strong erection. Complete erection was brought about by excitation at the second sacral root.

Other studies showed neural pathways for vasodilation through the sympathetic hypogastric nerves (Eckhard 1863; Bacq 1935). However, when Semans and Langworthy (1938) stimulated the middle group of peripheral branches from the hypogastric nerve in cats, a seminal emission was seen and stimulation of the lowermost group caused an incomplete erection or subsidence if erection was already present. However, excitation of a lower branch of the same nerve in the perineum resulted in a complete erection. In general, little importance has been attached to the role of the sympathetic nerves in erection. Contrary to such a role, erection occurring in cats and man in response to the administration of alpha-adrenergic blocking agents suggests that tonic sympathetic vasoconstrictor mechanisms may in fact have an inhibitory influence on erection and possibly contribute to detumescence (Domer et al 1978; Brindley 1983).

Electrical stimulation of the pudendal nerve caused detumescence

(Semans & Langworthy '1938). In the dog, Eckhard (1863) also found that stimulation of the pudendal nerve did not cause erection, but that destruction of the nerve abolished the dog's erectile response to mechanical stimulation of the penis.

It is evident that pathways originating above the sacral cord level also play a role in initiating erection. Root and Bard (1947) removed the entire sacral spinal cord from male cats and found that full penile erections would still occur. However, manipulation of the penis of these cats would not produce an erection. Similar observations have been made in man. In patients with lower motoneuron lesions involving the sacral spinal cord, reflexogenic erections are abolished but psychogenic erections may still occur via the sympathetic innervation to the penis (Bors & Comarr 1960). In patients with a spinal cord lesion above T12, psychogenic erections are abolished but reflexogenic erections persist. Under normal conditions it is likely that psychic and reflexogenic stimuli may act synergistically to produce erections.

Pharmacological studies in animals (Bacq 1935) have shown that erections elicited by stimulation of autonomic nerves are depressed by atropine and enhanced by anticholinesterase agents suggesting that at least a part of the postganglionic efferent input to the penis is cholinergic. However, the failure of atropine to completely block erection has also suggested the presence of non-cholinergic vasodilator nerves in the innervation of the penis. In 1966 Luduena and Grigas demonstrated that electrical stimulation of bretylium-contracted dog retractor penis muscle produced relaxation which was not inhibited by atropine. Erection in the

canine penis produced by pelvic nerve stimulation was shown to be reduced, but not abolished, by atropine (Dorr & Brody 1967) and in this same study it was found that acetylcholine administration failed to produce any signs of erection. In the rabbit Sjöstrand and Klinge (1979) reported the existence of two vasodilator pathways, both with pelvic ganglionic relays. These were the pelvic parasympathetic nerves, comprising mainly non-cholinergic postganglionic neurons and the sympathetic hypogastric nerves, containing some cholinergic postganglionic fibres. Following atropine infusion in normal young men, there was no change in the ability to produce a rapid and full erection, whether the erection was provoked by visual stimulation or by local vibration (Wagner & Green 1981). It was postulated, therefore, that acetylcholine may not be directly responsible for the vasodilation associated with erection but may rather serve as an intermediate in the liberation of an unidentified non-cholinergic vasodilator material.

While only a few reports of human pharmacological studies of smooth muscle effectors of penile erection exist, extensive studies have been carried out in other mammals. Relaxation of smooth muscle fibres in the inflow resistance vessels to the cavernous bodies, the walls and trabeculae of the erectile tissue as well as of those in the retractor penis leads to protrusion and engorgement of the penis. Of the many putative transmitters tested in vitro, VIP was the only one found to produce a relaxation of dog, cat and bull retractor penis muscle and rabbit and guinea-pig corpus cavernosum (Sjöstrand et al 1981). Smooth muscle strips from the corpus cavernosum of man, rabbit and monkey were also found to be dose-dependently inhibited by VIP (Willis et al 1981, 1983; Steers et al 1984).

The identity of the neurotransmitter responsible for the vasodilation associated with erection remains uncertain. As VIP was found to have a widespread distribution in the male genital tract (Chapter 3) and reported to cause in vitro relaxation in tissue from this region (see above), its functional role and the mechanisms which regulate its release in mammalian penile erection were studied.

MATERIALS AND METHODS (These experiments were carried out in collaboration with Dr A Dixson at London Zoo).

Anaesthesia was induced in seven adult Bennett's wallabies (Macropus rufogriseus) (16.5-22.4 kg) with intramuscular ketamine (5-10 mg/kg, Ketalar) and xylazine (1-2 mg/kg, Rompun, Bayer) and infused at intervals throughout the experiments in order to maintain deep anaesthesia. The corpora cavernosa penis and caudal or limb vein were cannulated by insertion of a heparinized 27-guage needle attached to a polythene catheter (0.97 mm o.d., Portex). Blood was collected into ice-chilled syringes containing heparin (10-20 units/ml blood, Hepsal, Weddel) and aprotinin (2,000 KIU/ml blood, Trasylol, Bayer) centrifuged and the plasma stored at -20°C until time of assay.

Penile samples were obtained from the corpora cavernosa, but since the dorsal penile veins lie just above the cavernosa, some samples may have been drawn from the vessels which are known to drain the

corpus spongiosum penis and corpus spongiosum glandis in various mammals (Watson 1964). The wallaby was used as the experimental model as its penis resembles that of man. It lacks an os penis and rigidity of the penis during erection is maintained by vascular mechanisms.

Erection was stimulated by either manipulation of the penis or using a rectal probe attached to a 60 Hz, low voltage (approx 4 V) sine-wave stimulator (Nuffield Laboratories, London Zoo). Repeated electrical stimulation (1-3 V) for 4 seconds alternating with 4 seconds of non-stimulation was sufficient to induce erection.

Concurrent levels of VIP in the penile and general circulation of the wallaby were measured. Blood samples were drawn from the flaccid penis as well as during erection and tactile stimulation occurred during all these sample collections (Exp I). The effects of tactile and electrical stimulation upon penile VIP levels in the wallaby were compared. Blood samples were obtained from the flaccid penis without stimulation, during electrically stimulated erection in the absence of tactile stimulation, during manually stimulated erection and during electrically stimulated erection accompanied by tactile stimulation (Exp II). The effects of increasing periods of manual stimulation upon levels of VIP in the penile circulation were examined. Seven wallabies were given 15, 30, 60, 120 and 300s of manual stimulation before collection of blood samples (Exp III). The rate of decline in plasma VIP levels after manipulation of the penis in wallabies was investigated. Cannulae were placed in the penises of five animals immediately after 60s of manipulation and varying degrees of erection. A single blood sample was drawn,

without tactile stimulation of the penis, 2-4 min after cannulation (Expt IV).

Mean levels and SEM values of VIP were calculated for each animal in experiments I-IV. Analysis of variance followed by the Scheffé test (Keppel 1973) was carried out using these mean values. Differences in mean levels of VIP were also compared in some experiments by means of a paired t-test.

RESULTS

VIP was present in the penile circulation of the wallaby at all times. Mean levels were 18.4 ± 2.5 pmol/l as compared to non-detectable levels in the general circulation ($t = 6.85$, $df = 6$; $p < 0.001$). Raised levels of VIP occurred in the flaccid penis of the wallaby as well as during erection but in all cases tactile stimulation had occurred during collection of blood samples.

In experiment II VIP levels in the flaccid penis of the wallaby were very low and did not differ from those measured in the tail or limb veins provided that no tactile stimulation occurred during collection of samples (Figure 7:1). Electrical stimulation of erection did not cause a significant increase in VIP levels (2.3 ± 0.9 pmol/l) unless accompanied with tactile stimulation (17.5 ± 1.4 pmol/l) ($p < 0.05$, Scheffé test). Manually stimulated erections caused VIP levels to rise markedly (25.1 ± 1.7 pmol/l, $p < 0.001$).

Experiment III showed that release of VIP into the penile circulation occurred continuously during manually-induced erection in the wallaby but that plasma levels of the peptide did not increase proportionally to stimulus duration. Mean increases in VIP after 15s of stimulation (20.3 ± 3.0 pmol/l) were similar to those after 300s (24.3 ± 2.4 pmol/l) and did not differ from levels after intervening stimulation times (analysis of variance, $F = 1.43$; $df = 4, 24$; not significant).

Once manual stimulation had ceased, VIP concentrations in the penile circulation fell rapidly. In experiment III, VIP levels in two blood samples drawn consecutively after manual extension of the flaccid penis fell from 25.5 ± 2.9 to 3.6 ± 1.3 pmol/l ($t = 5.86$; $df = 6$; $p < 0.001$). Experiment IV showed that such declines were not due solely to rapid withdrawal of blood and depletion of a 'pool' of VIP within the penile circulation. Mean VIP levels in single samples taken two to four minutes after manipulation and cannulation of the penis were 4.2 ± 1.8 pmol/l as compared to 21.2 ± 2.8 pmol/l in samples taken immediately after manual stimulation in the same subjects.

DISCUSSION

The level of VIP in the penile circulation of the wallaby was always found to be greater than in the peripheral circulation. The functional significance of this endogenous release of VIP is unclear.

The salient feature of this study is that the best or 'adequate' stimulus for release of VIP into the penile circulation appears to be manual stimulation of the penis. This suggests that the stimulation which occurs during copulation may also contribute to VIP release. Since VIP is a potent vasodilator, it may play some role in the maintenance of penile erection during sexual activity. It is proposed that local VIP-containing neurons are the source of the VIP released (see Chapter 3).

These findings are not restricted to the wallaby. In preliminary experiments on a number of other species, mean levels of VIP (pmol/l) in the penile circulation were greater than in the general circulation - cheetah (Acinomyx jubatus) = 44.6: Barbary sheep (Ammotragus lervea) = 12.6; sooty mangabey (Cercocebus atys) = 33; pigtail macaque (Macaca nemestrina) = 13.6; puma (Felis concolor) = 90; and chimpanzee (Pan troglodytes) = 2.5, 16.6 - compared to either very low or non-detectable levels in the general circulation, except for one chimpanzee in which a VIP level of 6.6 pmol/l was found. In all cases tactile stimulation had occurred during collection of penile blood samples.

Although VIP may be measured in the penile circulation of a variety of mammals, concentrations of the peptide exhibited some interspecific variability. In the cheetah and puma, VIP levels rose dramatically to 64 and 141 pmol/l respectively during manipulation of the erect penis. These very high VIP levels in two feline species are consistent with the anatomical observation of a dense VIP innervation in the penis of the domestic cat (see Chapter 3).

The finding that VIP release is best produced by manual tactile stimulation may explain a number of findings concurrently reported by other investigators. Virag et al (1982) reported a release of VIP during penile erection in man. They obtained blood samples from the cavernous body and/or deep dorsal vein of the penis before and after the bolus administration of papaverine (smooth muscle relaxant) into one of the cavernous bodies. The papaverine-induced erections were accompanied by an increase in the concentration of VIP from the penis. Where papaverine failed to produce an erection, no VIP release was detected. It is not clear whether VIP release produced, or was concomitant with, the penile erection. The differences in tactile stimulation in the two groups were not described in sufficient detail. A study carried out subsequently (Ottesen et al 1984) demonstrated a twenty-fold increase in the VIP concentration in cavernous blood during penile tumescence and erection in normal men (2) and men with erectile dysfunction (7). In the normal subjects erection was induced by means of visual sexual stimulation and in the other subjects by intracavernous injections of papaverine or a continuous infusion of saline. It may be that the tactile stimulation of the penis during sample collection could have caused the increased output of VIP. Alternatively the injection of papaverine or the possible abnormalities in these men who were being investigated for erectile dysfunction may have affected VIP release.

Recent evidence to support a role for VIP in penile erection was provided by Andersson et al (1984). They described in quantitative terms the sequence of changes of arterial inflow, venous outflow and tissue volume in the penis during pelvic nerve-induced erection in

the dog. Upon stimulation there was first a prompt and pronounced dilatation of the penile 'resistance vessels' causing a greatly increased arterial inflow of blood which caused no sign of erection and, hence, bypassed the cavernous bodies, thus increasing venous outflow to the same extent. Atropine caused no significant alteration to this response. The second event was the erectile response proper which started after a delay of about 20 seconds. It appeared to be caused by a sudden opening of low resistance 'shunt vessels' diverting blood into the cavernous bodies and causing rapid filling. Atropine clearly depressed this erectile response. These data suggest that the neurotransmitter mechanism for dilation of the 'resistance vessels' is mainly non-cholinergic in nature, whereas a cholinergic mechanism seems to contribute to the erectile response proper by an inhibitory influence on the penile 'shunt vessels' or the trabeculae of the cavernous bodies or both. In fact, during the pelvic nerve stimulation there was a substantial output of VIP from the penis which was correlated in onset and duration to the vasodilator response. In the same study, intra-arterial infusion of VIP elicited a moderate erection and a penile vasodilator response which resembled the neural response.

Ottesen et al (1984) also showed that intracavernous administration of VIP (200 pmol) in healthy men produced varying degrees of tumescence or erection, whereas low doses of VIP or saline had no effect. But Steers et al (1984) found that intracorporal injection of VIP had no effect on the monkey penis and administration of VIP into the internal iliac artery of the monkey, while having no effect on the flaccid penis, caused detumescence of the erect penis obtained by cavernous nerve stimulation.

From these studies and most other evidence it is concluded that VIP fulfils several of the criteria of a neuroeffector in penile erection in mammals. It is present in nerve endings around cavernous smooth muscle and blood vessels (see Chapter 3) and can be released by tactile stimulation of the penis and electrical stimulation of the pelvic nerve. When exogenously applied, it has been shown to mimic the action of the endogenously-released transmitter and displays its characteristics in pharmacological experiments.

PATHOLOGICAL ROLE OF VIP IN IMPOTENCE

Introduction

Penile impotence has many causes. A clear understanding of erectile dysfunction is difficult, since the mechanism for a normal erection is not fully understood, as has been previously emphasized. However, no matter where or how the sexual reflex is initiated, the arc must continue down to the pelvic nerves in order to produce erection. Thus any impairment of the pelvic nerves will interrupt the reflex, so that there will be a failure of dilatation of the penile arteries, of engorgement of the penis with blood and hence erection. For example, in diabetic peripheral autonomic neuropathy, the pelvic parasympathetic nerves may be involved, resulting in impotence.

Impotence in diabetes is a common and slowly progressive phenomenon

and may take from 6 months to 2 years to become manifest. In one study, a random sample of 200 diabetic men showed 59% to be impotent; of these 82% had neuropathy (Ellenberg 1971).

Having proposed a role for VIP in mammalian penile erection, we investigated the VIP immunoreactivity in the penises of men with diabetic impotence and compared these findings with those obtained in potent men. The concentration of VIP in the penile tissue was measured by radioimmunoassay. Immunocytochemistry was also carried out in order to localize the VIP-containing nerve fibres.

Materials and Methods

Fresh erectile tissue was obtained from the corpus cavernosum of 12 men at the time of operation (at Charing Cross Hospital, London). The control group, aged 30 to 48 years, consisted of 6 transsexual men undergoing gender reassignment. The remaining 6 patients had an erectile dysfunction which had prevented vaginal intromission. Erectile tissue was obtained from these men when a penile prosthesis was inserted.

Diabetes was considered to be the major aetiological factor responsible for the impotence of these six men. Three of the patients studied had other evidence of a sensory peripheral neuropathy (eg abnormalities of the ankle jerk reflex and loss of vibration sense) whilst in others it was associated with an autonomic neuropathy.

Absence or impairment of erectile dysfunction was confirmed by

studies of nocturnal penile tumescence, phalloarteriography, doppler studies and cavernosography. The details of all patients with diabetic impotence are summarised in Table 7:1.

The tissues were extracted for radioimmunoassay according to the method described in Chapter 2. Immunocytochemistry and haemotoxylin and eosin staining were performed on comparable sections from each case.

Results

No significant abnormality was seen in haemotoxylin and eosin stained sections of these penises from impotent men, although some thickening of the arteriolar and capillary walls was sometimes seen along with varying degrees of atrophic changes of the smooth muscle.

In the penises from potent men undergoing gender reassignment operations VIP-immunoreactive nerves were abundantly distributed throughout the erectile tissue and especially around the blood vessels. VIP concentration was 189.9 ± 45.9 pmol/g wet weight (mean ± SEM). In the tissues from the left and right corpora cavernosa of impotent diabetic men, the mean concentration was only 43.4 ± 9.9 pmol/g (see Figure 7:2). In these cases decreases in numbers of VIP-immunoreactive nerves, as shown by immunocytochemistry, were pronounced.

Discussion

The number of VIP-immunoreactive nerves and the concentration of

VIP in the penises of the men with diabetic impotence has been shown to be considerably reduced in comparison with potent men.

Limitations of this study were necessitated by the fact that samples were only obtained from two groups, ie potent men undergoing gender reassignment and diabetic impotent men undergoing prosthetic implantation. Transsexuals suffer as controls in view of prolonged oestrogen treatment they have received prior to penectomy. The effect of oestrogen treatment on levels of VIP in the penis is at present unknown. Secondly, the effect of diabetes alone (ie where there is no evidence of impotence) has not been studied. Nevertheless, in spite of these limitations, it may be reiterated that the control group was comprised of sexually potent men and the diabetic group of impotent men.

Crowe et al (1983) reported that there were fewer VIP-immunoreactive nerves in the penile tissue from streptozotocin-diabetic rats compared to control animals and also in the penis of an impotent diabetic man when compared to three non-diabetic impotent men. Since no normal human penises were examined, these findings may merely reflect the different extents of VIP decrease in penises from men with impotence of varied aetiology and severity of symptoms.

Other pathological studies on the impotent penis have shown varying morphological changes. Faerman et al (1974) analysed the autonomic nervous fibres of the corpora cavernosa of the human penis by light microscopy. In five autopsies of impotent diabetics, 4 showed morphological alterations of varying degrees. These included a diffuse thickening with rigidness of the nerves and vacuolated

thickenings and abnormalities of the calibre of nerve fibres. Fani et al (1983) also reported pathological changes in the long-term diabetic rat, and showed that damage to nerves and smooth muscle can best be appreciated with electron microscopy.

Melman et al (1980) measured the content of nordrenaline and choline acetyltransferase activity in the erectile tissue of impotent men. They found that the noradrenaline content was decreased in nearly all of them, with the most severe reduction occurring in the insulin-dependent diabetics. They found no change in the choline acetyltransferase activity. The interpretation of their findings is difficult, since activity of the sympathetic nervous system is not usually assumed to be involved in the mechanism of erection. Yet the results indicate that the insulin-dependent subject may have a defect in the sympathetic nervous system, possibly related to penile function or simply reflect a general reduction of neural elements or neurochemical content in the tissue. This would fit with the morphological findings of our study in which a marked neuronal depletion was displayed by the use of antibodies to the general neuronal marker neuron specific enolase (Marangos et al 1975). The lack of correlation between our findings that VIP is reduced in the diabetic penis and those of Melman et al (1980) in which choline acetyltransferase activity is unchanged suggests that VIP and acetylcholine do not always co-locate in fibres in the human penis, if at all (see Chapter 3).

Because the diabetic woman is at equal risk for the development of diabetic neuropathy, with comparable involvement of the pelvic autonomic nervous system, one might postulate similar impairment of

sexual function. However, there is little documented evidence about the relation of diabetes to female sexuality, even though the deleterious effects on sexual performance in the diabetic male are so well recognized.

In conclusion, the depletion of VIP-immunoreactive nerves seen in the impotent diabetic penis in this study may be either a primary or secondary change. It may be the result of the diabetes. Whatever the cause, the diminution in VIP levels would be suggestive evidence of a neurological basis for impotence and supports the contention that VIP may be a principal neurotransmitter involved in penile erection.

TABLE 7:1 Impotence in Diabetic Patients

AGE (Years)	DURATION OF IMPOTENCE (Years)	EVIDENCE OF NEUROPATHY	CORPUS CAVERNOSUM VIP CONCENTRATION (pmol/g)	
			Left	Right
62	3	YES	74.0	79.8
23	5	YES	39.0	42.4
44	4	NO	15.8	18.7
47	3	NO	36.3	18.2
53	4	NO	40.7	84.9
59	8	YES	41.2	28.5

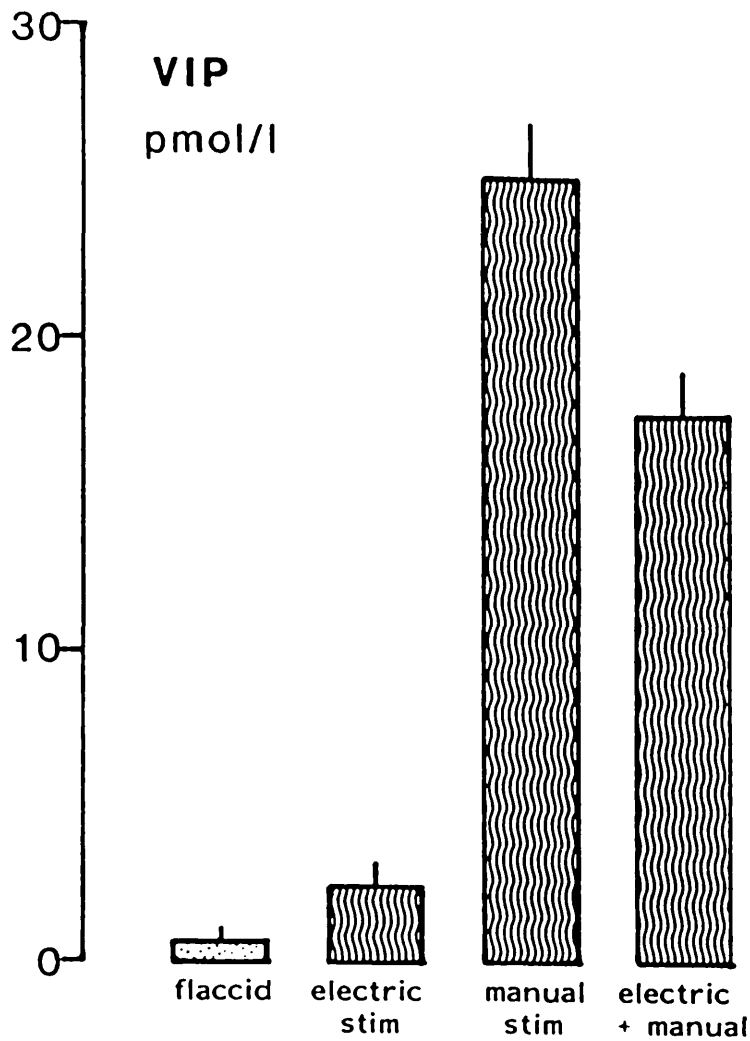


FIGURE 7:1 Plasma VIP levels in the wallaby penis (pmol/l) (mean ± SEM); responses to manual and electrical stimulation of erection.

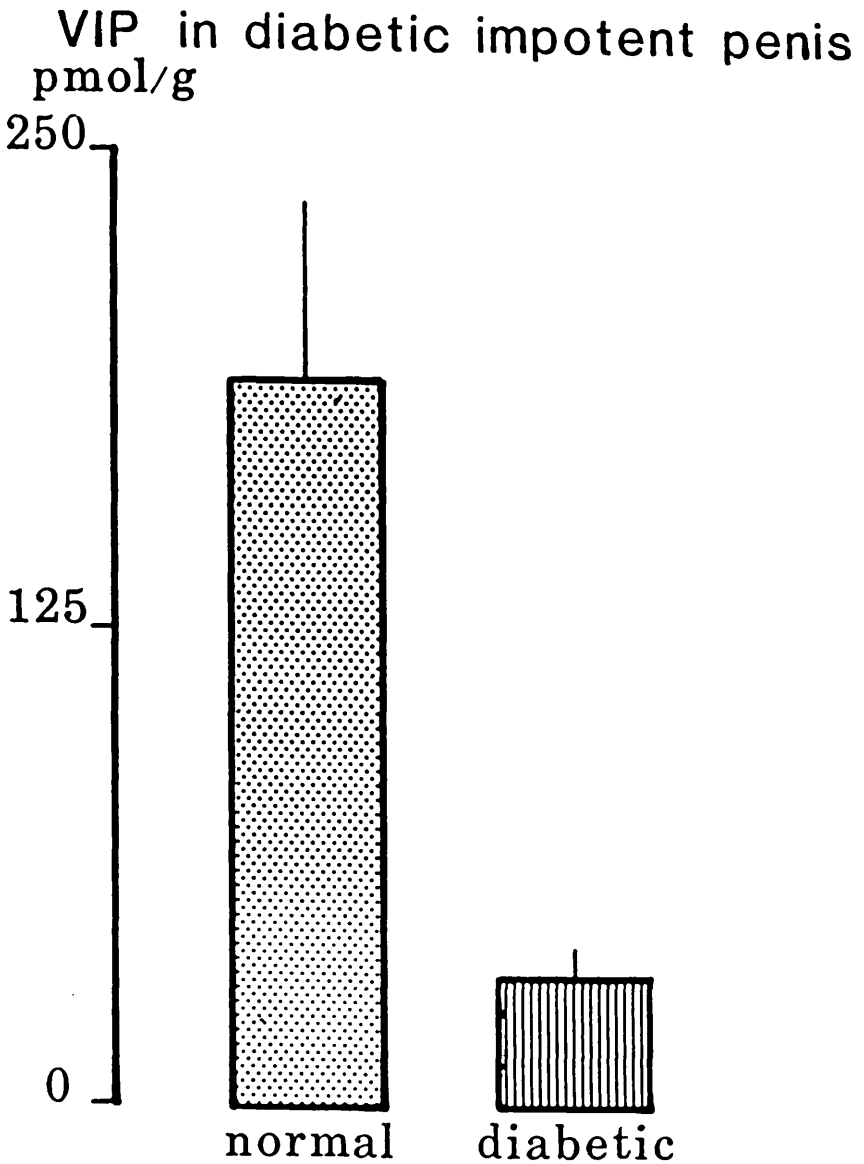


FIGURE 7:2 VIP concentration (pmol/g) (mean ± SEM) in normal and diabetic human penis.

CHAPTER 8

GENERAL DISCUSSION

This chapter is in two parts:- in the first part the salient findings of this thesis are discussed in the context of contemporary studies and directions of future research. In the second part, evidence is presented to define the extent to which VIP fulfils the criteria of a neurotransmitter in the mammalian genitourinary system.

The new finding presented in Chapter 7 is that the most adequate stimulus for the release of VIP into the venous outflow from the wallaby penis is tactile stimulation of the penis. The finding is clearly pertinent to other studies, which fail to document the extent to which tactile stimulation occurred during the course of the experiment. These include investigations which showed that penile erection in man induced by cavernosal injection of a smooth muscle relaxant is associated with a release of VIP (Virag et al 1982; Ottesen et al 1984). It has been proposed on the basis of our study, that the role of the vasodilator VIP is to maintain penile erection during sexual activity, particularly following the tactile stimulation which occurs during copulation.

The mechanism by which VIP may act in efferent pathways to the penis has been studied recently by Andersson et al (1984). Release of VIP correlated in onset and duration to the penile vasodilator response during pelvic nerve stimulation in the dog.

The increase of VIP seen in the rat urinary bladder following denervation supported the view that the major origin of VIP was from local ganglia. However, the study of VIP in human (and subsequently cat) spinal cord demonstrated that it marks a subpopulation of pelvic afferents. The studies presented in Chapter 5 show that within the spinal cord, the predominant source of VIP-containing fibres are pelvic afferents. This finding provides a new tool for neurobiologists and pathologists to study the central connections of pelvic afferents in physiological conditions and in disease.

One unanswered question of considerable importance to this thesis is the location of the VIP-containing afferent terminals within the peripheral organs. The difficulty in answering this question lies in the fact that the vast majority of fibres seen are efferents of local origin, which themselves respond to surgical or pharmacological manipulations of the sensory fibres, eg pelvic nerve transection in the cat fails to show an apparent change in VIP innervation of the bladder by radioimmunoassay or immunocytochemical methods, and in the rat bladder there is actually an increase of VIP following such a procedure. Sacral dorsal root ganglionectomy in adult cats and systemic treatment of neonatal rats with capsaicin have also failed to show the site of VIP afferent fibres. It is likely that some afferents from the bladder contain VIP, as bilateral high intensity stimulation of the vesical branches of the cat pelvic nerve has been shown to release VIP-like immunoreactivity into sacral spinal cord superperfusate (see Blank et al 1984, Publications). Two new approaches may resolve the problem. Tracer studies using double-staining methods are being attempted, as well as layered dissection of the organs concerned following sensory

denervation (it may be, for example, that most VIP-containing afferents derive from a particular region, such as the submucosal layer).

Studies with animal models of the VIP innervation in the penis and bladder led to proposals of their functional role. Apart from their potential value in diagnosis and management of human disease, studies of human impotence and unstable bladder proved to be a test of the proposed roles of VIP. The preliminary results showed reduced tissue VIP levels in the human diabetic impotent penis, and a dramatic decrease in the human unstable bladder. These findings are in accord with the proposed roles of VIP, and provide encouragement towards the development of therapeutic pharmacological agents that mimic VIP. These results show that a direct study of VIP innervation in patients with genitourinary disorders is both feasible and useful, and further such studies are at present under way in the genitourinary tract and post-mortem spinal cord.

Before reviewing the evidence relating to VIP, it is necessary to present the criteria which should be fulfilled in order to establish a substance as a neurotransmitter (Werman 1966). The substance should be present in presynaptic terminals of neurones; the neurones must possess the necessary mechanisms to synthesize the transmitter; the substance should be released upon activation of the presynaptic neurones, exogenously applied substance should mimic the action of the endogenously released material and should display an identical pharmacology; mechanisms for terminating the action of the candidate transmitter should be present; the effects of the substance, and those elicited by nerve stimulation should be blocked

by specific antagonist(s) and, finally, specific receptors for the transmitter substance must be demonstrated on postsynaptic membranes.

Within the genitourinary system, VIP increasingly fulfils the criteria of a neurotransmitter.

Numerous studies have been cited in Chapters 3 and 4 that demonstrated the presence of VIP cell bodies and fibre terminals in the genitourinary system.

The synthesis of VIP by neuronal tissue is probably the most difficult criterion for neurotransmitter status to demonstrate. Preliminary data indicated that ^{35}S-methionine injected into rat cerebral vesicles was incorporated into a large molecular weight precursor for VIP (Fahrenkrug 1980). Such experiments have not been carried out in the genitourinary system. Ribosomes responsible for synthesis of VIP, or its precursor, are found only in neuronal perikarya. The synthesized material then has to be transported from the cell body to the nerve terminals before it can be released to act post-synaptically. A number of pharmacological and surgical procedures that block axonal transport support the view that VIP is transported from cell bodies to terminals: neuronal cell bodies in many brain areas containing VIP-like immunoreactivity were found to stain much more intensely after prior treatment of the animal with the transport blocker, colchicine (Lorén et al 1979) and surgical interruptions of fibres containing VIP-like immunoreactivity resulted in an accumulation of the peptide on the cell body side of the cut (Marley & Emson 1982).

The release of VIP resulting from stimulation of efferent pathways in the genitourinary system has been documented in studies that have been extensively cited in this thesis (see Chapters 3,4 and 7).

Exogenously applied VIP has been shown to mimic the action of endogenously released VIP in the genitourinary system. Infusion of VIP in vivo (Fredericks & Lundquist 1983) has been shown to markedly inhibit contractile activity in the Fallopian tube, an effect which has been seen on local nerve stimulation (Helm et al 1982; Murcott & Carpenter 1977). VIP has also been shown to produce a dose-dependent reduction in spontaneous motor activity of human tubal strips (Walles et al 1980; Owman et al 1983) and urinary tract smooth muscle (Klarskov et al 1984; Sjögren et al 1985; Levin & Wein 1981; Andersson et al 1983; Larsen et al 1981).

Specific receptors for VIP have been demonstrated in the genitourinary tract - in myometrial membranes from porcine uterus (Ottesen et al 1982a).

Mechanisms for terminating the action of a transmitter could include an enzymatic inactivating system and/or a specific uptake mechanism into pre- or post-synaptic structures. These have been poorly studied for VIP and other neuropeptides in any region. As for other neuropeptides, it is reported that VIP proteases are likely to be widely distributed and are responsible for the termination of action of VIP rather than a specific uptake mechanism for the peptide (Straus et al 1982).

As yet, a specific antagonist which blocks the effects of exogenously applied VIP and those elicited by nerve stimulation causing a release of VIP is not available. However, VIP antiserum has been successfully applied to this end in organs other than pelvic organs. In the cat submandibular gland, local intra-arterial infusion of VIP antiserum (but not non-immune serum) reduced both the vasodilatation and salivary secretion induced by parasympathetic nerve stimulation (Lundberg et al 1982). In the cat and opossum lower oesophageal sphincter, and guinea pig gastric fundus and trachea, VIP antiserum inhibited relaxation induced by exogenous VIP and electrical stimulation of non-adrenergic non-cholinergic inhibitory nerves at a level that caused a release of VIP (Biancani et al 1984; Grider et al 1985; Goyal & Rattan 1980; Matsuzaki et al 1980).

Thus VIP is a strong candidate as a neurotransmitter mediating non-adrenergic non-cholinergic vasodilatation and smooth muscle relaxation in the genitourinary system, although a number of criteria remain to be fulfilled. In addition, it is possible that it acts as a neuromodulator or a trophic agent (see Burnstock 1981).

Finally, several immunocytochemical studies have demonstrated the presence of one or more neuroactive peptides in adrenergic and cholinergic autonomic neurones- VIP is present in acetyl-cholinesterase-staining post-ganglionic autonomic fibres (see Schultzberg 1983), and VIP and PHI may co-exist in the same neurone (see Chapter 1). First reports suggest that VIP-containing parasympathetic efferents in the genitourinary system do not stain

for acetylcholinesterase but, nevertheless, possible interactions between VIP and other released neurotransmitters must be considered, even if they are not co-released from the same neurone. A tentative model has been proposed by Lundberg et al (1982) for the interaction of VIP and acetylcholine released from neurones supplying blood vessels and exocrine glands, which may be applied to studies of the genitourinary system. They propose that VIP induces atropine-resistant vasodilatation and potentiates acetylcholine-induced secretion. In a study of the mechanisms of the interaction, VIP was found to enhance the binding of acetylcholine to muscarinic receptors on exocrine glands. The presence of PHI release alongside VIP will further complicate the model. Unravelling the physiological significance of co-existence of more than one putative transmitter within a neurone, and of interactions following release pose major new problems for future studies of VIP in the genitourinary system.

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