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CHARACTERIZATION AND REGULATION OF THE ANTERIOR PITUITARY RECEPTOR FOR VASOACTIVE INTESTINAL PEPTIDE

by

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ABSTRACT

Radioligand binding methods were employed to study the anterior pituitary receptor for vasoactive intestinal peptide (VIP) in support of its proposed action as a prolactin releasing factor. Following methodological studies to optimize binding conditions, the interaction of VIP with rat anterior pituitary membranes was found to be saturable, reversible, affected by guanine nucleotides, and dependent on pH, time, temperature and magnesium concentration. Binding was specific with VIP being 100 fold or more potent as compared to VIP homologous peptides. Using structurally modified analogs of VIP, comparison of VIP to different tissues suggest the existence of VIP receptor subtypes, with anterior pituitary binding resembling that seen in the brain. VIP binding was associated chiefly with lactotrope enriched fractions of anterior pituitary cells, obtained by unit gravity sedimentation. Prolactin secretion was stimulated in vitro by VIP in a dose dependent fashion with an ED_{50} of 1 nM. Nonlinear regression analysis of competitive binding studies indicated the presence of two binding sites, of high and low affinity. Regulation of these receptors was observed under several conditions including postnatal development, lactation and by testosterone. Only the low affinity receptor was detected prior to 3 weeks of age in female rats, while both receptors were present after 3 weeks to at least 1 year. Very little difference in the VIP receptor was observed during lactation in the female rat. Surgical castration of the male rat increased the number of high affinity binding sites, along with a decrease in affinity. These changes were not seen in sham operated animals, and could be prevented by administration of testosterone immediately after castration. These findings demonstrate specific binding sites for VIP in the rat anterior pituitary that localize to the lactotrope. Regulation of these receptors during development and other conditions associated with altered prolactin secretion is supportive of a biological role for VIP in lactotrope function.

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Ernst und Else

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
B	receptor bound radioligand
Bmax	maximum amount of binding sites per quantity of tissue
BSA	bovine serum albumin
CNS	central nervous system
cAMP	cyclic adenosine monophosphate
CD	Sprague Dawley rats
DMEM	Dulbecco's modified eagles medium
ED_{50}	dose of agonist producing 50% of maximal response
EDTA	ethylenediamine tetraacetic acid
F	free concentration of radioligand
icv	intra-cerebroventricular
IC_{50}	dose inhibiting maximal response by 50%
iv	intravenous
FSH	follicular stimulating hormone
GH	growth hormone
(r,b,h,)GRF	growth hormone releasing factor from rat, bovine and human species
GTP-γ-S	guanosine-5'-O-thiotriphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatograpy
K_D	equilibrium dissociation constant
LH	luteinizing hormone
(r,b,p,) PHI	peptide with N-terminal histidine and C-terminal isoleucine, from rat, bovine and porcine species
hPHI	human derived peptide with N-terminal histidine and C-terminal methionine (or also PHM)

PMSF	phenylmethylsulphonylfluoride
PRL	prolactin
RIA	radioimmunoassay
SEM	standard error of the mean
TFA	trifluoroacetic acid
TRH	thyrotropin releasing hormone
Tris	tris(hydroxymethyl)aminomethane
TSH	thyroid stimulating hormone
VIP	vasoactive intestinal peptide

INTRODUCTION

An effect of vasoactive intestinal peptide (VIP) on the secretion of prolactin (PRL) from the anterior pituitary has been observed. As PRL secretion appears to be regulated by a variety of factors, the biological relevance of these substances, including VIP, needs to be established. Purported to act directly on the anterior pituitary as a prolactin releasing factor (PRF), VIP action would require the presence of specific anterior pituitary receptors. The main objective of this study was to investigate whether such receptors could be identified and characterized utilizing radioligand binding studies. To facilitate understanding of the rationale for this study, the following will initially be discussed: 1. regulation of PRL synthesis and secretion from the anterior pituitary gland, 2. VIP, its receptors and role as PRF, and 3. the study of receptors, with emphasis on radioligand binding methods.

I. PROLACTIN

A. Localization and synthesis

PRL has been identified in the anterior pituitaries of almost all vertebrates examined and is secreted into serum in heterogeneous forms with molecular weights ranging from 22000 to >60000 daltons (1-3). Most prevalent in mammals is the lower molecular weight PRL consisting of a single polypeptide chain with about 200 amino acid residues (4,4). Synthesized as preprolactin on membrane bound polysomes, PRL is cleaved from this precursor and processed into granules for storage and release (5). This synthesis of PRL occurs primarily within cells, called lactotropes, that stain positively with acid dyes such as carmoisin, and comprise from 10 to 50% of adenohypophyseal cells depending upon the physiological state of the animal (6,7). Small numbers of anterior pituitary cells called mammosomatotropes, producing both PRL and growth hormone (GH), have also been identified (8,9). Although scattered throughout the anterior pituitary gland, lactotropes predominate at its posteromedial and posterolateral edges, frequently in close association with gonadotropes (6,10). Heterogeneous populations of lactotropes with respect to cell size and location, secretion of newly synthesized versus stored PRL, and responsivity to secretagogues have been identified using immunocytochemistry, electron microscopy and reverse hemolytic

plaque assay (11-14). PRL may undergo transformation (identified experimentally by altered chemical extractability) prior to secretion in response to certain stimuli such as suckling and dopamine (14,15).

B. Biological function

The physiological role of PRL is diverse (16) and remains to be fully elucidated. Well established is the effect of PRL on lactation, including development of the mammary ductal system in rodents (17) and lactogenesis in mammals generally (18). Once milk formation and secretion has been established, continuation of this process requires PRL in some but not all mammals (18,19). Suckling is a potent stimulus for PRL secretion in mammals (20-22).

Other actions of PRL including maintenance of the corpus luteum in rats (23), gonadotropic actions including stimulation of luteinizing hormone (LH) receptors in rat Leydig cells (24), inhibition of adrenal 5 α-reductase in rats (25), and parenting behaviour in birds (26) have been described. Receptors for PRL have been identified on many tissues including liver (27) and kidney (28), however the function of PRL at many target tissues is unclear (29).

C. Pattern of secretion

Circulating levels of PRL vary in response to many different physiological stimuli. In humans and other primates, normally low basal PRL levels show spontaneous fluctuations coupled temporally with LH secretion (30). Increases in PRL are seen daily during sleep (31), and secretion is in a pulsatile fashion (32). Differences in PRL secretion between males and females have been observed in many species, with sexual differentiation related to androgenization of the male hypothalamus and pituitary during neonatal life (33,34). Patterns of PRL secretion vary with reproductive cycles, pregnancy, lactation and suckling in the female (35). Anaesthetics like halothane and urethane increase PRL secretion (36), as do stressful situations such as hypoglycaemia, strenuous exercise and surgery (37). PRL release associated with surgical stress may be secondary to anaesthetic administration as surgery during epidural block does not change basal PRL (38). The patterns of PRL secretion during

the rat estrous cycle, lactation and development will be discussed in more detail as experimental evidence supports VIP involvement under these conditions (39).

1. Rat estrous cycle. The primary change in PRL during the estrous cycle in the female rat is a brief increase during the early part of proestrous, accompanied by similar surges of progesterone, LH, and FSH (40,41). Increases in follicular derived estrogens precede these rises (410) and seem necessary for PRL elevation as administration of estrogen antiserum reversibly blocks this surge (42). Estrogens mediate this rise in PRL at least partially via an indirect mechanism (43,44), and administration of VIP antiserum (45) or oxytocin antagonists (46) attenuate the proestrous PRL surge. VIP appears primarily involved in the early peak of the proestrous PRL surge, and not with the lower, sustained increase in PRL seen later in proestrous (46). Other factors such as decreases in hypophyseal portal blood levels of dopamine (47) and in anterior pituitary dopamine receptor number (48) may also participate in the proestrous PRL surge.

2. Lactation and suckling. Nipple stimulation, mechanically or by suckling, is an extremely potent stimulus of PRL secretion in lactating mammals, with elevations in PRL recorded within minutes of stimulation and lasting until stimulus removal and even beyond (37). In rats the hypothalamic paraventricular nucleus appears necessary in coupling suckling with the rise in PRL (49). Serotonin may mediate this neuroendocrine reflex as suckling induces depletion of hypothalamic serotonin within 5 minutes (50) and serotonergic neurotoxin attenuates the PRL response (51). As serotonin does not act directly on the anterior pituitary (52) this effect may be mediated via other factors including VIP. Hypothalamic mRNA for VIP increases during lactation (53) and VIP antiserum is able to partially blunt suckling induced PRL secretion (54). Antiserum to TRH (55) or administration of naloxone, an opiate receptor antagonist, (56) also attenuates but does not completely block the suckling induced rise in PRL.

3. Ontogeny. Development of lactotropes and PRL secretion demonstrates species variability. In contrast to early prenatal pituitary organogenesis and PRL secretion in human, bovine, ovine and porcine species, lactotropes in the rat first become detectable only very late

in gestation or at birth, and anterior pituitary PRL content and secretion remains low until at least 2 weeks after birth (32-38). Although PRL containing cells can be identified in the neonatal rat using immunocytochemistry, the majority of these cells do not spontaneously secrete PRL in the reverse hemolytic plaque assay until more than 2 weeks after birth (57-63). Cytodifferentiation of lactotropes in both rats and humans does not appear to require the hypothalamus although hypothalamic factors such as gonadotropin releasing hormone (GnRH) and LH have been found to stimulate lactotrope differentiation in vitro (58,64-66). Altered responsiveness prenatally and in the neonate, to modulators of PRL active in mature animals has been observed in sheep (stress, TRH, dopamine and serotonin) (57) and in rats (dopamine, VIP and TRH) (67). Aging results in increased numbers and secretory capacity of lactotropes in rats (68-70). PRL synthesis in the rat appears to be translationally and transcriptionally regulated (71).

D. Regulation of synthesis and secretion

Numerous reviews have been written about the neuroendocrine regulation of PRL (35,72-75). It has become clear that the effects of many factors are integrated to achieve measured PRL levels. An ever expanding number of substances have been reported to influence PRL secretion in vivo, in vitro or both. These substances may act directly at the anterior pituitary level on PRL synthesis, or as PRL release inhibitory factors (PIF) or PRL releasing factors (PRF). Indirect action at hypothalamic and pituitary levels via perturbations of the direct acting factors is postulated as the mechanism for others.

1. Prolactin inhibitory factors. As hypophyseal stalk sectioning (76) and pituitary autotransplantation without the hypothalamus (77) results in increased PRL secretion from the anterior pituitary, it has been widely accepted that PRL release from the anterior pituitary is under tonic inhibitory control by the hypothalamus. Dopamine is the undisputed major PIF thus far identified (78,79). It reaches the median eminence of the hypothalamus via the tuberoinfundibular system, and is released into hypophyseal portal blood in concentrations sufficient to inhibit PRL secretion in vivo (47,80). Specific, high affinity dopamine receptors (D_2) have been identified in anterior pituitaries of many species (78,81),

and dopamine acts in a dose dependent fashion to inhibit PRL release in vivo and in vitro (82,83). Antagonists of dopamine can prevent this effect (84,85). Further support of a biological role are changes in levels of dopamine or its receptor concomitant with alterations in PRL release observed under certain physiological conditions such as the estrous cycle of the rat (47,48). The inability of dopamine, at concentrations present in hypophyseal portal blood, to suppress to normal, the spontaneous secretion of PRL observed after median eminence lesions (86) or blockage of dopamine synthesis (82,87) indicates other PIFs may be operative.

The physiological relevance of other putative PIFs such as gamma amino butyric acid (88,89) and acetylcholine (90) is less definitive. A peptide PIF in hypothalamic extracts coeluting with GnRH from a Sephadex G-25 column identified as GnRH associated peptide (GAP) by Seeburg and colleagues initially demonstrated in vitro PRL inhibitory characteristics similar to dopamine (91). These actions of GAP have however been difficult to reproduce and physiological significance remains unclear (92,93).

2. Prolactin releasing factors. Despite evidence that PRL secretion is tonically inhibited, decreases in hypophyseal portal dopamine levels cannot account for PRL surges observed in such states as suckling and stress (73,94,95,115). As the role of other PIFs is unclear the presence of PRFs has been postulated to account for these rises in PRL. This is supported by the PRL releasing properties of hypothalamic extracts (96,116). A plethora of substances have been described possessing PRL releasing capabilities, including thyrotropin releasing hormone (TRH), VIP, peptide with amino terminal histidine and carboxy terminal isoleucine (PHI), oxytocin (105-109), galanin (97,98), relaxin (100), substance P (101), bombesin (102), neurotensin (103), and calcitonin (104). Most extensively studied and with considerable experimental support, TRH and VIP are considered prime candidates as physiologically relevant PRFs.

TRH, a known hypothalamic releasing factor for thyrotropin (TSH), is able to release PRL both in vitro and in vivo from the anterior pituitaries of different species (110-112). It is present in hypophyseal portal blood at concentrations sufficient to evoke PRL release

in vitro (113), and specific receptors for TRH exist in the anterior pituitary as well as on the PRL secreting tumour GH cell line (114). Very importantly, TRH is able to stimulate PRL release in the presence of physiological levels of dopamine (115), and its action has been found separate from that induced by dopamine receptor blockade in humans (85,117). Continuous intravenous (iv) infusion of the smallest doses of TRH capable of stimulating TSH release also increases PRL levels in humans (118), and iv administration of TRH antiserum attenuates the PRL rise occurring during proestrous in the rat (55). Although the evidence supporting TRH as a PRF is very strong, observations that TSH and PRL release are not always parallel, as has been observed during suckling (119), supports the existence of other PRFs. The limited influence of TRH on PRL secretion in male animals (118), the decreased sensitivity of this sex to dopamine along with lower dopamine levels than in females (78,120), indicates other PRFs are operative in the male.

The experimental evidence for VIP functioning as a PRF will be discussed in detail later in this dissertation. The effect of other members of the secretin family of peptides, to which VIP belongs, will be discussed at that time also.

3. Indirect control. Hypothalamic substances that stimulate PRL secretion in vivo, but fail to do so in vitro, include serotonin, histamine and the opioids. Serotonin injected iv or intracerebroventricularly (icv) increases circulating PRL (121) via an effect that can be blocked by prior administration of VIP (or PHI) antiserum (122,123). Activated serotonergic neurons in the midbrain projecting to the medial basal hypothalamus induce VIP release from the median eminence into hypophyseal portal blood (124,125). It appears serotonin acts through VIP to stimulate PRL secretion, and a role for this pathway has been postulated for the nocturnal as well as suckling induced surges in PRL (126). These rises in PRL can be attenuated by serotonergic blockade with methysergide or by VIP antiserum in rats (124,125,127).

The hypothalamus contains high concentrations of histamine with neuronal connections to the median eminence (128). Central administration of histamine appears to

exert its effects largely by inhibition of dopamine secretion into hypophyseal portal blood (78,129).

Opioids (endorphins, enkephalins and dynorphins) are present in both hypothalamus and pituitary,but appear to mediate their effect on PRL indirectly (131). They are involved in suckling (131) and stress (132) induced increases in PRL, and modulate hypothalamic dopamine (78) or serotonin (133). Although not able to stimulate PRL secretion directly beta endorphins appear to enhance the action of TRH on PRL release (134).

PRL acts to regulate its own secretion by feedback at the level of the hypothalamus (135). High PRL levels exert short loop feedback inhibition by increasing secretion of hypothalamic dopamine (78,136). Additionally, this feedback may involve PRFs, as PRL has recently been found to influence the hypophyseal portal blood levels of VIP and oxytocin (136).

As noted above some opioids influence the action of PRFs in the anterior pituitary. Somatostatin and estrogens also appear to exert their considerable effect on PRL at this level. The effects of other gonadal as well as adrenal steroids have not been as well studied.

Somatostatin, a hypothalamic GH release inhibiting factor, has specific receptors on lactotropes as well as somatotropes (137). Its action to decrease PRL secretion via these independent sites appears dependent on estrogen (137). Somatostatin is capable of antagonizing TRH and VIP stimulated PRL release (138,139). Contradictory effects of somatostatin on basal PRL release have been reported (139,140).

Estrogens have long been known to influence PRL. Many states of altered PRL secretion such as pregnancy, lactation and rat estrous cycling are also characterized by changes in sex steroids (35). Although species variability is seen, with primates less sensitive than rodents, in vivo estrogens stimulate PRL secretion, as well as the frequency and growth of PRL secreting tumours (52,72,141,142). In vitro they increase PRL mRNA, PRL synthesis, the number of lactotropes, as well as PRL secretion (143-146). These effects are likely exerted via specific nuclear estrogen receptors in the lactotrope (147) and involve

decreasing the sensitivity of the anterior pituitary to dopamine (148), and regulating anterior pituitary TRH receptors (173).

Progesterone appears to act at the hypothalamus with inhibitory or stimulatory effects on PRL depending on prior estrogen priming (149). The effects of testosterone on PRL have been controversial. Although most studies find stimulation of PRL release after administration of testosterone and decreased levels after castration of male animals (33,150,151,153), the opposite effect has been demonstrated (154) as well as none at all (152). These discrepancies may be due to in vivo but not in vitro conversion of testosterone to estrogen metabolites (43,153) as well as the physiological state of the animal (154). Single measurements of PRL may be too insensitive to detect changes in PRL secondary to testosterone, as Grosser and Robaire (155) have demonstrated that castration subtly influences the pulsatility, peak and nadir of PRL pulses. These actions may be directly on the lactotrope as morphological changes of these cells due to testosterone has been detected (156). Alternately the predominant localization of testosterone receptors to gonadotropes (157) and the interaction of dispersed gonadotropes and lactotropes on PRL release described by Denef (158), suggests that testosterone may exert an action indirectly via gonadotropes on PRL secretion.

An effect of glucocorticoids was also suspected as patients with Cushing's disease lose their nocturnal PRL surges (159). Glucocorticoids inhibit basal and certain stress (hypoglycaemia) mediated increases in PRL (36,160). In vitro studies have however found both attenuation (161) and augmentation (162) of VIP mediated PRL by glucocorticoids, making the action of glucocorticoids in PRL secretion unclear. Sex steroids and glucocorticoids appear to act via specific nuclear receptors in the anterior pituitary to influence transcription and/or translation of PRL (163). They appear also to influence production of other proteins influencing PRL secretion such as the lactotrope receptors for dopamine and somatostatin (87,164).

4. Paracrine and autocrine control. As mentioned before interactions between gonadotropes and lactotropes influence PRL secretion in vitro. The work of Denef on

enriched populations of dispersed anterior pituitary cells, documented the need of coincubation of gonadotropes with lactotropes to elicit the PRL stimulatory effects of GnRH and angiotensin II (165). These data strongly suggest a paracrine interaction with humoral factor(s) secreted from the gonadotropes acting on lactotropes to influence PRL release, with candidates including VIP, TRH, calcitonin, substance P and neurotensin. Autocrine regulation is also possible as lactotropes have been found to synthesize factors influencing PRL secretion. VIP and adenosine are two such substances. Adenosine receptors have been identified on GH cells and the agonist, R-2-phenyl-isopropyl adenosine, inhibits the ability of TRH and VIP to stimulate PRL release (166). The presence of PRL receptors on lactotropes (167) suggests an autocrine feedback loop may be operative.

5. Posterior pituitary control. Many of the factors capable of altering PRL secretion are found in the posterior pituitary. Vascular channels between posterior and anterior pituitary have been shown, and Ben Jonathan and coworkers have identified a posterior pituitary PRF distinct from known PRL secretagogues (168,169). Their finding that posterior lobectomy in rats elevated levels and interrupted normal cyclicity of PRL suggested that posterior pituitary dopamine, oxytocin, VIP, opioids as well as unidentified factors may be important in the regulation of PRL (170,171). The transient nature of the PRL rise after posterior pituitary lobectomy, as well as the quantitatively much smaller response when compared with stalk sectioning (171,172), however suggests that contribution from the posterior pituitary may be small or operative only in select circumstances such as suckling and rat proestrous (39,73).

E. Mechanism of secretion

Secretion of PRL from lactotropes appears to be mediated via calcium, adenylate cyclase activation, and phospholipid breakdown. The ultimate response is likely due to the cumulative effect of these factors. The action of TRH through its receptor is dependent on extracellular calcium and has largely been studied in the GH tumour cell line (174). It hydrolyses phosphatidylinositols generating inositol phosphates and diacylglycerol (175), substances that have been identified as intracellular messengers in other calcium dependent

systems. Although no absolute changes in phosphoinositides in pituitary membranes incubated with and without dopamine were found by Canonico et al. (184), others found increased hydrolysis after dopamine withdrawal (184,185). Dopamine is capable of inhibiting TRH mediated PRL release (115), and transient dopamine withdrawal in suckling rats results in augmentation of TRH mediated PRL secretion (186). This latter effect appears contingent on transformation of intracellular PRL by dopamine. VIP, on the other hand, stimulates adenylate cyclase in the anterior pituitary in a dose dependent fashion that correlates well with its ability to stimulate PRL release in vitro (177-179). Inhibition of VIP stimulated PRL secretion by somatostatin is tightly coupled with its inhibition of VIP stimulated cAMP accumulation (180). Dopamine action via specific receptors on lactotropes appears capable of interfering with both adenylate cyclase and phospholipid pathways (181). It reduces basal (182) and VIP stimulated (183) cAMP levels in anterior pituitary cell cultures, in a manner coupled to decreases in PRL secretion (182).

As detailed above, PRL secretion from the anterior pituitary is regulated by multiple factors interacting at various levels. The remainder of the thesis will deal specifically with the role of VIP in this regard. The definitive physiological relevance of this peptide can however, only be interpreted in the context of these other factors.

II. VASOACTIVE INTESTINAL PEPTIDE

A. Historical perspective

VIP was first identified by Said and Mutt from porcine small intestine in 1970 (187,188), although vasodilatory actions of intestinal extracts had been reported as early as 1902 by Bayliss and Starling (189). Purification of VIP was achieved using the method for secretin isolation (191), plus four additional separation steps, with vasodilatation in anaesthetized dogs utilized as the bioassay to follow purification (188). Immunohistochemistry has since identified VIP as a neuropeptide (190), widely distributed in the peripheral nervous system, supplying structures such as the gastrointestinal tract (192), exocrine glands e.g. salivary (193), urogenital tracts (194) respiratory tract (195,196) and vascular beds (196,197). In 1976 VIP was also found to be extensively distributed throughout the central nervous system (CNS) (190) and is now considered one of the ever growing list of gut-brain peptides (198.199). The actions of VIP that have been reported on different organ systems are as diverse as its distribution. Although exact physiological roles remain to be established, reported actions include vasodilation (200), bronchodilation (201), intestinal water and electrolyte secretion (203), pituitary PRL secretion (203) and depolarization of CNS neurons (204).

B. Chemistry and molecular biology

1. Structure. First sequenced by Mutt and Said in 1974 (205), VIP from hogs was found to be a carboxyl amidated peptide consisting of 28 amino acids with the following sequence:

His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2.

VIP appears well conserved in the animal kingdom and primary structures are identical in pigs (205), humans (206), cows (207), rats (208), goats and dogs (209). Conservative amino acid substitutions of 4 residues has been found in the quinea pig (209) (Table 1).

TABLE 1. Amino acid sequences of porcine, guinea pig, and chicken VIP

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Pig	His	Ser	Asp	Ala	**Val**	Phe	Thr	Asp	**Asn**	Tyr	**Thr**	Arg	**Leu**	Arg	Lys	Gln	Met	Ala	**Val**
Guinea pig	His	Ser	Asp	Ala	**Leu**	Phe	Thr	Asp	**Thr**	Tyr	Thr	Arg	Leu	Arg	Lys	Gln	Met	Ala	**Met**
Chicken	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	**Ser**	Arg	**Phe**	Arg	Lys	Gln	Met	Ala	Val

	20	21	22	23	24	25	26	27	28
Pig	Lys	Lys	Tyr	Leu	Asn	Ser	Ile	Leu	**Asn-NH2**
Guinea pig	Lys	Lys	Tyr	Leu	Asn	Ser	**Val**	Leu	Asn-NH_2
Chicken	Lys	Lys	Tyr	Leu	Asn	Ser	Val	Leu	**Thr-NH_2**

Differences in amino acids between chicken and guinea pig VIP and porcine VIP are in bold print.

Nonmammalian chicken VIP also differs from the common mammalian form by 4 amino acids (210). VIP has also been identified in more primitive species such as the nervous structures of the earthworm (211) and intestine of the dogfish (212).

VIP is a highly basic molecule with a molecular weight of 3381. Molecular variants, principally more acidic forms as well as larger precursor forms have been identified (213). These are found primarily in the gut, whereas brain and peripheral nerves supplying other tissues have predominately one form of VIP (214,215).

2. VIP family of peptides. VIP is a member of the secretin-glucagon family of peptides (Table 2). Of the 9 and 6 amino acid residues that share a common position with VIP in mammalian secretin and glucagon respectively, the majority are found in the amino terminal region of the molecules. These three peptides share amino terminal histidyl-seryl residues separated by 3 amino acids from a common phenylalanyl-threonyl sequence. These last two amino acids are also shared by another member of the secretin family, gastric inhibitory peptide (GIP). Other members of this expanding family that have been more recently identified, share even greater homology with VIP (Table 2). These include the growth hormone releasing factors (GRF), first isolated from human pancreatic tumour (hGRF) and since identified to be the same in human hypothalamus, as well as GRF from rats (rGRF) and cows (bGRF). Even greater similarities are seen with a 27 amino acid peptide first isolated by Tatemoto and Mutt from porcine small intestine (217). This peptide demonstrates species variability and is called PHI when derived from pigs, rats and cows, because of shared amino terminal histidines and carboxy terminal isoleucines (218). The equivalent human peptide, hPHI, is also called PHM as the carboxy terminal amino acid residue is methionine (219). Many biological actions of VIP are shared with these homologous peptides. Their actions are however species and target tissue specific and will be discussed more fully under VIP receptor structure activity relationships.

3. VIP gene. The human VIP gene has been mapped to chromosome 6 and contains 7 exons, each encoding a distinct functional domain of the VIP precursor or its mRNA (219). As genes found in close proximity on the same chromosome are often functionally related,

TABLE 2. Amino acid sequences of members in the secretin-glucagon 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
VIP	**His**	**Ser**	**Asp**	**Ala**	**Val**	**Phe**	**Thr**	**Asp**	**Asn**	**Tyr**	**Thr**	**Arg**	**Leu**	**Arg**	**Lys**	**Gln**	**Met**	**Ala**	**Val**	**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**

	22	23	24	25	26	27	28	29
VIP	**Tyr**	**Leu**	**Asn**	Ser	Ile	**Leu**	Asn-NH_2	
Secretin	**Leu**	**Leu**	Gln	Gly	**Leu**	Val-NH^2		
Glucagon	**Phe**	**Val**	Gln	**Trp**	**Leu**	Met	**Asp**	**Thr**
GIP	**Phe**	**Val**	**Asn**	**Trp**	**Leu**	**Leu**	**Ala**	Gln ...

Amino acid sequences common to VIP and secretin, glucagon, or gastric inhibitory peptide (GIP) are in bold type.

... only 29 N-terminal amino acids of GIP are shown.

TABLE 2 (continued)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
VIP	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln	Met	Ala	Val	Lys	Lys	Tyr
pPHI	His	Ala	Asp	Gly	Val	Phe	Thr	Ser	Asp	Phe	Ser	Arg	Leu	Leu	Gly	Gln	Leu	Ser	Ala	Lys	Lys	Tyr
rPHI	His	Ala	Asp	Gly	Val	Phe	Thr	Ser	Asp	Tyr	Ser	Arg	Leu	Leu	Gly	Gln	Ile	Ser	Ala	Lys	Lys	Tyr
bPHI	His	Ala	Asp	Gly	Val	Phe	Thr	Ser	Asp	Tyr	Ser	Arg	Leu	Leu	Gly	Gln	Leu	Ser	Ala	Lys	Lys	Tyr
pPHI	His	Ala	Asp	Gly	Val	Phe	Thr	Ser	Asp	Phe	Ser	Lys	Leu	Leu	Gly	Gln	Leu	Ser	Ala	Lys	Lys	Tyr

	22	23	24	25	26	27	28
VIP	Tyr	Leu	Asn	Ser	Ile	Leu	Asn-NH_2
pPHI	Tyr	Leu	Glu	Ser	Leu	Ile-NH^2	
rPHI	Tyr	Leu	Glu	Ser	Leu	Ile-NH_2	
bPHI	Tyr	Leu	Glu	Ser	Leu	Ile-NH2	
pPHI	Tyr	Leu	Glu	Ser	Leu	Met-NH2	

Amino acid seqences common to VIP and PHI peptides from porcine (p), rat (r), bovine (b) and human (h) species are underlined.

Heterologous amino acid sequences among the PHI structures are in bold type.

TABLE 2 (continued)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
VIP	His	Ser	Asp	Ala	Val	Phe	Thr	Asp	Asn	Tyr	Thr	Arg	Leu	Arg	Lys	Gln	Met	Ala	Val	Lys	Lys	Tyr
rGRF	His	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Arg	Ile	Leu	Gly	Gln	Leu	Tyr	Ala	Arg	Lys	Leu
bGRF	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Ser	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln	Leu	Ser	Ala	Arg	Lys	Leu
hGRF	Tyr	Ala	Asp	Ala	Ile	Phe	Thr	Asn	Ser	Tyr	Arg	Lys	Val	Leu	Gly	Gln	Leu	Ser	Ala	Arg	Lys	Leu

	23	24	25	26	27	28
VIP	Leu	Asn	Ser	Ile	Leu	Asn-NH2
rGRF	Leu	His	Glu	Ile	Met	Asn...
bGRF	Leu	Gln	Asp	Ile	Met	Asn...*
hGRF	Leu	Gln	Asp	Ile	Met	Ser...*

Amino acid seqences common to VIP and GRF peptides from rat (r), bovine (b) and human (h) species are underlined.

Heterologous amino acid sequences among the GRF structures are in bold type.

... only 28 N-terminal amino acids of GRF structures are shown.

* C-terminal NH_2.

of great interest is the finding that the human PRL gene has been mapped to a similar region on chromosome 6. The overall architecture of the VIP gene is similar to genes encoding peptides from the secretin-glucagon family (220). Complementary DNA sequencing of the mRNA for VIP, first done in human neuroblastoma cells, resulted in a translational product of 170 amino acids with VIP located near the carboxyl end of the precursor protein (219). In the centre of this precursor was a 27 amino acid peptide corresponding in structure to PHI (221). Hence these two peptides, VIP and PHI, share not only sequence homology but also a common gene. Cosecretion of VIP and PHI has since been documented (222), but as they are not always found in the same tissues differential tissue processing may occur (223).

Regulation of VIP gene expression has been found under various conditions including lactation and ontogeny (219). Modification of VIP expression could result in altered levels of VIP, and thereby potentially explain changes in PRL secretion seen during suckling and development. During lactation in female rats immunopositive VIP neurons in the hypothalamus increase in number (224). Transcriptional regulation is likely operative as 2 fold increases in VIP mRNA levels in the hypothalamus of lactating rats has been observed (225). Although not studied in prolactinomas, the observation that DNA rearrangements of the VIP gene in human epidermoid carcinoma-Hep#3 cells result in altered expression, suggest that VIP could potentially play a role in malignancies (219,220).

C. Distribution in the hypothalamic-pituitary axis

1. Hypothalamus. VIP has been identified in the hypothalamus of birds (226,227), rats (224,228,229,231,233), mice (229) cats (230), pigs (232), monkeys (233) and humans (234), using radioimmunoassay (RIA) and immunohistochemistry. Present in synaptosomes, (235,236) VIP has been localized to the paraventricular (PVN) and suprachiasmatic nuclei (SCN) of the hypothalamus (224,225). Lesions of the PVN in rats results in ablation of the PRL rise normally seen with stress, 5-hydroxy-L-tryptophan administration (237) and lactation (225), all conditions in which VIP has been implicated as mediator of PRL secretion. Neuronal projections from these nuclei to the median eminence have been reported in the transmission of central signals to the anterior pituitary for hormonal secretion, hence

transport of VIP from these nuclei to the median eminence and thereby to the anterior pituitary is possible. Indeed in human (238) and avian species (226,227) high values of immunoreactive VIP are found in the median eminence. This contrasts with low levels in the median eminence of rats in some studies (228), although significant amounts were detected by others in rodents (229). These discrepancies may be secondary to differing assay sensitivity, high turnover or low basal VIP levels as Mezey and Kiss were only able to identify VIP positive fibres in the median eminence of rats after pretreatment with colchicine (224).

Release of hypothalamic VIP would be necessary to mediate an effect on the anterior pituitary gland. Such release has been documented in vitro after nerve depolarization using potassium (236), and secondary to galanin (239), a neuropeptide that possesses PRL releasing capabilities (240). This localization and release of VIP from hypothalamic nuclei and the median eminence in different species, provides one criterion for VIP function as a classical hypothalamic hypophyseal factor.

Regulation of the concentration of other hypothalamic factors has been observed and implicated as a control mechanism by indirect acting substances on pituitary function. Hypothalamic VIP levels are also regulated under various conditions associated with altered secretion of PRL. The increase in number of VIP staining neurons in the PVN and median eminence of rats during lactation (224,225) could represent a mechanism by which the increase in PRL release, seen at this time, is controlled. The centrally mediated influence of steroids on PRL, could also be secondary to changes in hypothalamic VIP. Both estrogens and glucocorticoids have been found to decrease hypothalamic levels of VIP (224,241,242).

2. Hypophyseal portal blood. A common pathway by which hypothalamic peptides reach the anterior pituitary to exert their effects is via the hypophyseal portal circulation (portal) from the median eminence to the adenohypophysis. VIP has been measured in portal blood of rats after removal of the anterior pituitary, in concentrations averaging 19 times and up to 180 times those in peripheral arterial blood (243), indicating secretion of VIP into these vessels. Basal and stimulated portal VIP levels ranging from 400 to 3700 pg/ml (0.01 to 1.2

nM) have been measured in at least three independent laboratories (243,244,245). Similar portal blood VIP levels in rats were found after removal of the entire pituitary (244) or the gut (245), eliminating respectively the possibilities that these levels were secondary to retrograde flow of VIP contained in the pituitary, or from the largest peripheral source of VIP i.e. the gut. Dopamine, accepted as a hypothalamic factor, is present in portal blood at similar concentrations, suggesting that these levels of VIP are potentially capable of acting on the anterior pituitary (78).

Altered rates of VIP secretion into the portal circulation of rats have been found under some conditions known to influence PRL secretion. Serotonin or its precursor L-5-hydroxytryptophan (5HTP) injected icv, increase PRL secretion (246) and increase portal VIP levels significantly in the face of constant flow rates of portal blood (247). The administration of four anaesthetics, known to variably affect the release of PRL and other anterior pituitary hormones, results in differing concentrations of VIP in portal blood (245). Although VIP levels differ throughout the estrous cycle of rats no consistent increase in portal VIP is seen during the proestrous PRL surge (245). This lack of effect may be secondary to maximal stimulation of VIP release during the experiments secondary to surgical manipulation or administration of anaesthetics (245).

3. Anterior pituitary. VIP was identified in the anterior pituitary soon after its presence in the CNS was established (228,248) As peptide hormones acting through cell surface receptors are frequently internalized by the cells upon which they act, this was not surprising for a putative hypothalamic pituitary factor, and immunohistochemical localization of the intact VIP molecule to the lactotrope of the rat anterior pituitary (248) supported a direct action by VIP on the PRL secreting cell. Localization of VIP only to PRL containing cells of the rat anterior pituitary was found after separation of cells to 99% purity using fluorescence activated cell sorting (personal communication, Dr.D. Wynick, Royal Postgrad. Med. School, London, UK). More recently however, incorporation of [^{3}H]leucine into immunoprecipitatable and HPLC purified VIP by quartered rat anterior pituitaries, suggests this peptide is also synthesized in the anterior pituitary gland (249). The ability of VIP

antiserum to decrease PRL secretion from dispersed anterior pituitary cells in the absence or presence of exogenously administered VIP, also suggests that endogenous VIP exists and might account for the high "spontaneous" or basal secretion of PRL from lactotropes (250). Anterior pituitary derived VIP would also account for the dose dependent suppression of basal PRL secretion by VIP antiserum or antagonists seen by reverse hemolytic plaque (251). This putative locally synthesized VIP likely operates by an autocrine (versus paracrine) mechanism as the shortest distance from PRL secreting cells to other cells in the reverse hemolytic plaque assay was 7-10 times greater than the distance VIP is thought to diffuse during the experiment (251). These results are however controversial as Lam et al. (254) were only able to identify VIP in the anterior pituitaries of rats after they had been rendered hypothyroid using thionamide. These investigators did not find colocalization of VIP with PRL or TSH, and VIP immunoreactive cells were only one-tenth as abundant as lactotropes.

Indirect acting factors could hence influence PRL secretion by an effect on pituitary, portal blood and hypothalamic levels of VIP. Such effects have been observed secondary to steroids. In contrast to decreases in hypothalamic VIP, estrogen treatment of ovariectomized rats results in increased anterior pituitary VIP levels (242,252). Adrenalectomy, on the other hand, increases both hypothalamic and anterior pituitary VIP via an effect that is reversible with dexamethasone (241).

4. Posterior pituitary. The posterior pituitary content of VIP varies in different species. Rats contain very small amounts (228) whereas significant levels are found in the pig (232), dog, guinea pig and human (253). The elevation of PRL, and loss of PRL cyclicity and suckling induced surges after posterior pituitary lobectomy in the rat (168,171) implies that factors other than VIP are operative from the posterior pituitary in this species. This could be due to dopamine or other PRFs (78), however as noted above, VIP synthesized in the anterior pituitary could still potentially relay PRL release via an autocrine effect.

Localization and regulation of VIP levels suggest that it has the potential to act as a classical hypothalamic pituitary factor, locally via an autocrine mechanism, and in some

species by release from the posterior pituitary. The effects of VIP on anterior pituitary function will now be discussed.

D. Effect on secretion of anterior pituitary hormones

1. Prolactin.

a. In vivo studies. VIP injected peripherally or centrally (icv), stimulates PRL secretion in a dose dependent fashion in rats (255,256), rhesus monkeys (257), humans (258-260), turkeys (261), bantam hens (226) and doves (262). In addition to the PRL rise observed within minutes of VIP administration in all these studies, continuous infusion into rats of VIP is capable of prolonged PRL elevation for days (256). In contrast to mammals, control of PRL secretion in avian species is primarily stimulatory as hypothalamic stalk sectioning decreases PRL levels in birds. The presence of VIP in avian hypothalami, and potent in vivo PRL releasing abilities makes VIP a prime PRF candidate in birds (262). In vivo administration of the VIP fragments (VIP10-28 and VIP16-28) is ineffective in increasing PRL secretion (261). As PRL secretion increases in response to VIP both in intact and stalk sectioned monkeys (257) and turkeys (261), a direct effect on the anterior pituitary by VIP is likely.

In vivo studies employing VIP antiserum have resulted in altered PRL secretion primarily in PRL stimulated states. Administration of VIP antisera to rats results in ablation or attenuation of the PRL rise normally seen with suckling and ether stress (54,237,263), 5HTP (123,237), serotonin (122) and galanin (240) administration and during early proestrous (45). Although VIP antiserum alone does not modify basal PRL levels in male rats, pretreatment with this antiserum attenuates the increase in PRL normally seen after blockade of dopamine receptors (264). PRL pulse analysis reveals that VIP antiserum significantly decreases the pulse amplitude, trough and peak values normally induced by the dopamine antagonist, domperidone (264).

b. In vitro studies. VIP has been shown to directly stimulate PRL secretion in whole pituitaries from birds (226), hemipituitaries from rats (265-268) and bullfrogs (269), anterior pituitary fragments from monkeys (257), dispersed and cultured anterior pituitary cells from

rats (268,270,271) and humans (272), and lactotrope enriched fractions of anterior pituitary cells from rats (273). Original reports of nonresponsiveness of rat anterior pituitary tissue to VIP in vitro (255,274) was likely secondary to adsorption of the highly basic VIP molecule to glassware or its degradation during assay. These problems were circumvented by inclusion in the assay of bacitracin, an antibiotic/peptidase inhibitor (265,267). VIP dose dependent stimulation of PRL has been observed in most of the studies cited, with minimal effective doses ranging from 0.1 to 10 nM, and maximum stimulation seen with 1 to 10 μM VIP (257,265,267-273). The half maximal effective dose (ED_{50}) of VIP stimulated PRL release varies with tissue preparations. VIP is most potent in dispersed anterior pituitary cells, especially those enriched in lactotropes, with an ED_{50} of 2 to 7 nM (273,270,271).

c. Tumour tissue. VIP injection in normal humans increases peripheral PRL levels significantly in both men and women (258-260). The effect of VIP on PRL secretion in people with pathological hyperprolactinemia is however controversial. Lack of any PRL response to VIP in patients with prolactinomas has been promoted as a diagnostic tool by some investigators (258,259). Others, however, have found no difference in the percent PRL response to VIP above basal, between patients with prolactinomas and normal people (203). Investigators observing lack of responsiveness of prolactinomas to VIP agree that responsiveness is not restored by inhibition of dopamine. After successful adenomectomy in previously unresponsive women however, return to normal VIP induced PRL release was observed (260). Patients with hyperprolactinemia secondary to hypothalamic disease, e.g. craniopharyngiomas, vary in their responsiveness to VIP, depending perhaps on their degree of hyperprolactinemia (259).

In contrast to the variable results observed in vivo, VIP is able to stimulate PRL secretion from virtually all prolactinoma tissue studied in vitro (203,272). An interesting study by Spada et al. (275) sheds some light on these different observations, as marked differences between macro (>10 cm) and micro adenoma PRL responsiveness is found in vitro, to VIP stimulation as well as dopamine inhibition. Microadenomas are much more responsive to VIP, whereas the opposite is observed for dopamine (275).

Another prolactinoma product that has been used extensively in the study of PRL secretion are GH cells. GH cells are clonal strains of hormone producing cells originally established by Tashjian et al. (276) from a female Wistar rat with a GH and PRL secreting pituitary tumour. Since then many subclones have been established, including GH_3 and GH_4C_1 cells, which spontaneously synthesize and secrete variable amounts of hormones that are biologically active and immunologically indistinguishable from authentic rat PRL and GH. These cells respond to TRH, somatostatin, estradiol and cortisol in a manner analogous to normal anterior pituitary tissue, and have therefore been used extensively in studying the regulation of secretion of hormones.

PRL secretion from GH_3 and GH_4C_1 cells is stimulated by VIP in a dose dependent fashion (278-280). VIP stimulation is more potent in GH_3 cells with an ED_{50} of 0.2 to 0.5 nM (123,124) compared with 30 to 80 nM in GH_4C_1 cells (280). VIP stimulated PRL secretion from these cells occurs within approximately one minute (280) and is sustained whether VIP is administered in a pulsatile or continuous fashion (279).

Despite their usefulness, GH cells are tumour cells and differences from normal pituitary tissue has been detected and may limit extrapolation of data to normal physiology. Many GH cells do not respond to dopamine as they lack the specific receptors normally found on PRL secreting cells (281). Also the major benefit of a homogeneous population of cells compared with the multicellular composition of the normal pituitary may not be accurate as GH_3 cells have been found to be functionally heterogeneous by reverse hemolytic plaque assay (282). Some secrete PRL, others GH and a third group neither; with marked changes in the percentages of each of these three cells occurring after treatment with TRH, estradiol or cortisol (282).

d. Interaction with other factors. As secretion of PRL from the anterior pituitary is regulated in a complex manner by a variety of stimulatory and inhibitory factors, it is important to see that the actions ascribed to VIP act in concert with those of other neurotransmitters and peptides.

The ability of a PRF to stimulate PRL in the presence of the inhibitory actions of dopamine is important in establishing physiological relevance, as increases in PRL are frequently seen in the presence of significant portal blood levels of dopamine (78). Dopamine inhibits PRL secretion stimulated by VIP both in vivo and in vitro (255,257,272). VIP is able to partially overcome this inhibition in a dose dependent fashion, especially when VIP and dopamine are administered in vitro at equimolar concentrations (270,271). VIP interferes with dopamine mediated decreases in PRL pulse duration, peak and trough values in male rats (264). VIP and dopamine appear to act through separate receptors in the anterior pituitary as the dopamine antagonist, flupentixol, does not block the effects of VIP at concentrations that inhibit dopamine action (264). Dopamine likely antagonizes PRL secretion stimulated by VIP by inhibiting its stimulation of intracellular cAMP (182,183). Direct inhibition of adenylate cyclase is possible as the anterior pituitary D_2 receptor is coupled to a pertussis toxin sensitive GTP binding protein (283).

Transient removal of dopamine inhibition appears responsible for the depletional transformation of pituitary PRL that is seen with suckling, early pregnancy and during the rat estrous cycle (116,186). Although this effect of dopamine is insufficient to mediate the release of PRL seen (186), this transient removal and subsequent intracellular transformation of PRL, increases the responsiveness of PRL secretion to TRH administered subsequently in the presence of dopamine (115,116). No conclusive evidence exists to date whether VIP stimulation of PRL is also enhanced by this transformation of PRL. A similar increased responsiveness to VIP, as for TRH, after the temporary removal of dopamine is likely as the administration of non-TRH hypothalamic extracts along with TRH are necessary to obtain the sustained PRL rise normally seen after suckling (284). Also, dopamine removal stimulates Ca^{++} uptake of rat pituitary cells in culture (285), an action known to enhance the cAMP mediated increase in PRL secretion by VIP (286).

Despite the evidence strongly supporting TRH acting to stimulate PRL secretion, it is unlikely to be the only, or even the major, PRF. This supposition is based on several lines of evidence including: a. dissociation of TSH and PRL levels in vivo, even though PRL

responsiveness to exogenous TRH is maintained (119,287), b. PRL releasing activity exists in the hypothalamus that is distinct from TRH (96,116), and c. the inability of TRH to increase PRL in a sustained fashion (256,85,117). The actions of VIP and TRH on PRL secretion are likely complementary as: a. their effects are additive in vitro (266,279), b. their mechanisms of action are different, with VIP acting via adenylate cyclase (266) and TRH by hydrolysis of membrane phosphoinositides (175), and c. their pattern of stimulating PRL secretion differs, with the response to TRH occurring earlier and being transient, whereas the VIP induced response is sustained (256,279,280) This latter difference helps account for the observation that VIP can be less, more or equipotent as compared to TRH, in releasing PRL in vitro (257,266,270), as PRL levels are usually measured only once and at variable times after stimulation in different studies. As complete reversal of dopamine inhibition requires stimulation of both the cAMP and Ca^{++} messenger systems (181) it is possible to speculate that both VIP and TRH are active in physiological situations where a surge of PRL is followed by a sustained increase (78,186).

Somatostatin inhibits stimulation of PRL by VIP, by acting through specific anterior pituitary receptors (138). This inhibitory action of somatostatin is tightly coupled to inhibition of cAMP (138,180).

In addition to altering VIP levels in the hypothalamus and anterior pituitary as mentioned above (242), estrogens could potentially influence VIP stimulation of PRL by inducing lactotrope somatostatin receptors (137), increasing the number of lactotropes (143,144), and stimulating PRL synthesis (145,146).

Acute administration of glucocorticoids, specifically dexamethasone, abolishes VIP mediated PRL release in vitro (273). This effect correlates well with inhibition of cAMP stimulation by VIP, and changes only the maximal response and not the potency of VIP on PRL secretion (161). Opposite effects are observed after chronic dexamethasone treatment (162). In vivo effects of glucocorticoids on VIP stimulated PRL secretion correspond to those found in vivo (160).

Although testosterone increases PRL secretion from castrated rats of both sexes (43,151,152), marked differences between the sexes has been observed in basal and estrogen stimulated PRL secretion (33,154). An effect, if any, of testosterone on VIP mediated PRL secretion has not been reported.

2. Other hormones. The effect of VIP on other hormones secreted by the anterior pituitary has not been as extensively studied. Changes are not detected in FSH and TSH release secondary to VIP in both in vivo and in vitro studies (258,268,274,288). In vivo studies injecting VIP iv or icv into humans and rats increases peripheral levels of GH, LH and ACTH in some (274,288) but not all studies (203,258). These actions appear to be at the level of the hypothalamus as in vitro studies with VIP on anterior pituitary cells enriched in specific hormone secreting cells do not result in increased GH, LH or ACTH release (268,273). Possible mechanisms by which VIP may mediate these actions at a hypothalamic level include regulation of GnRH (274,289) and inhibition of hypothalamic somatostatin (290).

Stimulation of GH may however also be at the level of the anterior pituitary as VIP increases GH release when dispersed rat anterior pituitary cells are reaggregated and pretreated with dexamethasone (165,291). In the mouse VIP is able to increase GH from dispersed cells but only at a threshold of 0.1 μM (292).

VIP stimulation of GH and ACTH release in vivo and in vitro, has been reported in human adenomas (203) and in clonal pituitary tumour cells (203). As GH secreting tumours show paradoxical responses to many agents (203,277,293) the biological relevance of these observations is questionable.

E. VIP RECEPTOR

1. Distribution. As for other hormones and neurotransmitters, VIP mediates its actions via interaction with specific, high affinity receptors located in plasma membranes of target tissues, although nuclear VIP receptors have also been described (294). Binding sites for VIP have been described in many normal tissues including rat and guinea pig brain (295-298), rat and human intestine (299-303), rat and porcine liver (304,305), rat, human, guinea

pig, cat and rabbit lung (306-309), rat, cat and guinea pig pancreas (310-312), human sweat glands and monocytes (313,314), rat, bovine and porcine vasculature (315,316) rat ventral prostate, pineal, spleen, fat and lymphoid cells (317-321), porcine uterus (322) and bovine thyroid (323). VIP binding is also found on carcinomatous tissue from intestine (299), lung (306) and anterior pituitary (324-326). The transformed anterior pituitary tissue to which binding of VIP has been described are GH_3 and GH_4C_1 cells, and in a cursory fashion, a human prolactinoma. Identification of VIP receptors on normal anterior pituitary tissue utilizing radioligand binding methods to date consists only of a brief mention in a major study on rat brain VIP receptors that autoradiography reveals VIP binding to the anterior pituitary (327), and binding was seen to, but not characterized in, rat anterior pituitary cells (328).

2. Effector mechanisms. VIP mediates its actions through membrane receptors coupled to adenylate cyclase activation (177-179). The components thought to be operative in the transduction of a VIP stimulus into the cell in most, and likely all tissues, are specific receptors at the outer plasma membrane surface coupled to GTP binding proteins and the catalytic unit of adenylate cyclase.

Stimulation of adenylate cyclase activity and cAMP levels by VIP in a dose dependent fashion is found in anterior pituitaries from rats (161,177,268,329) and mice (292), human prolactinomas (275,326), and GH cells (178,178,278,280,330). This stimulation, potentiated by guanine nucleotides (177,329), is rapid and occurs prior to VIP mediated PRL secretion (178,278,280). The potency with which VIP stimulates cAMP compares with its ability to stimulate PRL release in vitro (178,280). The increase in sensitivity of PRL secretion to VIP from micro versus macro adenomas correlates with a similar sensitivity of cAMP stimulation to VIP (275). The cAMP response to VIP in rat anterior pituitary cells is biphasic suggesting action through more than one receptor is possible (329). The inability of VIP fragments, with deletions of amino terminal amino acids, to stimulate cAMP in rat anterior pituitaries (177) is in agreement with a lack of PRL responsiveness (261).

3. Pharmacological and biochemical properties.

a. Radioligand binding studies. Two binding sites, with differing affinity for VIP, have been identified in most tissues possessing VIP receptors. Equilibrium dissociation constants (K_D) for the high affinity site are generally subnanomolar, with a range reported from 0.06 nM (309) to 36 nM (297). The lower affinity site has reported K_D values ranging from 4.8 nM (309) to 480 nM (323). Site-to-site interactions (i.e. positive or negative cooperativity) have not been reported for the VIP receptor (303,315,320). Single binding sites have been reported in rat brain (295,331) and GH_3 cells (324).

Binding of VIP to its receptor is influenced by divalent cations (296,331,297) and pH (303,320,331,332). Pretreatment of receptor containing tissue with reducing agents such as dithiothreitol (333,334) or with trypsin (3305,335) decreases or abolishes VIP binding, indicating the protein nature and likely the presence of disulfide bonds in the receptor. GTP decreases the binding of VIP to its receptor at equilibrium (297,333,334,336,337) by increasing the rate of dissociation of VIP from the receptor (306,309).

b. Structure activity studies. In biological response and ligand binding studies the intact VIP(1-28) molecule has been found necessary for full potency and efficacy (296,299,338,339). Based on studies utilizing fragments of the VIP molecule, amino acids in positions 11-22 are needed for binding, whereas residues 1-10 and 23-28 seem to confer affinity (296,302,339). Of interest is the amino terminus of the VIP molecule. The His in position 1, a feature VIP shares with secretin, glucagon, PHI and rGRF (Tables II), is very important in the ability of VIP to act via its receptor. Deletion of His^1 or alteration of either the imidazole ring or the free α-amino group results in decreased potency and/or efficacy of VIP in activating adenylate cyclase in many tissues including the anterior pituitary (341,342). The potency of hGRF in binding to the VIP receptor is markedly enhanced when its amino terminal Tyr^1 is replaced by His^1 (300). L-amino acids in residues 1-4 are important for potency as individual substitutions with D-amino acids results in analogs less active than VIP, with potency decreasing the closer the D substitutions are to the amino terminus (343-345). The Val in position 5, which is common to VIP and PHI, also appears

important in binding to the VIP receptor as [Val^5]secretin is more potent then native secretin (305-306).

On the basis of binding interactions, three subclasses of receptors belonging to the glucagon-secretin family have been proposed (346). These are: a. the VIP-secretin group, also interacting with PHI and GRF, b. glucagon and c. GIP. No cross reaction with groups a and c has been observed, and very little between groups a and b. Although marked differences in potency and efficacy are described, the VIP receptor in most tissues appears to recognize the homologous peptides in the following order: VIP>PHI>GRF>secretin. Glucagon may or may not interact at very high concentrations.

Variability in binding of VIP analogs and homologous peptides to the VIP receptor from tissue to tissue in the same species (341,343), and in the same tissue from species to species (301,302) has been observed. To date no subclassification of VIP receptors has been possible on the basis of study with homologous peptides or VIP analogs. Although antagonistic properties have been reported for some VIP analogs, there is at present no good VIP antagonist, as those without efficacy have extremely low potency, whereas others possess variable efficacy depending on the tissue and species examined (299,300,308,344).

c. Affinity labelling and solubilization studies. Apparent molecular weights reported for the VIP receptor obtained by cross-linking experiments range from 16,000 to 159,000 (309,337,347-352,357). In tissues where VIP labels multiple proteins of differing molecular weight, the proteins differ in the effect GTP has on binding as well as the affinity for VIP. This is likely due to the presence of two distinct binding sites of high and low affinity, as described above (309,347-349,357). This is supported by solubilization from guinea pig lung membranes of two distinct VIP binding proteins with high and low affinity for VIP, with GTP inhibiting VIP binding only to the high affinity species (353). Photoaffinity labelling of HT-29 cells identified two VIP binding sites also, with only the higher affinity site coupled to adenylate cyclase (349). Although wide ranges are found, most tissues possess a major VIP binding protein with molecular weight around 47-64,000 (354). The discrepancies here may be due to differing carbohydrate moieties as the VIP receptor is a glycoprotein

(309,355). Coupling of the VIP receptor with the GTP proteins is likely phospholipid dependent as pretreatment with phospholipases (A_2 and C) attenuated the inhibitory effect of GTP on VIP binding (352). Whether the other proteins represent distinct receptors or complexes and fragments of the major form is not known.

4. Regulation. In addition to the regulation of VIP receptor binding found in vitro and detailed above, changes in both the number and the affinity of the VIP receptor have been detected in various tissues under different in vivo conditions. Decreases in the number of VIP receptors in the rat ventral prostate occur with increasing age and after castration (357,358). The effect of castration can be reversed by administration of testosterone (357). Increased light exposure and stress result in increased numbers of VIP receptors in pineal (359) and mononuclear cells (360) respectively. Changes in VIP receptor number and affinity in rabbit uteri is found during pregnancy and with estrogen therapy (361). Homologous desensitization, or down regulation of its own receptors, has been observed after administration of VIP in vitro (362).

5. Ontogeny. Changes in the levels of neurotransmitters has been observed during development, suggesting a possible role for some of these factors in maturation. Development of receptors has also been suggested as a mechanism regulating the onset of normal cellular function, such as hormone secretion. As a putative neurotransmitter, the development of VIP has been studied in the CNS and gastrointestinal tract, with marked differences observed in the two locations. Changes in the VIP receptor with age has also been observed in these two tissues.

a. Gastrointestinal tract. VIP has been identified in the intestinal tract of prenatal rats, however levels increase with age reaching maximum values by day 60 postpartum (364). Progressive attenuation of the cAMP response to VIP in the intestine occurs during pre and postnatal development. Along with this decrease in cAMP response, slight decreases in receptor affinity are seen along with apparent reductions in its molecular weight (365). These changes are not seen in the liver VIP receptor (365). A regulatory role is postulated for VIP in maturation of the intestine and liver of the fetal rat as specific VIP receptors are

expressed on these tissues prior to functional and morphological differentiation (365). Supportive of a maturational role in the intestine is the observation that differentiation of the colonic HT29-18 cells to enterocyte-like cells is also associated with attenuation of the cAMP response to VIP (366).

b. CNS. In contrast to the gut, VIP is not detectable in the CNS of fetal rats (367-371). When the hypothalamus is examined specifically, VIP also only becomes detectable at birth (367,371). After birth, VIP content in the hypothalamus suddenly rises between day 3 and day 5 and increases to reach maximum levels around day 40 (369). Similar patterns for VIP are observed in the cerebral cortex (369). Despite similar development of VIP, the pattern of VIP mRNA differs between hypothalamus and brain, with high levels found in the former at birth, whereas none is detected in the cerebral cortex (372). This raises the possibility that other VIP gene products may be expressed earlier and be developmentally regulated. The development of the other known product of this gene, PHI, however largely parallels that of VIP in the CNS (373). This pattern of hypothalamic VIP development compares with that found for GnRH (374) and TRH (375). The pattern of VIP development in the pituitary has not been studied.

The VIP receptor in the brain also appears to change with age as radioligand binding to brain membranes is low at day 2 pp, and rises markedly between days 7 and 17 postpartum (377). Differences in the postnatal development of the VIP receptor in regards to density and total number are seen comparing forebrain and hindbrain (377). Despite the low level of VIP binding at birth, VIP is able to stimulate cAMP in the rat brain at this time with activation constants for adenylate cyclase comparable to adult values (376).

As these patterns of postnatal VIP development in the hypothalamus correlate in time with the appearance of lactotropes in the anterior pituitaries of rats (63) and of PRL secretion (58-60) it is interesting to speculate that VIP may be developmentally involved in PRL regulation. Nothing is known regarding development of VIP receptors in the anterior pituitary.

F. Comparison with PHI

As mentioned above, PHI interacts with the VIP receptor to a variable degree depending upon the source of receptor and the species of PHI used. PHI and VIP are products of the same gene (221), frequently coexist in tissues (222), share structural homology and often mediate the same biological response (218). The relative physiological roles of PHI and VIP are unclear (213). Although PHI binds to the VIP receptor, specific PHI receptors have been identified in some (378,379) but not all tissues (346) containing VIP receptors.

With respect to anterior pituitary function, PHI is present in the hypothalamus (380,381), and stimulates cAMP and PRL release in a dose dependent fashion with lesser, greater or equal potency as compared to VIP (223,271,380-385). These discrepancies may represent species or experimental variability. Studies to date suggest a common receptor for VIP and PHI in the anterior pituitary, as stimulation of adenylate cyclase and PRL by these two peptides is additive, and not greater at maximum than with either agent alone (271).

III. RECEPTOR STUDY

A. Receptor theory

The presence of specific binding substances in biological tissues mediating a response to exogenous stimuli was first postulated independently by Ehrlich (386) and Langley (387) at the start of the twentieth century. This receptor concept has since expanded to include the action of endogenous substances on target tissues, and various theories have been proposed to explain the biological response resulting from such interactions. The occupancy theory, first proposed by Clark (388) and Gaddum (389) in the 1920's, initially postulated a linear relationship between the number of receptors occupied and the elicited response. This quantitative description was subsequently revised by Ariens (390) and Stephenson (391), to a nonlinear relationship between fraction of receptors occupied and response elicited. The underlying premise of this theory that the fraction of receptors (R) occupied by a drug (D) in a receptor-drug complex (RD), at any given time is related to the biological response (E) can be expressed mathematically by:

$$E = \alpha[RD] \qquad (1)$$

where α is a proportionality constant intrinsic to the drug. If maximal response is assumed when all receptors (R_T) are occupied by drug then:

$$E_{max} = [R_T] \qquad (2)$$

Assuming the simplest bimolecular model, the interaction can be described according to the law of mass action as:

$$[D] + [R] \underset{k_{-1}}{\overset{k_1}{<----->}} [RD] \text{ ----> } E \qquad (3)$$

where k_1 and k_{-1} are association and dissociation rate constants respectively. The dissociation constant at equilibrium (K_D) would be described by:

$$K_D = k_{-1}/k_1 = [D][R]/[RD] \qquad (4)$$

As conservation of mass also applies:

$$[R_T] = [RD] + [R] \qquad (5)$$

From equations 1-5 and according to the experimental observations made by Stephenson the following relationships can be derived:

$$f(S) = E/E_{max} = f(e[RD]/[R_T] = f(e[D]/([D] + K_D) \qquad (6)$$

where the response observed is some function (f) of the stimulus (S) and is dependent on the fraction of receptor occupied, and the efficacy (e) of the drug. A drug with high efficacy was defined as one that had to occupy only a small portion of receptors to elicit a maximal response (391). These mathematical relationships parallel those described for enzyme-substrate interaction (392) and for adsorption of gases onto metal surfaces (393). Although predicted by Stephenson because of the variable efficacy of agonists, the existence of "spare" receptors was verified using receptor antagonism of agonist dose-response studies (394). From a biological point of view these "spare" receptors can however be functional as they may be coupled to other biological responses. The response observed may also be limited by an intracellular effector (e.g. adenylate cyclase) and although each receptor could potentially mediate a response and is in that sense not "spare", occupation of a fraction will elicit a maximal response (395).

Another theory of receptor-ligand interactions, the rate theory, was proposed by Paton (396). This theory differs conceptually from the occupancy theory in that the response is thought not to be due to the fraction of receptors occupied at a given time, but rather dependent on the process of occupation of the receptor and hence proportional to the rate of drug-receptor interaction. Both theories have as a common base, however, the need for a physical receptor-ligand interaction in order for a response to occur.

The assumption of simple bimolecular interactions is not always justified on the basis of experimental observations, and the allosteric model of Monod and colleagues (397) has been applied to drug-receptor interactions (398). By this theory, drug-mediated changes in the equilibrium of a receptor possessing variable affinity states, could account for the ability of some drugs to elicit maximal responses at low receptor occupancy. This multiplicity of interaction has been expanded by the concept of receptor mobility within the cell membrane (399). This theory suggests that formation of the receptor-ligand complex changes the

mobility of the receptor, allowing it to interact with other receptors as well as with one or more effector systems (e.g. adenylate cyclase) to mediate a response. This theory allows for great flexibility in ligand-receptor interactions, that could account for many of the complex interactions observed in biological systems.

B. Radioligand binding studies.

Hormone and receptor interaction can be studied at different levels ,including biological effects and changes in intracellular mediators in response to variable amounts of hormone. The presence of "spare" receptors as indicated above, as well as binding sites not coupled to the response examined, necessitate studies other than biological response data for detailed investigation of hormone receptor interactions (400). Ligand binding studies can provide a more direct understanding of these interactions.

1. Radiolabelled hormones. Binding of hormones to biological tissues is followed using trace amounts (usually subnanomolar) of radiolabelled hormone. At these concentrations attaining high specific activity of the labelled hormone is vital in order to facilitate accurate measurements of radioactivity. Choice of a suitable isotope depends on specific activity, half-life, the ability to incorporate it into the hormone under study and retention of biological activity. ^{125}I, one of the preferred isotopes for proteins, can be incorporated primarily into Tyr, and His residues directly by catalysis (e.g. lactoperoxidase) or after oxidation (e.g. chloramine T, lactoperoxidase) and then spontaneous interaction of I_2 with the protein (401).

Separation of radiolabelled from damaged (e.g. oxidized) and unlabelled hormone is important to decrease non-receptor binding and increase the homogeneity of the radiolabelled hormone. Purification of the radiolabelled species to a monoiodinated hormone by e.g. HPLC, increases the chance of a homogeneous reaction. As iodination may interfere with the normal structure of the hormone and potentially alter hormone receptor interactions, establishing bioactivity of the iodinated species is important.

2. Receptor preparation. Radioligand binding studies require presentation of the tissue receptor in some form accessible for interaction with the hormone. Use of tissue

fragments is generally unsatisfactory as they contain very heterogeneous populations of cells and large amounts of hormone can sequester into extracellular spaces. To circumvent these problems, the tissues used most frequently are whole cells or membrane preparations (402). Receptors in membrane preparations, obtained after tissue homogenization and by separation from other cellular components using differential centrifugation and/or density gradients, are potentially subject to degradation by cellular proteinases (403). Solubilization of tissue components potentially allows isolation and purification of receptors. As membrane environments of the receptor (e.g lipids) may change during this processing, hormone receptor interactions can be altered. In view of the potential alterations in hormone receptor interactions secondary to receptor preparation, binding relationships observed in these studies must always be analyzed in conjunction with biological responses to the agent under study.

3. Receptor assay. Stability of the hormone under assay conditions must be ensured in order for binding data to be meaningful, and can be assessed by various methods including HPLC, protein precipitation and repeat binding (404). Degradation or adsorption of the hormone may be preventable by inclusion of protease inhibitors or BSA in the assay (403).

Separation of free ligand from that bound to the receptor at the end of assay is necessary and can be accomplished using filtration, centrifugation or dialysis (400). Important considerations in choosing a method include stability of the bound complex over the time required for separation, as well as reproducibility.

4. Receptor versus non-receptor binding. Hormones are capable of binding to various substances, of which receptors may represent only a small fraction in an assay. Hence hormone binding may be secondary to nonspecific or specific biological interactions. As not all biological interactions of hormones are receptor mediated (e.g. hormone internalization) it is important that binding ascribed to receptors is correlated with a biological response, as by definition hormone receptor interactions must be coupled to a physiological effect (405).

Nonspecific binding is generally assumed not to be displaceable, and the radiolabelled hormone normally bound to a set amount of tissue that can be displaced by excess native

hormone is assumed to be specific. Although a good starting premise, some nonreceptor binding can be displaced, hence a set of binding criteria have been established outlining properties of receptor binding (406). Taken in concert these criteria are capable of distinguishing receptor from nonreceptor binding and include binding saturability, reversibility, dependence on time and temperature, specificity with respect to binding of other peptides and affinity in keeping with biological responses (406).

5. Quantitative analysis of ligand binding studies. Analysis of radioligand binding studies is also based on some form of application of the law of mass action. If the assumptions are made that native and radiolabelled hormones interact in an identical fashion with a single receptor, a relationship of hormone (H) interacting with receptor (R) can be described as for drug-receptor interactions in equation 3:

$$[H] + [R] \underset{k_{-1}}{\overset{k_1}{<\!\!-\!\!-\!\!-\!\!-\!\!-\!\!>}} [RH] \qquad (7)$$

At equilibrium:

$$d[RH]/d(t) = 0 = k_1[H][R] - k_{-1}[RH] \qquad (8)$$

and:

$$[H][R]/[RH] = k_{-1}/k_1 = K_D \qquad (9)$$

Conservation of mass also applies to receptors hence:

$$[R_T] = [R] + [RH] \qquad (10)$$

Radioligand binding studies should be able to yield estimates for maximum receptor concentrations (R_T) and hormone affinity (K_D) under conditions of equilibrium according to these equations, provided the assumptions outlined above are met. Obtained from equation 10, ($[R_T]$ - [RH]) can be substituted for [R] in equation 9; and after algebraic rearrangements the following relationship can be described:

$$[RH] = [R_T][H]/([H] + K_D]) \qquad (11)$$

As the relationship defined by the coordinate system of known values ([RH] versus [H]) is nonlinear, the unknown parameters (K_D and R_T) are not easily derived. Many mathematical transformations attempting to linearize the data to allow graphical parameter estimations have

been described including those by Scatchard (407), Hill (408), Hofstee (409), Klotz (410), and Feldman (411). Frequently used, the Scatchard plot of bound ligand/free ligand (B/F) versus bound ligand (B), is obtained from rearrangement of equation 11 to :

$$B/F = (B_{max}-B)/K_D \qquad (12)$$

where bound ligand, B, = [RH] and free ligand, F, = [H], and $B_{max} = R_T$. With a simple bimolecular relationship between [H] and [R], this plot results in a straight line with K_D equal to the negative reciprocal of the slope of this line and B_{max} (or R_T) to the intercept of the abscissa.

Curvilinear Scatchard plots are however frequently seen, indicating simple bimolecular reactions are not operative. Possible explanations for this nonlinearity include ligand heterogeneity, ligand-ligand or receptor-receptor interactions, incorrectly defined nonspecific binding or multiple binding sites (404). The mobile receptor theory predicts such multiple interactions, however the mathematics involved in such complex isotherms [for review see (399)] are no longer amenable to graphical analysis. Towards this end computer aided analysis is a major asset, now readily available adapted to microcomputer use. Nevertheless it is important to appreciate that computer analysis cannot resolve information with greater precision than possessed by the original binding data obtained in the laboratory. Additionally, most commercially available analytical programs only compare the fit of algorithms defining binding at one versus multiple receptor sites, and the onus remains on the investigator to eliminate the other potential causes of nonlinearity of Scatchard plots. The method of analysis utilized by different computer programs varies, with many of the earlier ones performing linear regression on transformed data (412). Accuracy of all linear analysis methods decreases through transformation from nonlinear to linear coordinates, as errors from the dependent variable are introduced into the independent axis (413). This also creates problems using the least squares analysis in linear regression of transformed data as this statistical analysis assumes errors in the dependent variable are not correlated with errors in the independent variable. Nonlinear regression of original untransformed data is therefore the preferred method of analysis. Such a program designed by Munson and Rodbard (414)

and adapted to microcomputer use by McPherson (415), EBDA and LIGAND (Elsevier Biosoft version 3) was utilized in this study.

Nonlinear regression was based on the following algorithm:

$$[B] = \sum_{i=1}^{i=n} \{B_{max}[L]/(K_{Di} + [L]) + N[L]$$

where B, B_{max} K_D are as defined above, L refers to ligand, i the number of binding sites, and N the ratio of B/F at infinite F concentrations. N is essentially the gradient of the line relating nonspecific binding and free ligand concentration, so N[L] will give the amount of nonspecific binding at a particular point (416). This analytical program has the additional advantage of simultaneous multiple curve analysis.

RATIONALE FOR RESEARCH

At the time these investigations were undertaken a major gap in the study of VIP action on anterior pituitary PRL was obvious. A large body of literature provided evidence that VIP present in the hypothalamus could potentially mediate the observed stimulation of PRL by VIP. In contrast to the plethora of studies substantiating these observations, there was a distinct lack of studies characterizing the VIP receptor using radioligand binding techniques. In comparison these techniques had been copiously employed in the study of VIP receptors in other tissues. As the dose dependent stimulation of PRL and cAMP by VIP supported the presence of VIP receptors in the anterior pituitary, the paucity of radioligand binding studies of VIP in the anterior pituitary was likely secondary to a methodological problem. Indeed unsuccessful radioligand binding attempts had been reported. As the bioassays for PRL stimulation by VIP are replete with difficulty including imprecision, these radioligand binding studies were undertaken not only to provide further support for a biological role of VIP, but also potentially yield a more sensitive investigative tool.

A large portion of this work is devoted to a systematic evaluation of the various components of radioligand binding that could account for the failure of this technique in the study of VIP in the anterior pituitary. This involved screening of various anterior pituitary tissues, use of different VIP radioligands, as well as modifications in receptor preparation and assay conditions based on the study of VIP in other tissues.

The ability after these methodological investigations, to demonstrate displaceable binding of radiolabelled VIP was then necessarily followed by the study of the properties of this association to ensure criteria of receptor interaction were met. Once this was established a number of questions regarding the anterior pituitary VIP receptor were explored.

The effect of guanine nucleotides on the binding of VIP to this receptor was studied, along with the consideration of receptor heterogeneity. This latter question was addressed using a series of VIP analogs and homologous peptides and was pursued in conjunction with the study in our laboratory of VIP receptors in other tissues.

To verify a biological role for the binding of VIP, in vitro PRL release assays on dispersed anterior pituitary cells were conducted. The ability of members of the secretin-VIP family of peptides to compete for VIP binding in the anterior pituitary was compared with their ability to release PRL in vitro.

As the primary action of VIP in the anterior pituitary gland is on PRL secretion, radioligand binding studies were conducted on enriched populations of PRL containing cells to ascertain whether the stimulation of PRL secretion was mediated directly on the lactotrope.

Because receptor regulation has been reported as a mechanism by which the action of a hormone may be controlled, this possibility was addressed in several physiological conditions where evidence purporting action of VIP on PRL levels had accumulated. The rat model was chosen for convenience and reliability. The abundance of physiological studies available in these animals also assisted in interpretation of results. Regulation of the VIP receptor included study during lactation and postnatal development. The question of prenatal ontogeny, although interesting, was not addressed because of paucity of tissue. The serendipitous observation that anterior pituitary tissue from castrated male animals possesses different binding capabilities compared to normal animals, led to a series of binding studies characterising the binding parameters in these as well as in testosterone treated animals.

EXPERIMENTAL STUDIES

I. CONDITIONS OF TISSUE PREPARATION AND BINDING ASSAY

A. Materials and Methods

1. Materials. The source of materials is as follows: synthetic VIP, identical in structure to rat and bovine VIP, Peninsula Laboratories (San Carlos, CA); antipain, bacitracin, benzamidine, bovine serum albumin (BSA) (fraction V and RIA grade), methylated BSA, phenylmethylsulphonylfluoride (PMSF), polybrene, protamine (grade IV, from salmon sperm), soybean trypsin inhibitor (Type I-S, 10000 BAEE/mg protein), aprotinin (from bovine lung, 14.4 TIU/mg protein), sucrose, tris(hydroxymethyl)aminomethane (Tris), β-D-glucose, glucose oxidase (type V), lactoperoxidase solution from bovine milk (activity 70-100 units/mg of protein), adenosine 5'-monophosphate (from equine muscle, Type III), Sigma Chemical Co. (St. Louis, MO); ethylenediamine tetraacetic acid (EDTA), magnesium chloride, Fisher Scientific (Orangeburg, NY); trifluoroacetic acid (TFA), Pierce Chem. Co. (Rockford, IL); $Na^{125}I$ (100 mCi/ml) Amersham (Arlington Heights, IL), Hams F10 medium, fetal bovine and horse serum, GIBCO Canada (Burlington, Ont).

2). Rat anterior pituitary membrane preparation. Immediately after decapitation, pituitary glands were removed from 250-350 g female and male CD rats purchased from Charles River Inc. St-Constant, Que., and placed into 1^0C oxygenated Krebs-Ringer buffer, pH 7.4. All remaining processing of the glands was carried out on ice or at 4^0C. The anterior pituitaries were separated from the remainder of the gland by blunt dissection using forceps, and visible external vessels were removed. The anterior pituitaries were then cut into 1-2 mm fragments using two scalpel blades, washed three times with buffer A (25 mM Tris-HCl, pH 7.4, containing 2 mM EDTA and 2 mM magnesium chloride) and weighed. The overall schema of rat anterior pituitary processing from this point to membranes is

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Buffer designation:
Buffer A: 25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM $MgCl_2$.
Buffer A_2: 25 mM Tris-HCl, pH 7.4, 2 mM $MgCl_2$.
Buffer B: 0.25 M sucrose in 25 mM Tris-HCl, pH 7.4.
Binding buffer C: 25 mM Tris-HCl, pH 7.4, 1% BSA (fraction V), 0.25 mg/ml bacitracin, 2 mM $MgCl_2$.

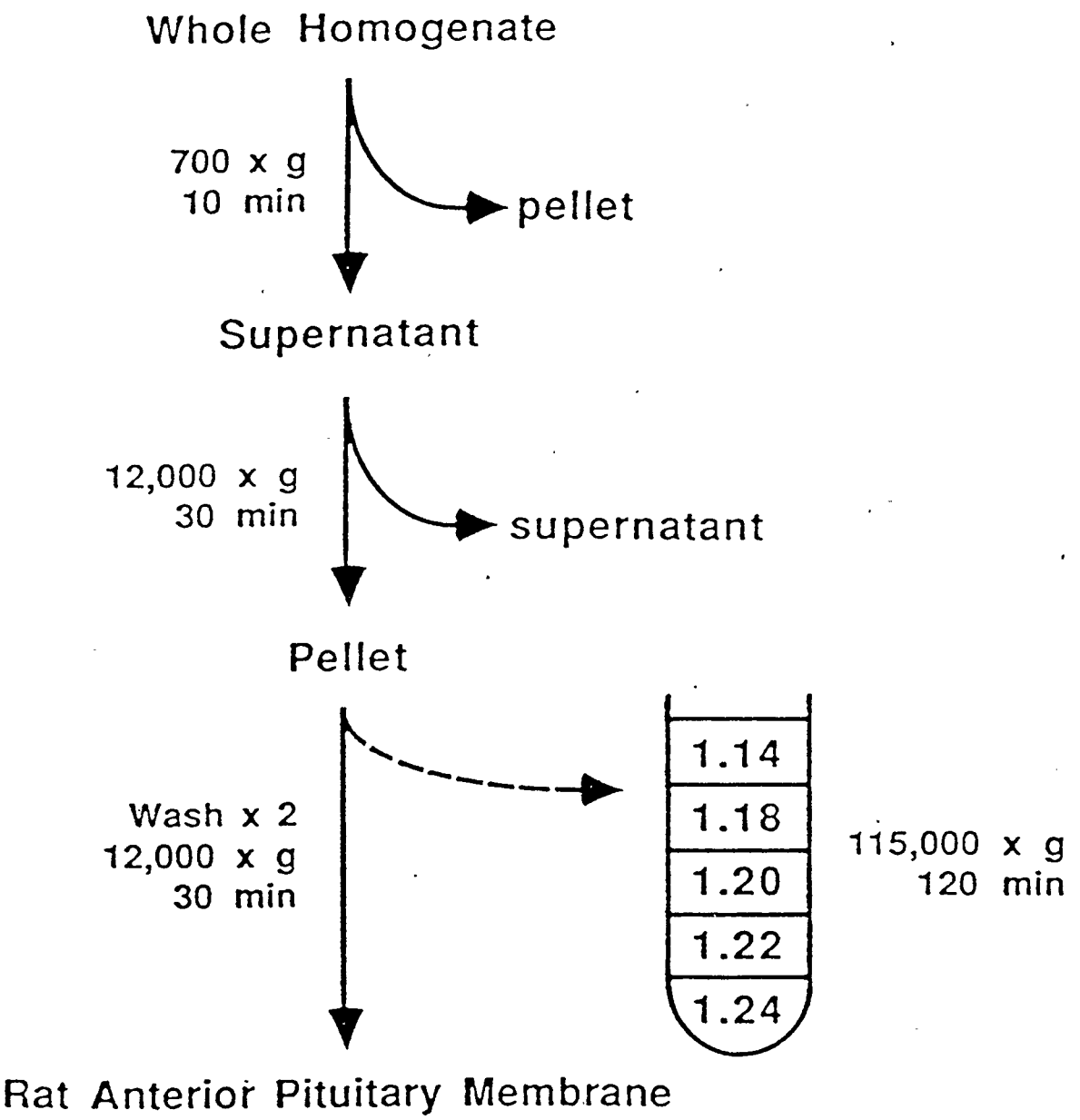
shown in Figure 1, and is based on the method of Bression et al. (417). The anterior pituitary fragments were suspended in buffer A, 150 ml/g tissue, and homogenized in a 40 ml glass-glass Dounce homogenizer using 15 strokes of pestle A, clearance 0.076 mm. This whole homogenate was centrifuged at 700 x g for 10 minutes, and the resulting supernatant was filtered through two layers of nylon gauze '53' and sedimented at 12,000 x g for 30 minutes. The 12000 x g pellet was washed twice with buffer B (0.25 M sucrose in 25 mM Tris-HCl, pH 7.4), and then resuspended in the same solution for storage at -70^{0}C. This tissue suspension is referred to as anterior pituitary membranes in this study. Anterior pituitary membranes were utilized for all binding studies on rat anterior pituitary tissue unless specifically indicated otherwise.

Rat anterior pituitary membranes were further purified by density centrifugation through a discontinuous sucrose gradient employing the method described by Baumann and Kuhl (418). Two ml samples of anterior pituitary membranes suspended in 1.1 M sucrose in 25 mM Tris buffer, pH 7.4, were layered onto discontinuous sucrose gradients of 2 ml each of 1.4, 1.56, 1.7, and 1.87 M sucrose in 25 mM Tris-HCl buffer, pH 7.4. Equivalent sucrose densities at 4^{0}C are 1.14, 1.18, 1.20, 1.22, 1.24 g/ml The gradients were centrifuged at 115,000 x g for 2 hours and tissue sedimenting at the 1.18/1.20 sucrose density interface was collected, diluted in buffer B and centrifuged at 115,000 x g for 30 minutes. The pellets were suspended in buffer B, and stored until binding assay at -70^{0}C.

During several preparations samples from the whole homogenate, the 700 x g pellet, the 12,000 x g supernatant, anterior pituitary membranes, and tissue at sucrose density interface 1.18/1.20, were kept and assayed for 5'-nucleotidase activity by the method of Goldfine et al. (419). Phosphate released during enzyme assay was measured using the method described by Ames (420). In these fractions and all tissue preparations used in binding studies, the protein concentration was determined using the method of Lowry et al. (421).

3). Bovine anterior pituitary membrane preparation. Pituitary glands were removed from steers and cows within 1 hour of death at XL-Beef Calgary, a local abattoir. The

FIGURE 1. Schema of rat anterior pituitary membrane preparation. Rat anterior pituitaries (20-40) were homogenized and subjected to differential centrifugation as indicated. Fractions not processed for membranes, i.e. 700 x g pellet and 12000 x g supernatant, were assayed along with membranes for protein, 5'-nucleotidase and VIP binding. The dashed line represents occasional further processing of the 12000 x g pellet through discontinuous sucrose gradients, with numbers in the tube referring to sucrose densities (g/ml).

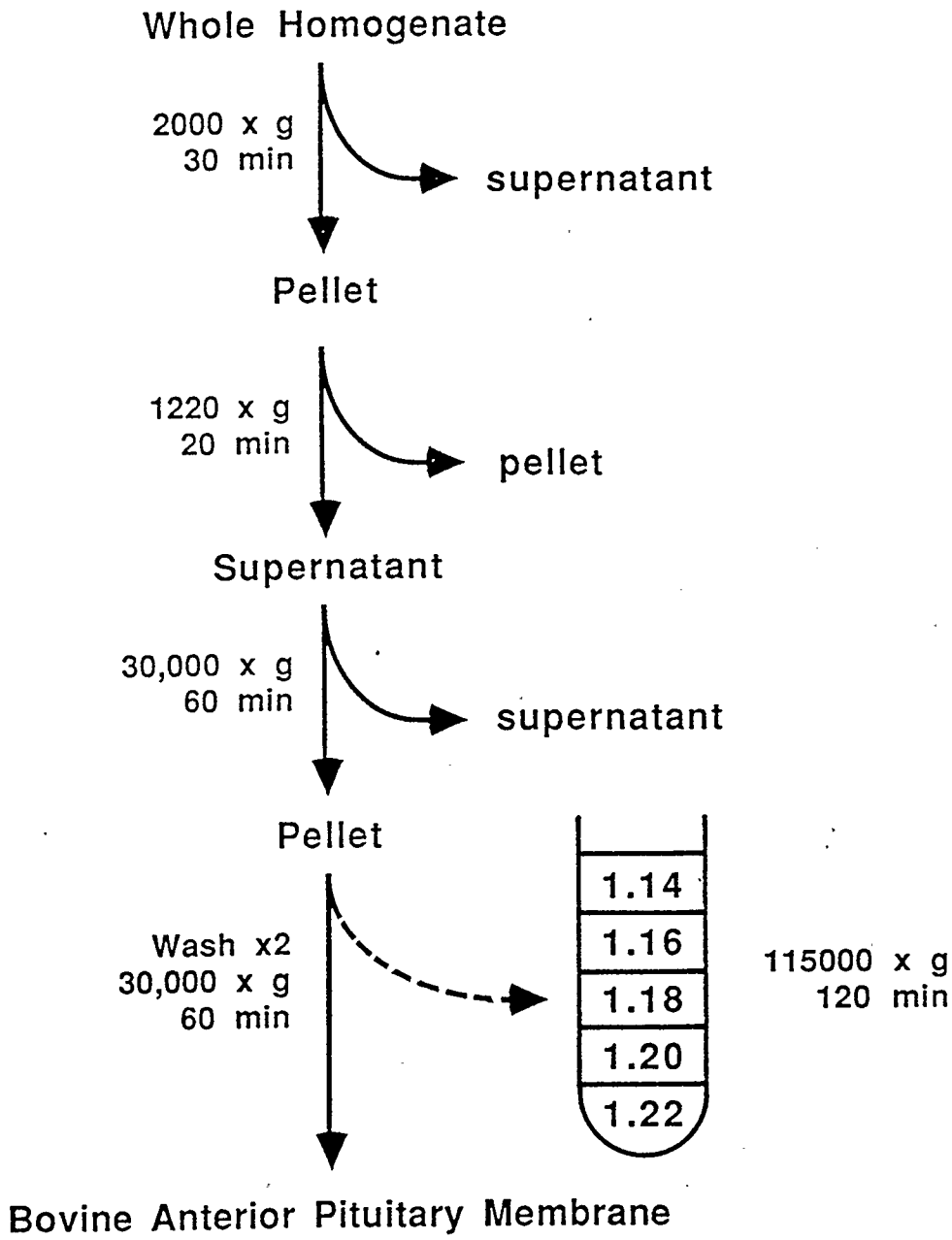

Whole Homogenate
700 x g
10 min
pellet
Supernatant
12,000 x g
30 min
supernatant
Pellet
Wash x 2
12,000 x g
30 min
1.14
1.18
1.20
1.22
1.24
115,000 x g
120 min
Rat Anterior Pituitary Membrane

glands were transported in 1^0C Krebs-Ringers solution, pH 7.4, and all tissue processing was conducted at 4^0C or on ice. The anterior pituitaries were dissected free from the rest of the gland, and all visible connective tissue and blood vessels were removed. Anterior pituitary membranes were prepared based on the method of Pourier et al. (422), and the basic schema is depicted in Figure 2. The anterior pituitaries were minced using scissors, washed three times with buffer A_2 (25 mM Tris-HCl, pH 7.4, with 2 mM magnesium chloride), and weighed. The minced tissue was homogenized in 5 volumes (wt/vol) of buffer A_2 using a Polytron homogenizer at speed setting 4.5 for 20 seconds 3 times with 1 minute pauses to minimize increases in temperature. After diluting this homogenate with additional buffer A_2 (25 vol/g original tissue) and stirring, it was filtered through 4 layers of cheesecloth and sedimented at 2000 x g for 30 minutes. The resulting pellet was suspended in 4 volumes of buffer A_2 and centrifuged at 1220 x g for 20 minutes. The supernatant from the 1220 x g spin was centrifuged at 30,000 x g for 60 minutes and the brownish top half of the resulting pellet was carefully removed, washed twice with, and then resuspended in buffer B (0.25 M sucrose in 25 mM Tris-HCl, pH 7.4) and stored until assay at -70^0C. This preparation is referred to as bovine anterior pituitary membranes.

For some studies these bovine anterior pituitary membranes were immediately further purified, based again on the method of Pourier et al. (422). Bovine anterior pituitary membranes, suspended in 8 ml of 1.4 M sucrose, were layered into discontinuous sucrose gradients containing 8 ml layers of 1.1, 1.25, 1.4 (membrane containing), 1.56 and 1.7 M sucrose. The corresponding sucrose densities at 4^0C are 1.14, 1.16, 1.18, 1.20, 1.22 g/ml. The gradients were centrifuged at 115,000 x g for 2 hours and tissue sedimenting at the 1.14/1.16 and 1.16/1.18 sucrose density interfaces was collected, diluted with buffer B and centrifuged at 30,000 x g for 30 minutes. The resulting pellets were suspended in buffer B, and stored for assay at -70^0C.

The following fractions were retained during preparation of bovine membranes; whole homogenate, 2000 x g supernatant, 1220 x g pellet, bovine anterior pituitary membranes and tissue from sucrose density interfaces; and assayed for protein concentration using the method

FIGURE 2. Schema of bovine anterior pituitary membrane preparation. Anterior pituitaries (2-3) were homogenized and subjected to differential centrifugation as indicated. Fractions not processed for membranes, i.e. 2000 x g supernatant and 1220 x g pellet, were assayed along with membranes for protein and VIP binding. The dashed line refers to occasional further processing of the 30000 x g pellet through discontinuous sucrose gradients, with numbers in the tube referring to sucrose densities (g/ml).


Whole Homogenate
2000 x g
30 min
supernatant
Pellet
1220 x g
20 min
pellet
Supernatant
30,000 x g
60 min
supernatant
Pellet
Wash x2
30,000 x g
60 min
1.14
1.16
1.18
1.20
1.22
115000 x g
120 min
Bovine Anterior Pituitary Membrane

of Lowry et al. (421), and for VIP binding. 5'-nucleotidase activity was not assayed and composition of these fractions was assumed to be the same as that identified by Pourier et al. (422).

4). GH_3 cell tissue preparation. GH_3 cells, a clonal cell line derived from a Wistar rat anterior pituitary tumour secreting PRL and GH, were purchased from the American Type Culture Collection (Rockville, IL) and kept in culture according to the methods described by Tashjian (423). Cells were grown in sterile Hams F10 medium, supplemented with L-glutamine, 25 % horse serum and 2.5 % fetal bovine serum, in 80 cm^2 polystyrene tissue culture flasks (Corning) at 36.5^{0}C in humidified 95% air/5% CO_2. Media was changed twice weekly and cells were subcultured when 80 % confluence was reached. At 60-80 % confluence cells were harvested for preparation by scraping with a rubber policeman, centrifugation at 200 x g for 10 minutes at 21^{0}C, and washing twice in buffer A (25 mM Tris-HCl, pH 7.4, with 2 mM magnesium chloride and 2 mM EDTA). Disrupted cell preparations were obtained based on the method of Hinkle and Tashjian (114), with all processing carried out at 4^{0}C. The cells were homogenized in buffer A using 50-60 strokes of pestle A, clearance 0.076 mm, in a 40 ml glass Dounce homogenizer, with less than 5 % of cells remaining intact. The homogenate was centrifuged at 10000 x g for 30 minutes, and the resulting pellet was washed twice and resuspended in buffer B (0.25 M sucrose in 25 mM Tris HCl, pH 7.4) for storage at -70^{0}C until assayed for VIP binding.

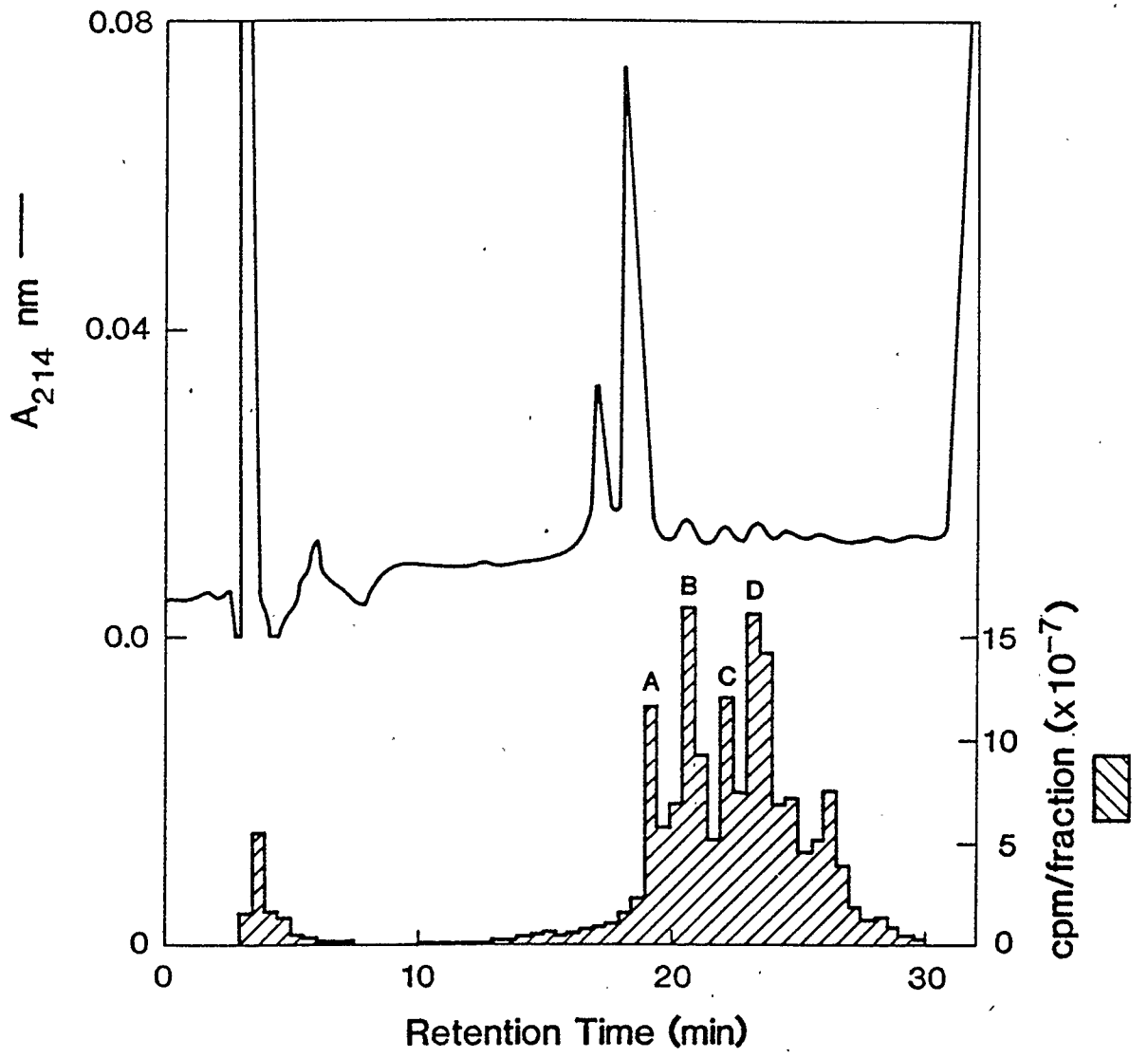
5. Radioiodination of VIP. For use in radioligand binding studies, a method of VIP radioiodination was employed that had been developed in our laboratory and used successfully in the study of vascular VIP receptors (315,316). Various methods of VIP radioiodination had been compared, with the lactoperoxidase-glucose oxidase method chosen because of excellent reproducibility, iodine incorporation and yield of monoiodinated, nonoxidized VIP derivatives (423). For radioiodination the following reagents were incubated for 10 minutes at 21^{0}C: 40 μl of 0.6 M sodium phosphate buffer (pH 7.4), 40 μl of 5 x 10^{-5} M VIP in 0.1 M acetic acid (6.66 μg), 1 μl undiluted stock lactoperoxidase solution, 1 μl of glucose oxidase as a 10% (vol/vol) dilution of the stock solution in 0.2 M sodium

phosphate buffer (pH 7.4), 10 μl of $Na^{125}I$ (10 mCi), and 10 μl β-D-glucose of 14 nM solution in 0.2 M sodium phosphate buffer (pH 7.4). As VIP contains two Tyr residues that can be iodinated and one Met residue susceptible to oxidation, the radioiodinated products were purified by reverse phase high performance liquid chromatography (HPLC) using a Waters Associated Apparatus with a C-18 μBondapak column (30 x 0.39 cm i.d.; Bondapak C-18/Corasil guard column) attached to a 214 nm absorbency detector. Product separation was achieved by HPLC using a linear gradient (1 ml/min) of 42-62 % solvent B (0.1% trifluoroacetic acid (TFA) in 60:40 acetonitrile/water) in solvent A (0.1 % TFA in water) run over 24 minutes, and followed by a 1 minute gradient to 100% solvent B and return to 42% solvent B by 30 minutes. 0.5 ml fractions were collected into glass tubes containing 5 μl of 10% (wt/vol) BSA (RIA grade) in water. The resulting major radioactive peaks had previously been identified using HPLC peptide mapping of tryptic digests and amino acid analysis (423), and the peaks labelled in Figure 3 represent the following: peak B, [Tyr(^{125}I)^{10}]VIP; peak C, [Tyr(^{125}I)^{22},Met(O)^{17}]VIP; peak D, [Tyr(^{125}I)^{22}]VIP. Peak A contains both unreacted VIP and [Tyr(125I)^{10},Met(O)^{17}]VIP. This monoiodinated derivative of VIP was prepared in a separate experiment by radioiodinating VIP that had previously been completely oxidized to [Met(O)^{17}]VIP. [Tyr(^{125}I)^{10},Met(O)^{17}]VIP was then isolated using HPLC (423). All 4 monoiodinated derivatives of VIP demonstated bioactivity in the vasorelaxant bioassay, with [Tyr(^{125}I)^{10}]VIP and [Tyr(^{125}I)^{22}]VIP having activities similar to that of unlabelled VIP (423).

As these monoiodinated derivatives of VIP had been shown to be free of unlabelled VIP and other iodinated contaminants, a maximal specific activity of 2200 Ci/mmol was assigned for use in radioligand binding studies.

6. Radioligand binding assay. The interaction of anterior pituitary tissue with VIP was examined by incubating radiolabelled VIP and anterior pituitary tissue in the absence or presence of unlabelled VIP. The initial screening binding assay consisted of incubating the following for 30 minutes at 21^{0}C: 55-65 pM [Tyr(^{125}I)^{10}]VIP, 10-30 μg of anterior pituitary membrane protein, with or without 0.1 μM native VIP, to a total volume of 0.2 ml

FIGURE 3. HPLC elution profile of radioiodinated derivatives of VIP. Radiolabelled derivatives were prepared by lactoperoxidase-glucose oxidase iodination of VIP. Separation of monoiodinated derivatives was achieved by HPLC through a C-18 μBondapak column, using a linear gradient (1 ml/min) of 42-62% solvent B (25.2-37.2% acetonitrile in water) over 24 minutes, followed by a short gradient to 100% solvent B and return to 42% solvent B. The elution profile in the upper panel is a function of absorbency at 214 nm, in the lower panel as radioactivity recovered in 0.5 ml fractions. For details see Experimental studies IA5. The labelled peaks represent: A, [Tyr(^{125}I)^{10}Met(O)^{17}]VIP plus unlabelled VIP; B, [Tyr(^{125}I)^{10}]VIP; C, [Tyr(^{125}I)^{22}(Met(O)^{17}]VIP; and D, [Tyr(^{125}I)^{22}]VIP.


0.08
0.04
0.0
0
A_{214} nm
A
B
C
D
15
10
5
0
cpm/fraction ($x10^{-7}$)
0
10
20
30
Retention Time (min)

of binding buffer (25 mM Tris-HCl, pH 7.4, 0.25 mg/ml bacitracin, 1% BSA (fraction V), and 2 mM magnesium chloride). Incubations were in polypropylene tubes that were precoated with 5 % BSA (fraction V) to decrease adsorbance of VIP. Nonspecific binding was defined by the amount of bound radiolabelled VIP not displaced by 0.1 μM native VIP. Specific binding was derived by subtracting nonspecific from total radioligand binding.

7. Modifications to membrane preparation and binding assay conditions. Initial screening studies of radiolabelled VIP binding to male rat anterior pituitary membranes, using the radioligand binding assay conditions outlined above, demonstrated a high degree of nonspecific binding, i.e. less than 20% of bound VIP radioligand was displaceable by 0.1 μM native VIP. To see if experimental problems could account for this lack of specific binding of VIP, various methodological studies were conducted and the effect on VIP binding was determined. The modifications studied were: 1). Binding of VIP to other sources of anterior pituitary tissues (i.e. female rat, GH_3 cells, and bovine tissue).; 2). Increased purification of rat and bovine membranes by sucrose density centrifugation as described above.; 3). The effect of inclusion of the following protease inhibitors during preparation of anterior pituitary membranes: to buffer A in the rat, phenylmethysulfonyl fluoride (PMSF) (0.5 mM); and to buffer A_2 in bovine tissue, EDTA, or EDTA (2 mM) plus antipain (10 μM), soybean trypsin inhibitor (523 BAEE/ml), pepstatin (1.5 μM), leupeptin (2.1 μM), benzamidine (10 mM) or PMSF (0.5 mM). Bovine anterior pituitaries were used primarily in these experiments as large quantities of tissue were readily available.; 4). Comparison of the following four different monoiodinated derivatives of VIP in binding to rat anterior pituitary membranes: [Tyr(^{125}I)^{22}]VIP, [Tyr(^{125}I)^{10}Met(O)^{17}]VIP, [Tyr(^{125}I)^{10}]VIP and Tyr(^{125}I)^{22}Met(O)^{17}]VIP.; 5). The effect of binding buffer pH, ranging from 6.0 to 10.0.; 6). The effect of magnesium chloride concentrations in the binding buffer of 0, 1, 2, 3 or 5 mM; 7). Comparison of binding with or without addition of bacitracin (0.25 mg/ml), PMSF (0.05 mM) or EDTA (2 mM) to the binding buffer.; 8). The effect of incubation temperature, 1^0C, 21^0C and 31^0C, over time, 5-120 minutes, on the binding of VIP to rat anterior pituitary membranes.

8. Separation of free and bound radioligand. Separation at the end of incubation of free radioligand from that bound to tissue was accomplished using centrifugation and filtration methods. For centrifugation, which was employed in all bovine and GH_3 cell as well as in some rat studies, incubations were conducted in 1.9 ml polypropylene microfuge tubes, and assays were stopped with 1 ml of ice-cold binding buffer and sedimentation at 16000 x g for 3 minutes at 4^0C using an Eppendorf microfuge. The resulting pellets containing the bound radioligand were washed twice with 1 ml of ice-cold binding buffer. Radiation contained in the pellet in the 7 mm tip of the microfuge tube was counted in a Beckman model 5500 gamma counter with a counting efficiency of 74 %. Because the amount of protein used per assay was small, making visibility of the pellet, loss of material and reproducibility problematic when using centrifugation, filtration was investigated as a means to separate bound and free radioligand.

As VIP, a highly basic molecule, binds nonspecifically to many substances, the retention of [Tyr(^{125}I)^{10}]VIP to filters of differing composition (cellulose acetate, cellulose acetate and nitrate, nylon, polycarbonate, glass) and from various manufacturers was checked in order to find one which retained an acceptably small amount of free radioligand. The binding of free VIP is likely due to its highly basic nature and the decreased retention reported by Bruns et al. (425) of filters presoaked in the polycationic agent polyethyleneimine (PEI) is thought to be due to modification of the surface charge of negatively charged filters by this agent. In an attempt to decrease this retention all filters tested were presoaked in binding buffer B (containing 1% BSA (fraction V) and bacitracin 0.25 mg/ml) for 1 hour prior to determination of [Tyr(^{125}I)^{10}]VIP retention in the absence of membrane protein. The retention of [Tyr(^{125}I)^{10}]VIP by Whatman glass microfiber (GF/B) filters and MSI cellulose acetate filters was examined after presoaking them for a minimum of 1 hour in 0.3% aqueous solutions of the polycationic agents: polybrene, protamine, PEI and methylated BSA (meBSA). The effect of presoaking glass fiber filters in these various agents on the specific binding of [Tyr(^{125}I)^{10}]VIP to rat anterior pituitary membranes was also compared.

The binding assays utilizing filtration were done in 12 x 75 mm polypropylene tubes and incubations were stopped with 2 ml of ice-cold binding buffer and filtration within 2 seconds using a 12 port Millipore filtration manifold under 10 mm Hg vacuum. The tubes were washed twice with 2 ml of binding buffer and after this was filtered, the filters were rinsed with 4 ml of cold binding buffer, dried under increased vacuum and radioactivity determined in the 5500 model Beckman gamma counter.

9. Radioligand stability. As degradation of radiolabelled VIP has been reported after incubation with other tissues especially at higher temperatures (304), the effect on [Tyr(^{125}I)^{10}]VIP of incubation with anterior pituitary tissue was evaluated. Reverse phase HPLC is a useful tool in assessing degradation as shorter elution times of degraded products compared with intact VIP are expected secondary to changes in size and hydrophobicity of the fragments (423). To evaluate the effect of membrane incubation on VIP radioligand stability, 20 μg of female rat anterior pituitary membranes were incubated with [Tyr(^{125}I)^{10}]VIP (60-65 pM) in binding buffer C at 21^{0}C for 30 minutes or 31^{0}C for 60 minutes. After incubation the membranes were sedimented at 16000 x g for 3 minutes at 4^{0}C, and the supernatant was applied to a C-18 μ-Bondapak column, and separated by reverse phase HPLC using a linear gradient (1 ml/min) identical to that used after radioiodination, i.e. 42-60% solvent B (25.2-37.2% acetonitrile) run over 24 minutes, with short gradient to 60% B and return to 42% B by 30 minutes. One ml fractions were collected and the profiles of radioactivity recovered from these experiments were compared to the results of [Tyr(^{125}I)^{10}]VIP, not incubated with membranes, analyzed in the same manner.

B. Results

1. Properties of anterior pituitary membrane fractions. As VIP receptors are localized to cell membranes in other tissues (295,344), it was desirable to obtain anterior pituitary tissue preparations consisting largely of membranes for use in binding studies. The techniques of differential and density gradient centrifugation for isolation of membrane fractions, first developed by Neville for the liver (426), have since been employed successfully in various tissues including the anterior pituitary. As the methods used in this

study had previously been shown by morphological and enzymatic studies to yield fractions enriched in plasma membranes (418,422), full characterization of the fractions was not performed. To ensure similar membrane purification was achieved 5'-nucleotidase activity, a commonly used plasma membrane marker (427), was followed during purification of tissue membranes. Figure 4 shows that the processing employed for male rat anterior pituitary tissue resulted in a 3 and 6 fold increase in the activity of this enzyme per mg of protein in the 12000 x g pellet and 1.18/1.20 g/ml sucrose density interface respectively, as compared with the whole homogenate. Other fractions obtained during this preparation had markedly lower levels of 5'-nucleotidase activity. These results correspond with the enrichment of plasma membranes found by Baumann and Kuhl (418) and rationalize labelling the 12000 x g pellet fraction as rat anterior pituitary membranes. Similar increases in 5'-nucleotidase activity were observed after preparation of anterior pituitary membranes from normal adult female rats or 1 day after surgical castration of male animals (Table 3). Differences in the absolute levels of 5'-nucleotidase activity were however seen between the two sexes, a result that has also been observed in other tissues (427).

Specific binding of [Tyr(^{125}I)^{10}]VIP to the whole homogenate and to each of the fractions during rat anterior pituitary membrane preparation followed the enrichment of plasma membranes (Table 4). Similar results were obtained using bovine anterior pituitary tissue. Nonspecific binding also largely followed purification of membranes, reflected by minimal changes in the percent of specific binding relative to total binding (Table 4). Significant losses of tissue protein occurred during processing of anterior pituitary membranes through sucrose density gradients, with protein yields in rats in the anterior pituitary membrane and the 1.18/1.20 sucrose interface fractions decreasing respectively from 11.9 ± 1.6 mg/g to 1.7 ± 0.3 mg/g wet weight of original tissue.

Specific binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary derived tissue from 4 different sources ranged from 15.8 to 22.8 fmol/mg tissue protein (Table 5). GH_3 cells and female rat anterior pituitary membranes bound slightly more, 22.8 and 21.5 fmol/mg respectively, than did bovine and male rat membranes, 15.8 and 16.9 fmol/mg respectively.

In all normal anterior pituitary derived membranes a large amount of nonspecific binding was observed, with specific binding of [Tyr(^{125}I)^{10}]VIP comprising less than 27% of total binding. In contrast, transformed tissue i.e. GH_3 cells, displayed relatively less nonspecific binding with specific binding comprising about 50% of the total. No difference in the specific or nonspecific binding of [Tyr(^{125}I)^{10}]VIP was observed whether rat anterior pituitary membranes were used immediately after preparation, or were frozen and thawed. Membranes were stable to VIP binding up to 3 months when stored at -70^{0}C.

2. Protease inhibitors during membrane preparation. Binding of VIP to brain (331) and intestine (335) is destroyed in the presence of trypsin indicating that the VIP receptor is susceptible to proteolytic degradation. VIP binding to rat brain and spleen studied by autoradiography also demonstrated increased binding in the presence of various proteinase inhibitors (317). As proteolytic activity has been reported in anterior pituitary homogenates (428), the addition of proteinase inhibitors during anterior pituitary membrane preparation, to putatively prevent receptor degradation during tissue processing, was evaluated with respect to binding of VIP (Table 6). In the absence of any added inhibitors the specific binding of VIP to rat and bovine anterior pituitary membranes was abolished although nonspecific binding was not affected. Inclusion during tissue preparation of a metallo proteinase inhibitor, i.e. the cation chelator EDTA, increased specific binding to 20-25% of total. Because of the susceptibility of the VIP receptor to trypsin, the addition of various serine proteinase inhibitors was evaluated. No improvement over the effect seen with EDTA was demonstrated with soybean trypsin inhibitor, benzamidine, or PMSF. The additional inclusion of inhibitors of thiol proteinases (antipain and leupeptin) or carboxyl proteinases (pepstatin) also did not increase the specific binding of [Tyr(^{125}I)^{10}]VIP. Nonspecific binding was unaffected by the addition of proteinase inhibitors.

3. Binding of different VIP radioligands. As variable effects on binding and bioactivity have been reported after iodination of the Tyr residues and oxidation of Met residue in VIP, the ability of the 4 different monoiodinated radioligands of VIP to specifically bind to rat anterior pituitary membranes was studied and the results are shown

FIGURE 4. 5'-nucleotidase activity in tissue fractions during male rat anterior pituitary membrane preparation. Protein concentration and 5'-nucleotidase activity were determined in all tissue fractions obtained during membrane preparation. Activity of 5'-nucleotidase is expressed as μg phosphate released per mg tissue protein. The sucrose gradient fraction refers to tissue sedimenting at sucrose density interface 1.18/1.20 g/ml,(see Fig. 1).

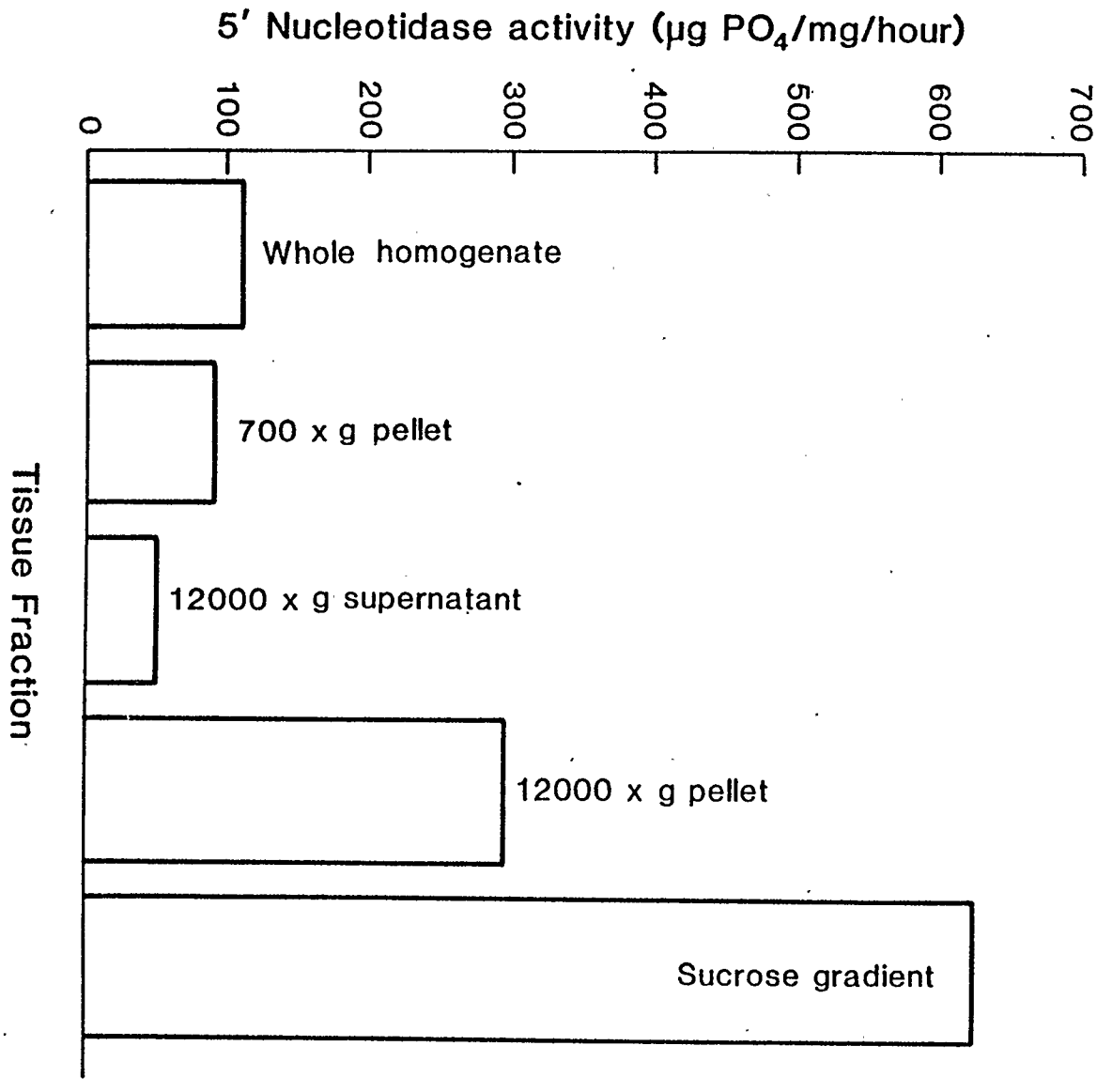

5′ Nucleotidase activity (µg PO_4/mg/hour)
0
100
200
300
400
500
600
700
Tissue Fraction
Whole homogenate
700 x g pellet
12000 x g supernatant
12000 x g pellet
Sucrose gradient

TABLE 3. Activity of 5'nucleotidase in homogenates and membranes prepared from anterior pituitaries of different sources.

Tissue source	5'nucleotidase activity (µg phosphate/mg protein/hour)		Relative enzyme activity (membranes/homogenates)
	Whole homogenates	Membranes	
Female rat	66 ± 6	184 ± 22	2.8
Male rat	132 ± 8	348 ± 64	2.6
Male rat (castrated)	158 ± 28	400 ± 58	2.5

5'nucleotidase activity and protein content were determined in whole homogenates and membranes (12000g pellets) prepared from anterior pituitaries of normal adult male and female rat, and 24 hours after castration of male animals.

Data are presented as mean ± SEM, n = 3-5, assayed in duplicate.

Enrichment is expressed as a ratio of 5'nucleotidase activity in membrane fractions relative to whole homogenates.

TABLE 4. Specific binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary tissue fractions prepared by differential centrifugation

Male Rat anterior pituitary			Bovine anterior pituitary		
	Specific binding [Tyr(^{125}I)^{10}]VIP			Specific binding [Tyr(^{125}I)^{10}]VIP	
Membrane fraction	(fmol/mg protein)	% of total binding	Membrane fraction	(fmol/mg protein)	% of total binding
Whole homogenate	4.1 ± 2.2	4.5 ± 2.0	Whole homogenate	5.1 ± 4.3	1.1 ± 1.0
700 g pellet	3.2 ± 1.5	2.1 ± 1.0	2000 g pellet	0.5 ± 0.4	3.3 ± 2.1
12000 g supernatant	1.1 ± 0.8	1.0 ± 0.8	1220 g supernatant	2.2 ± 1.1	3.3 ± 2.2
12000 g pellet	15.8 ± 3.3	23.7 ± 2.4	30000 g pellet	16.9 ± 1.9	21.5 ± 3.6
sucrose gradient	23.2 ± 4.1	28.7 ± 4.5	sucrose gradient	22.4 ± 1.1	29.4 ± 2.7

Anterior pituitary fractions (5-15 µg protein) prepared by differential and/or sucrose sucrose density gradient centrifugation, were incubated in buffer C with [Tyr(^{125}I)^{10}]VIP (55-65 pM) for 30 minutes at 21^{0}C. Specific binding was defined as total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 0.1 µM VIP. Sucrose gradient refers to tissue obtained from sucrose interfaces at 1.18/1.20 g/ml in the rat, and 1.14/1.16, 1.16/1.18g/ml in bovine tissue.
Data is presented as mean ± SEM, n = 3-6, assayed in duplicate.

in Table 7. Although female rat anterior pituitary tissue showed greater specific binding than did the male tissue for all 4 radioligands examined, the relative binding capabilities of each radioligand was similar in both sexes. VIP radioiodinated on Tyr^{10}, [$Tyr(^{125}I)^{10}$]VIP, demonstrated more than twice as much specific binding compared with iodination on Tyr^{22}, [$Tyr(^{125}I)^{22}$]VIP. Oxidation to methionine sulfoxide at position 17 of the iodinated Tyr^{10} radioligand, [$Tyr(^{125}I)^{10}Met(O)^{17}$]VIP, decreased specific binding to ~50% of that seen with the nonoxidized radioligand [$Tyr(^{125}I)^{10}$]VIP. The same oxidation in VIP radioiodinated at Tyr^{22} did not significantly alter the degree of specific binding observed with the nonoxidized molecule. As both oxidized radioligands contain an additional alteration from native VIP that did not potentiate specific binding, these radioligands were not used any further. Because of the clear advantage with respect to specific binding seen with radioiodination at Tyr^{10} as compared to Tyr^{22}, [$Tyr(^{125}I)^{10}$]VIP was used in all the remaining radioligand binding studies.

4. Radioligand binding conditions. Radioligand binding assays to VIP performed above room temperature are frequently associated with rapid degradation and loss of interaction in other tissues (303,304,320,325) In contrast, binding of [$Tyr(^{125}I)^{10}$]VIP to rat anterior pituitary membranes at 31^0C was not only more rapid but significantly greater than at 21^0C or 10C up to 120 minutes of incubation. (Figure 5). The specific binding of [$Tyr(^{125}I)^{10}$]VIP at 31^0C reached a maximum by 40 minutes and remained stable until 2 hours. In contrast, the specific binding seen at 1^0C and 21^0C was slower, and by 2 hours had achieved only 34 % and 71 % respectively, of the maximum attained at 31^0C. A similar improvement of specific binding of VIP with increased incubation temperatures has been observed in membranes prepared from rat brain (297,332).

An effect on the binding of [$Tyr(^{125}I)^{10}$]VIP secondary to binding buffer pH was observed at assays conducted at 21^0C and 31^0C (Figure 6A). Increases in specific binding occurred at both incubation temperatures from pH 6.0 to 7.4, with no further change up to pH 10.0 when assays were done at 21^0C. In contrast, specific binding done at 31^0C demonstrated an optimum centring around pH 8.0. This effect at 31^0C appeared to be secondary to a decrease in nonspecific binding seen at pH 8.0 while total binding remained

TABLE 5. Specific binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary membranes from different sources

Membrane source	Specific binding [Tyr(^{125}I)^{10}]VIP	
	fmol/mg protein	% of total binding
Female rat	21.5 ± 2.0	26.4 ± 1.4
Male rat	15.7 ± 2.3	23.7 ± 1.7
Bovine	16.9 ± 1.9	21.5 ± 3.6
GH_3 cells	22.8 ± 2.4	48.3 ± 3.1

Membranes (10-30 µg protein) were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) for 30 minutes at 21^{0}C. Specific binding was defined as total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 0.1 µM VIP.
Centrifugation was used to separate free from bound radioligand.
Data are expressed as mean ± SEM, n = 5-9, assayed in triplicate.

TABLE 6. Specific binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary membranes prepared in the presence of different proteinase inhibitors

	Specific binding [Tyr(^{125}I)^{10}]VIP					
	Female Rat		Bovine		GH_3 cells	
Proteinase inhibitor	fmol/mg protein	% of total binding	fmol/mg protein	% of total binding	fmol/mg protein	% of total binding
None	5.2 ± 3.8	3.3 ± 2.1	0	0	ND	ND
EDTA	20.9 ± 0.8	24.7 ± 1.3	16.4 ± 1.1	27.4 ± 3.6	ND	ND
EDTA,PMSF	21.5 ± 1.5	20.3 ± 2.3	17.8 ± 2.0	21.1 ± 5.0	22.8 ± 2.4	48.3 ± 3.1
EDTA,antipain, leupeptin,SBTI, pepstatin, benzamidine	ND	ND	15.4 ± 2.1	20.1 ± 2.5	18.6 ± 2.1	47.6 ± 3.5

Proteinase inhibitors concentrations were: EDTA 2 mM, PMSF 0.05 mM, antipain 10 µM, soybean trypsin inhibitor (SBTI) 523 BAEE/ml, leupeptin 2.1 µM, pepstatin 1.4 µM and benzamidine 10 mM. Inhibitors were added simultaneously for a preparation in the groups as indicated. For details see Experimental studies IA7.
Membranes (10-20 µg protein) were incubated in buffer B with [Tyr(^{125}I)^{10}]VIP (55-65 pM) ± 0.1 µM VIP at 21^{0}C for 30 minutes.
Data are presented as mean ± SEM, n = 3-6, assayed in duplicate, ND = not done.

TABLE 7. Specific binding of radiolabelled derivatives of VIP to anterior pituitary membranes

Membrane source	Specific binding (fmol/mg protein)			
	[Tyr(^{125}I)^{10}]VIP	[Tyr(^{125}I)^{10}Met(O)^{17}]VIP	[Tyr(^{125}I)^{22}]VIP	[Tyr(^{125}I)^{22}Met(O)^{17}]VIP
Female rat	21.5 ± 2.0	10.0 ± 0.5	8.5 ± 2.0	10.5 ± 2.0
Male rat	13.0 ± 3.5	6.0 ± 2.0	5.5 ± 2.0	3.0 ± 2.5
Male rat (castrated)	22.0 ± 3.0	16.0 ± 1.5	16.0 ± 3.5	15.0 ± 2.5

Membranes (10-20 µg protein) were incubated with radioligand (55-65 pM) for 30 minutes at 21^{0}C. Specific binding was defined as the amount of bound radioligand displaced in the presence of 0.1 µM VIP.

Radiolabelled VIP derivatives were purified using HPLC.

Anterior pituitaries of castrated rats were removed 1 day after surgery.

Data are expressed as means ± SEM, from 10 (female), 5 (male) and 3 (castrated male) determinations. Assays were performed in duplicate.

maximal at pH 7.4 and 8.0 (Figure 6B). Similar pH optimums have been reported for VIP binding in brain (331), intestine (303) and prostate (320).

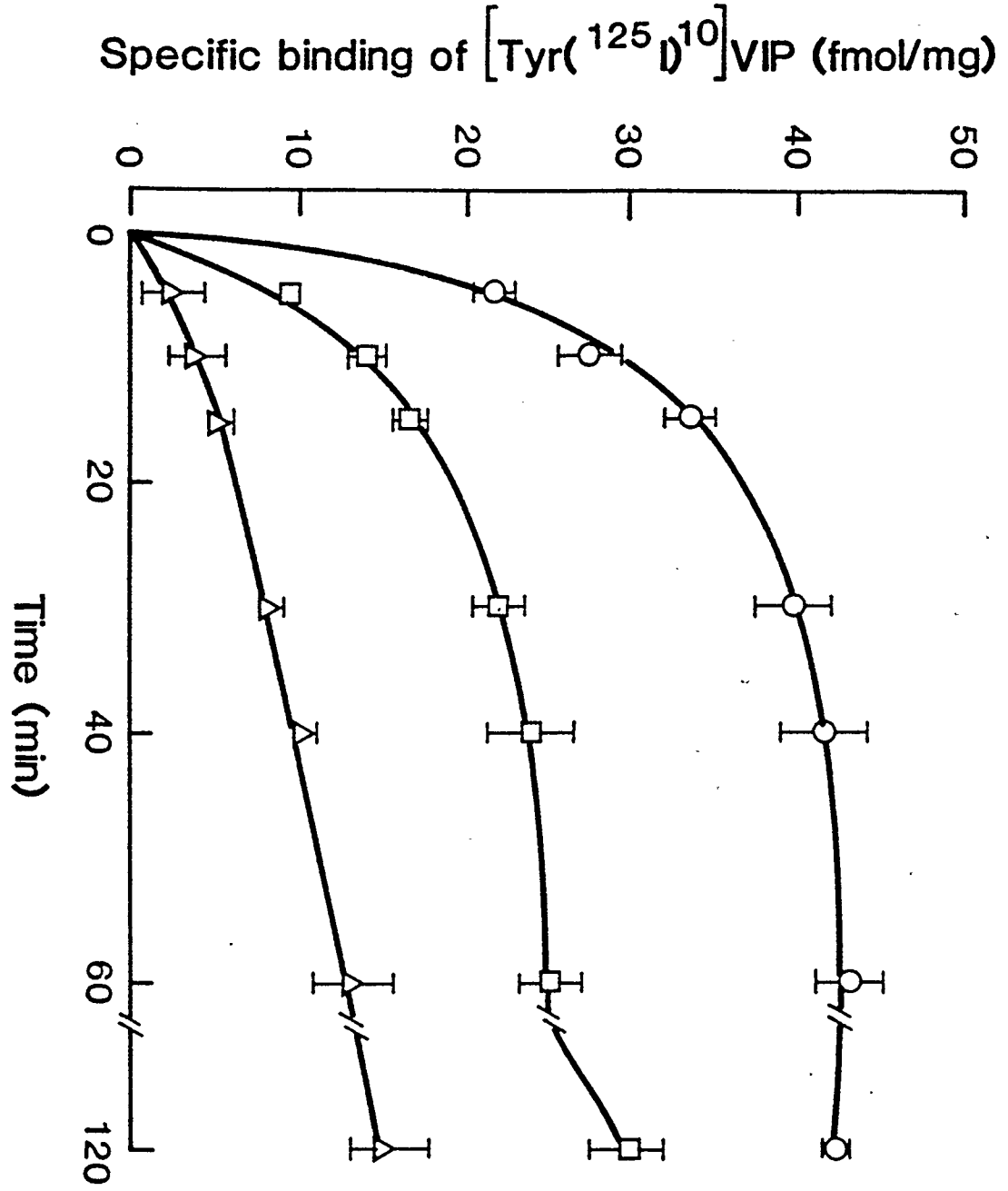
The effect of magnesium chloride ($MgCl_2$) added to the binding buffer was similar at both incubation temperatures (Figure 7A). Although the amount of specifically bound [Tyr(^{125}I)^{10}]VIP was consistently greater at 31^{0}C, a stable optimum was achieved at each temperature with 2, 3 or 5 mM $MgCl_2$. Specific binding decreased sharply at concentrations less than 2 mM $MgCl_2$. This effect of $MgCl_2$ was restricted to specific binding, as nonspecific binding was not appreciably altered over the concentration range checked (Figure 7B).

The necessity of bacitracin, an antibiotic thought to decrease adsorbance and/or degradation of VIP, and necessary for in vitro stimulation of PRL release by VIP (265), also improved the specific binding of VIP to rat anterior pituitary membranes (Table 8). Bacitracin, 0.25 mg/ml of binding buffer, increased specific binding of [Tyr(^{125}I)^{10}]VIP by 12.5 fmol/mg of rat anterior pituitary membrane protein. Its inclusion in the binding buffer also decreased the retention of [Tyr(^{125}I)^{10}]VIP to polybrene pretreated glass microfiber filters used to separate free from bound radioligand. In contrast the addition of PMSF or EDTA to the binding buffer did not change the specific binding of [Tyr(^{125}I)^{10}]VIP to rat anterior pituitary membranes.

5. Separation of free and bound radioligand. Centrifugation was successful in separating free from bound radioligand (see Table 5 for data generated using centrifugation). Poor pellet visibility and assay reproducibility using centrifugation required the use of larger amounts of membrane protein. In the face of limited quantities of tissue, especially from rat anterior pituitaries, filtration was evaluated as a means to separate free from bound VIP.

Despite presoaking in binding buffer C (containing 1% BSA (fraction V) and 0.25 mg/ml bacitracin) for a minimum of 1 hour, filters of many different compositions retained an unacceptably large amount of non-membrane bound [Tyr(^{125}I)^{10}]VIP (Table 9). Although cellulose acetate filters from some manufacturers were acceptable, their limited supply and variability from lot to lot necessitated investigation of other means to decrease retention of

FIGURE 5. Specific binding of [Tyr(^{125}I)^{10}]VIP as a function of temperature and time. Female rat anterior pituitary membranes (20 μg) were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) at 31^{0}C (○), 21^{0}C (□), or 1^{0}C (△) for the times indicated. Specific binding was defined as total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 0.1 μM VIP. Data points are mean ± SEM, n = 3-5, assayed in duplicate.


Specific binding of [Tyr(125I)10]VIP (fmol/mg)
0
10
20
30
40
50
Time (min)
0
20
40
60
120

free radioligand. Presoaking cellulose acetate or glass fiber filters in any of the four polycationic agents, polybrene, protamine, PEI or methylated BSA decrease retention of [Tyr(^{125}I)^{10}]VIP to an acceptable level (Table 9). As glass fiber filters are more economical, the effect of pretreatment with these four agent on the specific binding of [Tyr(^{125}I)^{10}]VIP was studied and results are shown in Table 10. Although no significant difference in specific binding was seen, methylated BSA resulted in slightly less specific binding and slightly greater retention of free [Tyr(^{125}I)^{10}]VIP and was hence not used further. Because PEI could potentially result in exposure to its carcinogenic monomer, ethylenimine (429) this agent was also eliminated. Polybrene was arbitrarily chosen over protamine for pretreatment of filters. Similar results have been obtained in our laboratory during study of the vascular VIP receptor (430).

Experiments including unlabelled VIP in the binding assay indicated that free [Tyr(^{125}I)^{10}]VIP retained on polybrene soaked glass fiber filters was not displaceable by this unlabelled VIP up to concentrations of 1.0 μM.

6. Stability of [Tyr(^{125}I)^{10} during binding. The integrity of [Tyr(^{125}I)^{10}]VIP during binding assay conditions is shown in the radioactivity recovery profiles from reverse phase HPLC in Figure 8. [Tyr(^{125}I)^{10}]VIP, eluting at 20-22 minutes, comprised the major peak of recovered radioactivity under all conditions. Degradation or alteration of the radioligand after incubation with rat anterior pituitary membranes, assessed by radioactivity eluting differently from the profile obtained from [Tyr(^{125}I)^{10}]VIP not reacted with membranes (Figure 8A), was less than 4 and 13 % for assays conducted at 21^{0}C for 30 minutes (Figure 8B), and 31^{0}C for 60 minutes (Figure 8C) respectively.

FIGURE 6. Effect of pH on the binding of [Tyr(^{125}I)^{10}]VIP to female rat anterior pituitary membranes. Anterior pituitary membranes were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) in binding buffer C at the indicated pH values. Specific binding was defined as the total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 0.1 μM VIP.

Panel A. Specific binding at 21^{0}C (□) and 31^{0}C (○) at different pH.

Panel B. Total (□), nonspecific (△) and specific (○) binding at 31^{0}C at different pH.

Data points in A and B are mean ± SEM, n = 4-6, assayed in duplicate.

Specific binding of [Tyr(125I)10]VIP (fmol/mg)
0
15
30
45
60
pH
6
7
8
9
10
A
Binding of [Tyr(125I)10]VIP (fmol/mg)
0
20
40
60
80
100
pH
6
7
8
9
10
B

FIGURE 7. Effect of magnesium concentration on the binding of [Tyr(^{125}I)^{10}]VIP to female rat anterior pituitary membranes. Anterior pituitary membranes were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) in binding buffer (Tris-HCl, pH 7.4, 1% BSA (fraction V), 0.25 mg/ml bacitracin) containing magnesium chloride (Mg^{++}) at the indicated concentrations. Specific binding was defined as the total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 0.1 μM VIP.

Panel A. Specific binding after incubation at 21^{0}C for 30 minutes (□) and 31^{0}C (○) for 60 minutes at the various concentrations of Mg^{++}.

Panel B. Total (□), nonspecific (△) and specific (○) binding after incubation at 31^{0}C for 60 minutes at the various concentrations of Mg++.

Data points in A and B are mean ± SEM, n = 4-6, assayed in duplicate.

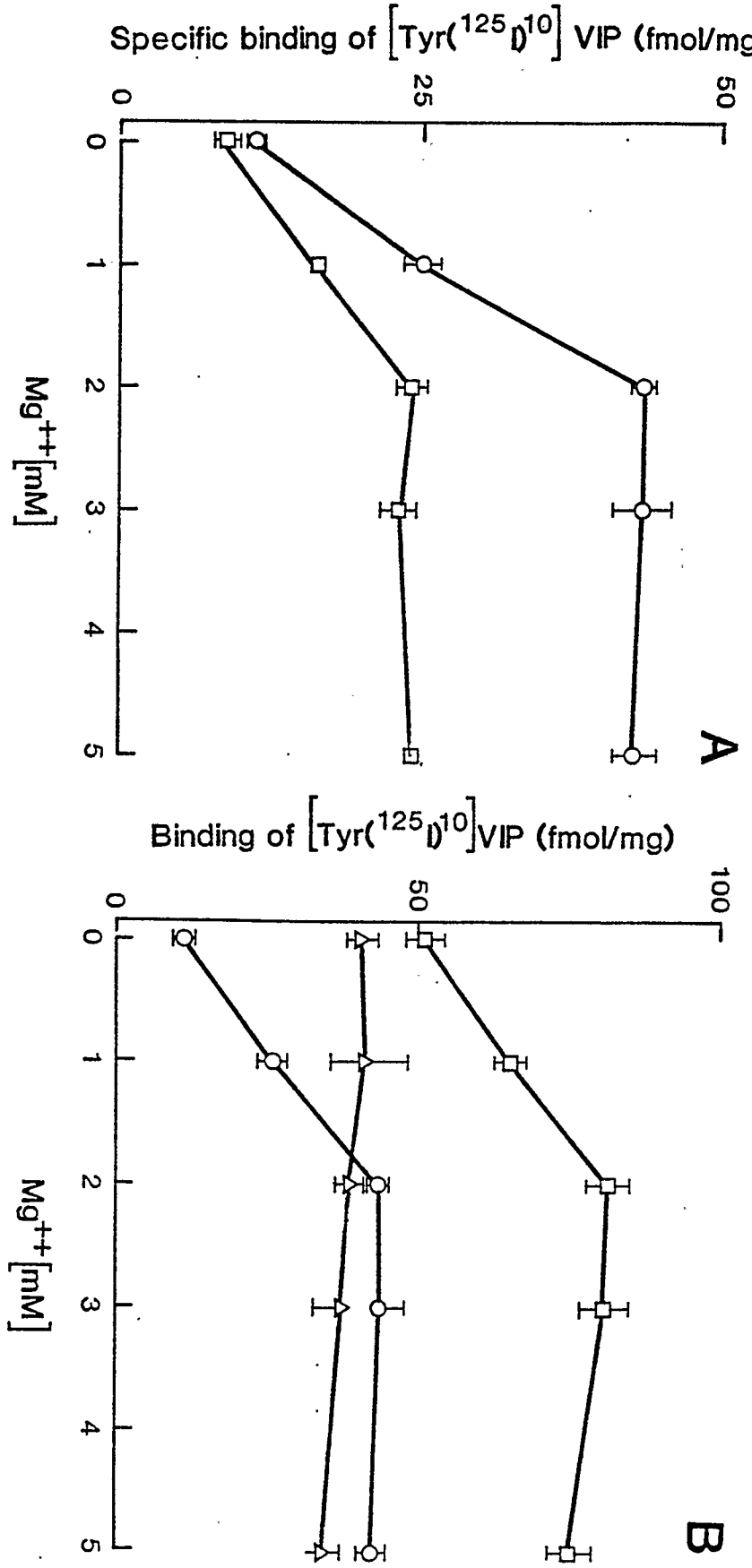

Specific binding of [Tyr(125I)10] VIP (fmol/mg)
0
25
50
0
1
2
3
4
5
Mg++[mM]
A
Binding of [Tyr(125I)10]VIP (fmol/mg)
0
50
100
0
1
2
3
4
5
Mg++[mM]
B

TABLE 8. Effect of bacitracin on the specific binding of [Tyr(^{125}I)^{10}]VIP and on its retention to glass fibre filters

	[Tyr(^{125}I)^{10}]VIP		
		retention on filter in absence of membranes	
Bacitracin (mg/ml)	Specific binding (fmol/mg protein)	fmol	% of total cpm added
0	31.0 ± 2.5	0.47 ± 0.02	3.9 ± 0.2
10	43.5 ± 1.5	0.31 ± 0.02	2.6 ± 0.02

Binding assays were conducted at 31^{0}C for 60 minutes with or without bacitracin added to the binding buffer (25 mM Tris-HCL, pH 7.4, 1% BSA (fraction V), 2 mM $MgCl_2$). Specific binding was determined by incubating [Tyr(^{125}I)^{10}]VIP (60-65 pM) with 20 µg female rat anterior pituitary membrane protein ± 0.1µM VIP.

Separation of free from bound radioligand was by filtration through glass fibre filters (GF/B) presoaked for > 1 hour in 0.3% aqueous polybrene. Retention of free [Tyr(^{125}I)^{10}]VIP to these filters was determined after identical incubation conditions in the absence of membranes.

Data are presented as mean ± SEM, n = 3, assayed in duplicate.

TABLE 9. The retention of [Tyr(^{125})^{10}]VIP to filters of differing composition, source and pretreatment

Filters			Retention on filters in absence of membranes	
Source	Composition	Treatment agents	fmol	% of total cpm added
Whatman	Glass fiber	polybrene	0.30 ± 0.04	2.5 ± 0.4
		protamine	0.29 ± 0.03	2.4 ± 0.3
		PEI	0.31 ± 0.02	2.6 ± 0.2
		meBSA	0.43 ± 0.05	3.6 ± 0.4
		buffer C	7.55 ± 0.21	61.6 ± 1.1
MSI	Cellulose acetate	polybrene	0.23 ± 0.02	2.0 ± 0.1
		protamine	0.30 ± 0.02	2.5 ± 0.3
		meBSA	0.43 ± 0.06	3.6 ± 0.5
Millipore	Cellulose acetate			
	lot a	buffer C	0.17 ± 0.01	1.4 ± 0.1
	lots b-d	buffer C	>3	>25
S&S, Amicon, MSI, Sartorius, Nuclepore	Nylon^{66}, polycarbonate cellulose acetate, cellulose acetate/nitrate	buffer C	>1	>10

The retention of [Tyr(^{125}I)^{10}]VIP (60-65 pM) was checked after presoaking filters ≥1 hour in buffer C (containing 1% BSA) or 0.3% aqueous solutions of polyethylenimine (PEI), protamine, polybrene, or methylated BSA (meBSA).

Filter pore size: 0.2, 0.22, 0.3 μm; glass fiber effective retention 1.0 μm.
Millipore filters lot a: C5D075A6, b-d: C4620205, F4H19120, H3B70738

Data are expressed as mean ± SEM, n = 3-13; of < minimum value per filter group tested, assays performed in duplicate.

TABLE 10. Effect of polycationic agents on the retention of [Tyr(^{125})^{10}]VIP bound to female rat anterior pituitary membranes by glass fibre filters

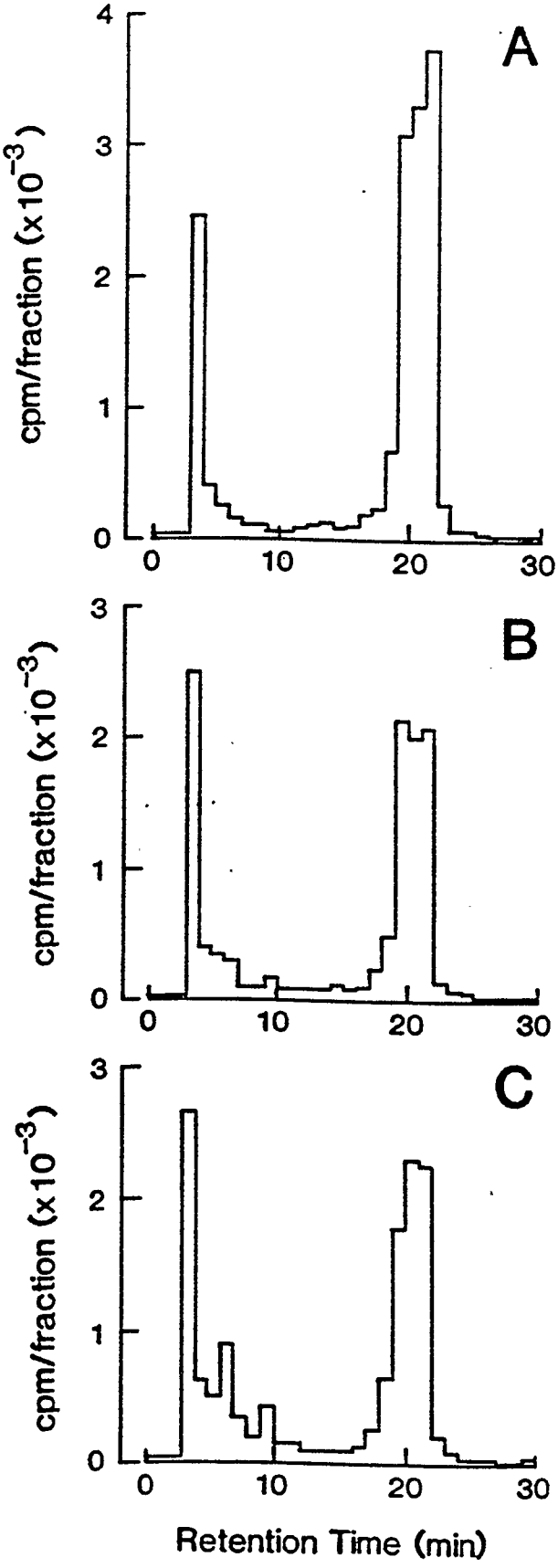
Polycationic treatment agents	Specific binding [Tyr(^{125}I)^{10}]VIP
polybrene	44.6 ± 2.6
protamine	43.7 ± 3.2
polyethylenimine	44.7 ± 4.3
methylated BSA	38.5 ± 6.7

Membranes (20 µg protein) were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) +/- 0.1 µM VIP at 31^{0}C for 1 hour.

Bound was separated from free radioligand by filtration through glass fibre filters (GF/B) presoaked ≥ 1 hour in 0.3% polybrene.

Data are expressed as mean ± SEM, n = 3, assayed in duplicate.

FIGURE 8. HPLC of [Tyr(^{125}I)^{10}]VIP before and after incubation with anterior pituitary membranes under assay binding conditions. [Tyr(^{125}I)^{10}]VIP (60-65 pM) was applied to a C-18 μBondapak column and eluted in the manner described in Fig. 3. [Tyr(^{125}I)^{10}]VIP was preincubated with 20 μg of female rat anterior pituitary membranes for 30 minutes at 21^{0}C (panel B) or 60 minutes at 31^{0}C (panel C), as compared to incubation in the absence of membranes (panel A). HPLC elution is expressed as radioactivity recovered per 1 ml fraction.


A
B
C
cpm/fraction (x10^{-3})
0
1
2
3
4
0
10
20
30
Retention Time (min)

II. CHARACTERISTICS OF VIP BINDING TO RAT ANTERIOR PITUITARY

A. Materials and Methods

1. Materials. The peptides used were obtained as follows: synthetic VIP, VIP(10-28), synthetic secretin, rat growth hormone releasing factor (r-GRF-(1-40)amide), bovine growth hormone releasing factor (b-GRF-(1-44)amide), human growth hormone releasing factor (h-GRF-(1-40)amide), pPHI and rPHI (peptides having amino terminal histidine and carboxyl terminal isoleucine from pigs and rats respectively), human PHI (hPHI or PHM, peptide with amino terminal histidine and carboxyl terminal methionine), ([4-Cl-D-Phe^6,Leu^{17}]VIP, and [N-Ac-Tyr^1,D-Phe^2]GRF(1-29)-NH_2, Peninsula Laboratories (San Carlos, CA); [Ac-His^1]VIP, [D-His^1]VIP, [Phe^1]VIP, [D-Arg^2]VIP, [D-Phe^2]VIP, [D-Ser^2]VIP, [D-Asp^3]VIP, and [D-Ala^4]VIP, VIP(2-28), gifts from Drs. D. Coy (Tulane University, New Orleans) and A. Fournier (Université du Quebec, Pointe Claire, Quebec); apamin, Serva (Heidelberg, FRG); and glucagon (bovine and porcine mixture), Sigma Chemical Co. (St.Louis, MO).

Guanosine-5'-0-thiotriphosphate tetralithium salt was purchased from Boehringer Mannheim Canada (Burlington, Ont).

2. Binding assay. Although VIP binding was observed in all tissues screened, the anterior pituitary from the rat was chosen for all further studies as this tissue is normal in contrast to the transformed and cultured GH_3 cells. Because the sex of the animal influenced binding of VIP, and was readily determinable in rats as compared to the cows and steers, bovine tissue was not examined any further. Additionally, the rat has been extensively used in the study of PRL regulation, and therefore the study of VIP binding to the anterior pituitary under different conditions could be interpreted with respect to these studies.

Based on the methodological experiments in section II, assay conditions optimizing specific binding were employed for further studies. Sufficient decreases in nonspecific binding had not been observed to justify the loss of tissue in processing to more purified plasma membrane preparations, hence rat anterior pituitary membranes prepared as described in section IIA in buffer A and by differential centrifugation only (Figure 1), was the anterior

pituitary tissue used in the remaining experiments. The standard radioligand binding assay consisted of incubating 55-65 pM [Tyr(^{125}I)^{10}]VIP, 20 μg of rat anterior pituitary membrane protein, plus or minus 0.1 μM unlabelled VIP, in binding buffer C (25 mM Tris-HCl, pH 7.4, containing 2 mM $MgCl_2$, 1 % BSA (Fraction V) and 0.25 mg/ml bacitracin) to a total volume of 0.2 ml. Incubations were conducted at 31^{0}C for 60 minutes in view of the increased specific binding observed at this temperature. Filtration through Whatman GF/B glass microfiber filters presoaked in 0.3 % aqueous polybrene as detailed in section IIA, was used to separate free from bound [Tyr(^{125}I)^{10}]VIP. Nonspecific binding, defined as the amount of [Tyr(^{125}I)^{10}]VIP not displaceable by 0.1 μM native VIP, was subtracted from total binding to calculate specific binding. This definition of nonspecific binding was used as very little additional displacement of [Tyr(^{125}I)^{10}]VIP occurred with higher concentrations of unlabelled VIP.

Dissociability of [Tyr(^{125}I)^{10}]VIP specifically bound to anterior pituitary membranes was tested by the addition of 0.1 μM unlabelled VIP after preincubation of the radioligand with 20 μg of membrane protein for 60 minutes at 31^{0}C. Remaining bound [Tyr(^{125}I)^{10}]VIP was determined after the addition of this excess unlabelled VIP at various time intervals up to 1 hour. Dissociation of a ligand from one binding site can be described by the following rate equation:

$$[RL] \xrightarrow{k_{-1}} [R] \ [L]$$

where [RL] is the initial concentration of ligand bound to the receptor, [R] and [L] are the free receptor and ligand concentrations respectively, and K_{-1} is the dissociation rate constant. As dissociation depends only on the concentration of ligand bound receptor, the dissociation can be described by the following first order rate equation:

$$[Rbt] = \sum_{i=1}^{i=n} [R_o i] e^{-k_{-1} i t}$$

where [Rbt] is the amount of radioligand bound at time t, [Ro] is the amount bound at time t=0 and k_{-1} is the dissociation constant for each site i out of a possible n sites. [For

derivation of this equation please refer to Weiland and Molinoff (431)]. Results from dissociation experiments were analyzed using the nonlinear curve fitting computer program KINETIC (Elsevier Biosoft, version 3.0, 1985), iterating to the above algorithm. Computer generated statistical testing by least squares was used in determining the most appropriate model.

Competitive inhibition of [Tyr(^{125}I)^{10}]VIP binding by VIP and some of its antagonists, fragments, structurally modified analogs and homologous peptides, as well as by apamin, was examined by incubation of the radioligand (55-65 pM) with increasing concentrations of these agents for 60 minutes at 31^{0}C. Results were expressed as the percent of binding relative to specific binding. Competitive binding of [Tyr(^{125}I)^{10}]VIP by VIP was analyzed according to Scatchard (407) and the curves were fitted using the nonlinear regression program LIGAND as described in the introduction.

The effect of the nonhydrolysable analog of GTP, GTP-γ-S, on radioligand binding was examined by the addition of 10 μMGTP-γ-S to the assay in the absence or presence of various concentrations of unlabelled VIP. Competitive binding curves obtained in the presence of GTP-γ-S were analyzed using LIGAND and binding parameters were compared with those obtained from studies done in the absence of GTP-γ-S.

B. Results

1. Properties of binding. In the presence of a fixed amount of anterior pituitary membrane protein (20 μg), specific binding of [Tyr(^{125}I)^{10}]VIP plateaus despite increasing concentrations of the radioligand (Figure 9). This contrasts sharply with nonspecific binding which increases in a linear fashion up to 490 pM [Tyr(^{125}I)^{10}]VIP. As these experiments were conducted at 21^{0}C, nonspecific binding was always much greater than specific binding even at lower concentrations of [Tyr(^{125}I)^{10}]VIP. They were not repeated at 31^{0}C as the results at the lower temperature were so definitive, and because of the undesirability of repeat exposure to high concentrations of radioligand.

Specific binding to [Tyr(^{125}I)^{10}]VIP (55-65 pM) was saturable in the face of increasing amounts of anterior pituitary membrane protein (Figure 10). A proportional

relationship between specific binding and membrane concentration was seen up to 50 μg, whereas nonspecific binding continued to increase up to at least 100 μg membrane protein. This was found at both 31^{0}C (Figure 10A) and at 21^{0}C (Figure 10B), however an increase in the absolute and relative amounts of specific versus nonspecific binding is seen at the higher temperature.

Sixty eight percent of [Tyr(^{125}I)^{10}]VIP specifically bound to female rat anterior pituitary tissue was reversible 1 hour after the addition of excess unlabelled VIP (Figure 11). The dissociation was rapid for the first 20 minutes and continued at a slower rate over the next 40 minutes. Transformation of these results to $\log_{10}$ of percent specific binding did not linearize the data (Inset, Figure 10). Analysis of this data using the nonlinear regression program KINETIC resulted in the statistically significant best fit being a bi-exponential model of dissociation. Compatible with this are two sites of VIP binding with the dissociation rate (k_{-1}) of one site being 0.051 ± 0.027 min^{-1}. The k_{-1} of the second site was very poorly defined at 0.001 ± 0.005 min^{-1}.

Unlabelled VIP was able to inhibit the binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary membranes in a concentration dependent manner (Figure 12A). The plots after Scatchard analysis (407) of displacement curves yielded upwardly concave curves indicating heterogeneity of binding (Figure 12B). Although numerous factors could be responsible for this observation, the use of purified [Tyr(^{125}I)^{10}]VIP makes radioligand heterogeneity unlikely. Negative cooperativity, although not excluded in this study is also unlikely as site-to-site interactions for VIP receptors has not been detected in other tissues (303,315,320). A very distinct possibility is the presence of more than one binding site for VIP in the anterior pituitary. This has been postulated in many other tissues, and supported by affinity labelling or receptor solubilization of two classes of VIP binding sites in brain (351), intestine (348), liver (337) and lung (307), with different structural and functional properties. When the data were analyzed using the least squares nonlinear regression computer program LIGAND, a statistically better fit was obtained for the two class binding site model compared with one or three binding sites. Equilibrium dissociation constants (K_D) of 0.19 ± 0.03 nM

and 28.0 ± 6.2 nM were obtained for high and low affinity binding sites respectively, from the means ± SEM of 7 experiments of curves analyzed individually. The corresponding maximum binding capacities (B_{max}) were 158 ± 34 fmol/mg and 11.7 ± 3.6 pmol/mg membrane protein. Competitive binding studies conducted at 21^{0}C resulted in binding parameters not significantly from those seen at 31^{0}C (Table 11).

2. Effect of GTP-γ-S. The inclusion of GTP-γ-S (10 μM) decreased specific binding of [Tyr(^{125}I)^{10}]VIP to only 22 % of that normally seen without the addition of GTP-γ-S (Figure 13). Analysis of these data using the LIGAND curve fitting program demonstrated that an equal fit was obtained statistically for the one and the two site binding models for VIP in the presence of GTP-γ-S (Table 12). In contrast the analysis of the displacement curve without adding GTP-γ-S highly favoured the 2 site model with K_D and B_{max} values like those obtained before (compare Tables 11 and 12). The effect of GTP-γ-S appeared to obliterate or severely decrease the number of high affinity binding sites. The addition of GTP-γ-S did not result in a detectable difference in the lower affinity binding site. The results in Table 12 are based on multiple curve analysis and hence standard errors are larger than for those examined by single curve analysis in Table 11. For more detailed discussion of these different analyses see section IIE Materials and Methods.

3. Competitive binding. Receptors for the VIP family of peptides (Table 1) are defined by their binding properties including specificity (346). VIP, able to inhibit 50 % of specifically bound [Tyr(^{125}I)^{10}]VIP to female rat anterior pituitaries at a concentration of 0.4 nM (IC_{50}), was at least 2 orders of magnitude more potent when compared with eight other members of the glucagon-secretin family (Figure 14). The rat derived peptides (rPHI and rGRF) as well as human PHI competed more effectively, with IC_{50} values of 63, 89 and 63 nM respectively, than did bGRF and pPHI (IC_{50} of 224 nM). Human GRF was less potent still with 1 μM displacing only 40 % of bound [Tyr(^{125}I)^{10}]VIP. Secretin and glucagon at 10 μM were able to displace 77 and 19 % of bound radioligand respectively. Apamin, a neurotoxic peptide isolated from bee venom lacking structural homology to VIP but able to competitively inhibit VIP binding in brain and uterus (432) inhibited [Tyr(^{125}I)^{10}]VIP binding

with an IC_{50} of 6.3 μM. Deletion of the 9 amino terminal amino acids (VIP_{10-28}) resulted in a peptide less than one-five hundredth as potent (IC_{50} of 224 nM) compared with intact VIP.

The relative ability of two VIP antagonists (300) to inhibit [Tyr(^{125}I)^{10}]VIP binding was the same in both male and female anterior pituitary tissue (Figure 15). Although both were not very potent, [4 Cl-D-Phe^{6},Leu^{17}]VIP was more effective at inhibiting radioligand binding (IC_{50} of 398 and 480 nM in female and male respectively) compared with [N-Ac-Tyr^{1},D-Phe^{2}]GRF(1-29)NH_2 (IC_{50} >1000 nM).

The ability of analogs of VIP, with single deletions or substitutions in the first 4 N-terminal amino acids, to competitively inhibit [Tyr(^{125}I)^{10}]VIP binding is shown in Figure 16. The corresponding IC_{50} values are given in Table 13, along with those obtained from competitive binding studies of the VIP receptor in rat brain, vasculature and liver. The studies on nonpituitary tissue were performed by Dr. Minta Huang, University of Calgary. In the anterior pituitary the amino terminal histidine appeared necessary for binding as VIP(2-28), with His^{1} deleted, was only about 1/10 as potent as the intact molecule. Amino acid substitutions at the His^{1} position also decreased potency, however to a lesser degree than did deletion. Rank order of potency of VIP analogs with alterations at position 1 was [N-Ac-His^{1}]VIP>[Phe^{1}]VIP>[D-His^{1}]VIP>VIP(2-28) (Figure 16A). Substitutions at positions 2, 3 and 4 also decreased the potency of all analogs examined relative to native VIP. Binding appeared to be more adversely affected with amino acid changes in positions 1 and 2 as compared with 3 and 4. The rank order for analogs of VIP modified in positions 2 to 4 was [D-Ala^{4}]VIP=[D-Asp^{3}]VIP>[D-Ser^{2}]VIP>[D-Arg^{2}]VIP>[D-Phe^{2}]VIP (Figure 16B).

Whereas the rank order of VIP structurally modified analogs was similar to that seen in the anterior pituitary in rat brain and liver, competitive binding in rat and bovine arteries differed considerably, especially in binding of analogs modified in positions 2 to 4 (Table 13). In the vasculature, the rank order of potency of VIP analogs modified at residues 2 to 4 was VIP>[D-Ser^{2}]VIP=[D-Asp^{3}]VIP>[D-Arg^{2}]VIP=[D-Phe^{2}]VIP=[D-Ala4]VIP.

FIGURE 9. Binding of VIP as a function of increasing concentrations of [Tyr(^{125}I)^{10}]VIP. Assays were conducted at 21^{0}C for 30 minutes with 20 μg of female rat anterior pituitary membrane protein. Data presented are total (□), nonspecific (△) and specific (○) binding curves from one representative experiment with each point determined in duplicate.

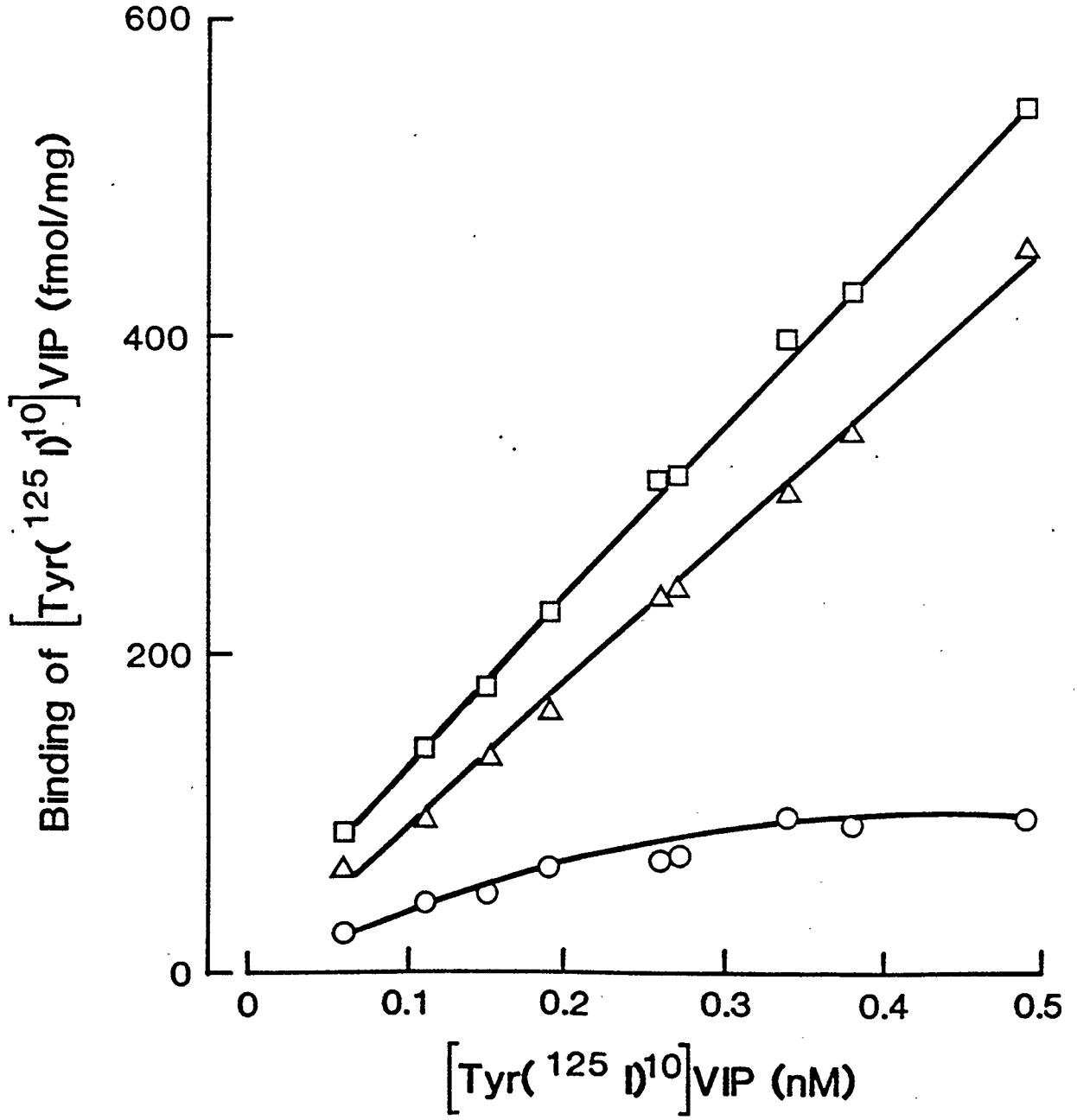

600
400
200
0
0
0.1
0.2
0.3
0.4
0.5
Binding of [Tyr(125 I)10]VIP (fmol/mg)
[Tyr(125 I)10]VIP (nM)

FIGURE 10. Binding of [Tyr(^{125}I)^{10}]VIP as a function of increasing female rat anterior pituitary membrane protein. Binding assays were performed with 55-65 pM [Tyr(^{125}I)^{10}]VIP at 31^{0}C for 60 minutes (panel A) or 21^{0}C for 30 minutes (panel B), and total ( ), nonspecific (△) and specific (○) binding was determined. Data points are mean ± SEM from 3-5 determinations in duplicate.

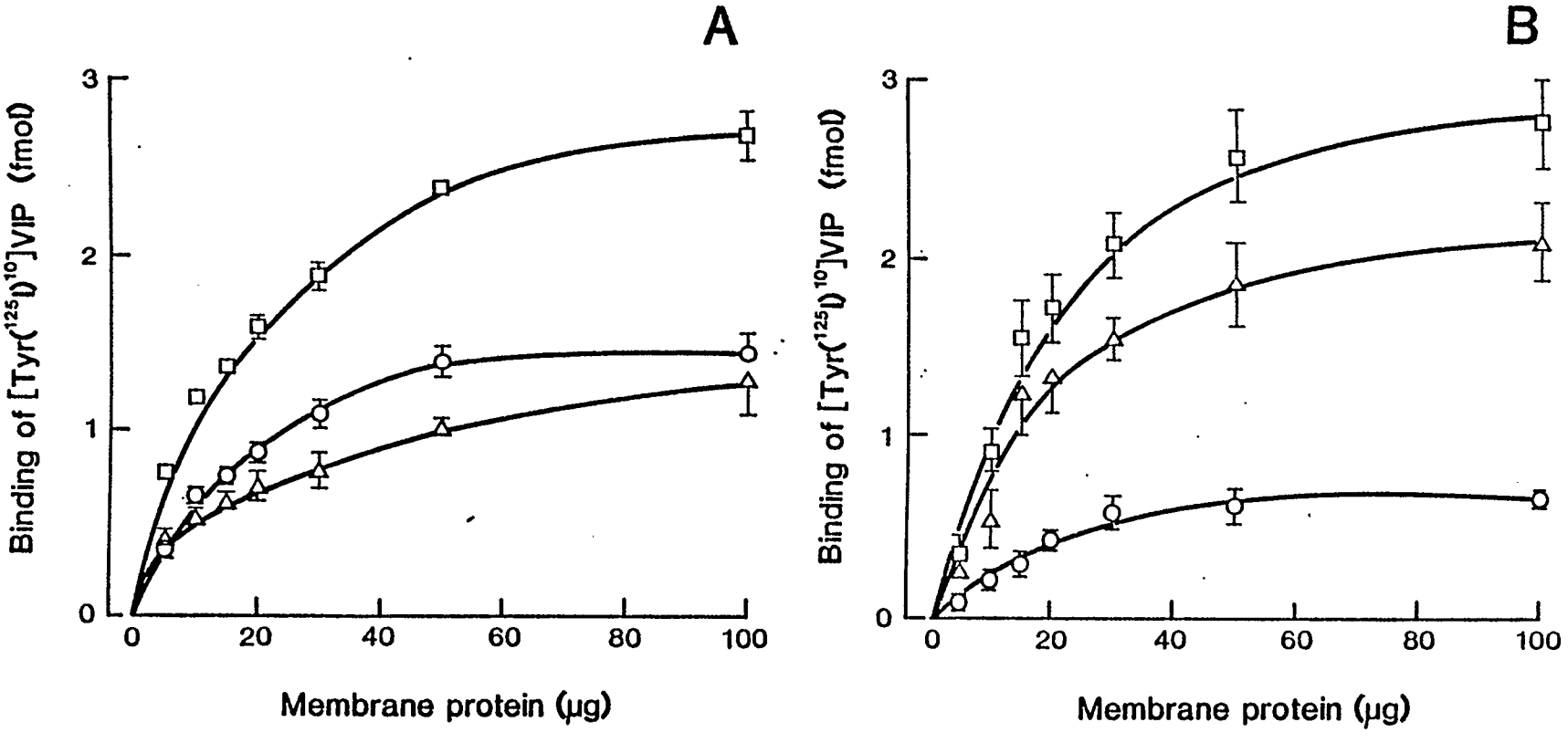

A
B
Binding of [Tyr(125I)10]VIP (fmol)
Membrane protein (µg)
0
1
2
3
0
20
40
60
80
100

FIGURE 11. Time course of the dissociation of [Tyr(^{125}I)^{10}]VIP bound to female rat anterior pituitary membranes. Membranes (20 μg protein) were incubated with [Tyr(^{125}I)^{10}]VIP (60-65 pM) for 60 minutes at 31^{0}C. Unlabelled VIP (0.1 μM) was then added and remaining bound radioligand determined at the time points indicated on the abscissa. the data points are mean ± SEM from 3 experiments assayed in duplicate.

Inset: Expression of the same data as the $\log_{10}$ of % initial specific binding on the ordinate.

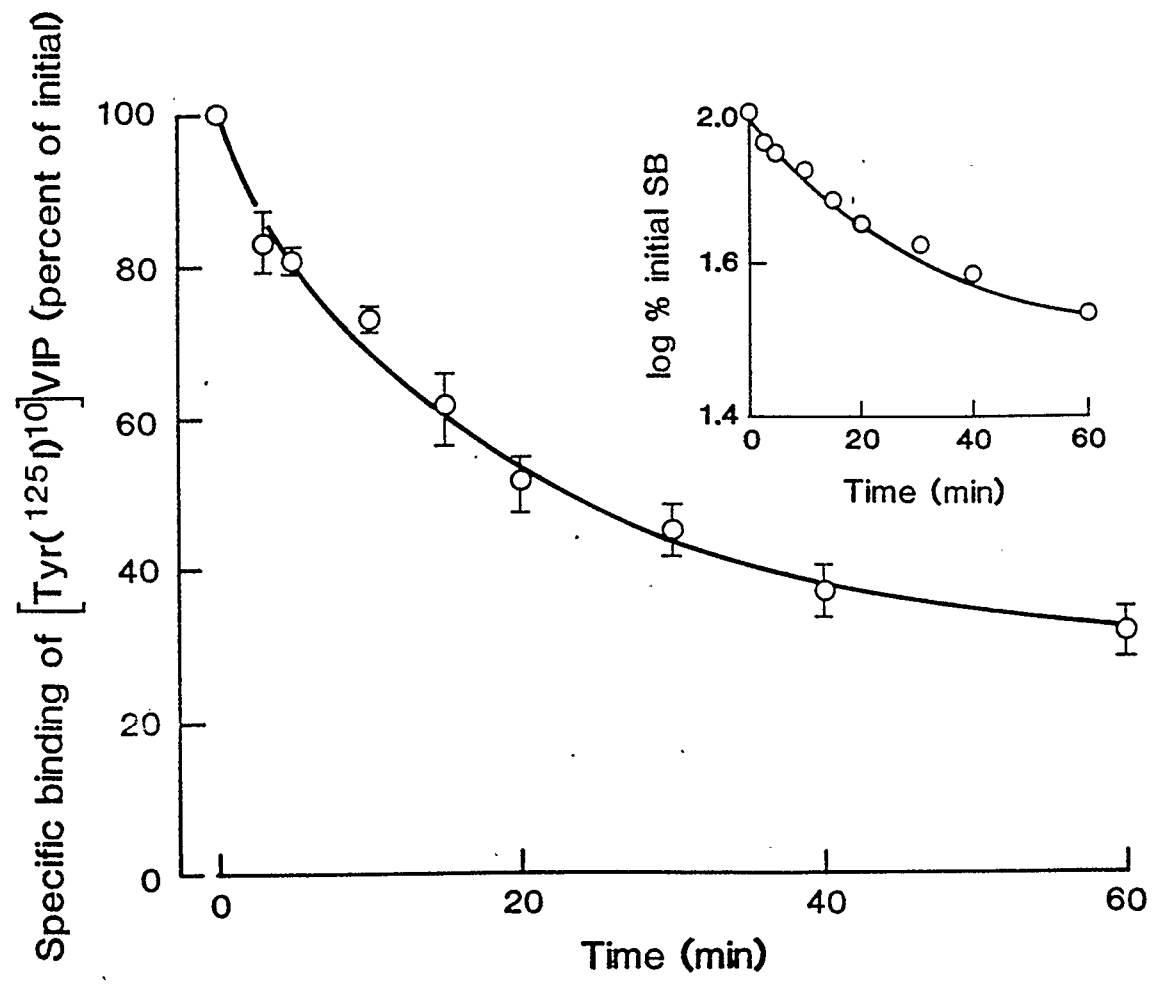
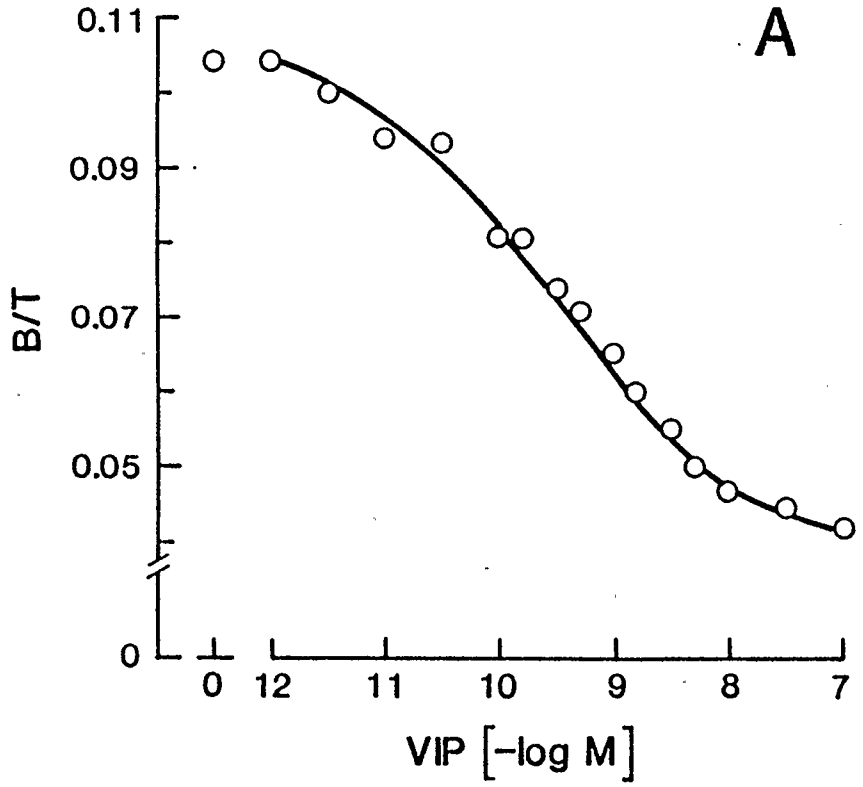

Specific binding of [Tyr(125I)10]VIP (percent of initial)
100
80
60
40
20
0
0
20
40
60
Time (min)
log % initial SB
2.0
1.6
1.4
0
20
40
60
Time (min)

FIGURE 12. Competition by unlabelled VIP for binding of [Tyr(^{125}I)^{10}]VIP to female rat anterior pituitary membranes. Membranes (20 μg protein) were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) in the presence of increasing concentrations of native VIP, for 60 minutes at 31^{0}C. B/T refers to specifically bound [Tyr(^{125}I)^{10}]VIP over total bound radioligand. Data points are the means of duplicate measurements from 1 study and is representative of 7 individual experiments.

Panel B. Scatchard analysis of data in panel A. B is bound [Tyr(^{125}I)^{10}]VIP, and B/F is bound over free radioligand.


A
0.11
0.09
0.07
0.05
0
B/T
0
12
11
10
9
8
7
VIP [-log M]

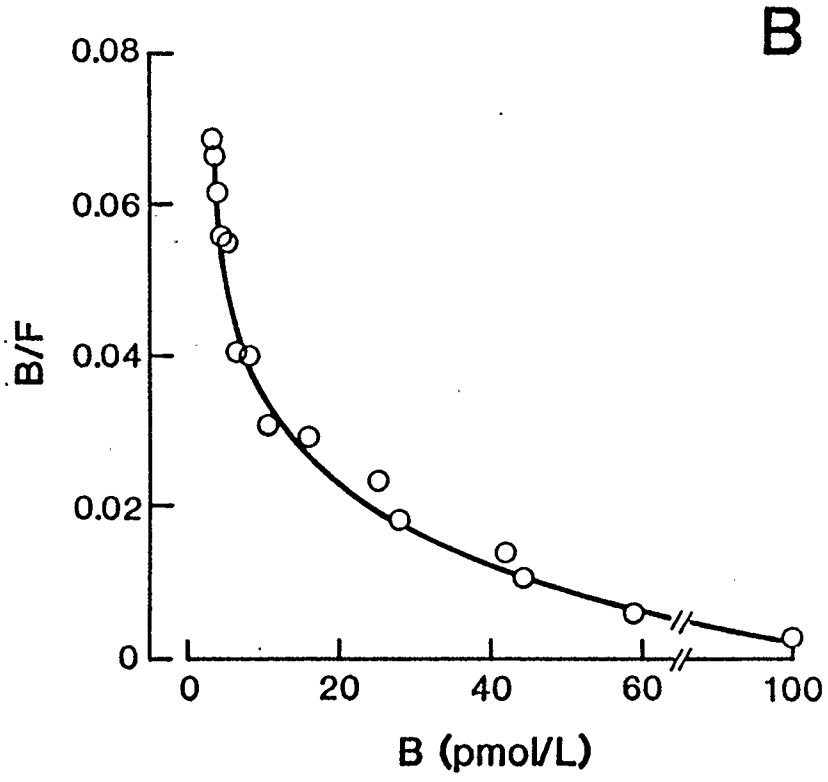

B
0.08
0.06
0.04
0.02
0
B/F
0
20
40
60
100
B (pmol/L)

Table 11. Equilibrium dissociation constants and maximum number of binding sites of the female rat anterior pituitary VIP receptor

	High affinity site		Low affinity site	
Assay temperature	K_D(nM)	Bmax(fmol/mg protein)	K_D(nM)	Bmax(pmol/mg protein)
21^0C	0.32 ± 0.08	224 ± 69	32 ± 15	25.4 ± 10.0
31^0C	0.19 ± 0.03	158 ± 34	28 ± 16	11.7 ± 3.6

Equilibrium dissociation constants (K_D) and maximum number of binding sites (Bmax) were obtained by nonlinear regression analysis using the computer program LIGAND.

Data are expressed as mean ± SEM, n = 7, assays performed in duplicate.

$p>0.05$, Student's t test, comparing parameter means between the two temperatures.

FIGURE 13. Effect of GTP-γ-S on the binding of [Tyr(^{125}I)^{10}]VIP to female rat anterior pituitary membranes. Membranes (20 μg protein) were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) in the absence (□) or presence (▨) of 10 μM GTP-γ-S, with increasing concentrations of native VIP, for 60 minutes at 31^{0}C. Data are presented as mean from 3 experiments performed in duplicate. Error bars reflect SEM.

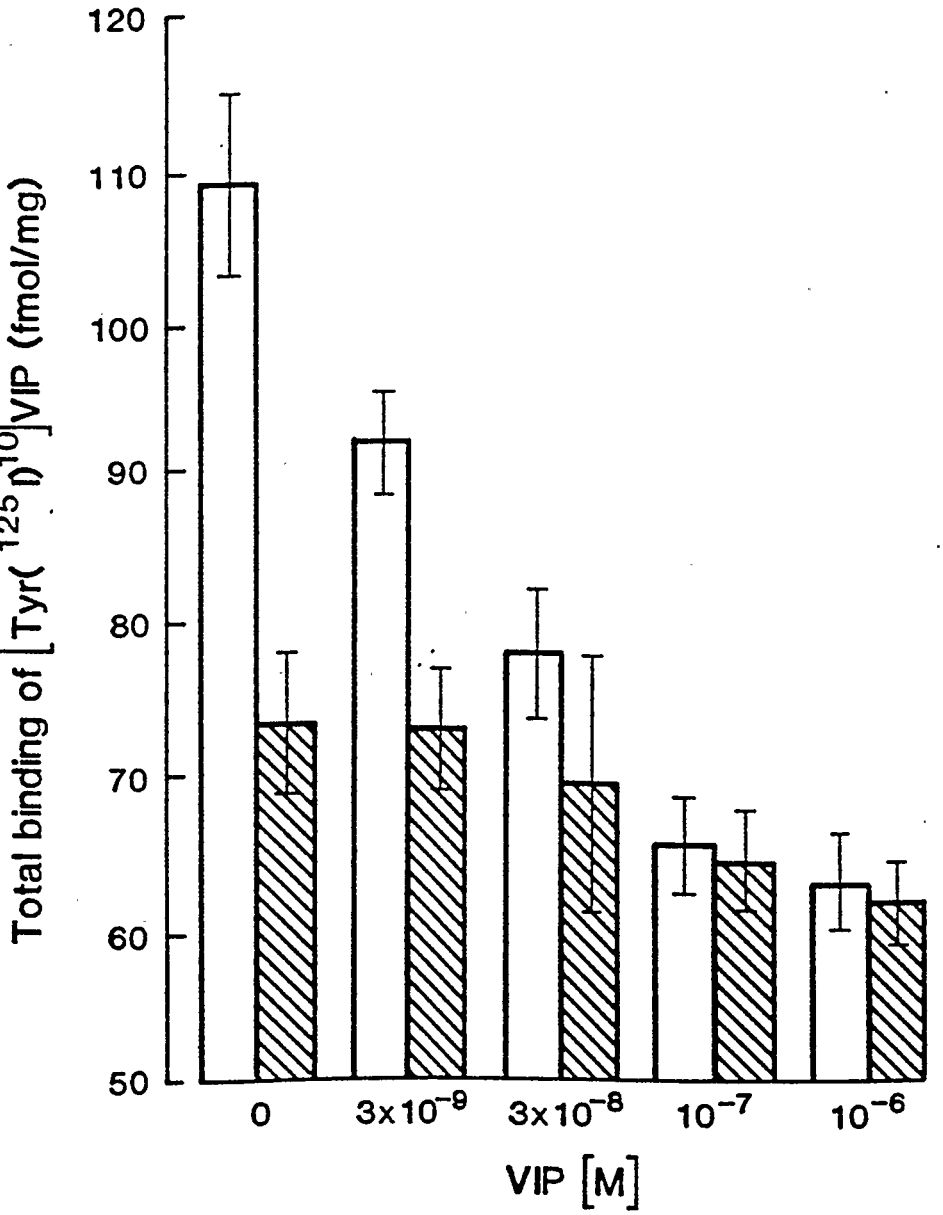

120
110
100
90
80
70
60
50
Total binding of [Tyr(125I)10]VIP (fmol/mg)
0
3x10-9
3x10-8
10-7
10-6
VIP [M]

Table 12. Equilibrium dissociation constants and maximum number of binding sites of the female rat anterior pituitary VIP receptor in the absence or presence of GTP-γ-S

GTP-γ-S (μM)	High affinity site		Low affinity site	
	K_D(nM)	Bmax(fmol/mg protein)	K_D(nM)	Bmax(pmol/mg protein)
0	0.20 ± 0.18	152 ± 11	45 ± 33	10.4 ± 2.8
10	----	----	28 ± 10	11.4 ± 4.2

Equilibrium dissociation constants $(K_D)_1$ and maximum number of binding sites (Bmax) were obtained by nonlinear regression analysis using the computer program LIGAND.
Data are expressed as mean ± SEM, n = 3, assays performed in duplicate.

FIGURE 14. Competitive inhibition of [Tyr(^{125}I)^{10}]VIP binding to female rat anterior pituitary membranes by VIP, apamin and VIP homologous peptides. [Tyr(^{125}I)^{10}]VIP (55-65 pM) was incubated with 20 μg of membrane protein for 60 minutes at 21^{0}C in the presence of increasing concentrations of VIP(1-28) (●), hPHI (○), rPHI (▲), rGRF (△), bGRF (◊), pPHI (□), VIP(10-28) (■), hGRF (♦), secretin (▽), apamin (▼) or glucagon (x). Results are expressed as a percent of maximum specific binding in the presence of these peptides. Data points are the mean of 3-6 assays in duplicate. SEM, which was less than 5% for all points, was omitted for clarity.

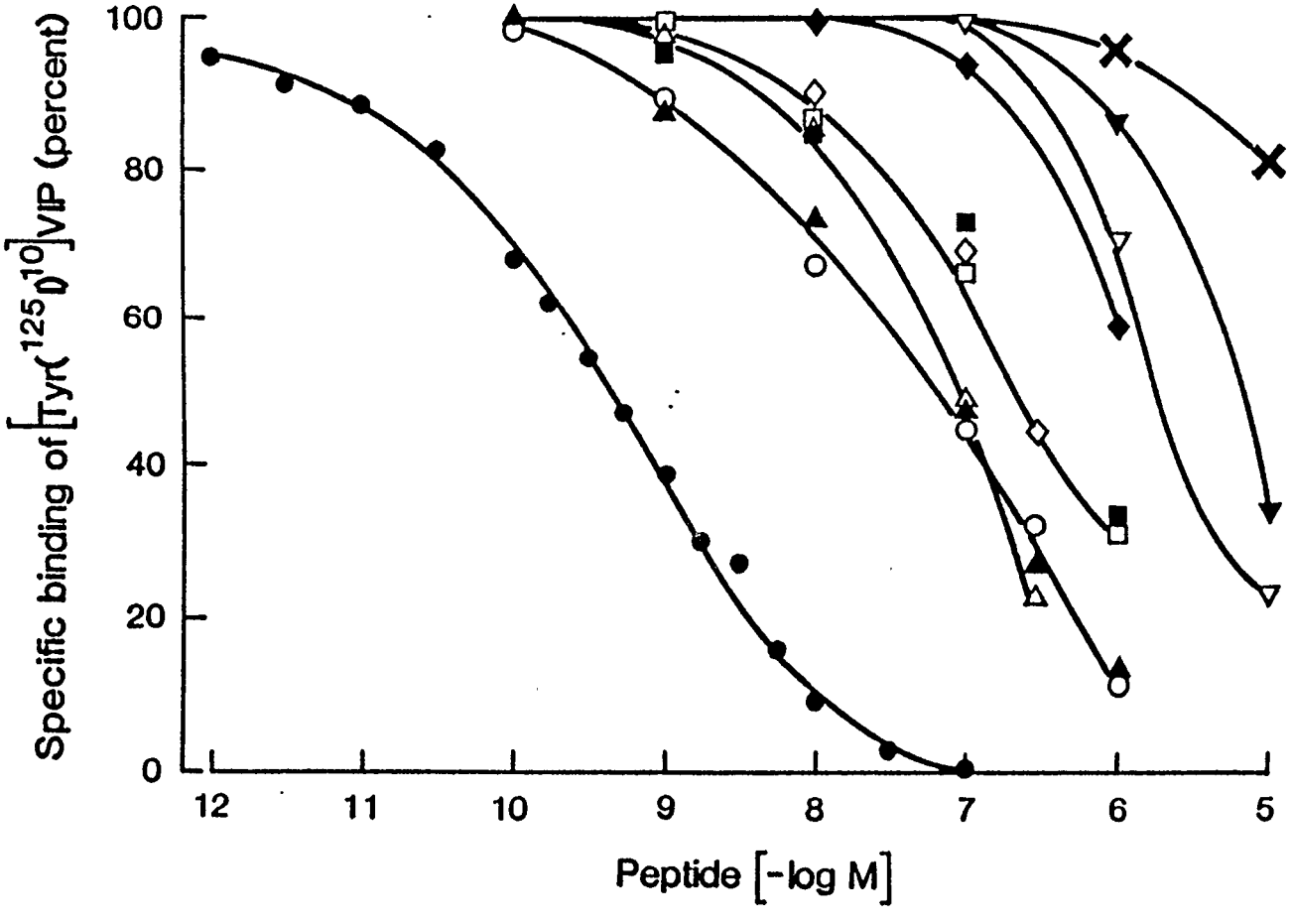

Specific binding of [Tyr(125I)10]VIP (percent)
100
80
60
40
20
0
12
11
10
9
8
7
6
5
Peptide [-log M]

FIGURE 15. Competitive inhibition of [Tyr(^{125}I)^{10}]VIP binding to anterior pituitary membranes by VIP antagonists. Radiolabelled VIP was incubated with 20 μg of female (panel A) or male (panel B) rat anterior pituitary membrane protein for 60 minutes at 31^{0}C in the presence of increasing concentrations of VIP(1-28) (●), [4-Cl-D-Phe^6,Leu^{17}]VIP (▲) and [N-Ac-Tyr^1,D-Phe^2]GRF(1-29)NH_2 (■). Data presentation is similar to that in Fig. 14. Each point is the mean of 3 experiments performed in duplicate. SEM is omitted and was less than 6% for each point.

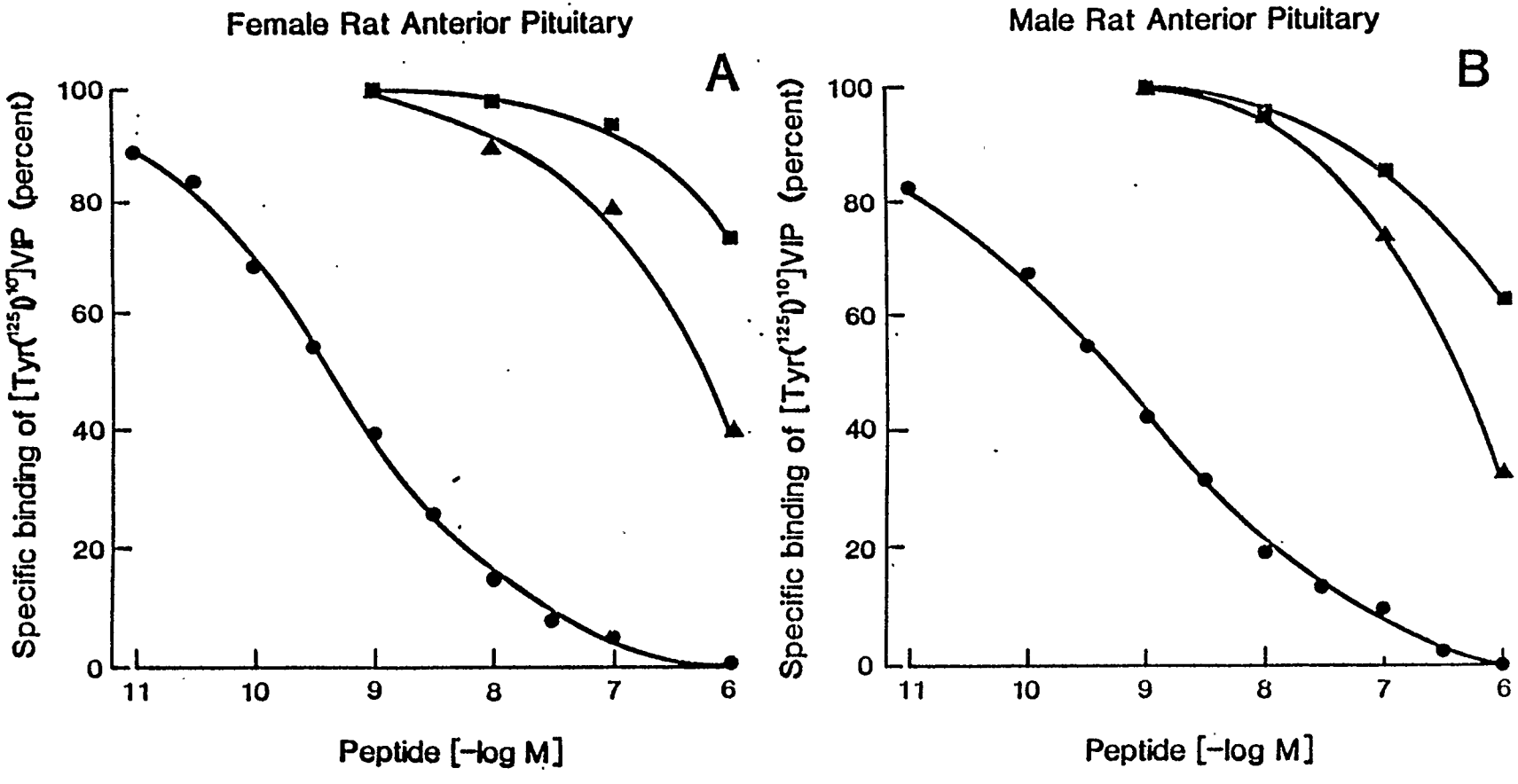

Female Rat Anterior Pituitary
A
Specific binding of [Tyr(125I)10]VIP (percent)
100
80
60
40
20
0
11
10
9
8
7
6
Peptide [-log M]
Male Rat Anterior Pituitary
B
Specific binding of [Tyr(125I)10]VIP (percent)
100
80
60
40
20
0
11
10
9
8
7
6
Peptide [-log M]

FIGURE 16. Competition for binding of [Tyr(^{125}I)^{10}]VIP to male rat anterior pituitary membranes by VIP analogs modified at amino acids residues 1 to 4. Data presentation is similar to that in Fig. 13.

Panel A: VIP(1-28) (●), [Nα-Ac-His^1]VIP (○), [Phe^1]VIP (▲), [D-His^1]VIP (△), and VIP(2-28) (■).

Panel B: VIP(1-28) (●), [D-Asp^3]VIP (▲), [D-Ala^4]VIP (○), [D-Ser^2]VIP (△), [D-Arg^2]VIP (□), and [D-Phe^2]VIP (■).

Data points are the mean of 3-6 experiments performed in duplicate. SEM is omitted for clarity and was below 6% of mean values.

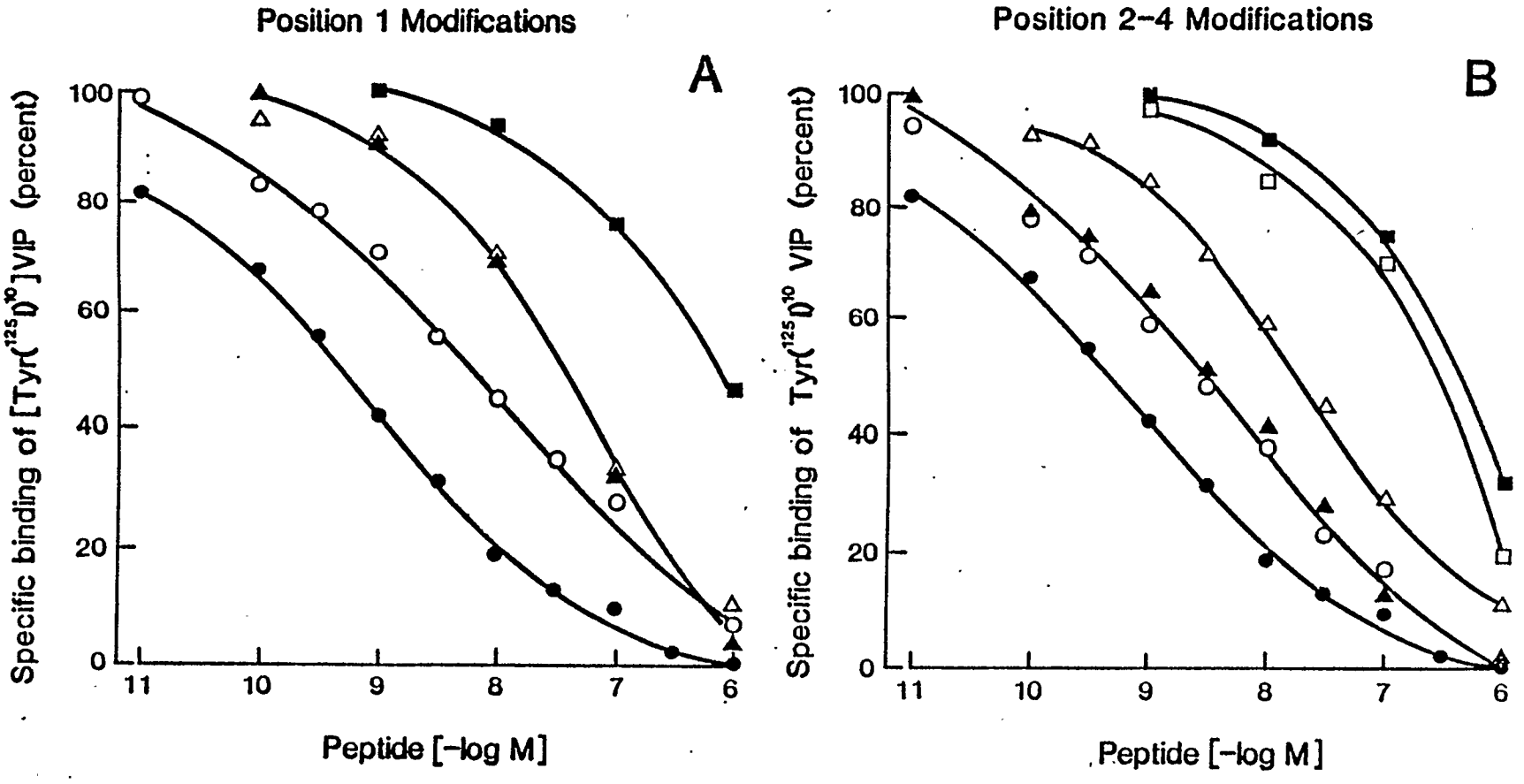

Position 1 Modifications
A
Specific binding of [Tyr(125I)10]VIP (percent)
100
80
60
40
20
0
11
10
9
8
7
6
Peptide [-log M]
Position 2-4 Modifications
B
Specific binding of Tyr(125I)10 VIP (percent)
100
80
60
40
20
0
11
10
9
8
7
6
Peptide [-log M]

TABLE 13. Comparisons of competitive binding of VIP and GRF analogs on rat and bovine tissues

Peptides	bovine coronary artery		rat mesenteric artery		rat pituitary		rat brain		rat liver	
	IC_{50} nM	Potency	IC_{50} nM	Potency	IC_{50} nM	Potency	IC_{50} nM	Potency	IC_{50} nM	Potency
VIP	0.25	100	0.55	100	0.56	100	1.2	100	0.37	100
[Nα-Ac-His^1]VIP	0.47	53	0.78	71	6.6	8.5	6.1	20	3.8	9.7
[Phe^1]VIP	5.5	4.5	5.8	9.5	31	1.8	72	1.6	8.9	4.2
[D-His^1]VIP	26	0.96	48	1.1	31	1.8	72	1.6	12	3.0
des-His^1-VIP	160	0.16	250	0.22	480	0.12	>1000	<0.12	280	0.13
[D-Ser^2]VIP	0.91	27	1.8	31	20	2.8	18	6.7	6.3	5.9
[D-Phe^2]VIP	13	1.9	16	3.4	480	0.12	280	0.42	12	3.0
[D-Arg^2]VIP	13	1.9	16	3.4	350	0.16	180	0.67	85	0.43
[D-Asp^3]VIP	0.91	27	1.8	31	3.7	15	10	12	2.4	15
[D-Ala^4]VIP	13	1.9	22	2.5	3.7	15	10	12	0.83	45
[4-Cl-D-Phe^6, Leu^{17}]VIP	213	0.12	141	0.39	480	0.12	390	0.26	162	0.22
[N-Ac-Tyr^1,D-Phe^2] GRF(1-29)NH_2	740	0.03	890	0.06	>1000	<0.06	>1000	<0.12	790	0.05

The IC_{50} is the concentration of peptide which reduced maximal specific binding of [Tyr(^{125}I)^{10}]VIP by 50%. Potency is the ratio of IC_{50} for an analog over the IC_{50} of VIP multiplied by 100.

III. [TYR(^{125}I)^{10}]VIP BINDING TO LACTOTROPES

A. Materials and Methods

1. Materials. The source of the products was as follows: Percoll, Pharmacia (Dorval Que); acetone, methanol, 1,2 dichloroethane, sodium iodide, cupric sulphate, acetic acid, Fisher Scientific (Orangeburg NY); trypan blue, light green SF yellowish (synonymous with wool green) and orange G dyes, bovine pancreatic trypsin (fraction III), dopamine hydrochloride, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), mercuric chloride, formaldehyde, phosphotungstic acid, polyethylene glycol 8000, Sigma Chem. Co. (St. Louis MO); carmoisine dye, ICN Biomed. (Irvine, CA); RIA kit for rat PRL, National Institute for Arthritis, Diabetes, Digestive Diseases and Kidney (USA); normal rabbit serum and goat antiserum against rabbit gamma globulin, Antibodies Inc. (Davis CA).

The following tissue culture supplies were purchased from GIBCO Canada (Burlington, Que): Dulbecco's Modified Eagles Medium (DMEM) with L-glutamine, medium 199 with Hank's balanced salt solution, penicillin-streptomycin (10,000 U/ml and 10,000 μg/ml respectively),.1% nonessential amino acids, horse serum, fetal bovine serum and 25 cm^2 polystyrene tissue culture flasks.

2. Sedimentation of dispersed anterior pituitary cells. In order to determine whether the binding of VIP seen in the anterior pituitary is directly to PRL secreting cells, separation of the heterogeneous population of anterior pituitary cells by cell sedimentation was undertaken in an attempt to obtain fractions enriched in lactotropes. The theory of cell sedimentation has been extensively reviewed by Miller (433). The sedimentation velocity of a particle (cell) depends upon its size and density, as well as the density and viscosity of the fluid through which it is falling. This relationship is described by the general sedimentation law (Stokes' equation):

$$u = 2(P_p - P_m)gr^2/9n$$

where u is sedimentation rate, P_p and P_m are cell and medium densities respectively, g is the earth's gravitational acceleration, r is the particle radius and n is the medium viscosity. Sedimentation at unit gravity separates cells primarily on the basis of size, as sedimentation

is a squared function of cell diameter and a linear function of cell density. This is particularly useful in separating anterior pituitary cells as their density can vary with hormonal content and great overlap in the densities of the different anterior pituitary cells has been found (434). As unit gravity sedimentation does still depend on cell density, variable sedimentation patterns are seen under different physiological states (435). Other potential problems with velocity sedimentation of cells, such as streaming, have been fully discussed by Pretlow (436).

In order to separate anterior pituitary cells, single cell suspensions must be obtained. Dissociation of rat anterior pituitary cells was achieved using mechanical and enzyme dispersion based on the method of Rotzstejn et. al. (273). Anterior pituitaries were minced into 1 mm fragments using two scalpel blades and placed into 1 ml/gland of 25 mM HEPES buffer, pH 7.3, containing Spinners salts, 0.1% trypsin (fraction III), and 0.1% BSA (RIA grade). Dissociation was conducted at 36.5^{0}C in a 25 ml siliconized Spinner flask. Fragments were continuously agitated by a teflon impeller at 100 rpm and trituration every 20 minutes through siliconized Pasteur pipettes, for 1-1.5 hours until greater than 95% of cells were individual using light microscopy. After dispersion cells were sedimented at 200 x g for 10 minutes at 21^{0}C, washed twice with medium 199 containing 25 mM HEPES, pH 7.4, 0.5% BSA (faction V), 250 U/ml penicillin and 250 μg/ml streptomycin, and processed separately for culture, [Tyr(^{125}I)^{10}]VIP binding, PRL release or separation by cell sedimentation.

Dispersion of anterior pituitaries yielded 3.1 x 10^6 cells/gland from female rats (mean, n=17) and 2.2 x 10^6 cells/gland (mean, n=3) from male animals. Only cells from adult, random cycling CD female rats were used in cell sedimentation studies. Cell viability assessed by trypan blue exclusion was greater than 90%.

a. Unit gravity sedimentation. Dispersed anterior pituitary cells from rats were separated by unit gravity sedimentation following the methods described by Hymer (434). A 920 ml continuous BSA (fraction V) gradient (0.9-2.2%) prepared in medium 199 supplemented with 25 mM HEPES, pH 7.4, 5 μg/ml dopamine, 25 U/ml penicillin and

25 μg/ml streptomycin, was generated over an hour in a 1000 ml reorienting Celsep^{TM} cell separation chamber, on top of 70 ml of cushion solution (7% sucrose and 0.9 % NaCl). Dispersed cells (16-17 x 10^6) were suspended in 20 ml of medium 199 containing 25 mM HEPES, pH 7.4, 0.5 % BSA (fraction V), 10 μg/ml dopamine, 25 U/ml penicillin and 25 μg/ml streptomycin, and carefully layered onto the gradient and covered with 25 ml of phosphate buffered sodium chloride (0.9%, pH 7.3). The chamber was then oriented to the raised from the horizontal position and cells were allowed to sediment for 80 minutes at 21^{0}C. After sedimentation the chamber was reoriented to the horizontal position, and sequential 30 ml fractions were collected by displacement through the top of the chamber via cushion solution introduced in the bottom. Cells recovered in each fraction were sedimented at 150 x g for 15 minutes at 21^{0}C, and resuspended in medium 199 supplemented with HEPES 25 mM, pH 7.4, and 0.5% BSA (fraction V). The cells were immediately processed for cytochemistry or prolactin content, or were established as a monolayer culture to determine [Tyr(^{125}I)^{10}]VIP binding after 2 days. Cell counts were performed manually in quadruplicate using a Neubauer haemocytometer and cell viability was assessed throughout by exclusion of trypan blue dye.

b. Isopycnic sedimentation. This method of separation depends largely on differential cell densities, and for reasons cited above is theoretically less ideal in isolating lactotropes. This has been borne out by some investigators (437), however Velkeniers et al. achieved lactotrope enrichment up to 70% using this method (438). As this technique possesses certain advantages over unit gravity sedimentation, i.e. time, this method was also employed in an attempt to obtain purified populations of lactotropes for binding studies. Following the method described by Velkeniers et al. (438), 3 x 10_6 cells dispersed cells were suspended in 1 ml of Percoll in 0.15 M NaCl (1.020 g/ml), and layered onto a discontinuous gradient of 1 ml Percoll/NaCl solutions fractions with densities of 1.045, 1.060, 1.080, 1.090 g/ml. The gradients were then centrifuged at 2000 x g for 15 minutes at 21^{0}C, and cells sedimenting at each of the density interfaces were collected. Virtually nothing sedimented at densities above 1.080 g/ml. Cells recovered from the gradients were washed 3 times with DMEM (pH 7.4)

to remove Percoll, and were then assayed for cellular PRL content or cultured for 2 to 4 days to determine [Tyr(^{125}I)^{10}]VIP binding.

3. Identification of cells.

a. Cytochemical. Dispersed anterior pituitary cells, both unfractionated and those separated by unit gravity sedimentation, were subjected to differential cell staining in order to identify lactotrope number and distribution. Cells were concentrated and fixed for staining based on the method described by Schneidermann (439). Approximately 100,000 cells were sedimented onto moistened Gelman DM 800 filters (25 mm diameter, 0.8 μm pore size, copolymer of acrylonitrile and polyvinyl chloride, Gelman Instruments Co, Ann Arbor, MI) using a single port Millipore filtration apparatus. Cells were washed free of medium using 10 ml of phosphate-buffered sodium chloride (0.9 %, pH 7.3) and then fixed by the passage of 10 ml each of phosphate buffered formaldehyde and formaldehyde sublimate over 10 minutes. After removal from the filtration manifold, the filters were consecutively put in two methanol baths for 1 minute each, and then an acetone:methanol:1,2-dichloroethane (1:1:1) bath until softening. They were then placed cell side up onto glass slides and air dried at 50^{0}C. This processing rendered the filters translucent 'by chemical melting and fusion of the polymer' (439). These fixed cells were then stained using the method described by Brookes (440). This method consisted of hydration for 5 minutes using iodine and tap water, mordanting for 1 hour at 44^{0}C in 10% aqueous cupric sulfate, followed by rinsing in running and then distilled water. The cells were then stained with carmoisine (1% in 1% acetic acid) for 30 minutes. Distilled water and then 95 % ethanol rinses were followed by 30 minutes of staining in orange G (0.5% in 2 % phosphotungstic acid in 95 % ethanol). After a water rinse, cells were restained with carmoisine for 5 minutes, rinsed with water and stained with light green SF yellowish (0.5% in 0.5 % acetic acid) for 10 minutes. Cells were rinsed with water again, placed in 1% acetic acid for 2 minutes, passed through 3 changes of ethanol and mounted under a cover slip. Cells were examined and counted using light microscopy and cell types were expressed as percentages of total number of cells. Lactotropes are stained red

due to carmoisine, somatotropes orange with orange G and basophils green with light green (440). Samples from each fraction were processed in duplicate.

b. RIA for prolactin. Measurement of PRL was carried out using a rat PRL RIA kit provided by the National Institute of Arthritis, Diabetes, Digestive Diseases and Kidney (NIADDK).

Purified rat PRL, [NIADDK-rPRL-I-5(AFP-4459B)], was iodinated based on the method of Greenwood et al (441). Five μg of rPRL were radioiodinated with 1 mCi of ^{125}I in the presence of 10 μg chloramine T in 0.5 M sodium phosphate buffer, pH 7.4. The reaction was stopped after 60 seconds by the addition of 60 μg of sodium metabisulfite. Bound was separated from free ^{125}I by gel filtration on a Biogel P-30 (mesh 100-200) column (1.5 x 100 cm) and eluting with 0.15 M sodium chloride in 0.05 M sodium phosphate buffer, pH 7.5. the elution profile of radioactivity is shown in Figure 17. The first peak eluted contained the radioiodinated product, and the later free ^{125}I. Two fractions from the protein peak containing maximal radioactivity were pooled and stored at -70^{0}C.

The procedure for RIA was based on the recommendations in the assay kit from the NIADDK. The standard rat PRL was rPRL-RP-3, and 0.078 to 20 ng were used in the standard dose response curve. Bound was separated from free[^{125}I]rPRL using polyethylene glycol precipitation or the double antibody technique with normal rabbit serum and goat anti-rabbit gamma globulin. The inter-assay coefficient of variation was 11.1% using 1.25 ng of rPRL-RP-3 (n=17), and the intra-assay coefficient of variation was 6.0% at the same concentration (n=8). The competition of binding of iodinated PRL to rat PRL antiserum by the rat PRL standard and PRL containing extracts is shown in Figure 18.

4. Culture and [Tyr(^{125}I)^{10}]VIP binding. Monolayer cultures of anterior pituitary cells were established immediately after dispersion or after cell sedimentation based on the method described by Dobson and Brown (442). After dispersion or sedimentation cells were washed twice with and then suspended in DMEM supplemented with 10% and 2.5% (vol/vol) horse and fetal bovine serum respectively, 1% (vol/vol) non-essential amino acids, 200 U/ml penicillin and 200 μg/ml streptomycin. For [Tyr(^{125}I)^{10}]VIP binding studies, 5-8 x 10^5 cells

were cultured in 5 ml of supplemented DMEM medium in 25 cm^2 polystyrene tissue culture flasks (GIBCO) at 36.5^{0}C under humidified 95% air/5% CO_2. Culture conditions for PRL release studies are discussed in a later section. At 24 hours the majority of non red blood cells were attached to the bottom of the flask and had lost the round appearance characteristic of acutely dispersed cells. Approximately 10% and 20% of attached cells were spindle shaped after 2 and 4 days of culture respectively.

As the VIP receptor in other tissues is susceptible to trypsin, binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary cells was checked immediately after dissociation using trypsin and compared with the binding to cells after 1, 2, 3 or 4 days of culture (Table 14). Specific binding of [Tyr(^{125}I)^{10}]VIP was abolished immediately after dissociation, whereas nonspecific binding was not affected. Recovery of specific binding was seen within 1 day and remained stable up to 4 days. Because of this effect using trypsin, the binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary cells dispersed using hyaluronidase and collagenase was examined. A similar loss of specific binding was seen using these enzymes for dissociation. All subsequent [Tyr(^{125}I)^{10}]VIP binding studies to trypsin dispersed cells were conducted after 2 days of culture.

Cells were scraped from the culture flasks using a rubber policeman, sedimented at 200 x g for 10 minutes at 4^{0}C and washed twice with 0.25 M sucrose in 20 mM Tris buffer, pH 7.4, to remove culture media as its presence inhibits binding. Cell clumping was observed during resuspension of washed cells in any of the following buffers, pH 7.4: a. 0.25 M sucrose in 20 mM Tris with or without 0.5% (wt/vol) BSA (fraction V), b. 25 mM HEPES plus Spinner's salts with or without 0.5% BSA, c. binding buffer C, and d. 25 mM Tris-HCl, 2 mM EDTA, 2 mM magnesium chloride with or without 0.5% BSA. As this clumping prevented reproducible binding assays, cells were ruptured prior to assay. Hand held glass or teflon homogenizers resulted in large losses of cells, so cells were fractured in 0.25 M sucrose in 20 mM Tris-HCl by sonication at 4^{0}C (Branson Sonifier 185, output 7, 30 second intervals) until less than 5% of cells remained intact by light microscopy. Aliquots were

assayed for protein concentration using the method of Lowry et al. (421), and the remainder was stored at -70^{0}C.

In the actual binding assay, disrupted cells (4-8 μg of protein) were incubated with [Tyr(^{125}I]^{10}VIP (60-65 pM) in the presence or absence of 0.1 μM VIP, in binding buffer C to a total volume of 200 μl. Filtration as described previously was used to separate bound from free radioligand. Specific binding of [Tyr(^{125}I)^{10}]VIP, defined as the amount of bound radioligand displaceable in the presence of 0.1 μM VIP, to dispersed, unfractionated cells was compared with binding to cells obtained after separation by isopycnic or unit gravity sedimentation. The pattern of specific binding to sedimented cells was compared to lactotrope distribution as determined by cytochemistry and PRL profile.

B. Results

1. Unit gravity sedimentation. On the basis of Brookes staining two thirds of dispersed anterior pituitary cells from female rats were acidophils, while the remaining were basophilic or chromophobic. Cell viability assessed by trypan blue exclusion after dispersion and separation was greater than 90%.

Unit gravity sedimentation allowed separation of populations enriched in PRL containing cells. The profile of PRL content centring around fraction 21 (Figure 19), likely reflects cellular rather than released PRL as dopamine was used to inhibit release throughout the study (435). Only 30 to 35 % of dispersed anterior pituitary cells prior to separation were identified by Brookes stain as lactotropes. After sedimentation through the CELSEP chamber, cells with histochemical appearance of lactotropes comprised between 45 to 65% of cells in fractions 16 to 24. In contrast 20 to 25% of cells were identified as lactotropes in fractions 13 to 15 and 25 to 26, with less than 10% in the remaining fractions. Fractions 25 to 33 were enriched in somatotropes. Basophils and chromophobes were found throughout, but in larger numbers in fractions 23 to 30 and 11 to 16 respectively. The majority of red blood cells sedimented in fractions 6-10. Seventy nine % of the prolactin content and 88% of the cells loaded onto the gradient were recovered from the CELSEP chamber after sedimentation (mean, n=3).

Negligible specific binding of [Tyr(^{125}I)^{10}]VIP was detected before fraction 13 and very little after fraction 28 (Figure 19). Although specific binding was found in all fractions between these two, when expressed relative to cell number or amount of protein per assay, a peak of specific binding centred around fraction 21 was seen. The profile of specific binding of [Tyr(^{125}I)^{10}]VIP associated closely with that of PRL containing cells as identified cytochemically and by cellular PRL content by RIA (Figure 19). When the CELSEP chamber was unloaded in three fractions, cells from lactotrope containing fractions 16-26 bound 2.5 times more [Tyr(^{125}I)^{10}]VIP per mg protein than did unfractionated cells. In contrast the binding ratio for cells from lactotrope poor fractions 1-15 was 0.73, and 27-33 was 1.1, when compared with unfractionated cells (Table 15). The effect of increased temperature on the binding of [Tyr(^{125}I)^{10}]VIP was similar in the dispersed cells as seen in anterior pituitary membranes.

2. Isopycnic sedimentation. Cellular PRL content, measured by RIA, was similar in all cell fractions separated by isopycnic sedimentation in a discontinuous Percoll gradient. This can logically be interpreted as inability of this technique to separate PRL containing cells. This is probable on the theoretical grounds that separation on the basis of density is unlikely to differentiate between various anterior pituitary cell types (433). An alternate possibility that PRL was released during tissue processing despite the presence of dopamine, was considered especially as these results differ from those found by Velkeniers et al. (438). The binding of [Tyr(125I)^{10}]VIP to cells collected from each fraction was examined after 2 days of culture. Although specific binding of [Tyr(^{125}I)^{10}VIP to fractions 1.020/1.045 and 1.080/1.090 was half that bound to fractions 1.045/1.060 and 1.060/1.080 (Table 16), there was little enrichment in the specific binding to these latter fractions when compared with binding to unfractionated cells. As the distribution of lactotropes identified by Velkeniers et al. (438) were primarily in the 1.045/1.060 and to a lesser extent the 1.060/1.080 fractions, these results suggest that binding of VIP could correspond to the isolation of lactotropes. Very little change from unfractionated cells was seen in the percent specific binding in the

1.045/1.060 and 1.060/1.080 fractions indicating that nonspecific binding was not appreciably decreased during fractionation.

Because of the inability to demonstrate lactotrope enrichment during this separation method, as well as only small differences in specific binding of [Tyr(^{125}I)^{10}]VIP, this technique was not pursued any further. After the binding studies on cells separated by sedimentation were completed, a subsequent more extensive report was published using isopycnic sedimentation through a discontinuous Percoll gradient, achieving lactotrope enrichment up to 70% (458). Using this method, these investigators identified two populations of lactotropes, sedimenting in the density ranges of 1:055 to 1.065 and 1.070 to 1.080 g/ml. These densities correspond to those from the discontinuous gradient (1.045/1.060 and 1.060/1.080 g/ml) binding the greatest amount of [Tyr(^{125}I)^{10}]VIP (Table 16).

FIGURE 17. Gel filtration chromatography of iodinated rat prolactin. Prolactin, iodinated using chloramine T, was separated from unreacted iodine on a Biogel P-30 column (1.5 x 100 cm), by eluting with 0.15 M sodium chloride in 0.5 M sodium phosphate buffer, pH 7.5 at 4^0C. Profile of radioactivity recovered is shown per 3 ml fractions (fractions 1-13) and per 1.6 ml fractions (fractions 14-100).

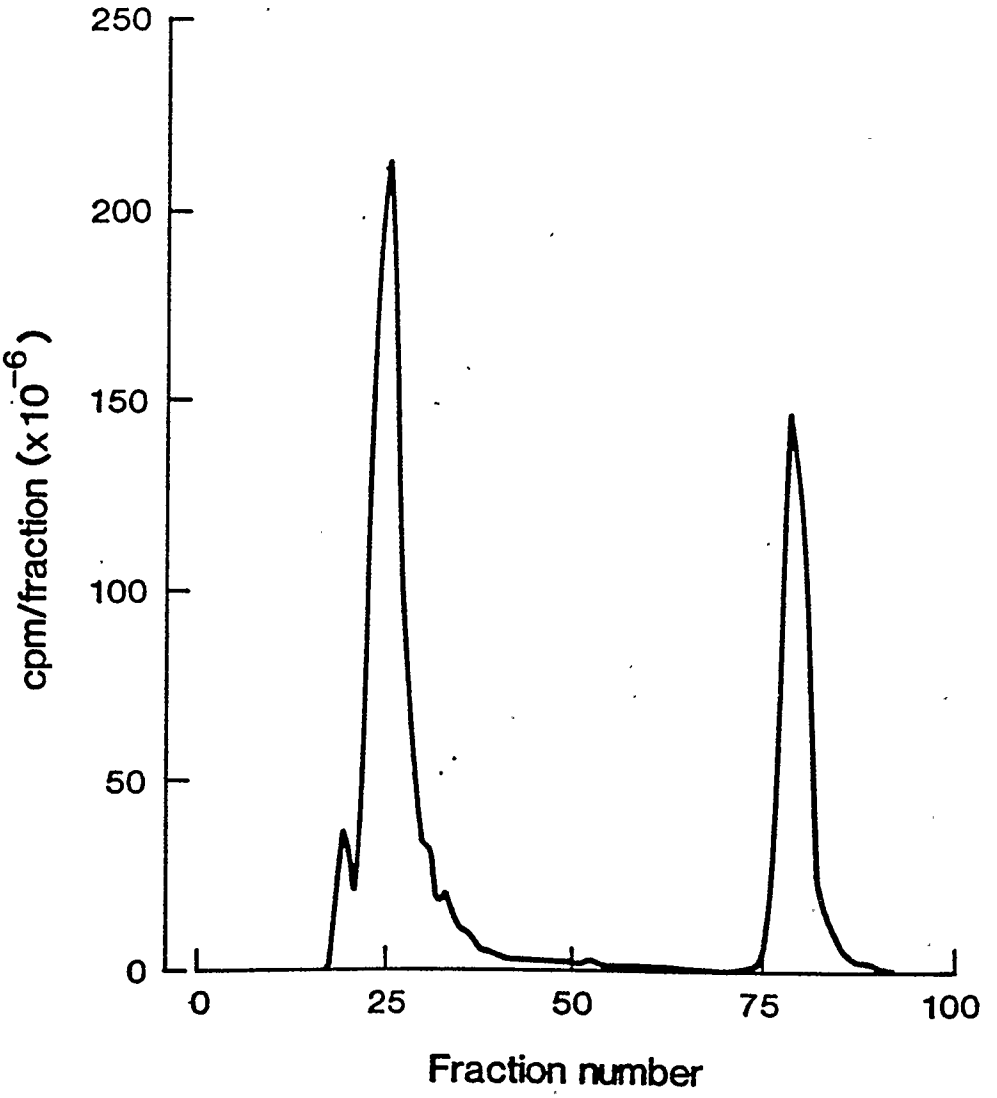

250
200
150
100
50
0
cpm/fraction (x10^{-6})
0
25
50
75
100
Fraction number

FIGURE 18. Binding of [^{125}I-PRL] to antiserum as a function of prolactin from various sources. B/Bo, is the ratio of radioiodinated prolactin bound in the presence of rat prolactin (NIADDK-rPRL-RP-3) or other samples to that bound in their absence. Each point on the standard curve (ng rPRL-RP-3/assay on abscissa) (O) is the mean ± SEM from 17 experiments performed in duplicate. The remainder are dilution curves (μl/assay on abscissa) of rat serum (■), or prolactin contained (●) or released from cultured anterior pituitary cells under basal conditions (△). The data points on these curves are the mean of 3 determinations in one experiment.

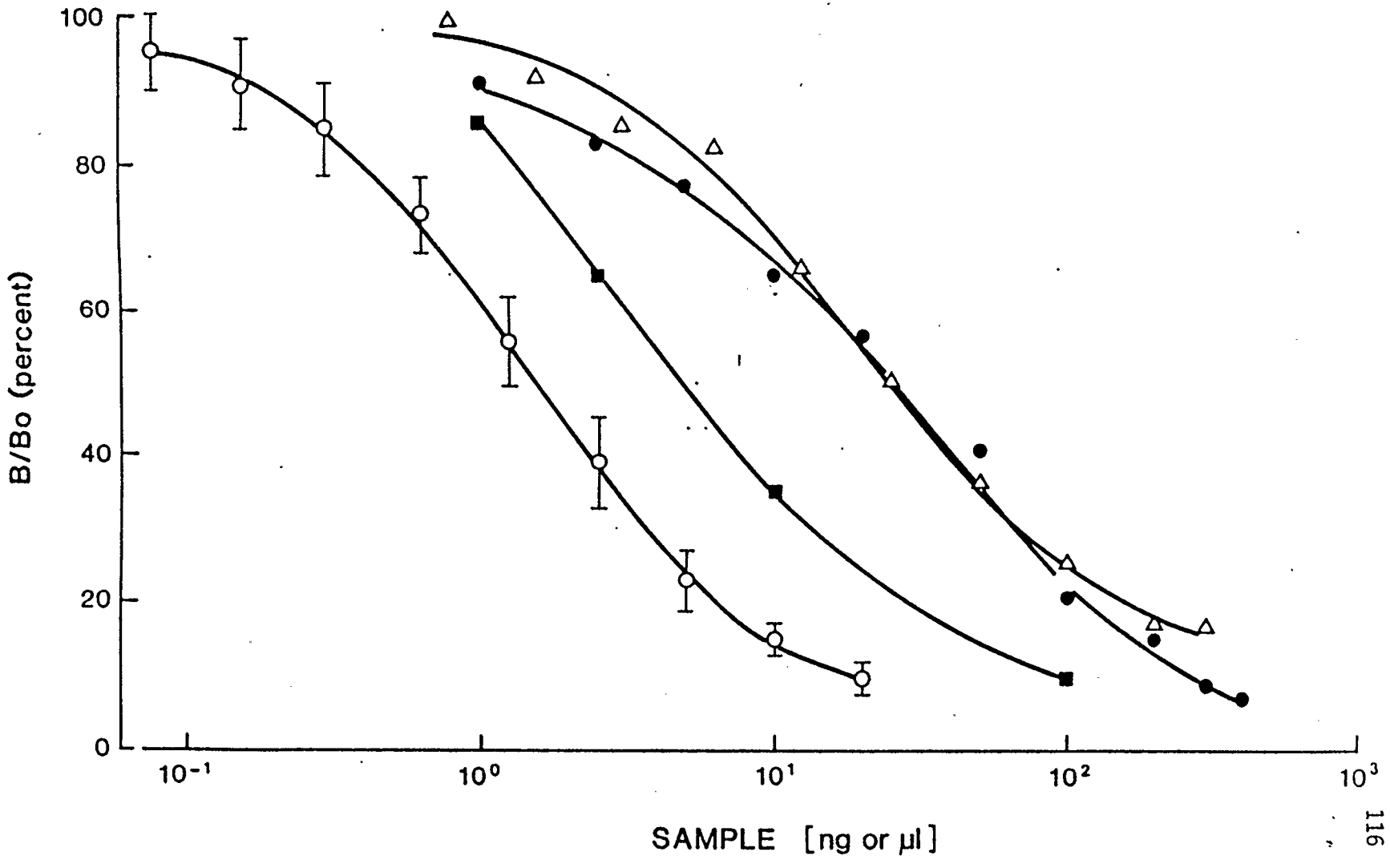

100
80
60
40
20
0
B/Bo (percent)
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
SAMPLE [ng or µl]

TABLE 14. Effect of culture on the specific binding of [Tyr(^{125}I)^{10}]VIP to dispersed female rat anterior pituitary cells

Time in culture (days)	Specific binding [Tyr(^{125}I)^{10}]VIP	
	fmol/mg protein	% of total binding
0	0	0
1	8.3 ± 1.0	20.4 ± 2.5
2	11.6 ± 1.2	17.0 ± 2.7
3	10.1 ± 0.1	20.8 ± 4.2
4	10.6 ± 2.0	21.7 ± 3.2

Dispersion and culture of anterior pituitaries is described in Experimental studies IIIA4.

For binding, ruptured cells (4-8 µg protein) were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) in the absence or presence of 0.1 µM VIP at 21^{0}C for 30 minutes.

Data are expressed as mean ± SEM, n = 3-4, assayed in duplicate.

FIGURE 19. Specific binding of [Tyr(^{125}I)^{10}]VIP to dispersed female anterior pituitary cells separated by unit gravity sedimentation. Cells were separated through a BSA gradient (0.9-2.2%) using a CELSEP chamber (see Experimental studies IIIA2). Cells, unloaded through the top of the chamber, were collected in 30 ml fractions and cell count (□) and cell prolactin content (○) are shown as the mean per fraction as a percent of the total applied to the gradient, n = 3. Specific binding of [Tyr(^{125}I)^{10}]VIP to cells from each fraction after 2 days of culture (●) are the mean of 2 experiments performed in duplicate. Cells recovered refer only to non red blood cells.

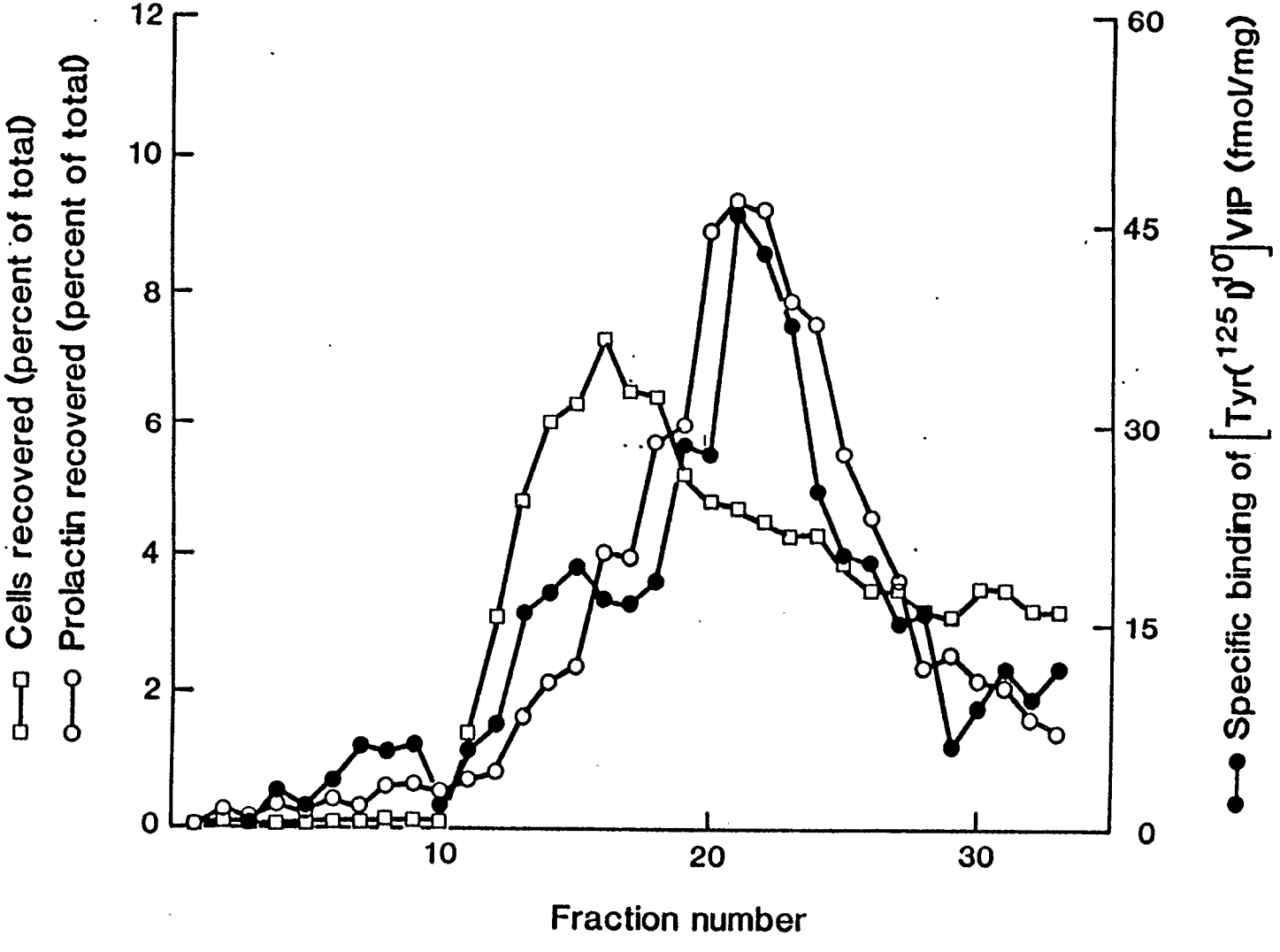

Cells recovered (percent of total)
Prolactin recovered (percent of total)
Specific binding of [Tyr(125I)10]VIP (fmol/mg)
Fraction number
0
2
4
6
8
10
12
15
30
45
60
10
20
30

TABLE 15. Specific binding of [Tyr(^{125}I)^{10}]VIP to dispersed female rat anterior pituitary cells separated by unit gravity sedimentation

Assay temperature	CELSEP fraction	Specific binding [Tyr(^{125}I)^{10}]VIP	
		fmol/mg protein	% of total binding
21^{0}C	unfractionated	11.6 ± 1.2	17.0 ± 2.7
	A	8.4 ± 1.7	14.5 ± 2.5
	B	21.2 ± 3.4	48.7 ± 2.9
	C	9.3 ± 0.7	12.2 ± 1.6
31^{0}C	unfractionated	18.7 ± 1.5	27.3 ± 4.6
	B	47.8 ± 3.4	75.9 ± 6.8

Unit gravity sedimentation of cells was through a BSA (fraction V) gradient (0.9-2.2%) using a 1 litre CELSEP chamber (see Experimental studies IIIA2).
Cells were unloaded through the top and collected in 3 fractions: A, 0-480 ml; B, 481-780 ml; C, 781-1000 ml.

After 2 days culture, ruptured cells (4-8 µg protein) were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) +/- 0.1 µM VIP at 21^{0}C for 30 minutes or 31^{0}C for 1 hour.

Data are expressed as mean ± SEM, n = 3-5, assayed in duplicate.

TABLE 16. Specific binding of [Tyr(^{125}I)^{10}]VIP to dispersed female rat anterior pituitary cells separated by centrifugation through a Percoll density gradient

Percoll density gradient interface (g/ml)	Specific binding [Tyr(^{125}I)^{10}]VIP	
	fmol/mg protein	% of total binding
unfractionated	10.6 ± 2.0	21.7 ± 3.2
1.020/1.045	5.8 ± 2.9	9.3 ± 4.7
1.045/1.60	14.1 ± 0.1	27.9 ± 9.1
1.060/1.080	12.7 ± 1.6	21.9 ± 1.0
1.080/1.090	5.7 ± 2.3	16.4 ± 3.0

Cells collected at each interface were cultered 2 days prior to assay.

For binding, ruptured cells (4-8 µg protein) were incubated with [Tyr(^{125}I)^{10}]VIP 55-65 pM) in the presence or absence of 0.1 µM VIP at 21^{0}C for30 minutes.

Data are expressed as mean ± SEM, n = 4 except 1.080/1.090 n =2, assayed in duplicate.

IV. EFFECT OF VIP ON PRL RELEASE IN VITRO

A. Materials and Methods

1. Cell culture. Anterior pituitary glands from adult female CD rats were subjected to mechanical and enzymatic dispersion as described for cell sedimentation studies (see Methods, IIC). Prior to studying basal and stimulated PRL release, these cells were suspended in culture medium, dispensed into the wells of 24-well multiwell plates (1 ml/well, Dow Corning) and maintained as monolayer cultures at 36.5^{0}C under humidified 95%air/5% CO_2. As cell culture conditions are known to influence the synthesis and secretion of hormones, including PRL (282), the effect of different culture media, cell culture density, and duration of culture on basal and VIP stimulated PRL release was compared. Cells at a density of 500,000/ml were cultured for 2 days before PRL release studies in one of the following media: a.DMEM, b.DMEM plus 20 mM HEPES, and c. Hams F10. All media was at pH 7.4, and supplemented with horse serum (10%)(vol/vol), fetal bovine serum (2.5%)(vol/vol) nonessential amino acids (1%) (vol/vol), penicillin (250 U/ml) and streptomycin (250 μg/ml). Medium b (supplemented DMEM plus 20 mM HEPES) was then used for cell culture to study the effect of culture duration and seeding density on PRL release. Cells were cultured for 1, 2, or 4 days at a density of 500,000 cells/ml, or cells were seeded at a density of 2 x 10^5, 5 x 10^5, or 1 x 10^6 cells/ml and cultured for 2 days prior to PRL release studies.

Dose response studies of VIP and effects of its homologous peptides on PRL release were studied in anterior pituitary cells cultured for 2 days in medium b at a density of 500,000 cells/ml.

2. Bioassay. The bioassay of PRL release from these monolayer cultures was based on the methods described by Vale and Grant (459). The medium from the anterior pituitary cultures was aspirated and the cells were gently washed with 1 ml of incubation medium (medium 199 with 20 mM HEPES, pH 7.4 and 0.5 mg/ml bacitracin). For the actual bioassay, 900 μl of incubation medium was added to each well followed by 100 μl of one of the following: 1). synthetic VIP, final concentrations ranging from 10^{-12} to 10^{-6}M; 2). rPHI,

secretin or glucagon, final concentrations of 10^{-7}M, 10^{-5}M and 10^{-5}M respectively; or 3). incubation medium only; to assess basal PRL release. After incubation for 5 hours at 36.5^{0}C, the culture medium was aspirated, centrifuged at 700 x g to remove cells, and the supernatant was stored at -24^{0}C until assay for PRL by RIA. Four or more wells of each condition per experiment were studied in a minimum of 3 separate experiments. RIA of rPRL was done in duplicate on three dilutions from each sample and results are expressed as a percentage of basal PRL released.

Basal and VIP (0.1 μM) stimulated PRL release was compared when bacitracin (0.5 mg/ml) or BSA (fraction V)(1%, wt/vol) was added to the incubation medium (medium 199 buffered with HEPES, pH 7.4). This was also compared to the release seen when Krebs Ringer buffered with sodium phosphate, pH 7.4, plus BSA (1%,wt/vol) (KRP), was used as the incubation medium. The effect of incubation time on PRL release was studied at 1, 2, and 5 hours.

B. Results

1. Effect of assay conditions. Both basal and VIP (0.1 μM) stimulated PRL release were affected by the culture and incubation conditions of anterior pituitary cells. Composition of the culture medium changed both basal and VIP stimulated release, with HEPES buffering of DMEM resulting in the lowest basal secretion, and the greatest percentage change upon addition of VIP (Figure 20A). The basal secretion of PRL also changed with duration of culture, increasing in a linear fashion between 1, 2, or 4 days of culture (Figure 21A). Although VIP stimulated PRL secretion also increased with culture duration, this increase was not linear and the stimulation of PRL by VIP as a percentage of basal PRL released was greatest at 2 as compared to 1 or 4 days of culture. The inclusion of bacitracin in the incubation medium was necessary in order to demonstrate stimulation of PRL secretion by VIP (Figure 20B). This is in agreement with that found by other investigators (267). BSA, frequently added to incubation medium in secretion studies of anterior pituitary hormones, also allowed stimulation of PRL by VIP, whether added to medium 199 or KRP incubation medium (Figure 20B). BSA also markedly increased basal

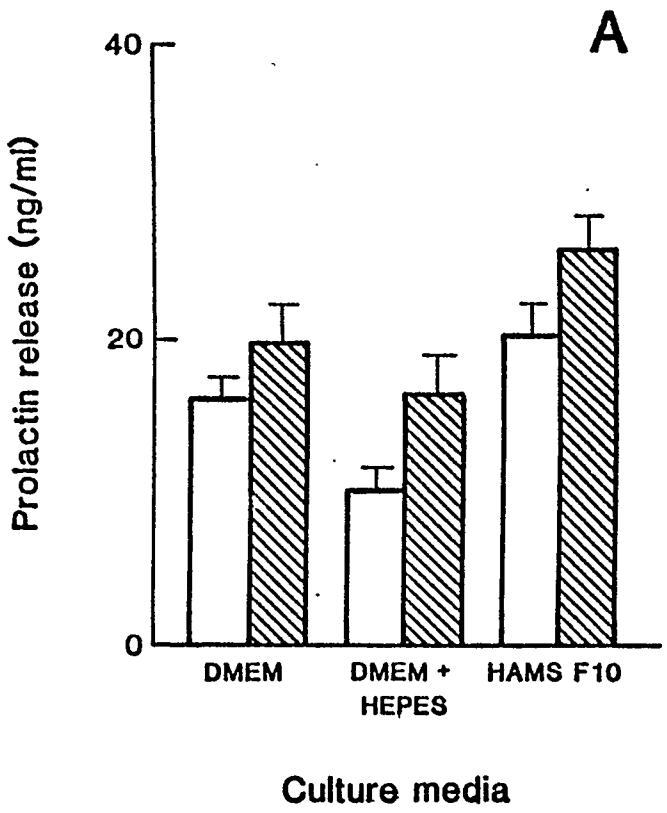
PRL secretion however, an effect that was not observed when bacitracin was used. A relatively greater response to VIP stimulated as compared with basal PRL secretion, was seen when the time of incubation was 5 hours as compared with 1, 2, or, 3 hours (Figure 21B), although both basal and stimulated PRL release increased with increasing incubation time. Increased basal and VIP stimulated PRL secretion was also observed with increasing number of cells per sample well, at both 1 (Figure 22A) and 5 hours (Figure 22B) of incubation.

2. Effect of VIP and related peptides. Because incubation conditions differentially affected basal and VIP stimulated PRL secretion, dose response studies were conducted under conditions that selectively decreased basal PRL secretion. This included culturing of cells for 2 days prior to bioassay in culture medium b, inclusion of bacitracin to the incubation medium, and incubation time of 5 hours. Although stimulation of PRL by VIP increased with increasing number of cells up to 10^6 cells per well, only 5×10^5 cells per well were used as too many animals were required if greater cell seeding density was employed. Under these conditions the basal release of PRL was 121 ± 4 ng/10^5 cells/ml/hour (mean ± SEM, n=35).

PRL release was stimulated by VIP in a dose dependent fashion with an ED_{50} of 1 nM (Figure 23). An effect was seen at 10^{-10}M VIP, with the response at a maximum by 10^{-7}M. A shift to the right in the PRL release response curve to stimulation by rPHI was seen as compared with VIP stimulation (Figure 23).

The relative PRL stimulating abilities of peptides from the secretin family are shown in Figure 24). At 10^{-7}M, VIP and rPHI stimulate PRL release above basal levels by 48 and 28% respectively. Secretin, at 10^{-5}M was only able to stimulate PRL secretion by 14%, whereas glucagon was totally ineffective at the same concentration. Dose response studies of PRL stimulated by peptides other than VIP and rPHI were attempted, however the low percent stimulation seen by these substances and variability inherent in this bioassay did not allow discrimination of effect at low concentrations. By one way analysis of variance and Student-Newman-Keuls' test, $p<0.05$ comparing VIP with secretin or glucagon, or rPHI with glucagon. Comparison between the other means was not statistically significantly different.

FIGURE 20. The effect of different culture and release media on prolactin release from cultured female rat anterior pituitary cells. Dispersed cells (500,000 cells/ml) were cultured in one of 3 different media (panel A) for 2 days. Basal (□) and VIP (0.1 μM) (▨) stimulated prolactin release after 1 hour incubation in medium 199 + HEPES + bacitracin release medium was measured by RIA. Panel B shows the basal (□) and VIP stimulated (▨) release of prolactin after 1 hour from cells (500000/ml) cultured for 2 days in DMEM + HEPES, using 4 different release media. For details regarding the exact media composition, culture and release conditions see Experimental studies IVA. Data are the mean ± SEM from 8 experiments performed in duplicate.


A
40
20
0
Prolactin release (ng/ml)
DMEM
DMEM + HEPES
HAMS F10
Culture media

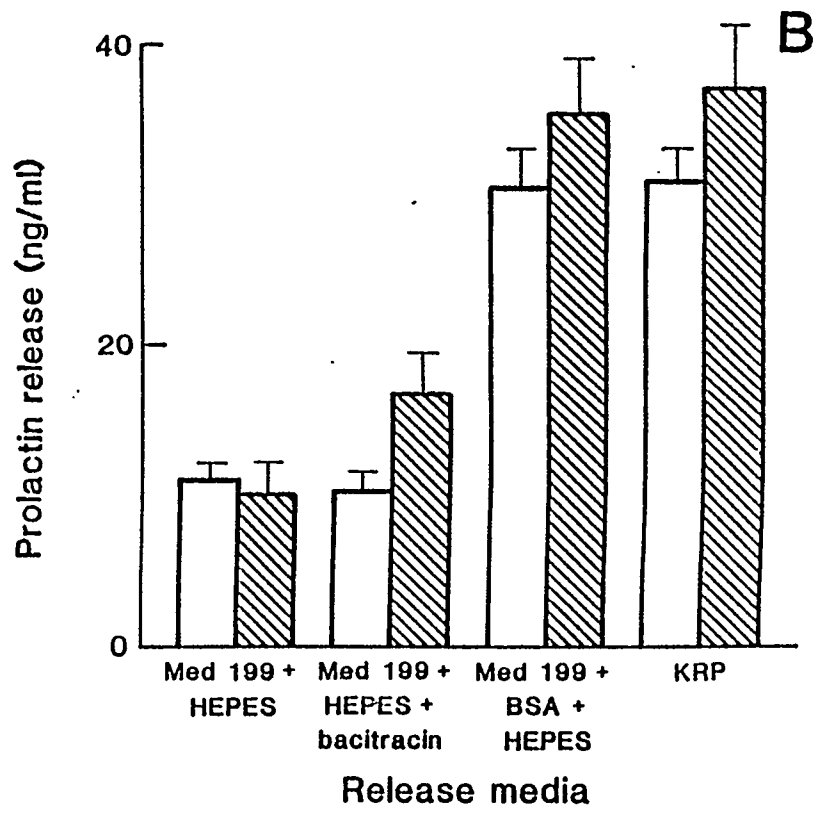

B
40
20
0
Prolactin release (ng/ml)
Med 199 + HEPES
Med 199 + HEPES + bacitracin
Med 199 + BSA + HEPES
KRP
Release media

FIGURE 21. The effect of culture duration and length of bioassay on the release of prolactin from cultured female rat anterior pituitary cells.

Panel A: Dispersed cells (500000/ml/well) were cultured up to 4 days in DMEM + HEPES (see Fig. 20A) before basal (○) and VIP (0.1 μM) (□) stimulated release after 1 hour in Med 199 + HEPES + bacitracin (see Fig. 20B) was determined by RIA.

Panel B: Cells were cultured for 2 days in the same manner as for panel A, and prolactin release was measured after 1, 2, 3 or 5 hours of incubation in medium 199 + HEPES + bacitracin, in the absence (○) or presence of VIP (0.1 μM) (□).

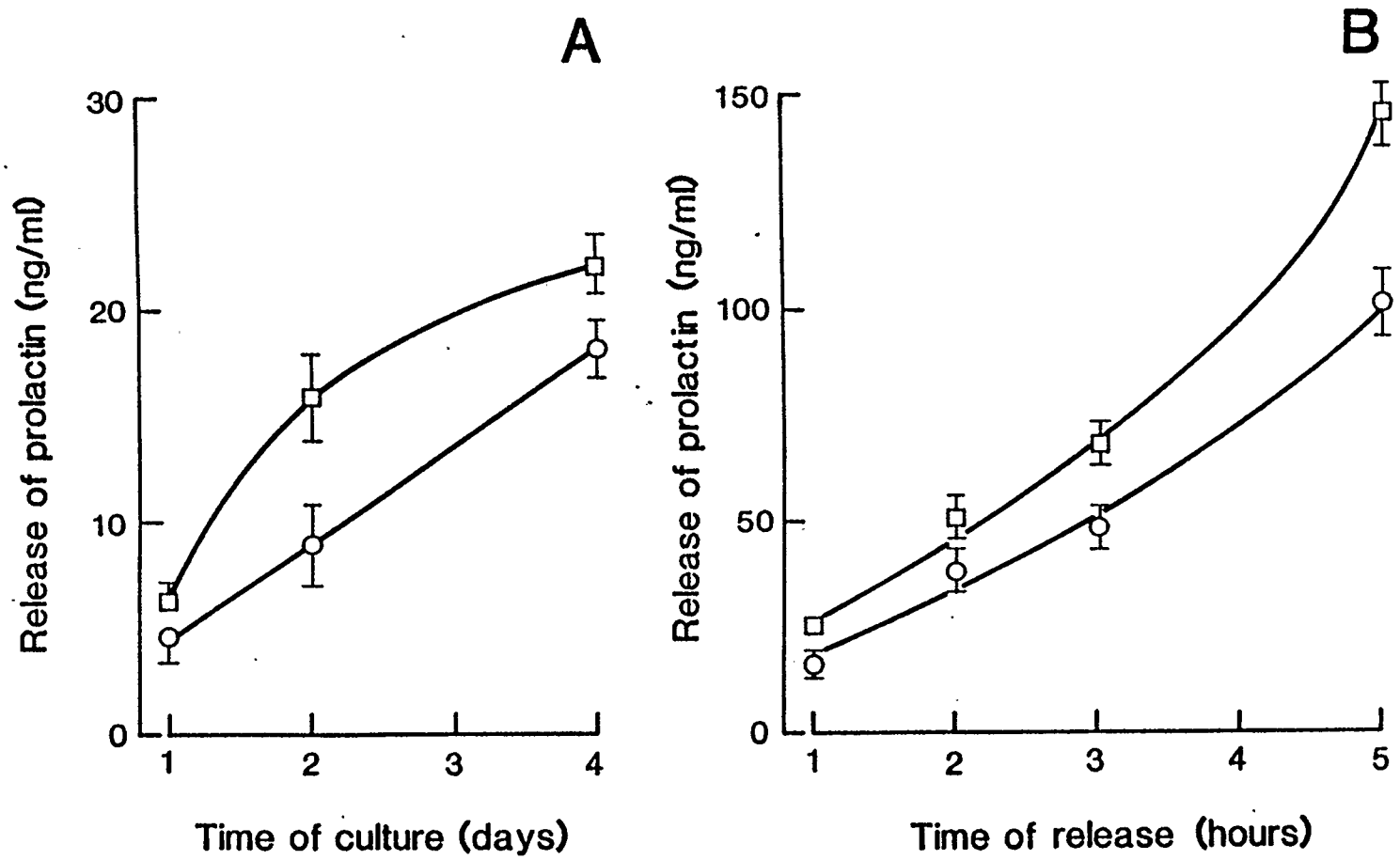

A
B
Release of prolactin (ng/ml)
30
20
10
0
1
2
3
4
Time of culture (days)
150
100
50
0
1
2
3
4
5
Time of release (hours)

FIGURE 22. Effect of cell number and duration of bioassay on the release of prolactin from cultured female rat anterior pituitary cells. Dispersed cells at increasing concentrations were cultured for 2 days in 1 ml of DMEM + HEPES (see Fig. 20A). Prolactin release was measured by RIA after 1 hour (panel A) or 5 hours (panel B) incubation in Med 199 + HEPES + bacitracin release medium (see Fig. 20B) in the absence (○) or presence of 0.1 μM VIP (□).

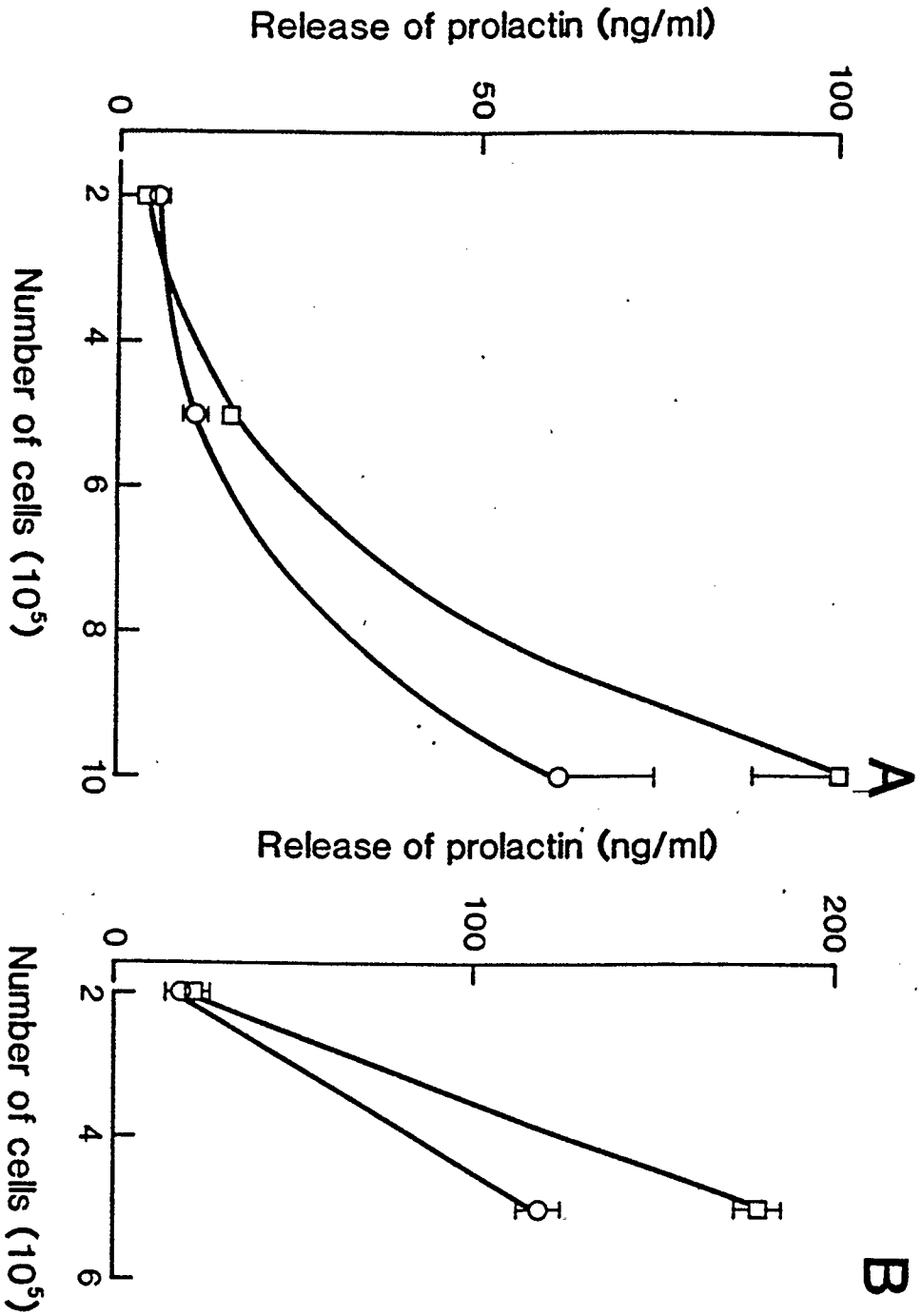

Release of prolactin (ng/ml)
0
50
100
2
4
6
8
10
Number of cells (10^5)
A
Release of prolactin (ng/ml)
0
100
200
2
4
6
Number of cells (10^5)
B

FIGURE 23. Effect of VIP and rPHI on prolactin release from dispersed, cultured female rat anterior pituitary cells. After 2 days culture, cells (5 x 10^5/ml/well) were incubated for 5 hours with increasing concentration of VIP (○) or rPHI (△) and prolactin release was determined by RIA, and is expressed as percent released above basal (i.e in the absence of added peptides). Data points are the mean ± SEM from 3 (○) and 2 (△) separate experiments using 3-5 wells at each concentration per experiment. RIA for prolactin on samples from each well was performed at 3 dilutions, assayed in duplicate.

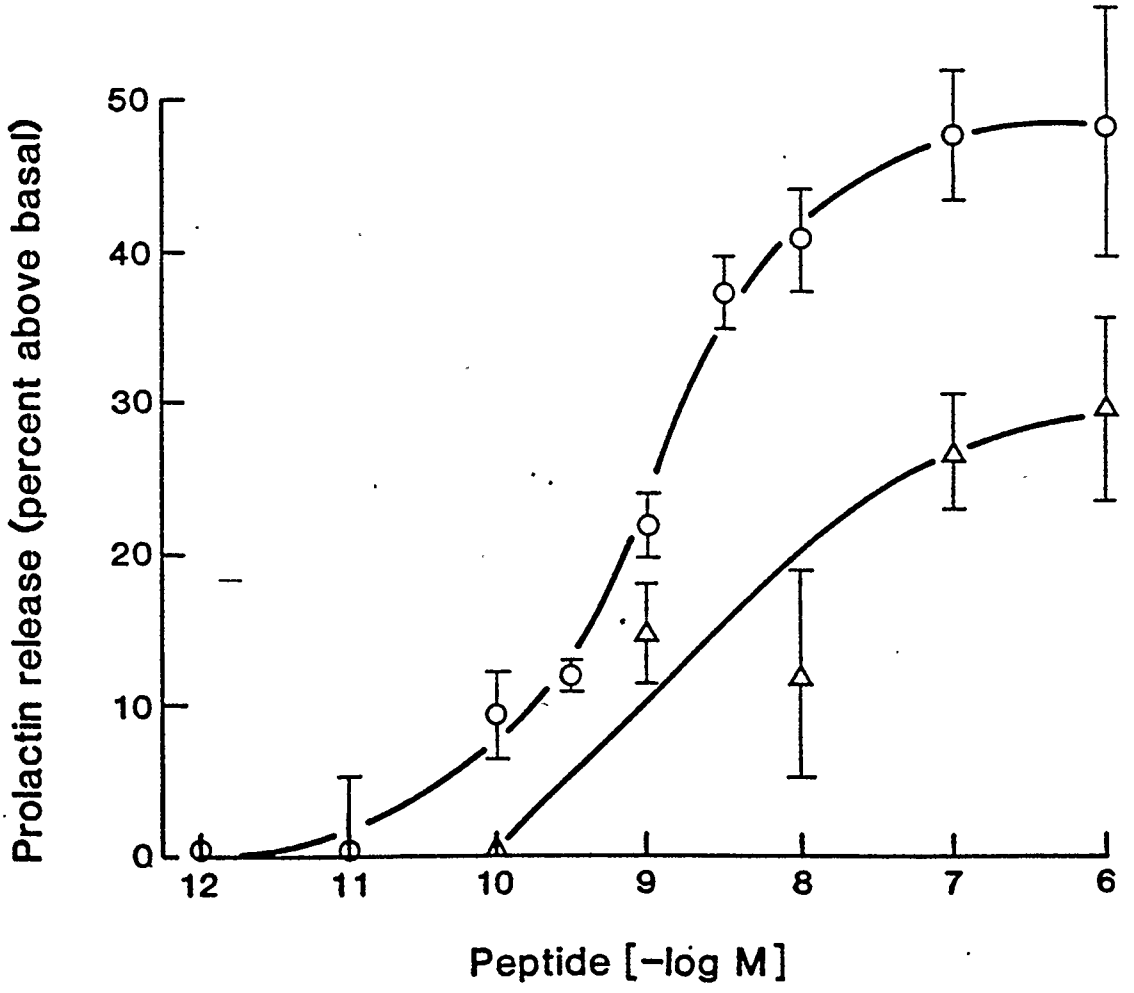
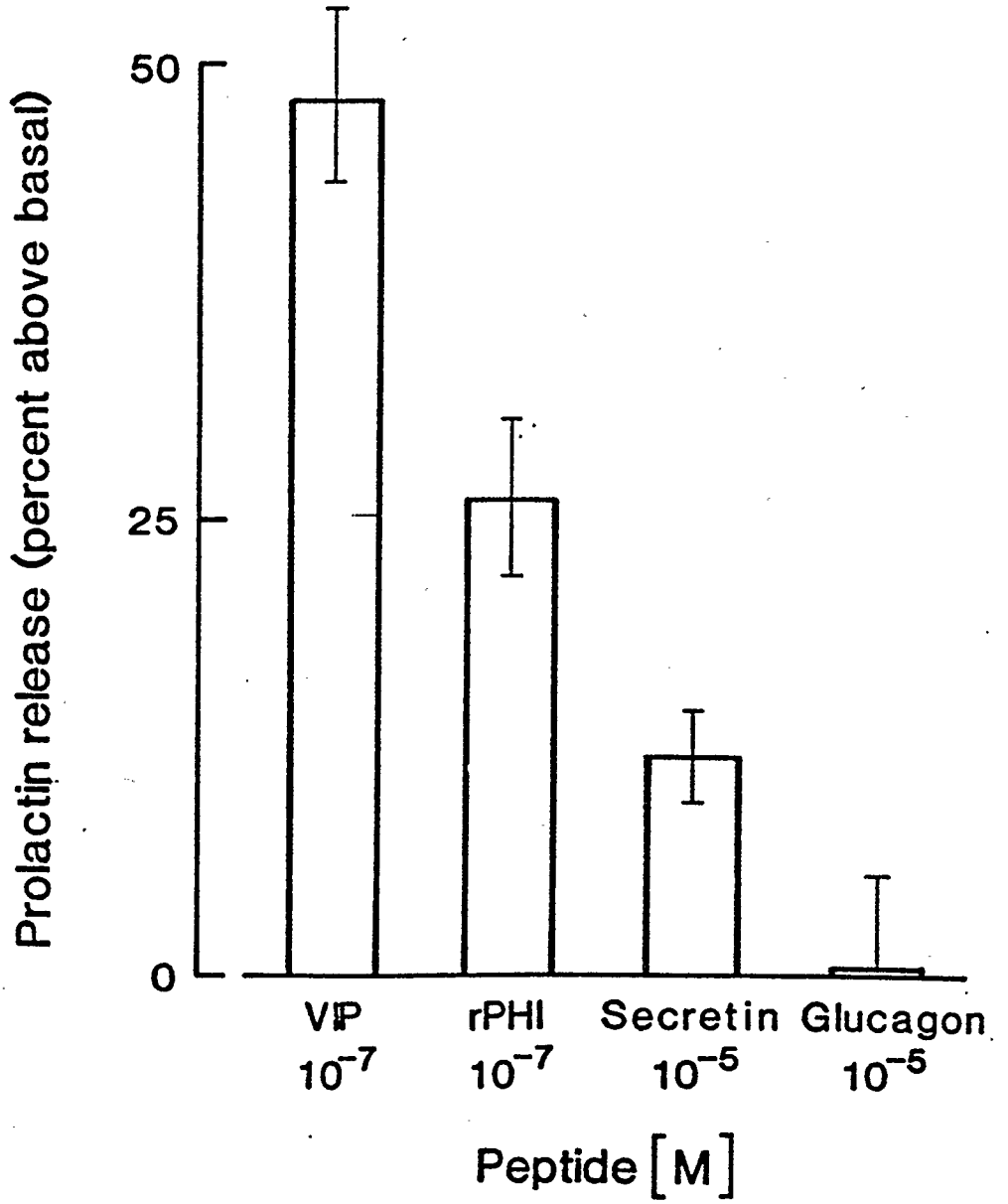

Prolactin release (percent above basal)
50
40
30
20
10
0
12
11
10
9
8
7
6
Peptide [−log M]

FIGURE 24. Effect of VIP and homologous peptides on the release of prolactin from cultured female rat anterior pituitary cells. Experimental conditions were similar to those in Fig. 23. The release of prolactin is shown in the presence of the different peptides, at the indicated concentrations, as a percentage above that secreted in their absence (i.e. basal). Data are the mean ± SEM of 3 (VIP) and 2 (other peptides) separate experiments with 3-5 wells per experiment.


Prolactin release (percent above basal)
50
25
0
VIP
10^{-7}
rPHI
10^{-7}
Secretin
10^{-5}
Glucagon
10^{-5}
Peptide [M]

V. REGULATION OF THE ANTERIOR PITUITARY VIP RECEPTOR

B. Materials and Methods

1. Anterior pituitary membrane preparation. Anterior pituitary glands from both male and female animals were removed and processed as described previously (see Methods IIA, Figure 1). Membranes were prepared by differential centrifugation and sucrose density gradient separation was not employed. Protein concentration was measured in each preparation by the method of Lowry et al. (421).

For developmental studies anterior pituitary glands were obtained from 1, 2, 3, 5, 6, 8, and 10-12 week old virgin female CD rats. As the pituitary glands of 1, 2, and 3 week old animals were exceedingly small, a dissecting microscope was used to aid in separation of the anterior pituitary from the rest of the gland. The weights of animals in each age group was checked and corresponded to that expected for CD rats at the respective ages. Anterior pituitaries were also examined from parous CD rats ranging in age from 32-60 weeks.

Lactating female CD rats, 1 and 2 weeks post partum, were separated from their pups for 1.5-2 hours, then sacrificed by decapitation and the anterior pituitary glands were removed and also processed in the fashion described above.

Anterior pituitary membranes were also prepared from 5 groups of adult male CD rats (250-350 grams) under the following conditions: 1). 1 day after surgical castration under halothane anaesthesia, 2). 2 weeks after surgical castration under halothane anaesthesia, 3). 1 day after a sham operation under halothane anaesthesia, 4). 1 day after surgical castration under halothane anaesthesia, followed by immediate subcutaneous injection of 4 μg/g body weight depo-testosterone cypionate (Upjohn Chem. Co.) suspended in mineral oil, and 5). normal adult male rats.

2. Binding assays and analysis of results. The radioligand binding assays for each of the above groups of anterior pituitary membranes was the same. Membranes (10 and 20 μg) of protein) were incubated in buffer B with [Tyr(^{125}I)^{10}]VIP (60-65 pM) in the absence or presence of VIP (0.1 μM) for 60 minutes at 31^{0}C. Specific binding was determined as previously defined. Specific binding of VIP was examined in the presence of increasing

membrane protein. The ability of VIP, 1.0 pM to 1.0 μM, to compete with [Tyr(^{125}I)^{10}]VIP binding was examined in each tissue under the same assay conditions.

Competitive binding data were examined using Scatchard transformations and plots, and analyzed by nonlinear regression using the computer program LIGAND. As detailed previously the algorithm fitted by this program is:

$$[B] = (B_{maxi}[L]/K_{Di}) + N[L]$$

were B and L are bound and free radioligand respectively, K_{Di} and B_{maxi} are the equilibrium dissociation constant and total receptor concentration at site i respectively, and N is the ratio of bound/free at infinite free concentration. For each experiment, curve fitting to six possible models was examined: 1, 2 or 3 distinct binding sites +/- N. Statistical analysis regarding the degree of fit was computer generated using the runs test and least mean squares. On the basis of these statistical analyses relative probability of each of these models was obtained, and the best fitting model could be determined.

For each group of membranes examined two types of analysis using LIGAND was performed. In the first, or single curve analysis, every experimental curve was individually analyzed. The resulting parameter values, i.e. K_D, B_{max} and L, were then analyzed for each group with respect to mean values and standard error of the mean (SEM). These group means were compared statistically using one way analysis of variance (ANOVA), Duncan's new multiple range test and Student-Newman-Keuls' test. As this type of analysis reflects only inter assay variability, a second analysis was performed. This involved computer analysis of all competitive binding curves from each group of animals at the same time. From these analyses the best fit model and parameter values obtained were compared with those from pooled single curve analysis. Computer generated standard errors for each parameter derived from multiple curve analysis provides an estimate of intra assay variability. Although such standard errors, obtained from nonlinear regression are at best only approximations, they were used to estimate 95% confidence intervals for each parameter. As a check whether intra assay variability could account for the differences observed using

single curve analysis, these confidence intervals were compared for parameters that had been deemed statistically significantly different during the first analysis.

B. Results

1. Postnatal development. Marked changes were found in the binding of VIP to anterior pituitary membranes early in postnatal life. At one and two weeks of age less VIP was bound per mg of tissue protein as compared with older animals (Figure 25). A dramatic increase in specific binding of VIP as a function of increasing membrane protein concentration was seen at 3 weeks of age. This amount of binding per quantity of anterior pituitary tissue declined gradually with increasing age after 3 weeks, until in the 32-60 week group the binding saturation curve was superimposable with that at 2 weeks (Figure 25).

When these changes in binding with age were studied with respect to VIP receptor number and affinity, a major change was again observed from 2 to 3 weeks after birth. The ability of VIP to competitively inhibit [Tyr(125I)^{10}]VIP binding to anterior pituitary membranes from 2, 3, and 10-12 week old female rats is shown in Figure 26. The curve from 2 week old animals differs appreciably in shape from the other two. In contrast to the ability of VIP to compete with [Tyr(^{125}I)^{10}]VIP binding at subnanomolar concentrations in 3 and 10-12 week old animals, very little competition is found in the 2 week old animal until greater than nanomolar levels of VIP are used. Although the curve shapes from 3 and 10-12 week old animals are more similar, increased amounts of nonspecific as well as specific binding was seen at 3 compared with 10-12 weeks (Figure 26). When this data was plotted as a percent of specific binding, the shift to the right of the curve from 2 week old animals becomes obvious (Figure 27A).

When data shown in Figure 26 was transformed and plotted according to Scatchard analysis (407), nonlinear, upwardly concave curves were generated for 3 and 10-12 week old animals (Figure 27B). In contrast this transformation of competitive binding curves from 2 week old animals results in nearly linear plots. Small upward shifts and to the right of curves from 10-12 week old animals was seen at 3 weeks of age.

For the purposes of clarity, curves from the other age groups have been omitted from these graphs. These three curves are however representative of those observed at all age groups examined, with competitive binding curves from 1 week old animals superimposable on those from 2 week old rats. The binding curves from the remaining age groups, i.e. 5, 6, 8 and 32-60 week old rats, possess the same shape, and fall between the curves obtained from 3 and 10-12 week old animals.

The binding parameters and model probability generated by computer multiple curve analysis of the competitive binding curves from 2, 3, and 10-12 week old animals is shown in Table 17. Equal probability of a 2 binding site model and a 1 site model with nonspecific binding is seen in 2 week old animals. Despite the similar probability of these two separate models, the following points argue strongly against defining a second, lower affinity site in these animals. The precision of parameters generated for the low affinity site in the two site model from two week old animals is exceedingly poor with computer generated standard errors around 100% (i.e K_D, 429 ± 466 nM; B_{max}, 188 ± 167 pmol/mg protein). The high K_D values generated for this site would suggest it to be of little biological relevance, as actions mediated by a receptor with such low affinity would require concentrations of VIP likely in excess of those present physiologically. As well the ability to resolve binding with such a low affinity is also questionable when, as in this study, filtration is used to separate free from bound radioligand. Finally, the linear shape of the competitive binding curve after Scatchard analysis (Figure 27B) argues against receptor heterogeneity. Analysis of data from 1 week old animals paralleled those from 2 week old animals, and in view of the logic presented above, the one binding site model was appropriated for these two groups of animals.

In contrast to the findings above, nonlinear regression analysis from 3 and 10-12 week old rats indicated 2 binding sites were statistically more probable as compared to the one site model +/- N (Table 17). This is in concordance with the nonlinear Scatchard plots from these animals (Figure 27B). The two site model was found most probable in all groups 3 weeks or older. As the probability in all groups of a 3 binding site model (+/- N) was zero

or iteration was impossible, parameters generated from these fits are omitted from Table 17.

Using one way ANOVA the binding parameters from the single binding site of 1 and 2 week old animals were compared with those from the low and then the high affinity binding sites from older animals. Comparison of means generated by single curve analysis, indicated no significant difference in the binding affinity or capacity of the single site when compared with the low affinity site from animals 3 or more weeks of age (Table 18). In contrast the single site parameter means from 1 and 2 week old animals differed significantly when compared to the high affinity site parameters in all the other groups (Table 19). This suggests that the single site seen at 1 and 2 weeks of age corresponds to the low affinity binding site seen in older animals.

Although differences in affinity and binding capacity of the high affinity VIP receptor are observed between 3 and 10-12 weeks of age, the significance of this is questionable. Using Duncan's and Student-Newman-Keuls' tests, means from computer analysis of individual binding curves indicated significant differences in K_D values from 3 and 5 compared to 6 and 10-12 week old rats. (Table 18). A significant change in number of binding sites was seen between 3 and 10-12 week old animal. As these comparisons take into account only inter assay variability, parameter means were generated by simultaneous, multiple curve computer analysis of each group and compared with the corresponding ones obtained by pooled, single curve analysis. (Table 20). Regardless of which analysis was implemented, similar parameter means were derived (Table 20). Reflected by multiple analysis standard errors, parameter values demonstrate considerable intra assay variability (Table 20). Approximate 95% confidence intervals were calculated from these standard errors and are shown for the single site from 1 and 2 week old animals and for the high affinity site from the remaining animals (Table 21). Readily obvious is the lack of overlap in confidence intervals of either parameter from 1 and 2 week old animals with corresponding parameters from the high affinity site, indicating that intra assay variability cannot account for the differences in the single site versus high affinity site parameters using pooled, single curve

FIGURE 25. Specific binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary membranes as a function of development and protein concentration. [Tyr(^{125}I)^{10}]VIP (55-65 pM) was incubated at 31^{0}C for 60 minutes, with increasing membrane protein concentrations from 2 (●), 3 (□), 5 (■), 6 (○), 8 (△), 10-12 (▲), and 32-60 (○) week old female rats. Specific binding was defined as total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 0.1 μM VIP. Data points are the mean ± SEM of 3-4 experiments performed in duplicate.

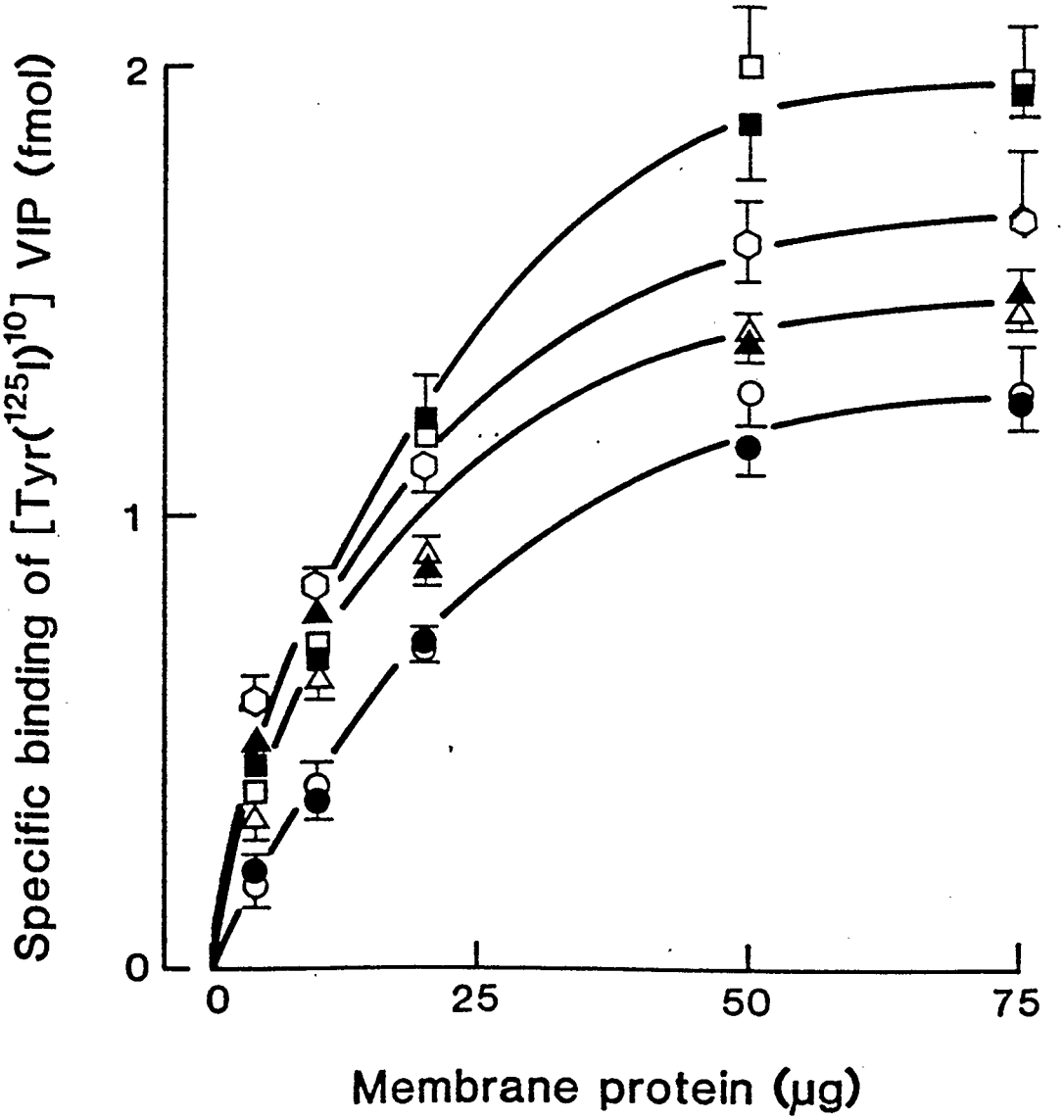

Specific binding of [Tyr(125I)10] VIP (fmol)
2
1
0
0
25
50
75
Membrane protein (µg)

FIGURE 26. Competition of unlabelled VIP for the binding of [Tyr(^{125}I)^{10}]VIP to female rat anterior pituitary membranes during development. Membranes (20 μg protein) from 2 (●), 3 (□) and 10-12 (○) week old rats were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) in the presence of increasing concentrations of VIP. B/T is the amount of radioligand bound over the total radioactivity added to the assay. Data points are the mean ± SEM from 4 (●,□) and 7 (○) experiments, performed in duplicate.

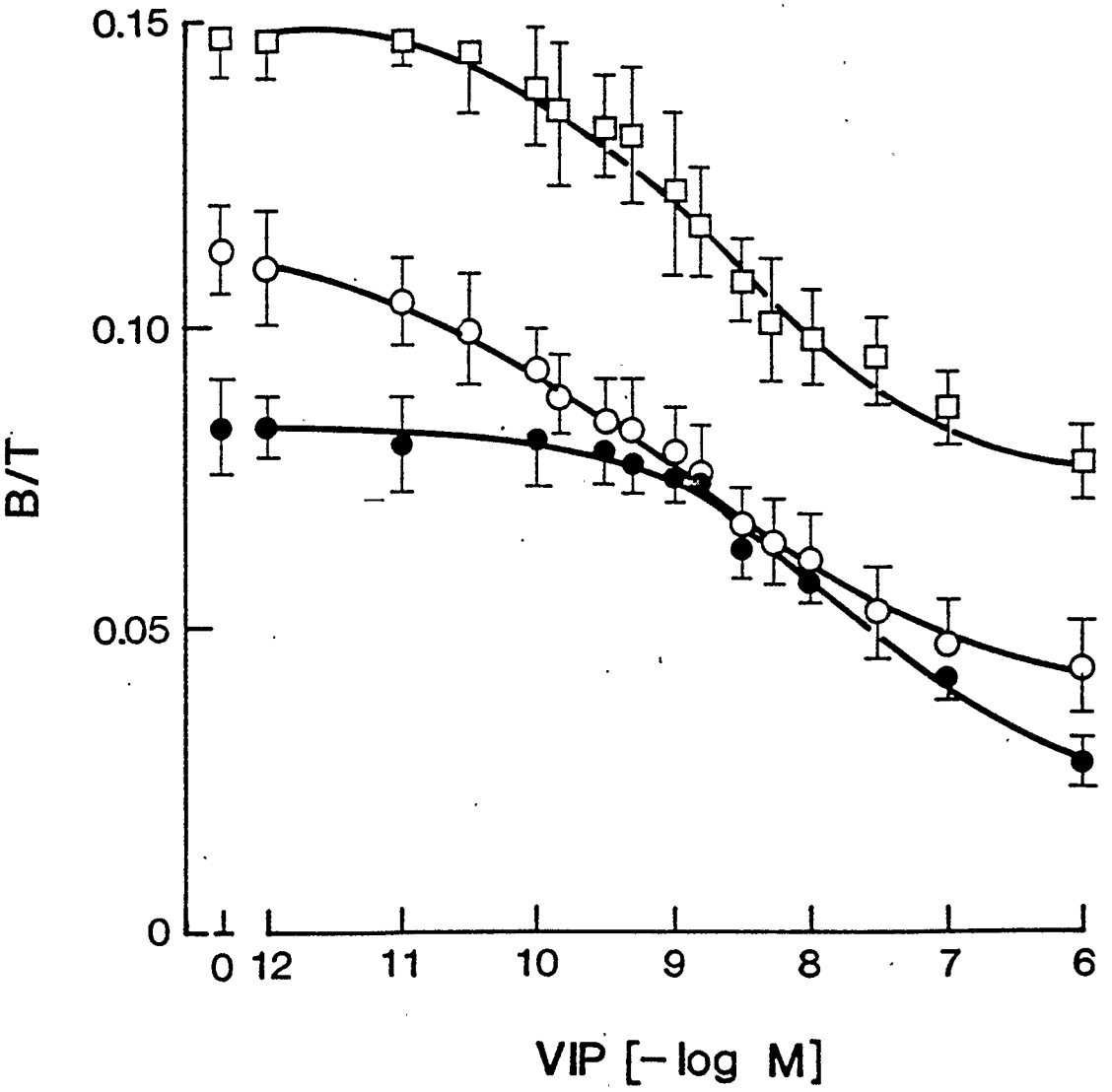

0.15
0.10
0.05
0
B/T
0 12
11
10
9
8
7
6
VIP [– log M]

FIGURE 27. Scatchard plots and percent specific binding of [Tyr(^{125}I)^{10}]VIP in the presence of increasing concentrations of VIP to anterior pituitary membranes from female rats during development.

Panel A: Data from Fig. 26 are plotted as a percentage of specific binding remaining in the presence of increasing concentrations of unlabelled VIP. Note that specific binding is defined here as the amount of total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 1.0 μM VIP.

Panel B: Scatchard plot derived from 1 representative curve at each age group shown in Fig. 26. B/F, ratio of specifically bound over free ligand.

Data are from 2 week (●), 3 week (□) and 10-12 week (○) old female rats.

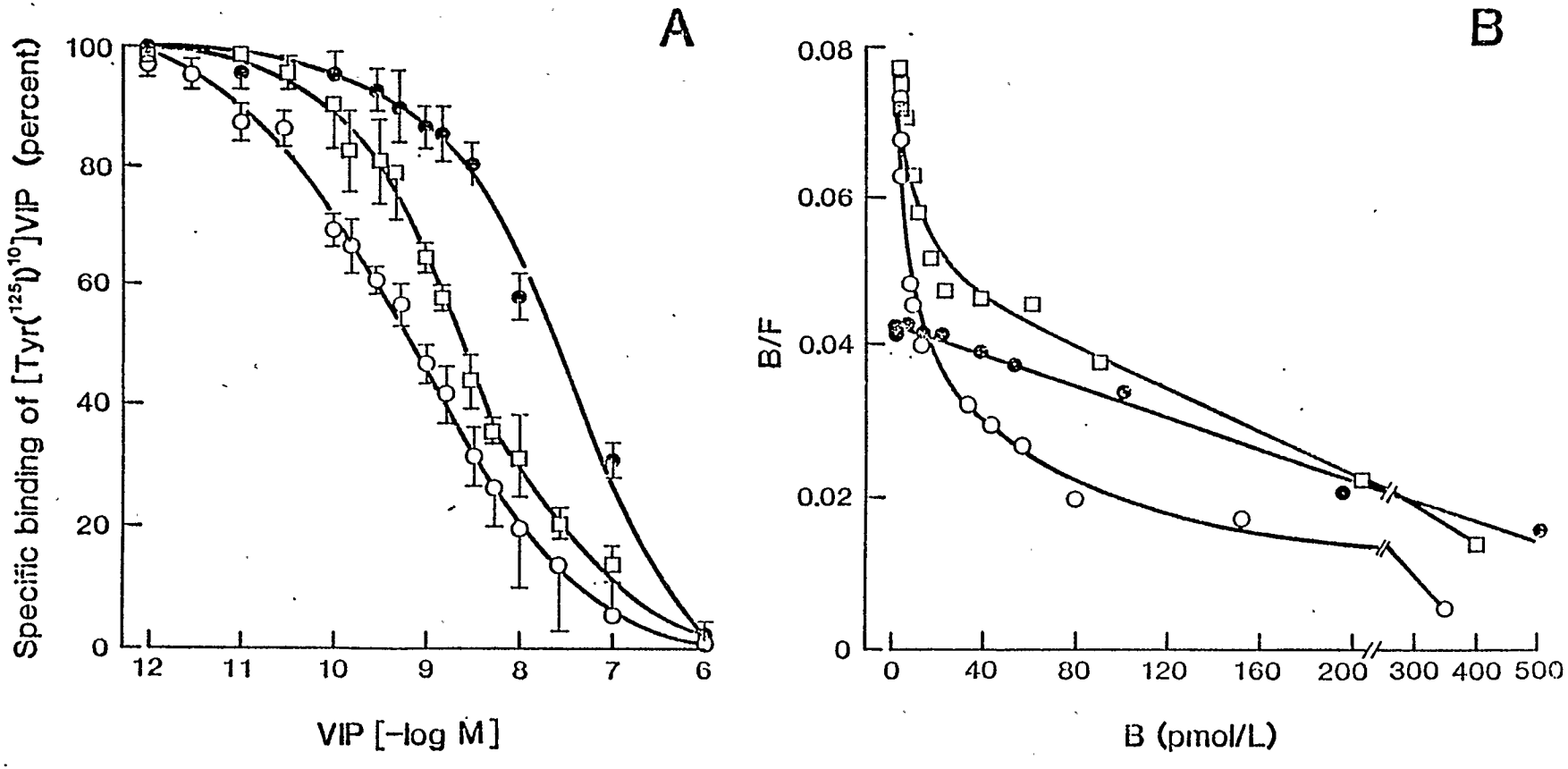

A
Specific binding of [Tyr(125I)10]VIP (percent)
100
80
60
40
20
0
12
11
10
9
8
7
6
VIP [-log M]
B
B/F
0.08
0.06
0.04
0.02
0
0
40
80
120
160
200
300
400
500
B (pmol/L)

TABLE 17. Binding parameters and model probability from computer nonlinear regression of VIP competitive binding curves to female rat anterior pituitaries during development

Age (weeks)	Number of sites	Binding site 1		Binding site 2		N	Probability
		K_D(nM)	Bmax(pmol/mg protein)	K_D(nM)	Bmax(pmol/mg protein)		
2	1	40.5 ± 4.9	50 ± 8	+---	----	----	0
	*1	5.9 ± 0.7	5.9 ± 1.1	----	----	0.016 ± 0.001	1
	*2	4.6 ± 1.3	4.2 ± 1.6	429 ± 466	180 ± 160	----	1
	2	5.4 ± 2.1	5.2 ± 7.0	676 ± 392	200 ± 72	0.006 ± 0.410	0
3	1	11.3 ± 1.8	14 ± 20	----	----	----	0
	1	1.5 ± 0.2	2.0 ± 0.2	----	----	0.009 ± 0.001	0
	2	0.56 ± 0.13	0.68 ± 0.16	70 ± 17	28 ± 4	----	0.13
	*2	0.41 ± 0.19	0.45 ± 0.24	14 ± 11	6 ± 3	0.006 ± 0.001	1
10-12	1	6.2 ± 0.9	6.5 ± 1.4	----	----	----	0
	1	5.1 ± 1.6	5.9 ± 1.6	----	----	0.003 ± 0.001	0
	*2	0.23 ± 0.11	0.25 ± 0.11	28 ± 16	12 ± 4	----	1
	2	0.28 ± 0.15	0.30 ± 0.10	39 ± 45	16 ± 10	0.001 ± 0.001	0

Nonlinear curve fitting to 6 models: 1, 2, or 3 binding sites ± N. Best fit of each model tested is shown

N is factor of nonspecific binding (see Experimental studies VA2).

Computer generated probability using approximate F test based on weighted least squares.
Parameters for 3 binding sites omitted as probability was 0 for all membranes.

Data are computer generated mean ± SEM, n = 4 except 10-12 weeks n = 7, * = most probable model.

Table 18. Equilibrium dissociation constants and maximum number of binding sites of the female rat anterior pituitary VIP receptor during development

Age (weeks)	Number of sites	High affinity site K_D(nM)	High affinity site Bmax(fmol/mg protein)	Low affinity site K_D(nM)	Low affinity site Bmax(pmol/mg protein)
1	1	----	----	8 ± 2	9.2 ± 2.7
2	1	----	----	6 ± 1	5.9 ± 1.1
3	2	0.38 ± 0.11c	438 ± 180c	35 ± 15	14.1 ± 6.0
5	2	0.47 ± 0.03[ab]	381 ± 56	48 ± 22	5.4 ± 0.7
6	2	0.27 ± 0.02	308 ± 54	33 ± 3	8.4 ± 1.3
8	2	0.29 ± 0.03	291 ± 46	20 ± 3	9.1 ± 2.6
10-12	2	0.19 ± 0.03[abcd]	158 ± 34[c]	28 ± 16	11.7 ± 3.6
32-60	2	0.40 ± 0.10[d]	236 ± 62	42 ± 5	13.8 ± 3.3

Binding parameters values were obtained by computer nonlinear regression analysis (LIGAND) of individual competitive binding curves.

Data are expressed as mean + SEM, n = 4 except 10-12 weeks n = 7.

Comparison of means within each parameter group was by one way analysis of variance and: Duncan's new multiple range and Student-Newman-Keuls' (S-N-K) tests.
In both Duncan's and S-N-K tests: $^{a}p<0.05$.
In Duncan's test only: $^{b}p<0.01$, $^{cd}p<0.01$.

Table 19. Equilibrium dissociation constants and receptor concentrations of the high affinity binding site of the female rat anterior pituitary VIP receptor during development

		Single or high affinity binding site	
Age (weeks)	Number of sites	K_D(nM)	Bmax(fmol/mg protein)
1	1	8.2 ± 2.0[A]	9200 ± 2700[A]
2	1	5.9 ± 0.7[B]	5900 ± 1100[B]
3	2	0.38 ± 0.11	438 ± 180
5	2	0.47 ± 0.03	381 ± 56
6	2	0.27 ± 0.02	308 ± 54
8	2	0.29 ± 0.03	291 ± 46
10-12	2	0.19 ± 0.03	158 ± 34
32-60	2	0.40 ± 0.10	236 ± 62

Binding parameters, obtained by nonlinear regression analysis (LIGAND) of individual competitive binding curves, are of the single site in 1 and 2 week old rats, and from the high affinity site of the other ages.

Data expressed as mean + SEM, n = 4, except n = 7 in 10-12 week old rats.

[A]$p<0.01$ compared to all means in parameter group except age 2 weeks,
[B]$p<0.01$ compared to all means in parameter group except age 1 week, one way analysis of variance and Student-Newman-Keuls' test.

TABLE 20. Comparison between parameters of binding to rat anterior pituitary VIP receptors during postnatal development as generated by single versus multiple curve computer analysis

Age (weeks)	Analysis	High affinity site		Low affinity site	
		K_D(nM)	Bmax(fmol/mg protein)	K_D(nM)	Bmax(pmol/mg protein)
1	single	----	----	8.2 ± 2.0	9.2 ± 2.7
	multiple	----	----	7.7 ± 1.5	7.2 ± 1.5
2	single	----	----	5.9 ± 0.7	5.9 ± 1.1
	multiple	----	----	5.8 ± 0.7	5.8 ± 0.7
3	single	0.38 ± 0.11	438 ± 180	34.9 ± 14.5	14.1 ± 6.0
	multiple	0.41 ± 0.19	446 ± 236	13.8 ± 10.8	5.7 ± 3.0
5	single	0.47 ± 0.03	381 ± 56	47.9 ± 21.6	5.4 ± 0.7
	multiple	0.47 ± 0.26	384 ± 198	49.2 ± 23.5	13.1 ± 3.8
6	single	0.27 ± 0.02	308 ± 54	33.3 ± 3.4	8.4 ± 1.3
	multiple	0.27 ± 0.11	400 ± 120	70.0 ± 25.9	26.0 ± 6.0
8	single	0.29 ± 0.03	291 ± 46	19.8 ± 2.5	9.1 ± 2.6
	multiple	0.26 ± 0.12	380 ± 160	18.2 ± 4.7	10.0 ± 1.4
10-12	single	0.19 ± 0.03	158 ± 34	28.0 ± 16.1	11.7 ± 3.6
	multiple	0.23 ± 0.11	252 ± 109	23.0 ± 5.8	9.4 ± 1.8
32- 60	single	0.40 ± 0.10	236 ± 62	41.9 ± 5.0	13.8 ± 3.3
	multiple	0.40 ± 0.17	326 ± 127	41.6 ± 9.7	19.0 ± 2.8

Single analysis refers to computer nonlinear regression (LIGAND) of individual experiments, with mean ± SEM calculated for each group. Multiple analysis is computer nonlinear regression of all curves in one age group simultaneously, with computer generated mean ± SEM.

TABLE 21. Confidence intervals of computer generated binding parameters of female rat anterior pituitary VIP receptor during development

Age (weeks)	Single or high affinity binding site	
	K_D(nM)	Bmax (fmol/mg protein)
1	5.10 - 11.63	4720 - 10940
2	4.53 - 7.41	4480 - 7440
3	0.16 - 1.05	146 - 1320
5	0.12 - 1.45	161 - 1310
6	0.12 - 0.61	220 - 740
8	0.11 - 0.64	160 - 880
10-12	0.08 - 0.60	112 - 660
32-60	0.17 - 0.94	160 - 680

Approximate 95% confidence intervals were calculated for binding parameters from standard errors generated by computer multiple curve nonlinear regression analysis.
For details regarding analysis see Experimental studies VA.

analysis (Table 19). In contrast the differences in high affinity site affinity and capacity seen after 3 weeks of age, that are statistically different using single curve analysis method, may be secondary to intra assay variability as approximate confidence intervals for these parameters show marked overlap (Table 21).

These analyses of VIP binding to anterior pituitary membranes from female rats of different postnatal age support the following conclusions: 1). only one VIP binding site is present at 1 and 2 weeks of age, 2). 2 VIP binding sites are present in animals 3 weeks of age and up to at least one year of age, 3). the low affinity site is present from 1 week of age until 1 year of age, 4). the high affinity binding site is not detectable at 1 and 2 weeks of age, while it is present at 3 weeks and up to at least 1 year of age, with minor changes in receptor number and affinity between 3 weeks of age and adulthood a possibility, and 5). conversion of some low affinity to high affinity sites from 2 to 3 weeks is not supported on the basis of changes in receptor number, however resolution of number of low affinity sites is too poor to rule out this possibility.

2. Lactation. Specific binding of VIP to anterior pituitaries from lactating female rats is less per mg of tissue protein as compared to similar aged nonlactating, nulliparous female rats (Figure 28). Specific binding per anterior pituitary gland was however not different in lactating (13.7 fmol/anterior pituitary) and nonlactating adult female rats (13.6 fmol/anterior pituitary). This discrepancy is likely due to the increased weight (23 ± 4 versus 16 ± 1 mg/anterior pituitary gland,) and membrane protein yield (0.23 versus 0.20 mg protein/anterior pituitary) (X ± SEM, n=4) in anterior pituitary glands from lactating versus nonlactating rats respectively.

Competitive binding studies corroborate this difference in specific binding (Figure 29), however very little difference in the shape of the curve and potency of VIP is detectable. The similarity becomes obvious when competitive binding data is plotted as a percent of specific binding (Figure 30A), and after Scatchard analysis (Figure 30B). In the later plot, curves from both groups of animals are upwardly concave and LIGAND generated best fit models indicate 2 binding sites. K_D values of the high affinity binding site do not change,

FIGURE 28. Specific binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary membranes from lactating and nonlactating adult female rats . [Tyr(^{125}I)^{10}]VIP (55-65 pM) was incubated at 31^{0}C for 60 minutes with membranes from lactating (△) and nonlactating (○) adult female rats. Specific binding was defined as the amount of bound radioligand displaced in the presence of 0.1 μM VIP. Data points are the mean ± SEM of 3-4 experiments performed in duplicate.

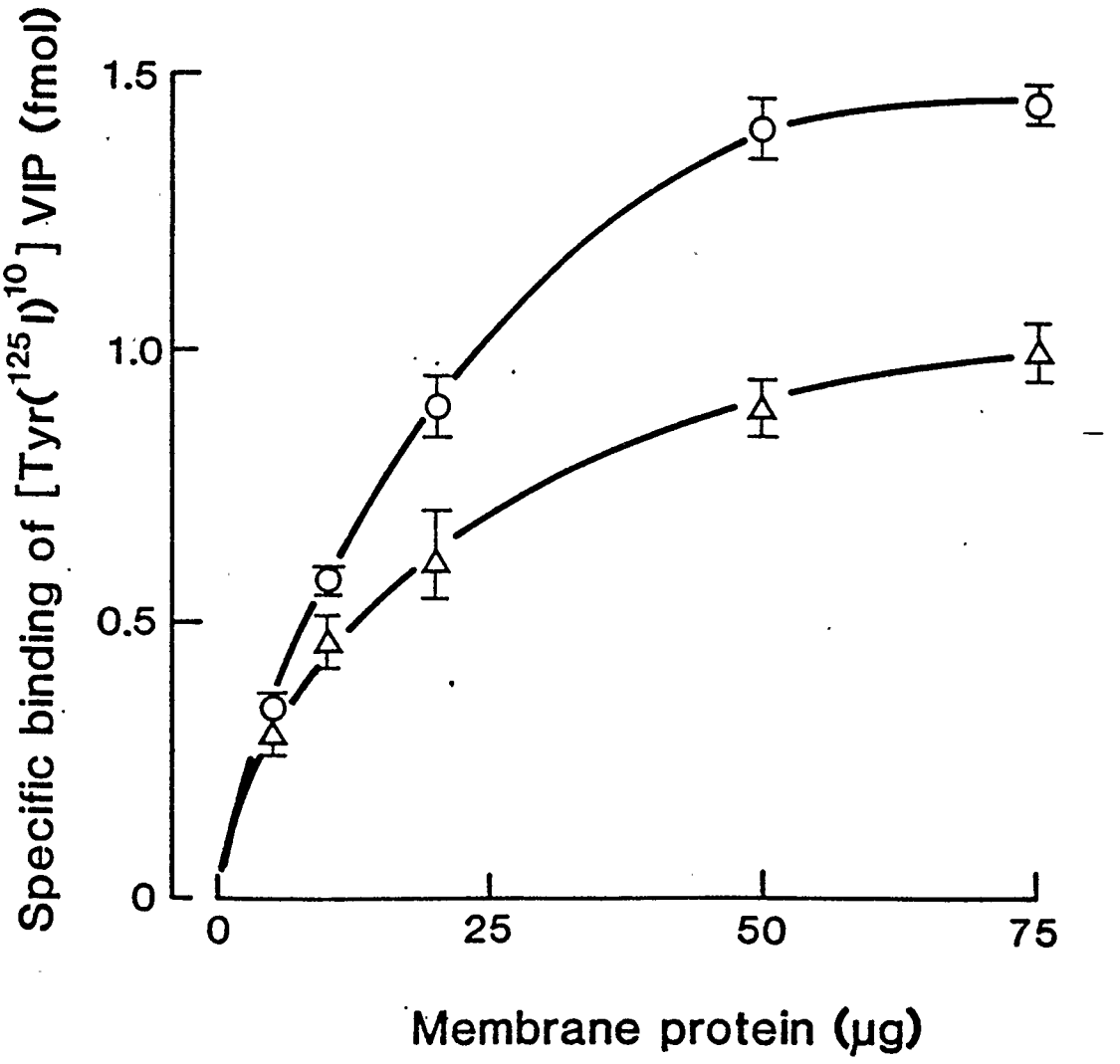

Specific binding of [Tyr(125I)10] VIP (fmol)
1.5
1.0
0.5
0
0
25
50
75
Membrane protein (µg)

FIGURE 29. Competition of unlabelled VIP for the binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary membranes from lactating and nonlactating female rats. Membranes (20 μg protein) from lactating (△) and nonlactating (○) rats were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) in the presence of increasing concentrations of VIP. B/T is the amount of radioligand bound over the total radioactivity added to the assay. Data points are the mean ± SEM from 4 (△) and 7 (○) experiments, performed in duplicate.

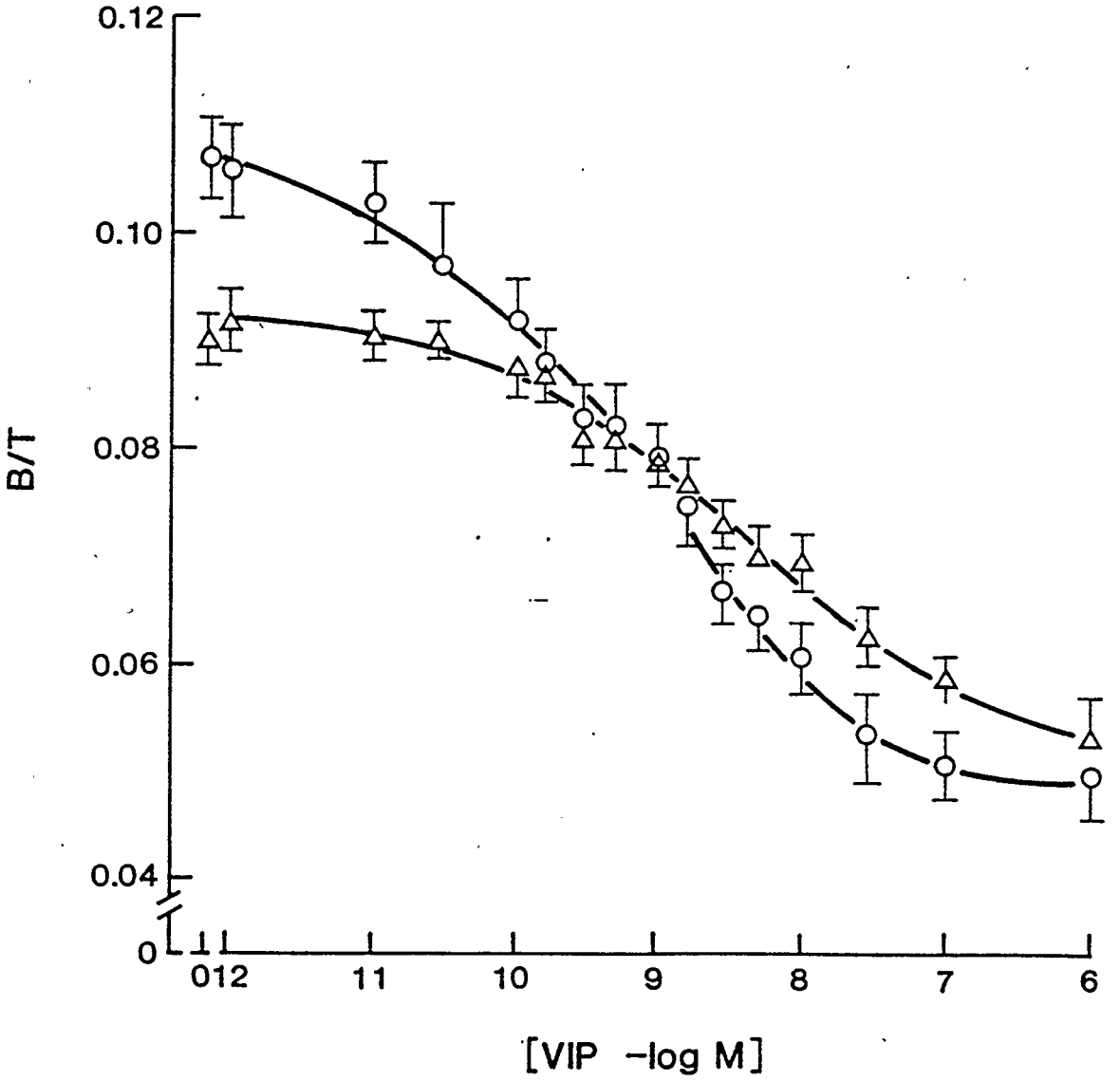

0.12
0.10
0.08
0.06
0.04
0
B/T
012
11
10
9
8
7
6
[VIP −log M]

FIGURE 30. Scatchard plots and percent specific binding of [Tyr(^{125}I)^{10}]VIP in the presence of increasing concentrations of VIP to anterior pituitary membranes from lactating and nonlactating female rats.

Panel A: Data from Fig. 29 is plotted as a percentage of specific binding remaining in the presence of increasing concentrations of unlabelled VIP. Note that specific binding is defined here as the amount of total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 1.0 μM VIP.

Panel B: Scatchard plots derived from 1 representative curve from each group shown in Fig. 29. B/F, ratio of specifically bound over free ligand.

Data are from lactating (△) and nonlactating (○) adult, female rats.

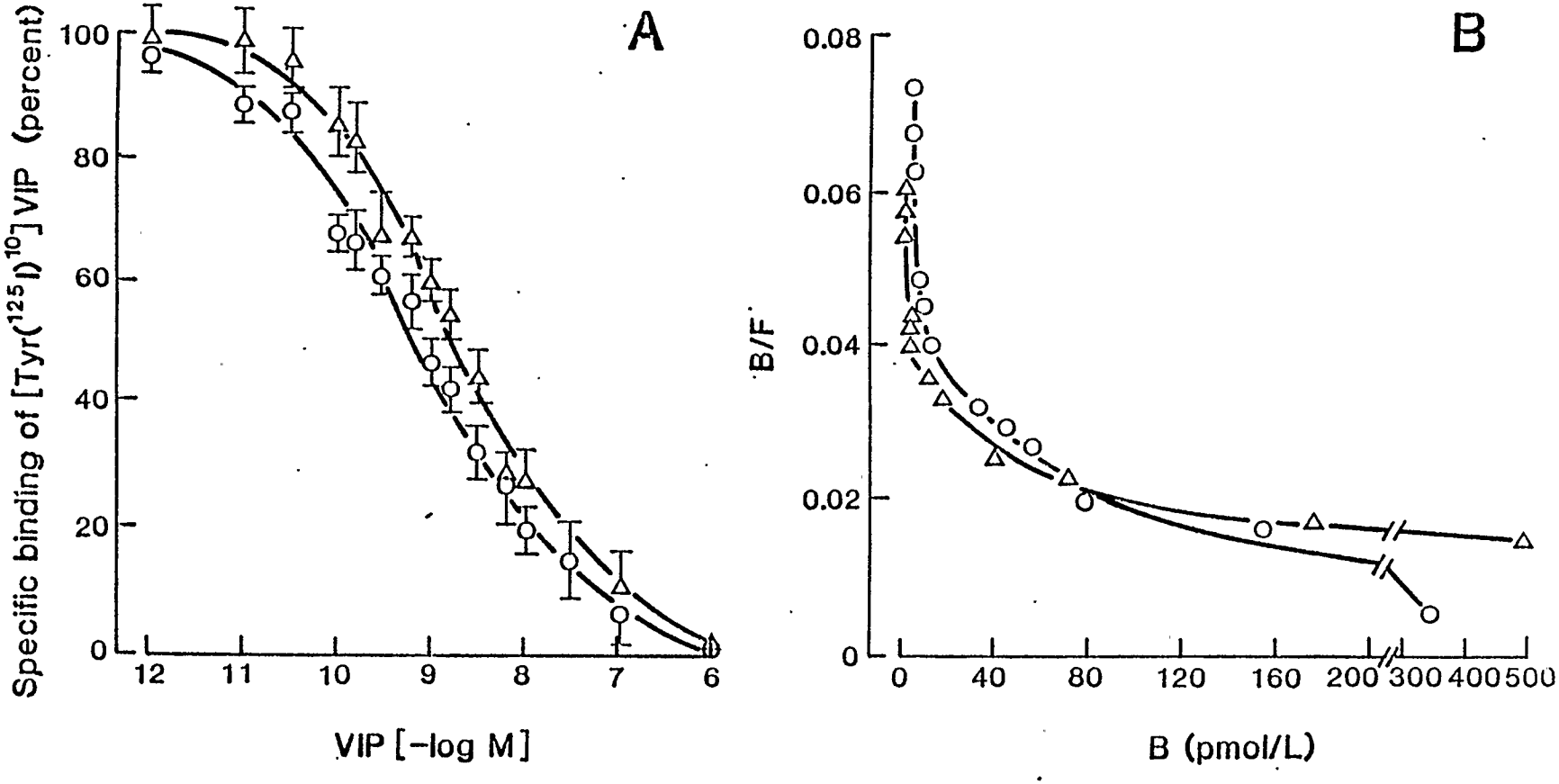

A
Specific binding of [Tyr(125I)10]VIP (percent)
100
80
60
40
20
0
12
11
10
9
8
7
6
VIP [-log M]
B
B/F
0.08
0.06
0.04
0.02
0
0
40
80
120
160
200
300
400
500
B (pmol/L)

Table 22. Equilibrium dissociation constants and maximum number of VIP binding sites in the anterior pituitary from lactating as compared to nonlactating female rats

Membrane source	High affinity site		Low affinity site	
	K_D(nM)	Bmax(fmol/mg protein)	K_D(nM)	Bmax(pmol/mg protein)
Lactating	0.19 ± 0.03	158 ± 34[a]	69 ± 14[b]	35.2 ± 7.8[a]
Nonlactating (10-12 weeks old)	0.18 ± 0.03	85 ± 19[a]	28 ± 16[b]	11.7 ± 3.6[a]

Equilibrium dissociation constants (K_D) and maximum number of binding sites (Bmax) were obtained by nonlinear regression analysis using the computer program LIGAND.

Data are expressed as mean ± SEM, n = 4 (lactating), n = 7 (nonlactating); assays performed in duplicate.

[a]$p<0.01$, [b]$p<0.05$, Student's t test, comparing binding parameter means.

however all other parameters change significantly (Table 22). When compared with the nulliparous adult random cycling nonlactating female, there is a decrease in the number of high affinity binding sites during lactation. The low affinity binding sites also changes, with a decrease in affinity and an increase in number during lactation. It is likely the combination of these that results in the marked decrease in VIP bound per mg of protein (Figure 28).

3. Effect of castration and testosterone. Differences in specific binding of VIP to male and female rat anterior pituitaries per quantity of tissue protein, were abolished 24 hours after surgical castration of the male animals (Figure 31). Competitive binding studies in anterior pituitary tissue from castrated males 24 hours after surgery demonstrated increases in total as well as specific binding when compared with normal animals (Figure 32). A decrease in competitive ability was seen using low concentrations of VIP in the castrated animals (Figure 32). This difference in potency in castrated animals is seen by up and rightward displacement of specific binding competed for by subnanomolar concentrations of VIP (Figure 33A). Scatchard plots of these data were nonlinear, upwardly concave (Figure 33B). Again a shift up and to the right was seen after castration.

The results of nonlinear regression analysis of these binding studies, along with those 2 weeks after surgical castration, 24 hours after sham castration, and testosterone treated castrated animals, are shown in Table 23. Analysis favoured interpretation of the two binding site model in each group of animals. One way ANOVA and the Newman-Keuls' test revealed no difference in binding parameters between normal adult males and sham operated animals. One day after surgical castration a significant increase in the K_D to 0.67 ± 0.07 nM from 0.13 ± 0.02 nM was seen, along with a 6-7 fold increase in the number of binding site of this high affinity receptor. Similar changes were seen in the high affinity receptor 2 weeks after castration. These changes were prevented by the administration of testosterone cypionate, with these animals not differing significantly from control or sham operated animals. Although binding to 1 day and 2 week castrated animals was indistinguishable at the high affinity site, changes were seen at two weeks and not 1 day after castration at the

low affinity site. Significant increases in the K_D and receptor number were seen at the low affinity site 2 weeks after castration in comparison with all other groups studied. This site was not significantly different amongst the other groups. Confidence intervals (95 %), derived from computer generated standard errors of multiple curve analysis, revealed no overlap in the majority of parameters for which significant differences were found with ANOVA and Student-Newman-Keuls' test (Table 24). A small overlap of 0.01 nM in the 95% confidence interval of K_D values from castrated and castrated-testosterone treated animals was observed. A somewhat greater overlap of 26 nM was observed for the low affinity site K_D in the two groups of castrated rats. These finding indicate that conclusions drawn from the one way analysis of variance are statistically valid and not due to intra assay variability.

Although binding of VIP to females and castrated males were similar with regards to protein concentration curves, the binding parameters of castrated animals did not resemble that of female rat (Table 23). Instead a 3-4 fold decrease in affinity along with tripling of the binding capacity of the high affinity site was observed in the castrated male as compared to the female. These reciprocal changes can account for the similar amounts of VIP bound per mg of membrane protein from female and castrated rats. Although binding capacities of normal female rats were higher than in normal male rats, this difference did not reach statistical significance, and no difference was discernable in the binding parameters of normal male and female rats.

FIGURE 31. Specific binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary membranes from normal female, male and castrated male rats. [Tyr(^{125}I)^{10}]VIP (55-65 pM) was incubated at 31^{0}C for 60 minutes, with increasing membrane protein concentrations from normal adult female (○), male (△) and surgically castrated male (1 day) (□) rats. Specific binding was defined as total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 0.1 μM VIP. Data points are the mean ± SEM of 3-4 experiments performed in duplicate.

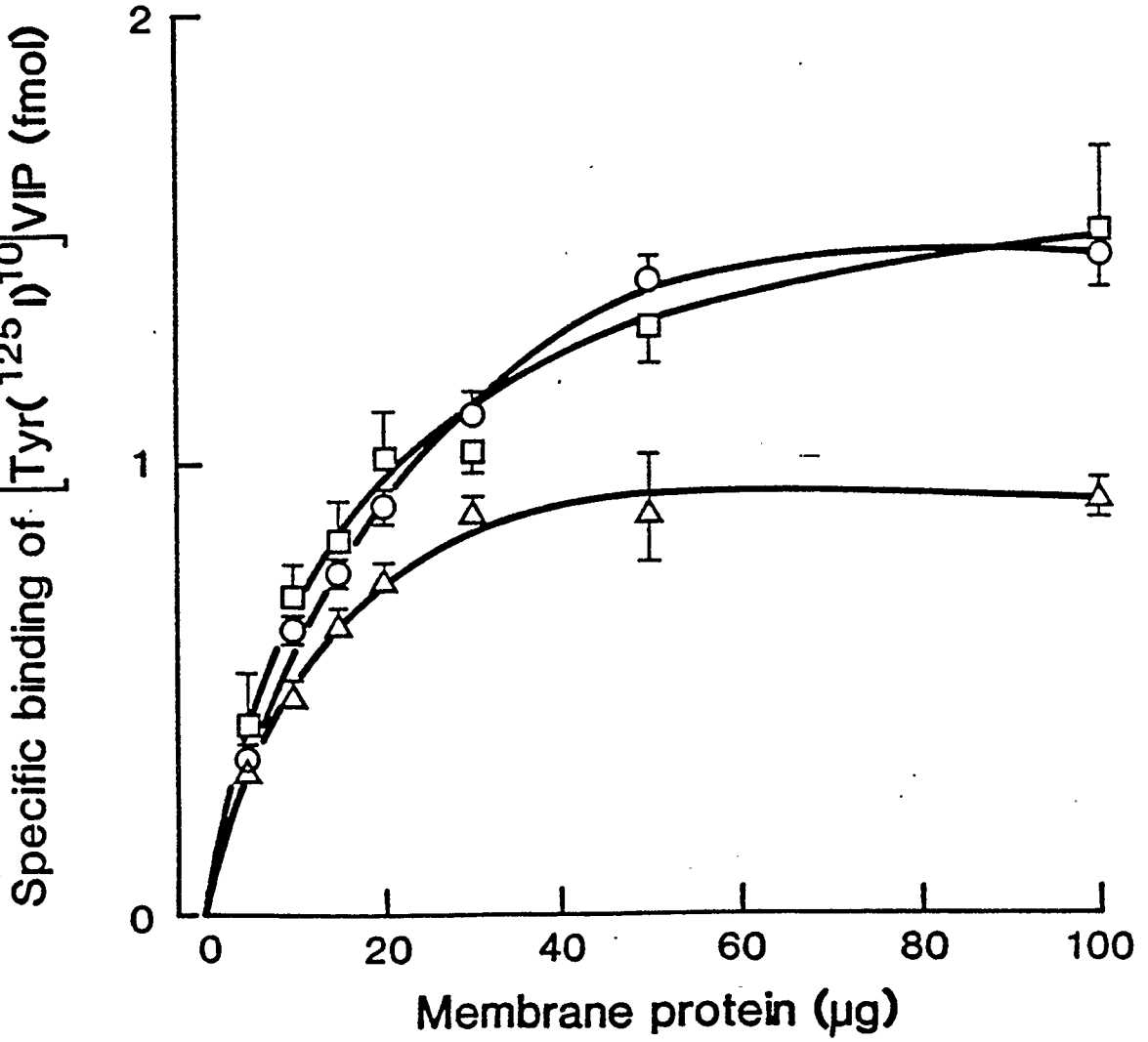

Specific binding of [Tyr(125I)10]VIP (fmol)
2
1
0
0
20
40
60
80
100
Membrane protein (µg)

FIGURE 32. Competition of unlabelled VIP for the binding of [Tyr(^{125}I)^{10}]VIP to anterior pituitary membranes from normal and castrated adult male rats. Membranes (20 μg protein) from normal (○) and surgically castrated (1 day) (□) rats were incubated with [Tyr(^{125}I)^{10}]VIP (55-65 pM) in the presence of increasing concentrations of VIP. B/T is the amount of radioligand bound over the total radioactivity added to the assay. Data points are the mean ± SEM from 7 (○) and 6 (□) experiments, performed in duplicate.

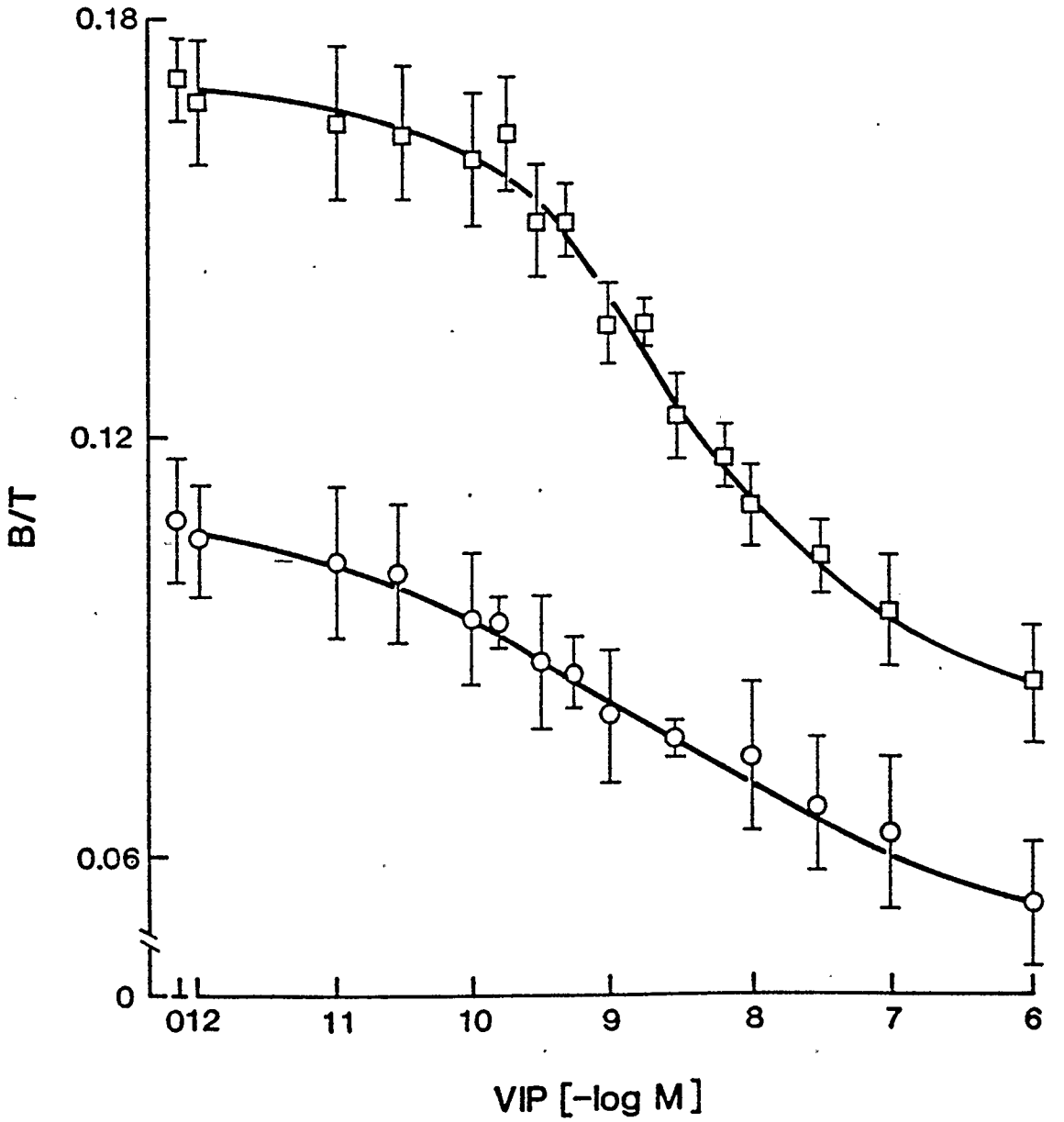

0.18
0.12
0.06
0
B/T
012
11
10
9
8
7
6
VIP [-log M]

FIGURE 33. Scatchard plots and percent specific binding of [Tyr(^{125}I)^{10}]VIP in the presence of increasing concentrations of VIP to anterior pituitary membranes from normal or castrated male rats.

Panel A: Data from Fig. 32 is plotted as a percentage of specific binding remaining in the presence of increasing concentrations of unlabelled VIP. Note that specific binding is defined here as the amount of total bound [Tyr(^{125}I)^{10}]VIP displaced in the presence of 1.0 μM VIP.

Panel B: Scatchard plot derived from 1 representative curve of each group shown in Fig. 32. B/F, ratio of specifically bound over free ligand.

Data are from adult normal (O) and 1 day castrated (□) male rats.

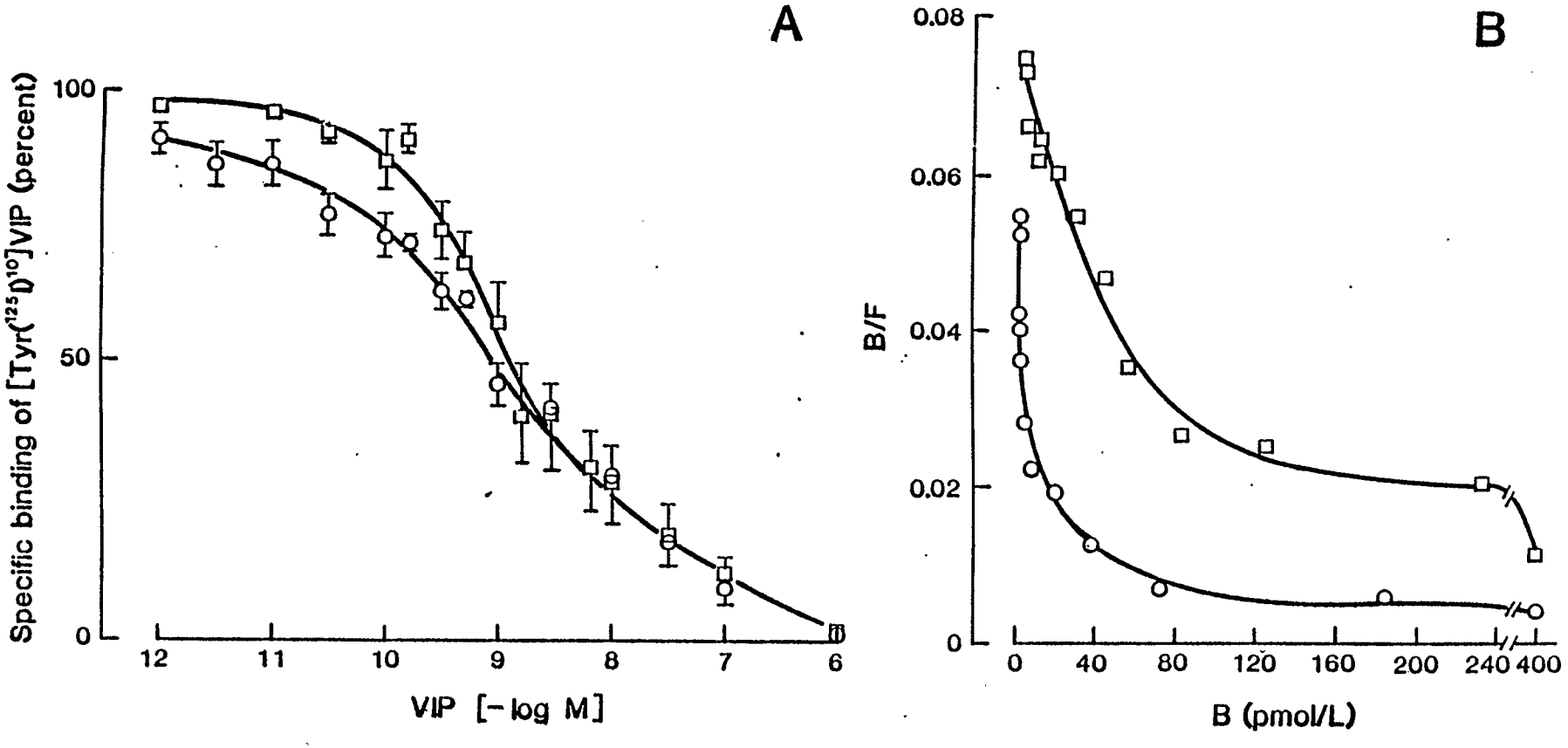

A
B
Specific binding of [Tyr(125I)10]VIP (percent)
100
50
0
12
11
10
9
8
7
6
VIP [- log M]
B/F
0.08
0.06
0.04
0.02
0
0
40
80
120
160
200
240
400
B (pmol/L)

TABLE 23. Equilibrium dissociation constants and maximum number of binding sites of the anterior pituitary VIP receptor in normal, castrated and testosterone treated rats

	High affinity site		Low affinity site	
Membrane source	K_D(nM)	Bmax(fmol/mg protein)	K_D(nM)	Bmax(pmol/mg protein)
Male, normal	0.13 ± 0.02	71 ± 9	45.2 ± 13.4	15.0 ± 6.8
Male, sham	0.15 ± 0.03	92 ± 20	40.8 ± 16.6	13.2 ± 4.1
Male, castrated (1 day)	0.67 ± 0.07[A]	470 ± 112[A]	41.2 ± 8.9	13.9 ± 14.6
Male, castrated (2 weeks)	0.59 ± 0.03[B]	418 ± 35[B]	115.2 ± 34.0[C]	63.0 ± 30.0[C]
Male, castrated, testosterone treated	0.20 ± 0.04	150 ± 34	46.5 ± 11.5	18.2 ± 4.2
Female, normal	0.19 ± 0.03	158 ± 34	28.0 ± 16.1	11.7 ± 3.6

Binding parameters were derived by computer nonlinear regression (LIGAND).
Anterior pituitary membranes from adult normal rats, 1 day after sham surgical castration, 1 day or 2 weeks after surgical castration and 1 day after surgical castration plus testosterone cypionate (4 μg/g body weight).

Data are expressed as mean ± SEM, n=4 (sham), 5 (testosterone), 6 (castrated), 7 (normal).

[A] $p<0.01$ compared to all means in group except 2 week castrated,
[B] $p<0.01$ compared to all means in group except 1 day castrated,
[C] $p<0.05$ compared to all means in group, using one way ANOVA and Student-Newman-Keuls' test.

TABLE 24. Confidence intervals of computer generated binding parameters of the male rat anterior pituitary VIP receptor

Membrane source	High affinity site		Low affinity site	
	K_D(nM)	Bmax(fmol/mg protein)	K_D(nM)	Bmax(pmol/mg protein)
Male, normal	0.05 - 0.32	25 - 95	33 - 67	12 - 18
Male, sham	0.07 - 0.35	47 - 180	27 - 51	10 - 17
Male, castrated (1 day)	0.36 - 1.39	205 - 695	56 - 112	11 - 15
Male, castrated (2 weeks)	0.36 - 0.87	340 - 801	86 - 291	39 - 100
Male, castrated testosterone treated	0.09 - 0.39	91 - 214	29 - 47	11 - 17

Approximate 95% confidence intervals were calculated for binding parameters from standard errors generated by multiple curve, nonlinear regression analysis.

For details regarding analysis see Experimental studies VA.

DISCUSSION

This study demonstrates the anterior pituitary gland VIP receptor as an entity using radioligand binding techniques. Criteria substantiating that binding of VIP to the anterior pituitary is receptor mediated included saturability to limited concentrations of radioligand or receptor, specificity with respect to homologous peptides, reversibility, and interaction with sufficient affinity to facilitate biological action. Changes observed in this binding under conditions associated with altered anterior pituitary secretion of PRL, add import to the physiological significance of this interaction.

As for many VIP receptor containing tissues (297,332,303,316), dissociation of VIP from anterior pituitary membranes was incomplete. The retention effect, described by Silhavy et al. (443), of a ligand by protein containing high concentrations of binding sites in the face of low dissociation constants, could account for this lack of complete reversibility. Kinetic analysis suggested dissociation from more than one binding site, with the dissociation rate constants generated for two sites in the anterior pituitary gland comparable to those in the rat cerebral cortex (332).

This heterogeneity of interaction was also observed during competitive binding studies, and transformation of experimental data did not result in linearization, e.g. Scatchard analysis (407). Of the many possible explanations for this diversity (399), radioligand heterogeneity is unlikely as pure unoxidized, monoiodinated VIP was used, with stability of [Tyr(^{125}I)^{10}]VIP under conditions of assay verified by HPLC. Although not specifically excluded in this study, negative cooperativity is also doubtful, as site to site interactions between VIP receptors has been experimentally excluded in other tissues (303,315,320). In concert with biphasic dose response curves of adenylate cyclase activation (329), and affinity labelling of VIP binding sites with differing molecular weight and functional properties (347-352), the most plausible explanation of VIP binding heterogeneity in tissues including the anterior pituitary, is the presence of more than one distinct binding site. Computer nonlinear regression analysis of competitive binding curves supported this premise in anterior pituitary tissue from adult rats, providing as the statistical best fit a two binding site model. The

equilibrium dissociation constant of the higher affinity site, K_D of 0.19 ± 0.03 nM, compares with that found in other VIP responsive tissue (332,309,320,316). Concentrations of VIP in hypophyseal portal blood sufficient to interact with this site have been reported in basal and stimulated states (243-245). In this, as well as other studies, the dose response curves of PRL release by VIP exhibit potencies, e.g. ED_{50} of 1-7 nM (270,271,273), compatible with an action of VIP on PRL release at the high affinity receptor.

The significance of the low affinity receptor is harder to define. Although portal blood levels appear too low (243-245) to effect an action via these receptors with a K_D of 28 nM, de novo synthesis of VIP in the anterior pituitary gland (249), suggests that locally acting concentrations of VIP could be sufficiently high to mediate an effect. In addition to the possibility of a second specific VIP receptor, viable explanations for the second binding site for VIP include interactions with another peptide receptor, as has been shown for the low affinity, secretin preferring VIP binding site in the pancreas (311). Attractive possibilities are receptors for members of the secretin-glucagon family, particularly GRF and PHI. These two peptides have been identified in the hypothalamic hypophyseal system (223,380), and specific receptors for GRF have been found in the anterior pituitary (444). VIP, capable of stimulating GH release in vitro, is much less potent than GRF and may mediate this action via the GRF receptor (445). As in lung, the low affinity VIP binding site may be a GRF preferring receptor (352). Although no cross reactivity with the rat anterior pituitary GRF receptor by VIP (up to 0.1 μM) was detected by Siefert et al. (444), the radioligand binding conditions used such as absence of bacitracin and lower incubation temperatures, might not favour VIP binding. The lack of specific GRF receptors in GH_3 cells (324), which possess only one VIP binding site, also endorses the possibility that the low affinity site may be the GRF receptor. The question of specific PHI receptors in the anterior pituitary has not been addressed to date, however VIP is able to cross react with PHI preferring receptors in other tissues containing high affinity specific VIP receptors (378,379).

The marked decrease in specific binding of VIP in the presence of GTP-γ-S is supportive of receptor coupling to adenylate cyclase (333,336,337). Similar decreases in

binding have been reported in other tissues (356,297,347,336), and are associated with decreases in receptor affinity secondary to increased dissociation rates of VIP from its receptor (306,309). In the anterior pituitary inclusion of GTP-γ-S resulted in virtual disappearance of the high affinity receptor with no discernable change at the low affinity site. A similar decrease in the number of high affinity VIP binding sites secondary to GTP has been reported in rat lung (352). Selective effects of guanine nucleotides on VIP binding to the high and not low affinity VIP binding sites have also been found in affinity labelled (356,347) or solubilized VIP receptors (353). As the effect of guanine nucleotides is most frequently reported to be on receptor affinity, it is interesting to speculate that in the anterior pituitary inclusion of GTP-γ-S may convert the high affinity receptors to ones indistinguishable from the low affinity receptors. Although no changes were seen in the low affinity site, the poor resolution secondary to the very little degree of specific binding remaining in the presence of GTP-γ-S, may mitigate against any differences being discernable.

Anterior pituitary VIP receptors were highly specific when tested against eight members of the secretin-glucagon family of peptides. The receptor recognized peptides from rat (rGRF and rPHI) with greater affinity than peptides endogenous to bovine (bGRF) or porcine (pPHI) species. This contrasts with bovine and rat vasculature (316), and intestine (300) where recognition properties of VIP receptors were not necessarily greater for peptides endogenous to the tissue. Human derived peptides were variably potent, hPHI and rPHI being equipotent, while hGRF was one-sixteenth as potent as rGRF. This likely reflects the greater degree of structural homology of hPHI with VIP (Table 2). Substitution of the amino terminal His^1 residue, as occurs in the hGRF and bGRF but not rGRF, has been found to decrease potency of adenylate cyclase activation in most rat tissues studied including the anterior pituitary gland (341). This effect may partially be due to their lower affinity for the VIP receptor. The importance of the amino terminus is evident by the low potency of VIP(10-28), an effect that has been observed for VIP binding (299), adenylate cyclase activation (341,343) and biological activity (340). The importance of other areas of the

molecule in binding to the VIP receptor of the anterior pituitary was apparent in the different potencies of the PHI peptides where isolated changes (Tyr to Phe, Ile to Leu in positions 10 an 17 respectively) between rPHI and pPHI resulted in a 3.5 fold decrease in receptor binding. The differences in specific binding observed between the various monoiodinated radioligands of VIP also attest to the importance of the central and carboxyl end regions of the molecule in VIP binding. Like brain and uterus, but unlike liver (482) and vascular receptors (315,316), the anterior pituitary receptor recognized apamin, a polypeptide from bee venom, lacking structural homology to VIP. Other differences between tissues is observed in that secretin cross reacts with the VIP receptor in anterior pituitary, pineal cells, intestine, prostate, pancreas, uterus and brain, but not with vessels or thyroid (315,316,323,322,296,318,304).

The amino terminal region of VIP was necessary for binding to its receptor in the anterior pituitary, as has been shown in other tissues (299,344309,315). Sequential replacement by D-isomers of the four naturally occurring amino terminal amino acid residues demonstrated greater reductions in affinity the closer the substitution was to the amino terminus, i.e. potency order was VIP>[D-Ala4]VIP=[D-Asp^3]VIP>[D-Ser^2]VIP>[D-His^1]VIP. The His^1 residue appears particularly important, with the magnitude of the perturbation of this residue dramatically influencing analog potency. Acetylation of the α-amino group of His^1 resulted in an analog with greater potency than substitution of His^1 with the large, hydrophobic Phe residue. These both were more potent than the [D-His^1]VIP, and the least affinity is seen when His^1 is deleted (i.e. VIP(2-18). Substitution of Ser^2 with a positively charged Arg residue or the hydrophobic Phe residue also decreases affinity for the VIP receptor. When the affinities of these analogs to the anterior pituitary VIP receptor is compared to that exhibited towards other rat tissues (Table 13), it becomes obvious that their rank order of potency is the same in brain and pituitary, with only minimal differences in the liver. Very similar rank orders have been observed in lung (344). In contrast, the vascular VIP receptor displayed a different sequence of affinity, with [D-Ala^4]VIP among

the least potent analogs tested compared to being one of the most potent in other tissues. The vascular receptor also discriminated between [D-His^1]VIP and [Phe^1]VIP whereas the others did not. The two VIP antagonists examined demonstrated low affinity in all tissues, with no discrimination between tissues, or between male or female derived anterior pituitary membranes. The difference in selectivity of the vascular receptor becomes interesting when considering the inability of secretin to cross react with these receptors (315,316). It may be that the vascular VIP receptor is more sensitive to the postulated beta turn structure of the first 4 amino terminal residues (341), reflected in the lower affinity of peptides differing at amino acid residue 4, i.e. [Ala^4]VIP and secretin. Whether the local tissue membrane environments confer distinct recognition properties or alterations in molecular structure exist, it is tempting to speculate that these differences could represent VIP receptor subclasses. Heterogeneity in the molecular sizes of VIP binding sites has been identified (350-353), however further molecular characterization will be necessary to clarify the issue of VIP receptor subclasses.

Significant differences exist between VIP receptors in normal anterior pituitary tissue compared with those found in two different subclones of the PRL secreting GH cell lines. Two binding sites are identified in both normal tissue and GH_4C_1 cells (325), whereas GH_3 cells have only VIP receptor (324). Binding of this single site is considerably less (K_D of 17 nM) than for the high affinity site in the other two tissues, and is quantitatively similar to the low affinity VIP site identified in normal anterior pituitary tissue (K_D of 28 nM). Even in the tissues with two binding sites the K_D values differ ten fold. Specificity also differs, as rGRF does not recognize the GH_4C_1 cell VIP receptor, while this was amongst the most potent of the homologous peptides examined in normal tissue. All tissues display inhibition of VIP binding by pPHI, however the absolute potencies vary with VIP being 1.3, 16 and 560 times more potent than pPHI in GH_3, GH_4C_1 cells and normal rat anterior pituitary tissue respectively. These observed differences may reflect changes in the VIP receptor secondary to neoplastic transformation or culture conditions of the GH cell lines (282), heterogeneity of normal anterior pituitary tissue, or assay conditions such as the use of whole cell versus

subcellular fractions (299,350). These findings illustrate some limitations that exist in extrapolation from transformed to normal tissue.

The dose dependent stimulation of PRL by VIP in this study agreed with previous investigations (267-272), and potency of this effect corresponded well with the affinity of VIP binding to anterior pituitary membranes. The rank order of PRL release in response to VIP homologous peptides, i.e. VIP>rPHI>secretin>glucagon, agreed with the sequence observed for VIP binding to the anterior pituitary. At low concentrations, rPHI and pPHI have been observed to stimulate less PRL release compared with similar doses of VIP (271,381). This difference is obliterated at high concentrations of the peptides, and agrees with the present finding that although less potent than VIP, all PHI peptides tested can effectively compete with [Tyr(^{125}I)^{10}]VIP binding when they are present at high concentration (271). Micromolar levels of secretin are needed to stimulate PRL release, and glucagon may or may not be active at similar levels (266). This finding is in agreement with their low affinity for the VIP receptor in this study. The excellent correlation in rank order between binding of the structural analogs of VIP and their ability to stimulate anterior pituitary adenylate cyclase (343), supports coupling of the VIP receptor identified by radioligand binding to the known effector system of VIP (376).

The observation in some studies that PHI may be more (384) or equipotent (271) to VIP could conceivably be explained by the various conditions of PRL release assay employed during investigation, as conditions of culture and bioassay in the present study, were found not only to influence basal PRL release but also that stimulated by VIP. Various conditions could conceivably favour the actions of different peptides. These discrepancies with the in vitro PRL release assay emphasize the usefulness of another investigative technique, i.e. radioligand binding. The preferential stimulation by VIP of newly synthesized versus stored PRL (11), might account for the nonlinear increase in VIP stimulation of PRL over basal release, seen between 1 and 5 hours.

Association of [Tyr(^{125}I)^{10}]VIP binding to lactotrope enriched fractions agrees with a direct effect of VIP on PRL secreting cells to effect release of this hormone (268,270-

273). The presence of VIP receptors on lactotropes supports the observation that endogenous VIP could act in an autocrine fashion to mediate PRL secretion (251). The binding of [Tyr(^{125}I)^{10}]VIP to fractions not enriched in PRL containing cells is likely due to residual lactotropes in those fractions. Alternately, as previously discussed, this binding may reflect cross reactivity with another receptor such as GRF. The lack of binding to fractions containing primarily red blood cells is in agreement with that found by Wiik et al. (314).

The methodological studies performed in this study also provide some information regarding the nature of the anterior pituitary VIP receptor. As in rat intestine (335) and brain (331), GH_3 cells (325), and lung from several species (353), exposure of rat anterior pituitary tissue to trypsin abolishes VIP receptor binding with no effect on nonspecific binding. The protein nature of the anterior pituitary binding site is further supported by abolition of binding after subjection to collagenase digestion. This susceptibility to metallo proteinases, i.e. collagenase, of which several have been reported in the anterior pituitary (446), might account for the beneficial effects of EDTA during anterior pituitary receptor preparation. The beneficial effects of bacitracin during binding are harder to define as diverse actions of this peptide have been described (447). Decreased degradation or adsorption of VIP during assay has been postulated (267), however the interaction of bacitracin with membrane lipids (447), coupled with enhanced membrane labelling of ^{125}IVIP in its presence, suggests this peptide/antibiotic could function in more complex manner, such as alteration of the VIP receptor environment. The ability of bacitracin to bind divalent cations, such as are needed to activate metallo proteinases (447), may also contribute to the enhanced binding in its presence, and explain the lack of effect of EDTA when included with bacitracin during the binding assay. The addition of bacitracin alone has no effect on PRL release from rat anterior pituitaries (267). (267). The pH effect on VIP binding to the anterior pituitary has been observed in other VIP containing tissues (331,332,303). It is interesting to speculate that the increase in specific binding seen under slightly alkaline conditions may be secondary to altered reactivity of the amino terminal His^1, whose functional groups, i.e. an α-amino and an imidazole group, have pK_a values of 7.59 and 7.88

respectively (342). A similar effect has been observed for glucagon (448), and the required integrity of the amino terminal His^1 in binding (341) and adenylate cyclase activation would support a role for the reactivity of these two groups involved in binding of VIP to its receptor. The increase in binding seen in the presence of magnesium supports coupling of the anterior pituitary receptor to GTP binding proteins (296,331,297).

Altered anterior pituitary responsiveness to VIP secondary to regulation of its receptor appears probable in light of the changes observed in this study. Regulation of their receptors with age has been described for numerous hormones, including glucagon, thyroid, adrenalin, LH and insulin (451). The postnatal appearance of the high affinity site for VIP is particularly interesting. As this receptor is likely involved in PRL secretion in adult animals, it is possible to speculate that development of the VIP receptor reflects some control in the release of this hormone in the rat. Indeed, a marked decrease in responsivity to VIP of PRL release from lactotrope enriched populations in 2 week old as compared to adult animals has been described (273). The inability of stress or serotonin, thought to act via VIP (122,123,54) to increase PRL secretion prior to 3 weeks of age in the rat also supports a role for this high affinity receptor. Disparity in the time of lactotrope development is observed between species (57), and has also been reported in rodents (205). In the rat, anterior pituitary PRL has been detected as early as day 16 of gestation (453,58), or not until after birth (60). Regardless of when first detectable, anterior pituitary levels of PRL remain low after birth until 2-3 weeks of age and then peak to adult levels around day 40 (367-369). Circulating levels of PRL parallel this increase in pituitary PRL content, with minimal amounts detectable at birth, and increasing values observed after 2 weeks, peaking to adult levels between 27 to 50 days of age (58-60). This correlation between increases in pituitary and circulating levels of PRL, and the first appearance of the high affinity VIP receptor between 2 and 3 weeks of age, intimates that the presence of the receptor may be necessary for PRL secretion. The demonstration of adult levels of hypothalamic VIP in rats as early as 15 days of age suggests sufficient levels are available to act at the receptor (369,371).

Several interpretations as to the role of VIP acting via this receptor during the postnatal development of lactotropes and PRL secretion can be entertained. In contrast to intestine and liver (165,166), high affinity VIP receptors are not present prior to cell maturation, as PRL is detectable prior to appearance of the high affinity binding site. This would suggest a mature cell is required to express the VIP receptor, and that VIP may not be involved in lactotrope differentiation. An opposing argument can be made that VIP may be involved in the differentiation of mammosomatotropes to lactotropes. Using the sequential reverse haemolytic assay the majority of PRL secreting cells at 5 days of age also secrete GH (449), in contrast to very few cosecretors detectable in the adult animal. Closer analysis of the time period that this change occurs relative to the development of the VIP receptor, still needs to be addressed. Another intriguing potential role for VIP could be reversal of the translational block in PRL mRNA that has been detected in the neonatal rat (71). The inability of the majority of PRL containing cells to spontaneously secrete PRL until after 20 days of age (63), corresponds well with the appearance of the high affinity receptor and lends support to the premise that VIP functions as an autocrine factor to mediate basal PRL release (250,251). As the role for the low affinity VIP receptor is not defined, putative actions during development are even more speculative for this binding site. The earlier responsivity to regulatory factors of other anterior pituitary hormones, e.g. GH (450) and TSH (375), would allow cross reaction with other peptide receptors. Alternately, this site could play a morphogenetic role, with the high affinity site representing a switch in function for VIP postnatally (451). Interestingly for the other major PRF, TRH, very poor correlation in development of hypothalamic levels, binding to the pituitary and ability to stimulate TSH is seen with developmental changes in PRL in the rat (452).

The insufficiency of withdrawal of dopamine blockade to mediate PRL rises seen during suckling/lactation (35,186), indicates PRFs may be involved. Stimulation by TRH is operational (284), but the relative role of these and other agents has not been satisfactorily resolved. The literature regarding VIP action during suckling indicates an effect on both the acute rise and on the maintenance of PRL release during suckling (54) mediated via a

neuroendocrine reflex (49,50) likely involving serotonin. The decrease in high affinity VIP receptors in this study may reflect down regulation of the VIP receptor after the stimulus of suckling. The difference in binding of VIP becomes more marked when considered on a per gland basis, and as increased anterior pituitary size during lactation is secondary to increases in lactotrope number as well as size (69), this reduction in binding presumably reflects less VIP binding sites per PRL secreting cell. This decrease could reflect ligand stimulated down regulation or internalization of the VIP receptor (362), as anterior pituitary glands were removed shortly after suckling. VIP stimulation of PRL may be regulated more at the level of the peptide rather than via the receptor as increases in hypothalamic VIP mRNA, in both the PVN and the SCN (53) have been observed during lactation in the rat. In contrast to the PVN (49), the SCN appears not to be directly involved in lactation (35), hence VIP may have indirect as well as direct effects on PRL release.

Castration of male animals is most frequently associated with decreased levels of PRL (43,151,153) although increases (154) and no effect (152) have been seen. These discrepancies may reflect the multitude of factors operational in an in vivo system, as variable effects of castration on PRL are seen in the face of different levels of FSH, estrogens and testosterone (152). Intuitively the increase in binding of VIP after castration would suggest increases in PRL secretion should occur if VIP functions as a PRF. As the increase in VIP binding subsequent to castration involved a greater increase in the number compared to the decrease in receptor affinity, the increase in binding per quantity of protein can be explained. Altered coupling of the VIP receptor to its effector system could plausibly explain this discrepancy between the increase in binding and the decrease in PRL secretion. Such incongruity between ligand binding and coupling to the adenylate cyclase system has been observed in the well studied β-adrenergic receptor (453). Castration also affects the prostatic VIP receptor, without a concomitant change in the ability of VIP to stimulate adenylate cyclase in this tissue (357,358). The rapidity, i.e. within one day, of change after castration, in the high affinity VIP receptor in male rats has also been observed in the anterior pituitary GnRH receptors (454). The effects of castration were not secondary to

anaesthetic administration and or surgical stress, both conditions associated with elevations of VIP (245). In contrast testosterone was able to prevent the changes induced in this receptor, in a fashion similar to that seen in the GnRH receptor (454). The decline in testosterone after orchidectomy in rats to less than 4% of normal within 2 hours, and to undetectable levels by 1 day (455), defends the possibility that deficiency of this steroid could account for the changes in the VIP receptor. In concert with the anterior pituitary GnRH receptor, additional increases in binding were seen during the second week after castration (454), in the VIP receptor affecting only the low affinity site however.

Testosterone has been shown to influence the number and/or the affinity (456-458) of different receptors in various tissues. The mechanism by which this is accomplished is however unclear. In the anterior pituitary, morphological changes in lactotropes has been observed secondary to testosterone administration (156). Changes in membrane fluidity as has been observed secondary to testosterone in other tissues (357,358), could also be operational in the anterior pituitary, thereby potentially influencing the binding of VIP to its receptor as well as coupling to adenylate cyclase. Phospholipids play a very important role in the interaction of VIP with its membrane receptor (457), and as testosterone is capable of regulating the biosynthesis of lipids including phospholipids (357) this could be a potential mechanism by which changes are exerted. Direct receptors for testosterone are present in the anterior pituitary, however their localization primarily to the gonadotrope (157), suggests that the ability of this steroid to affect VIP binding may be an indirect one. The need to co-culture gonadotropes with lactotropes in order to see the response in PRL secondary to GnRH administration in vitro (158,165), would be compatible with an effect by testosterone on the gonadotrope influencing PRL secretion via a paracrine mechanism. Paracrine factor(s) from the gonadotrope (165) could potentially relay this effect by changes in the lactotrope VIP receptor. The inability of dihydrotestosterone to stimulate PRL secretion in vitro (153), despite the presence of 5α-reductase in the anterior pituitary, suggests that testosterone may influence. PRL release and potentially the VIP receptor only by aromatization to estrogen (153). Estrogens are definitely involved in the regulating PRL secretion in the female

(143-146), and effects of this steroid on VIP binding in the genital tract has been observed (361). The effect of estrogen on VIP binding in the anterior pituitary is not known.

Regardless of whether a direct or indirect mechanism is operational, it is possible to speculate that the sexual differences in PRL secretion (35), including postnatal development, may be secondary to changes at the level of the anterior pituitary VIP receptor. The observation that orchidectomy of rats attenuates PRL pulse peaks and amplitudes, and in a delayed fashion frequency (155), in a manner analogous to that produced by VIP antiserum when uncovered from the overriding effect of dopamine (264) intimates a similar effect on the fine tuning in of PRL secretion.

In conclusion, these studies demonstrate the presence of highly specific anterior pituitary receptors for VIP that localize to the lactotrope. Their regulation by guanine nucleotides and testosterone, as well as during postnatal ontogenesis, supports an integral function for the VIP receptors in the secretion of PRL from the anterior pituitary gland.

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