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EFFECT OF VASOACTIVE INTESTINAL PEPTIDE (VIP) AND OTHER PEPTIDES ON cAMP ACCUMULATION IN RAT BRAIN

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Abstract—The effects of a number of peptides which have recently been demonstrated to be present in brain, were determined on adenylate cyclase activity in homogenates and on cAMP and cGMP levels in rat brain slices. Substance P, luteinizing hormone releasing factor, thyrotropin releasing factor, somatostatin, glucagon, and neurotensin were without effect in any of these tests. When slices from a number of regions of rat brain were incubated in the presence of 0.5 μM vasoactive intestinal peptide (VIP), a significant increase in the accumulation of cAMP over basal values was observed. There were no changes in cGMP levels. VIP also caused an increase in cell-free adenylate cyclase activity of striatal, cortical and hippocampal homogenates, and this response was considerably increased in the presence of guanylyl-imidodiphosphate (GMP-PNP). The phosphodiesterase inhibitor isobutylmethylxanthine caused a 3 to 6-fold increase in basal levels of cAMP in brain slices, but VIP was still able to elicit a further increase, indicating that its effects on cAMP accumulation were probably due to activation of adenylate cyclase. The increase in cAMP in cortical and hypothalamic but not striatal slices was affected by alterations in the calcium concentration of the incubation medium. When tissue slices were incubated in the presence of VIP and a variety of antagonist drugs (propranolol, phenoxybenzamine, α-flupenthixol, naloxone), no alteration in the VIP induced increase in cAMP was observed. Furthermore, when VIP was incubated in the presence of agonists (dopamine, noradrenaline, isoproterenol, prostaglandin E_1, adenosine), the induced increase in cAMP levels was additive to that caused by VIP. These results support a role for VIP as a neuromodulatory or neurotransmitter compound in the central nervous system, (CNS), mediating its action through the adenylate cyclase/cAMP system.

Evidence suggests that peptides may have neurotransmitter or neuromodulatory roles in the central nervous system (CNS). This includes such peptides as luteinizing hormone releasing factor (LHRH), thyrotropin releasing factor (TRH), somatostatin, substance P, neurotensin, and more recently vasoactive intestinal peptide (VIP) which was initially isolated from the gastrointestinal tract [1, 2] and subsequently detected in brain [3–5]. Evidence for this hypothesis stemmed initially from studies in which the peptides were observed to have effects on behaviour [6–9]. Further evidence from biochemical experiments has demonstrated (1) their selective distribution in brain [10–13], (2) localization in nerve terminals [13–17] and (3) release from nerve endings [18, 19].

The postsynaptic actions of a number of neurotransmitters may be mediated through alterations in the intracellular concentration of adenosine-3′,5′-monophosphate (cAMP) in target cells [20, 21]. Furthermore, some of the peptides present in CNS have been reported to stimulate adenylate cyclase activity [22, 23]. Therefore, the actions of a number of CNS peptides were determined on cAMP and cGMP accumulation in rat brain slices and adenylate cyclase activity in brain homogenates.

MATERIALS AND METHODS

Male Sprague–Dawley rats (fed *ad lib.* and weighing approximately 250 g) were killed by decapitation and the brains rapidly removed and placed on ice. Brain regions were dissected using the method of Glowinski and Iversen [24] and Jessell and Iversen [25].

Slices were prepared and incubated according to the method of Forn *et al.* [26]. The tissue was cross-chopped at 260 μm × 260 μm intervals at an angle of 45° with a McIlwain tissue chopper. The slices were suspended in Krebs–Ringer bicarbonate (NaCl 124 mM, KCl 5 mM, $CaCl_2$ 0.8 mM, $MgCl_2$ 1.3 mM, KH_2PO_4 1.4 mM, glucose 10 mM and $NaHCO_3$ 26 mM; gassed for 10 min with 95% O_2 and 5% CO_2 to obtain a final pH of 7.4) and incubated at 37° in a shaking water bath with two buffer changes. After 1 hr, aliquots (approximately 10 mg tissue wet wt) were removed and added to tubes containing Krebs–Ringer bicarbonate, with or without drugs, in a total volume of 250 μl. After a 10 min incubation, the reaction was stopped by boiling. An aliquot of the supernatant was removed for the measurement of cAMP using the protein binding assay of Brown *et al.* [27], in which an extract from bovine adrenal cortex is used as the source of the binding protein. The assay was linear for standards of cAMP in the range of 0.2–10.0 pmoles. The remainder of the

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supernatant was used for the determination of cGMP by radioimmunoassay according to Minneman and Iversen [28]. The specificity of the cGMP antiserum prepared in rabbit was checked with cAMP, ATP, GTP and GDP all of which failed to cross react with cGMP when present in a 1000-fold molar excess. The lower limit of sensitivity of the radioimmunoassay was approximately 0.1–0.2 p-moles cGMP/assay sample; the dilution of antiserum used was 1 : 3000.

Adenylate cyclase determinations were carried out by the method of Kebabian *et al.* [29]. Tissue was homogenized in 25 vol. 2 mM Tris maleate (pH 7.4) containing 2 mM EGTA. Fifty μl aliquots of this homogenate were added to assay tubes containing 250 μl of buffer consisting of 80 mM Tris maleate (pH 7.4), 2 mM $MgSO_4$, 10 mM theophylline and 0.2 mM EGTA plus the drugs as indicated. The tubes were incubated with shaking at 30° for 2.5 min and then transferred to a boiling water bath, followed by centrifugation to sediment the denatured protein. Ten μl aliquots of the supernatant solution were taken for analysis of cAMP by the method of Brown *et al.* [27].

Protein was determined by the method of Lowry *et al.* [30]. Statistical comparisons were made by Student's '*t*' test; P values of less than 0.05 were considered significant.

RESULTS

The effect of peptides on cAMP and cGMP accumulation in tissue slices. Table 1 shows that, of the peptides tested, VIP was the only one which elicited a significant increase in cAMP accumulation in hypothalamic slices; none of the peptides altered cGMP levels in slices. The releasing factors LHRH, TRH and somatostatin were also tested at several other concentrations ranging from 10^{-8} to 10^{-4} M without effect. Substance P failed to cause an increase in cortical, striatal, nigral and spinal cord slices from 10^{-8} to 10^{-5} M when using a range of incubation times from 2 to 15 min; no stimulation of adenylate cyclase activity by substance P in homogenates was observed under similar conditions. Neurotensin, glucagon and ACTH were tested at 10^{-6} to 10^{-4} M in cortical and striatal slices, as well as hypothalamic slices, without effect. In an attempt to inhibit degradation of the exogenous peptides by peptidases present in the slices, bacitracin, a peptidase inhibitor, was added to the assay tubes in some experiments; however, no increase in cAMP was observed in its presence.

Table 1. Effect of peptides on cAMP and cGMP accumulation in hypothalamic slices

Peptide	Conc. (μM)	% of Basal cAMP	% of Basal cGMP
VIP	0.5	210±29†	103±10
LHRH	10	116±19*	109±29
TRH	10	81±7*	93±23
Somastostatin	10	90±6*	85±5
Substance P	10	93±18*	95±6*
Neurotensin	10	102±6	NT
Glucagon	10	114±8	NT
ACTH	10	126±10	NT

Slices for the determination cAMP and cGMP accumulation were prepared as described in Methods; they were then incubated in the presence and absence of the above peptides at the indicated concentrations. Basal values for cAMP and cGMP were 11.3±0.9 and 0.18±0.02 pmoles/mg protein, respectively. Each value represents the mean±S.E. of four to eight determinations.

* Done in the absence and presence of 30 μg/ml bacitracin. NT—Not tested.

Significance of difference from basal: † $P<0.01$.

Table 2. Effect of 0.5 μM VIP in different brain regions

Region	Peptide	pmoles cAMP/mg protein	% Control
Hypothalamus	VIP	23.95±3.30†	210
	Basal	11.40±1.15	100
Striatum	VIP	3.35±0.30*	209
	Basal	1.60±0.14	100
Cerebral cortex	VIP	24.10±1.75†	169
	Basal	14.20±0.90	100
Hippocampus	VIP	5.65±0.60‡	141
	Basal	4.00±0.41	100
Cerebellum	VIP	79.80±5.50	119
	Basal	67.10±2.30	100
Brain stem	VIP	9.95±1.05†	195
	Basal	5.10±0.43	100
Thalamus	VIP	26.85±3.21†	211
	Basal	12.75±1.38	100

Regions were dissected as described and the cAMP accumulation in the slices determined. Each value represents the mean±S.E. of four to eight determinations. Significance of difference from basal; * $P<0.001$; † $P<0.01$; ‡ $P<0.05$.

Differential effect of VIP on cAMP accumulation in rat brain regions. The stimulation of cAMP accumulation by VIP (0.5 μM) was of a similar magnitude in hypothalamus, thalamus, striatum and brainstem (Table 2). Smaller increases were observed in the cerebral cortex and hippocampus and there was no significant effect of VIP on cAMP accumu-

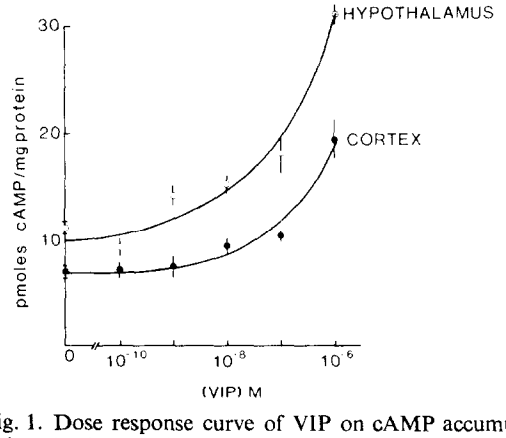

Fig. 1. Dose response curve of VIP on cAMP accumulation in hypothalamic and cortex slices. Slices were prepared as described in Methods and were then incubated for 10 min in the presence of various concentrations of VIP. Each value represents the mean±S.E. of four to eight experiments.

Table 3. Effect of 1 μM VIP on adenylate cyclase activity in homogenates in the presence and absence of GMP-PNP

Region	VIP	cAMP (pmoles/mg protein) −GMP-PNP	cAMP (pmoles/mg protein) +GMP-PNP
Striatum	−	47.3 ± 1.5	36.3 ± 1.9
	+	52.6 ± 2.1 (111%)	46.1 ± 2.2†(127%)
Hippocampus	−	20.1 ± 1.5	35.5 ± 4.0
	+	28.5 ± 1.1*(142%)	64.9 ± 3.5*(183%)
Cortex	−	31.0 ± 1.6	60.4 ± 2.8
	+	38.2 ± 1.0†(123%)	106.0 ± 6.3*(175%)
Hypothalamus	−	46.3 ± 1.4	81.3 ± 5.6
	+	50.6 ± 1.7(109%)	77.4 ± 3.8 (95%)
Cerebellum	−	15.7 ± 1.0	16.8 ± 3.0
	+	17.7 ± 1.7(113%)	21.5 ± 2.9(128%)

The adenylate cyclase assay was done as described in Methods. Prior to the addition of the ATP the incubation mix containing the homogenate and/or VIP and/or GMP-PNP was left to stand for 30 min. Each value represents the mean ± S.E. of six to twelve determinations. Significance of difference from basal (no VIP): *$P<0.001$; †$P<0.01$.

lation in the cerebellum. A dose response curve for VIP on cAMP accumulation in cortex and hypothalamus showed that significant increases in cAMP occurred at concentrations of VIP as low as 10 and 1 nM, respectively (Fig. 1).

The effect of VIP on cell-free adenylate cyclase activity. Homogenates of a number of brain regions were prepared and the adenylate cyclase activity determined in the absence and presence of 1.0 μM VIP (Table 3). A small but statistically significant increase was observed in homogenates of the hippocampus and striatum but no effect was seen in cortex, hypothalamus and cerebellum. Other investigations have shown that incubation in the presence of guanine nucleotides can lead to a marked stimulation of hormone induced adenylate cyclase [31]. When the guanine nucleotide GMP-PNP was added, alterations in basal adenylate cyclase activity were observed together with a potentiation of the VIP induced increase (Table 3).

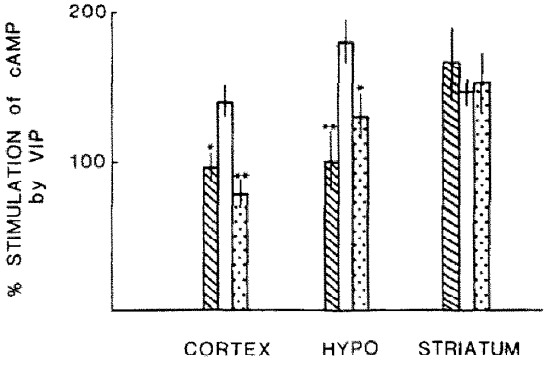

Fig. 2. Effect of Ca^{2+} on the VIP induced increase in cAMP accumulation in tissue slices. Cortical, hypothalamic (hypo) or striatal slices were preincubated in Krebs bicarbonate. Aliquots of the slices were then added to tubes containing no $CaCl_2$ + 0.5 mM EGTA (hatched bars), 0.8 mM $CaCl_2$ (normal Krebs) (open bars) or Krebs containing 4.0 mM $CaCl_2$ (dotted bars) with or without 0.5 μM VIP. The procedure was then continued as described in Methods. Control values (in pmoles cAMP/mg of protein) were 6.39 ± 0.69 for cortex, 13.26 ± 1.78 for hypothalamus and 4.44 ± 0.33 for striatum. Each bar represents the mean ± S.E. of 10 experiments. Significance of difference from Krebs containing 0.8 mM $CaCl_2$; ** $P<0.01$; * $P<0.05$.

Effect of alterations in calcium concentration on the VIP induced increase in cAMP in hypothalamic and cortex slices. Slices were preincubated for 1 hr at 37° in normal Krebs bicarbonate buffer pH 7.4. At the end of this time, 10 mg aliquots of the slice suspension were added to tubes containing Krebs with no $CaCl_2$ plus 0.5 mM EGTA, to tubes containing normal Krebs (0.8 mM Ca^{2+}) or to tubes containing Krebs with 4.0 mM $CaCl_2$ (Fig. 2). The maximal response to VIP was obtained in 0.8 mM $CaCl_2$ for cortical and hypothalamic slices; no alterations in cAMP accumulation were observed in striatal slices.

Effect of the phosphodiesterase inhibitor isobutylmethylxanthine on the VIP induced increase in cAMP accumulation in slices. When striatal, hypothalamic or cortical slices were incubated in the presence of 2 mM isobutylmethylxanthine, the percentage increases in cAMP accumulation elicited by VIP were similar to those observed in its absence (Table 4). There was a 3 to 6-fold increase in basal cAMP levels in the slices in the presence of isobutylmethylxanthine, indicating effective phosphodiesterase inhibition.

Effect of antagonists and agonists on the VIP induced increase in cAMP accumulation in tissue slices. To determine whether the effects of VIP on cyclic AMP accumulation were mediated indirectly through previously defined receptors in brain, the effect of various antagonists was tested on the VIP-induced increase in cAMP in striatal, hypothalamic and/or cortex slices. α-Flupenthixol (1 μM) or fluphenazine (50 μM), dopamine receptor blockers, had no effect on cAMP levels after VIP (0.5 μM) (Fig. 3). Naloxone (1 μM), an opiate receptor blocker, phenoxybenzamine (50 μM), an α-adrenoceptor blocker, or propranolol (10 μM), a β-

Table 4. Effect of the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX) on the VIP induced increase in cAMP accumulation in brain slices

Region		No IBMX		2 mM IBMX	
		cAMP	% control	cAMP	% control
Striatium	VIP	7.0 ± 0.7*	248	40.5 ± 4.4*	227
	Basal	2.8 ± 0.3	100	17.9 ± 0.7	100
Hypothalamus	VIP	13.9 ± 1.4*	201	48.0 ± 4.1*	187
	Basal	6.9 ± 1.0	100	25.6 ± 1.8	100
Cerebral cortex	VIP	15.7 ± 1.3†	144	43.7 ± 3.7†	133
	Basal	10.8 ± 1.5	100	32.8 ± 1.7	100

Slices were prepared as described and then incubated in the absence and presence of 0.5 μM VIP. Each value represents the mean ± S.E. of five determinations. Significance of difference from basal: *$P < 0.001$; †$P < 0.01$.

adrenoceptor blocker, were similarly without effect on the VIP-induced increase in cAMP accumulation in the three types of slices tested.

In a further attempt to characterize the receptor involved in the stimulation of cAMP by VIP the interactions of various agonists known to cause increases in cAMP levels with VIP were measured. Figure 4 shows that the effects of 10 μM noradrenaline and 0.5 μM VIP from all three regions were additive on cAMP accumulation in tissue slices. Similar results were observed with 100 μM dopamine, 5 μM prostaglandin E_1, 1 μM isoprenaline and 50 μM adenosine; in each case, the increase induced by these compounds was additive with the VIP induced increase.

DISCUSSION

Vasoactive intestinal peptide, a 28-residue peptide originally isolated from the gastro-intestinal tract [1, 2] and subsequently found to be present in CNS, was the only peptide of the series tested which stimulated the accumulation of cAMP in rat brain slices or homogenates. The cAMP response to VIP in slices varied among different brain regions, with the largest increase in the hypothalamus and striatum and no effect in the cerebellum. Because the regional distribution of VIP has not yet been determined in rat brain, it was not possible to correlate the per cent increases in cAMP with the regional levels of VIP.

Recently it has been demonstrated that hormone stimulated activation of adenylate cyclase could be potentiated in the presence of guanine nucleotides [31]. Exposure of the brain homogenates to guanylylimidodiphosphate was also found to facilitate the VIP induced stimulation of adenylate cyclase in several brain regions. The finding that VIP still caused an increase in tissue cAMP in the presence of the phosphodiesterase inhibitor isobutylmethyl-

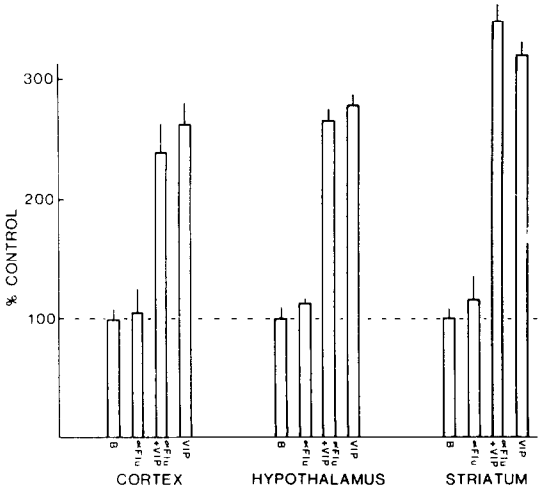

Fig. 3. Effect of α-flupenthixol on the VIP-induced increase in the cAMP accumulation in tissue slices. Slices were prepared as described in Methods and were then incubated in the absence or presence of the drugs as indicated. Each value represents the mean ± S.E. of four to eight experiments. Results are expressed as per cent control. Control values (B = basal; in pmoles cAMP/mg of protein) were 9.8 ± 0.9 for cortex, 10.4 ± 1.1 for hypothalamus and 2.3 ± 0.2 for striatum. The concentration of VIP was 0.5 μM and α-flupenthixol (α-Flu) was 1 μM.

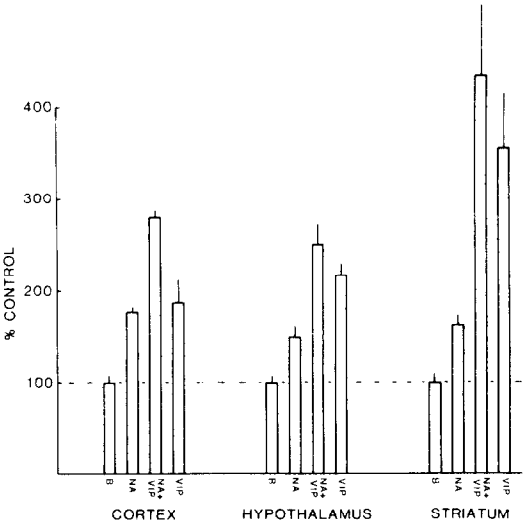

Fig. 4. Effect of agonists on the VIP-induced increase in cAMP accumulation in tissue slices. Legend as for Fig. 3. Control values (in pmoles cAMP/mg of protein) were 9.3 ± 0.8 for cortex, 8.6 ± 0.5 for hypothalamus and 3.1 ± 0.3 for striatum. The concentration of VIP was 0.5 μM and noradrenaline (NA) was 10 μM.

xanthine, and that VIP activated cell-free adenylate cyclase activity in homogenates suggests that the effects of VIP are probably due to stimulation of adenylate cyclase rather than to a decrease in cyclic nucleotide phosphodiesterase activity.

It has been demonstrated that there is a calcium-dependent regulation of adenylate cyclase by a calcium binding protein. Low calcium concentrations increase the accumulation of cAMP in intact cells while high concentrations are inhibitory [32–34]. Furthermore, Schwabe and Daly [35] demonstrated a decreased accumulation of cAMP in cortical slices after α-adrenergic agonists in the presence of EGTA. Thus, induced increases in the accumulation of cAMP might be expected to depend on the calcium concentration in the medium. This appears to be the case with the VIP induced increase in cAMP in hypothalamic and cortical slices, although a different mechanism may be involved in striatal slices.

In order to determine whether VIP was mediating its effect possibly through dopaminergic, adrenergic or opiate receptors, the effects of various antagonists were tested on the VIP induced increase in cAMP accumulation in slices; however, no blockade was observed in any case. Similarly when VIP was incubated together with various other agents known to stimulate adenylate cyclase activity the increases in cAMP levels were additive. Therefore a receptor, different from others known to be linked to cerebral adenylate cyclases appears to be involved in the VIP induced increase in cAMP.

These results further support a role for VIP as a neurotransmitter or neuromodulator in the CNS and suggests that, as in the periphery, its central actions may be mediated through an adenylate cyclase/cAMP system.

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