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Vasoactive intestinal polypeptide (VIP)-sensitive adenylate cyclase in rat brain: regional distribution and localization on hypothalamic neurons†

R. W. KERWIN*, SARAH PAY, K. D. BHOOLA AND C. J. PYCOCK

Department of Pharmacology, Medical School, University of Bristol, Bristol BS8 1TD

The regional distribution of the vasoactive intestinal polypeptide (VIP)-sensitive stimulation of adenylate cyclase activity was studied in homogenates of rat brain. The order of sensitivity of this peptide on enzyme activity was: olfactory bulb > hippocampus > thalamus > occipital cortex ≃ frontal cortex ≃ midbrain ≃ hypothalamus > striatum > cerebellum ≃ brain stem > spinal cord. With neurotoxin-induced lesions the possible location of VIP-sensitive adenylate cyclase within the anterior hypothalamus was investigated. Intrahypothalamic 6-hydroxydopamine blocked the stimulatory effect of VIP on hypothalmic adenylate cyclase, but neither kainic acid nor 5,7-dihydroxytryptamine lesions had any effect. Similarly, there was a 71% reduction in the sensitivity of VIP-sensitive adenylate cyclase activity in animals whose hypothalamic noradrenergic innervation had been interrupted by transection of the ascending dorsal and ventral bundles. Biochemical analyses of the lesions suggested that loss of VIP action on adenylate cyclase was associated with loss of hypothalamic noradrenaline-containing neurons. These results may give insight into those regions of rat brain where VIP may have a neurotransmitter role, and point to a possible noradrenergic localization of the VIP-sensitive adenylate cyclase within the hypothalamus.

Originally characterized as an intestinal peptide (Said & Mutt 1970), vaso-active intestinal peptide (VIP) is also found in the central nervous system (c.n.s.) (Said & Rosenberg 1976) but its possible physiological role there is not known. This octacosapeptide binds specifically and with high-affinity to guinea-pig brain synaptic membranes (Robberecht et al 1978, 1979). It is concentrated in synaptosomal fractions of brain tissue (Giachetti et al 1977) and can be released from these (Giachetti et al 1977) and brain slices (Emson et al 1978) by depolarizing stimuli in a calcium-dependent fashion. VIP has been shown to stimulate adenylate cyclase activity in guinea-pig brain preparations (Deschodt-Lanckman et al 1977). Although some knowledge of the regional distribution of this enzyme is known in rat brain (Quik et al 1978), detailed knowledge of the distribution of the peptide itself in rat brain has only recently been demonstrated (Besson et al 1979). We have therefore extended observations on the regional distribution of VIP-sensitive adenylate cyclase activity in homogenates from various areas of the rat brain.

The hypothalamus is considered to be a site where VIP may be an important neuropeptide (Emson et al 1978). Other studies investigating the origin of hypothalamic VIP have shown that in addition to intrinsic VIP, a relatively high proportion of the peptide (> 40%) is present in nerve endings originating from neuronal cell bodies located outside the hypothalamus (Besson et al 1979). Using neurotoxin and lesion techniques, we have investigated upon which neuronal elements within the hypothalamus the VIP-sensitive adenylate cyclase may reside. Enzyme activity has subsequently been measured following prior intrahypothalamic injections of the neurotoxins kainic acid, 5,7-dihydroxtryptamine (5,7-DHT), 6-hydroxydopamine (6-OHDA) and following transection of the dorsal ventral noradrenergic bundle.

METHODS

Preparation of animals for lesion studies

Male Porton rats, 200 ± 10 g, were anaesthetized with chloral hydrate (300 mg kg^{-1}, i.p.) and immobilized in a Kopf stereotaxic frame for the stereotaxic injection of neurotoxic substances. All neurotoxins were delivered bilaterally into the anterior hypothalamus (co-ordinates A + 1·0, L ± 1·0, V −8·6; Pellegrino & Cushman 1967). Three groups of animals were prepared. One group received 2 μg kainic acid (Sigma) bilaterally in 2 μl 50 mM phosphate buffer (pH 7·4). A second group received 8 μg 6-OHDA hydrobromide (Sigma) dissolved in

* Correspondence.

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2 μl 0·1% nitrogen-bubbled sodium metabisulphite solution. The third group of animals received 10 μg 5,7-DHT (Sigma) bilaterally also in 2 μl sodium metabisulphite solution. Control animals received injections of the appropriate vehicle alone. In one group of animals the noradrenergic innervation of the hypothalamus was interrupted by a stereotaxic knife cut (1 mm wide) of the ascending dorsal and ventral bundles placed bilaterally at the junction between brain stem and midbrain (coordinates A + 4·6, L ± 1·5, V −8·5; Pellegrino & Cushman 1967).

Biochemical characterization of lesion

Anterior hypothalamic tissue was taken using a modification of the dissection described by Cox et al (1978). A 2 mm block of tissue was removed representing anterior hypothalamus at the level of 0·5–2·5 mm anterior to bregma according to the atlas of Pellegrino & Cushman (1967). This tissue contains predominantly arcuate nucleus, anterior hypothalamus and anterior and medial preoptic nucleus. The tissue is inevitably contaminated with fibres of the lateral hypothalamus and median forebrain bundle and some mesolimbic structures such as nucleus accumbens and olfactory tubercle.

The extent of the lesions in the anterior hypothalamus induced by the neurotoxins was determined in animals one week after surgery by either measuring concentrations of hypothalamic transmitter substances or assessing high-affinity uptake of transmitter to indicate loss of pre-synaptic terminals. Accordingly, concentrations of dopamine and noradrenaline were determined by radioenzymatic assay (Cuello et al 1973) and 5-hydroxytryptamine (5-HT) by fluorimetric techniques (Curzon & Green 1970). High-affinity uptake of radiolabelled transmitters (final concentration 5×10^{-8} M) was measured into small (0·2 × 0·2 mm) hypothalamic slices (Shaskan & Snyder 1970; Kerwin & Pycock 1979) using [^{3}H]5-HT (sp. act. 15 Ci mmol^{-1}), [^{3}H]γ-aminobutyric acid ([^{3}H]GABA, 54 Ci mmol^{-1}, [^{3}H]noradrenaline (10·7 Ci mmol^{-1}) or [^{3}H]dopamine (5 Ci mmol^{-1}). For a study of noradrenaline uptake, 0·5 μM benztropine was present in the incubation medium in order to restrict uptake into dopaminergic terminals (Coyle & Snyder 1969): for [^{3}H]dopamine uptake 0·5 μM desmethylimipramine was included in the incubation medium to restrict uptake into noradrenergic terminals (Tassin et al 1974). (All radiochemicals were purchased from the Radiochemical Centre, Amersham). Results of the uptake experiments are expressed as d min^{-1} mg^{-1} weight wet tissue. Tests of statistical significance in biochemical parameters between lesioned and sham-operated groups utilized Student's *t*-test.

Preparation of enzyme

For a study of the regional distribution of VIP-sensitive adenylate cyclase unlesioned rats were used. Animals were killed by cervical dislocation and their brains rapidly removed: brain regions were dissected on ice using the method of Glowinski & Iversen (1966). For the lesion studies, anterior hypothalamic region from neurotoxin-injected or vehicle-injected controls was dissected out one week after surgery. Tissue was homogenized in 5 vol of 50 mM Tris HCl buffer (pH 7·4) containing 0·25 M sucrose, 5 mM $MgCl_2$, 25 mM KCl, 2 mM EGTA and 6 mM theophylline. After filtering through two layers of nylon mesh, the homogenates were centrifuged at 1600 *g* (4 °C, 10 min). The pellets were used as the enzyme source and were resuspended in 20 vol of homogenizing medium.

Determination of basal and VIP-stimulated adenylate cyclase activity

Adenylate cyclase activity was determined using a modification of the method of Albano et al (1973). The standard assay system contained in a final volume of 75 μl 1·5 mM ATP, 10 μM GTP, 3 mM $MgCl_2$, 10 mM NaCl, 10 mM KCl, 1 mM EGTA, 6 mM theophylline in 50 mM Tris. HCl buffer (pH 7·4) and was either with or without VIP. The reaction was started by the addition of 25 μl of enzyme preparation and incubated at 30 °C. In initial experiments reactions were carried out using enzyme prepared from whole rat brain (mesencephalon + diencephalon) at differing concentrations of VIP (Penninsula Laboratories, U.S.A.: 0·1–10 μM) and was incubated for various lengths of time (0·5–30 min). This synthetic source of VIP was considered to be highly pure since it gave a single peak on analysis by high pressure liquid chromatography. Routine reactions for the study of regional activity were incubated for 10 min using 2 μM VIP. Enzyme activity was terminated by placing the incubation tubes in a Grant heating block (100 °C) for 3 min. Following freezing and thawing, the samples were suspended in 1 ml of Tris HCl buffer (50 mM, pH 7·4) and centrifuged for 2000 *g* for 10 min. Fifty μl samples were taken for the determination of cyclic (c)AMP by the method of Brown et al (1971). Protein was measured by the method of Lowry et al (1951). All results are

expressed as pmol $cAMP^{-1}$ mg $protein^{-1}$ 10 min and are mean ± s.e.m. of quadruplicate determinations, each assayed in triplicate.

RESULTS

Extent of hypothalamic lesions

All three neurotoxic substances induced significant changes in hypothalamic transmitter function as indicated by reduction in transmitter concentrations and high-affinity uptake mechanisms reflecting loss of pre-synaptic terminals. Kainic acid produced a significant loss of hypothalamic dopamine concentration (sham, 0·92 ± 0·09 μg g wet $weight^{-1}$; lesion 0·26 ± 0·12 μg g wet $weight^{-1}$; n = 4, $P < 0·01$), but no significant change in either 5-HT concentrations (sham 0·58 ± 0·07 μg g wet $weight^{-1}$; lesion 0·52 ± 0·07 μg g wet $weight^{-1}$; n = 4) or noradrenaline concentrations (sham 0·48 ± 0·08 μg g wet $weight^{-1}$; lesion 0·41 ± 0·07 μg g wet $weight^{-1}$; n = 4). Such results were supported by a significant reduction of [^{3}H]-dopamine uptake (to 52% of sham, $P < 0·05$) (Fig. 1), but no changes in the high-affinity uptake of either [^{3}H]noradrenaline or [^{3}H]5-HT. In addition kainic acid significantly reduced [^{3}H]GABA uptake in the hypothalamus (to 43% of sham, $P < 0·01$) (Fig. 1).

6-OHDA induced marked falls in hypothalamic concentrations of both dopamine (sham 0·84 ± 0·28 μg g wet $weight^{-1}$; lesion 0·33 ± 0·12 μg g wet $weight^{-1}$; n = 4, $P < 0·05$) and noradrenaline (sham 0·43 ± 0·20 μg g wet $weight^{-1}$, lesion 0·08 ± 0·04 μg g wet $weight^{-1}$; n = 4, $P < 0·01$). This lesion induced no changes in hypothalamic 5-HT concentrations (sham 0·63 ± 0·16 μg g wet $weight^{-1}$, lesion 0·57 ± 0·17 μg g wet $weight^{-1}$; n = 4). 6-OHDA caused significant reduction of uptake of both catecholamines ([^{3}H]dopamine to 49% of control, $P < 0·05$ [^{3}H]noradrenaline, to 28% of control, $P < 0·01$) (Fig. 1). Uptake of [^{3}H]5-HT and [^{3}H]GABA was unaltered.

5,7-DHT caused marked falls in hypothalamic 5-HT concentrations (sham 0·68 ± 0·16 μg g wet $weight^{-1}$, lesion 0·30 ± 0·12; n = 4, $P < 0·05$). Noradrenaline concentrations were unchanged (sham 0·48 ± 0·14 μg g wet $weight^{-1}$, lesion 0·41 ± 0·16 μg wet $weight^{-1}$; n = 4): dopamine concentrations were slightly reduced, but not significantly (sham 0·73 ± 0·15 μg g wet $weight^{-1}$, lesion 0·59 ± 0·11 μg g wet $weight^{-1}$; n = 4). This lesion significantly reduced high-affinity [^{3}H]5-HT uptake (to 49% of control, $P < 0·05$) (Fig. 1). Uptake of neither [^{3}H]GABA nor the [^{3}H]catecholamines were significantly affected by the 5,7-DHT lesion. Lesion of the ascending noradrenergic bundles produced a 57% depletion of hypothalmic noradrenaline (control; 0·46 ± 0·055 μg g wet $weight^{-1}$; lesion 0·198 ± 0·02 μg g wet weight, $P < 0·05$) with no change in hypothalamic 5-HT concentrations (control; 0·43 ± 0·033 μg g wet $weight^{-1}$, lesion 0·46 μg g wet $weight^{-1}$).

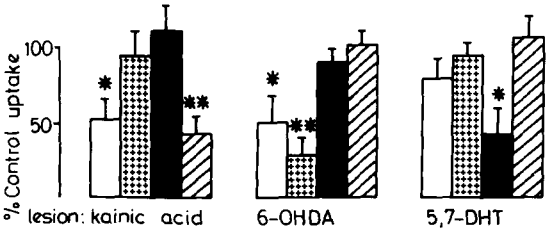

FIG. 1. Effect of intrahypothalamic injections of neurotoxins on high-affinity uptake of [^{3}H]dopamine (open columns), [^{3}H]noradrenaline (dotted columns), [^{3}H]5-HT (solid columns) and [^{3}H]GABA (hatched columns). Uptake was conducted into hypothalamic slices, using 5 × 10^{-8} M transmitter one week following injection of kainic acid, 6-hydroxydopamine (6-OHDA), 5,7-dihydroxytryptamine (5,7-DHT) or vehicle. The results shown are a mean of 4 determinations: vertical bars denote standard errors. Results are calculated as d min^{-1} mg^{-1} wet weight tissue, and for each of the lesions have been expressed as a percentage of the control (sham-operated) observations. Control uptake results: dopamine 3677 ± 692 d min^{-1} mg $tissue^{-1}$; noradrenaline 13407 ± 3721 d min^{-1} mg $tissue^{-1}$; 5-HT 15004 ± 2252 d min^{-1} mg $tissue^{-1}$; GABA 7434 ± 1329 d min^{-1} mg $tissue^{-1}$. *$P<0·05$, **$P<0·01$ (Student's t-test).

Regional activity of VIP-sensitive adenylate cyclase

Experiments were initially performed in whole brain in order to investigate the time course of VIP activation of adenylate cyclase and to determine the most satisfactory dose of VIP that would give a reliable maximal response to allow subsequent comparison of regional activity. VIP stimulated adenylate cyclase activity in whole brain in a partially dose related fashion from 0·1–10 μM (Fig. 2). The lowest dose giving a reliable maximal stimulation was 2 μM. In time-course studies it was seen that 2 μM VIP increased adenylate cyclase activity over the corresponding baseline level at all incubation times studied (30 s–30 min). The stimulation was linear with time reaching a maximum at 10 min. Thereafter the degree of stimulation declined gradually reaching about half the maximal level of stimulation at 30 min (Fig. 2).

After incubating enzyme prepared from different regions of rat brain for 10 min with 2 μM VIP, it was found that the peptide stimulated adenylate

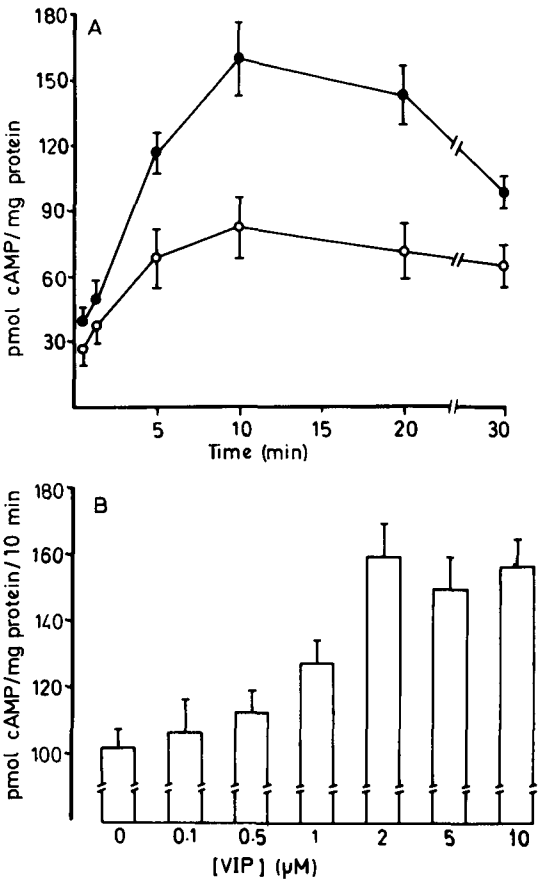

FIG. 2. VIP-induced stimulation of adenylate cyclase activity in homogenates of rat forebrain. (A) Time course for the production of cyclic AMP shown by basal activity (○—○) and in the presence of 2 μM VIP (●—●). (B) Variation of concentration of VIP, range 0·1–10 μM, on the production of cyclic AMP (cAMP). Each point is the mean of 4 determinations: vertical bars denote standard errors.

Table 1. Effect of 2 μM VIP on adenylate cyclase activity in homogenates prepared from various rat brain regions. The adenylate cyclase assay was performed as described in Methods. Each value represents pmol cAMP mg^{-1} protein/10 min and is the mean ± s.e.m. of four determinations. Significance of difference from baseline values are indicated in the right-hand column (Student's *t* test). n.s. = not significant.

Region	Baseline	VIP	% stimulation	*P*
Olfactory bulbs	425 ± 26	1076 ± 38	153	<0·01
Hippocampus	186 ± 13	373 ± 13	101	<0·01
Thalamus	147 ± 3	267 ± 11	81	<0·01
Occipital cortex	466 ± 33	795 ± 8	71	<0·01
Midbrain	159 ± 9	271 ± 13	70	<0·01
Frontal cortex	182 ± 11	305 ± 21	67	<0·01
Hypothalamus	353 ± 3	575 ± 14	61	<0·01
Striatum	313 ± 19	483 ± 23	54	<0·01
Cerebellum	142 ± 15	191 ± 5	34	<0·05
Brain stem	128 ± 1	165 ± 19	29	<0·05
Spinal cord	164 ± 9	161 ± 11	(−1·8)	n.s.

cyclase activity in all regions studied except the spinal cord. There were, however, marked regional variations, the degree of stimulation being highest in the olfactory bulbs, thalamus and hippocampus with intermediate levels of stimulation in the cortical areas, hypothalamus, midbrain and striatum. Low levels of stimulation were noted in the cerebellum and brain stem. These results are shown in Table 1.

Hypothalamic location of VIP-sensitive adenylate cyclase

In studies on the hypothalamic enzyme, it was found that the ability of VIP to stimulate adenylate cyclase activity was markedly reduced in 6-OHDA-lesioned animals compared with sham vehicle-injected (0·1% sodium metabisulphite solution) controls (Table 2.) However, neither 5,7-DHT nor kainic acid lesions caused any change in the responsiveness of hypothalamic enzyme activity to 2 μM VIP when compared with the relevant sham-operated controls (Table 2).

Following transection of the ascending noradrenergic bundles there was a 71% reduction in the ability of 2 μM VIP to stimulate hypothalamic enzyme activity (mean control stimulation = 145 pmol cAMP mg^{-1}/10 min; mean stimulation in lesioned animals = 45 pmol cAMP mg^{-1}/10 min: significance of difference at $P < 0·01$, n = 6).

DISCUSSION

The receptor actions of many neurotransmitters may be mediated through activation of adenylate cyclase (for review, see Greengard 1976). A regional assessment of adenylate cyclase sensitivity to VIP might, therefore, give insight into the regions of the c.n.s. where VIP may play an important role in

Table 2. Effect of 2 μM VIP on adenylate cyclase activity in homogenates from rat hypothalamus in sham or neurotoxin-lesioned animals. Rats received bilateral injections of neurotoxins or vehicles into the anterior hypothalamus one week before biochemical assay. The adenylate cyclase assay was performed as described in Methods. Each value represents mean ± s.e.m. of four determinations. Significance of difference from baseline values are indicated in the right-hand column (Student's *t* test). n.s. = not significant.

Lesion		Baseline	+ VIP	% stimulation	*P*
Sham	Sodium metabisulphite	405 ± 21	681 ± 13	68	<0·01
	Phosphate buffer	454 ± 20	688 ± 61	51	<0·01
Lesion	6-OHDA	410 ± 22	484 ± 34	18	n.s.
	5,7-DHT	389 ± 17	690 ± 30	77	<0·01
	Kainic acid	439 ± 22	733 ± 13	67	<0·01

neurotransmission, complementing studies on the distribution of the peptide itself (Fuxe et al 1977; Besson et al 1979; Lören et al 1979). Thus areas where VIP has been assigned an important role on the basis of other types of study, such as from electrophysiological recordings in the hippocampus (Kelly et al 1979), a proposed physiological role in thermoregulation in the hypothalamus (Clark et al 1978), and biochemical studies on this neuropeptide in the striatum (Quik et al 1979), showed high concentrations of enzyme activation. Conversely, those regions relatively devoid of this peptide, such as brain stem, cerebellum and spinal cord (Besson et al 1979), showed relatively little enzyme activity.

Although the rank order of activity differs slightly from the reported regional distribution of the peptide, there is some correlation between adenylate cyclase activity and regional distribution. Thus, the hippocampus and cortical regions and thalamus having large amounts of this peptide (Besson et al 1979; Lören et al 1979) show high activity and regions devoid of the peptide such as brain stem cerebellum and spinal cord only show very slight activity. However, the high activity of olfactory bulb homogenates is anomalous and requires further study.

Furthermore, the distribution of adenylate cyclase in this study accords well with the distribution of VIP receptor binding reported by Taylor & Pert (1979). It should be noted that one group (Borghi et al 1979) failed to show any VIP sensitive adenylate cyclase activity in the caudate nucleus but showed significant activity in cerebellum. However, our study showing activity in the caudate nucleus and none in the cerebellum is in agreement with others (Quik et al 1978) and with the regional distribution of the peptide (Besson et al 1979; Lören et al 1979).

We studied the hypothalamus to define upon which neuronal elements the VIP-sensitive enzyme may reside in this region. 6-OHDA is toxic to catecholamine-containing neurons, thus the loss of enzyme activity along with a concomitant fall in dopamine and noradrenaline concentrations points to a possible catecholaminergic location of the enzyme in the hypothalamus. Kainic acid, a neurotoxin acting at cell soma, produced loss of dopamine and GABA uptake but no change in sensitivity to VIP. Furthermore, since there are believed to be no dopaminergic afferents to the hypothalamus (Ungerstedt 1971) which would be unresponsive to kainic acid, this would possibly point to a more specific localization of the VIP-stimulated adenylate cyclase activity upon noradrenergic elements.

This hypothesis is further strengthened by the observation that a lesion of the ascending noradrenergic afferents to the hypothalamus, which was associated with 57% loss of noradrenaline was accompanied by a significant reduction of the hypothalamic enzymes's sensitivity to VIP. The absence of any loss of sensitivity in animals treated with the neurotoxin 5,7-DHT precludes the presence of any VIP-sensitive adenylate cyclase on 5-HT-ergic neurons in the rat hypothalamus.

It could be suggested that such a change in the hypothalamic response might reflect reduced phosphodiesterase activity following lesion. This seems unlikely. In the standard hypothalamic reaction system 6 mM theophylline maximally inhibits phosphodiesterase since further addition has no effect on responsiveness. Thus it is unlikely that such a reduction in activity would be manifest in this system. Furthermore, it seems unlikely that such an effect would be specific to one particular type of lesion.

In summary the regional distribution of the VIP sensitive adenylate cyclase in the rat brain may provide further insight into those regions where VIP may have a transmitter role. Secondly, lesion studies in the hypothalamus point to a possible noradrenergic localization for the enzyme in this area.

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