VASOACTIVE INTESTINAL PEPTIDE : LEVELS AND
FUNCTIONAL RECEPTORS IN RAT BRAIN BEFORE AND AFTER WEANING

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Summary

The average level of VIP was found to be 17 pmol/g wet weight in the brain of the newborn rat. This level remained constant during the first two weeks after birth then increased progressively to 40 pmol/g wet weight at 20 days, a value comparable to that found in adult animals. VIP was already able to stimulate brain membrane adenylate cyclase activity at birth. The stimulation with 10^{-6} M VIP allowed a 2.5-fold increase in basal activity in membranes from 1 to 14-days-old pups as compared to a 1.7-fold stimulation in membranes from adult brain. The apparent activation constant for VIP adenylate cyclase stimulation was $4 \cdot 10^{-7}$ M at all ages. The efficiency of VIP activation amounted to as much as 70 % of that of fluoride at birth and to 35 % only of fluoride activation in brain membranes from adult rats.

The distribution of the octacosapeptide vasoactive intestinal peptide (VIP) in discrete areas is relatively well documented (1) but the precise neuroanatomy of VIPergic pathways and the role(s) of VIP in neurons are still largely unknown. Five lines of evidence suggest, however, that VIP is a neurotransmitter in both brain and parasympathetic nerves in the digestive tract : 1) VIP is present in relatively large concentration in central and peripheral neurons (1-4); 2) VIP is secreted by chemical depolarization from a brain synaptosomal fraction (3,5); 3) a topical administration of VIP on cortical brain areas elicits electrical signals (6); 4) specific binding sites for VIP, coupled to a VIP-sensitive adenylate cyclase system, are present in brain membranes (7-9) and the distribution of VIP parallels that of dopamine during the subcellular fractionation of brain homogenate (3). The purpose of the present study was to document the level of VIP and the presence of functional VIP receptors in rat brain during postnatal development.

Materials and methods

1. Animals.

Birth dates of albino Wistar rats were recorded 22 days after mating by twice-daily inspections of the animal house. The litters of newborn rats were adjusted to 10 pups. Rats were weaned at 25 days post-partum but they began to nibble at the standard chow offered to the mothers when 18-day-old.

Abbreviation : VIP, vasoactive intestinal peptide.

2. VIP assay.

The levels of brain VIP were estimated by a radioreceptor assay based on the competition between ^{125}I -labeled VIP and unlabeled VIP or brain extracts for binding to isolated guinea pig pancreatic acinar cells.

For brain extracts, the animals were sacrificed by decapitation. The brain was dissected out and the cerebellum, the cerebral trunk, and the hypophysis were discarded. Brain extracts from 6, 3, or 1 animal(s) were prepared, respectively, when provided by 1-3 days-old, 4-7 days-old, or older rats. The homogenate was immediately boiled for 10 min in a water bath and centrifuged after cooling for 45 min at $100\,000 \times g$. The clear supernatant was stored in liquid nitrogen before assay.

Isolated guinea pig pancreatic acinar cells were prepared following the procedure of Amsterdam and Jamieson (10) using crude collagenase and hyaluronidase digestion as previously described (11). The dispersed acinar cells were resuspended in Krebs-Ringer bicarbonate buffer containing 0.1 mM calcium in order to obtain a cell concentration of 10^7 cells/ml.

Porcine VIP was iodinated by the chloramine T procedure previously described (11). The labeled peptide, purified by adsorption chromatography on cellulose, was stored at -20°C until use.

For the radioreceptor assay, 200 μl aliquots of dispersed acinar cells were incubated with 25 μl of ^{125}I -labeled VIP ($\pm 20\,000$ cpm/assay) and 25 μl of increasing concentrations of unlabeled VIP or 25 μl of an appropriate dilution of brain extract. After 15 min at 37°C , the samples were filtered on EHWP Millipore filters (cellulose acetate, $0.45 \mu\text{m}$ pore size). Each filter was quickly washed three times with 2 ml of ice-cold Krebs-Ringer bicarbonate buffer, transferred to vials, and counted in a Packard gamma counter. Nonspecific binding occurring in the presence of 1.10^{-6} M VIP represented less than 2 % of the total. Using this technique, 0.05 pmol of VIP could be measured with accuracy. Successive dilutions of brain extracts gave proportional results and there was virtually no interference between ^{125}I -VIP and VIP fragments or parent peptides (11).

3. Brain membrane adenylate cyclase assay.

3.1/ Brain membrane preparation : 10 % (w/v) homogenates of rat brains were prepared in ice-cold 0.32 M sucrose in a glass homogenizer by 4 up and down strokes with a Teflon pestle driven at $900 \text{ rev} \times \text{min}^{-1}$. Each homogenate was centrifuged for 10 min at $1000 \times g$ at 4°C . The supernatant was centrifuged for 20 min at $16\,000 \times g$. The resulting pellet was resuspended in distilled water and then diluted two-fold with a buffer (pH 7.4) composed of 25 mM Tris-HCl, 4 mM MgCl_2 , and 1 mM 2-mercaptoethanol (Buffer A). This 6 ml suspension was layered onto a discontinuous sucrose density gradient made of five 6 ml layers of, respectively, 1.2 M, 1.0 M, 0.8 M, 0.6 M and 0.4 M sucrose and centrifuged for 2 hours at $25\,000 \text{ rev/min}$ and 4°C in the SW 27 rotor of a Beckman ultracentrifuge. The 1.2-1.0 M sucrose interface was collected with a syringe, diluted 20-fold with buffer A and centrifuged for 10 min at $50\,000 \times g$. The final membrane pellet was rehomogenized in an appropriate volume of buffer A and was stored in liquid nitrogen after protein determination (12). Typically, the activity of adenylate cyclase was determined simultaneously on six preparations of purified brain membranes provided, respectively, by rats sacrificed on day 1 ($n = 7$), 7 ($n = 4$), 14 ($n = 2$), 21 ($n = 1$), 28 ($n = 1$) and 80 (adult male of 250 g on an average, $n = 1$).

3.2/ Adenylate cyclase assay : adenylate cyclase activity was estimated by the conversion of [α - ^{32}P]ATP into cyclic [^{32}P]AMP. Membranes (10 to 100 μg protein) were incubated for 7 or 14 min depending on the protein available, and in the absence or in the presence of VIP or NaF as previously described (7). The reaction was stopped by adding 0.5 ml of a 0.1 % sodium dodecylsulfate solution containing 1 mM ATP, 2 mM cyclic AMP and cyclic [$8\text{-}^3\text{H}$]AMP (20 000 counts/min). The separation of cyclic AMP was achieved by the double column procedure of Salomon et al. (13). Under all conditions tested, the cyclic [^{32}P] AMP produced was linearly correlated to time and protein concentration.

4. Materials.

Essentially pure natural porcine VIP was supplied by Dr. V. Mutt (GIH Laboratory, Karolinska Institutet, Stockholm, Sweden). Carrier-free Na^{125}I (300 mCi/ml), $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ (10 Ci/mmol), and cyclic $[8\text{-}^3\text{H}]\text{AMP}$ (20-30 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, England). The origin of reagents used for isolation of pancreatic cells was published elsewhere (11). All other reagents were of the highest grade available.

FIG. 1

Developmental accumulation of VIP in rat brain determined by a radioreceptor assay. The results are expressed in pmol/g wet weight VIP by reference to a standard curve established with essentially pure porcine VIP. The number of brain extracts investigated is mentioned. For animals younger than 10 days, several brains were pooled for a single determination as described under Materials and Methods. B = birth. A = 240- to 260-g male adults.

Results

Levels of brain VIP in neonatal stages of development.

Significant levels of VIP were already present at birth (Fig. 1). The mean concentration of 17 pmol/g wet weight brain remained constant during the first postnatal week, then increased in 7- to 17-day-old pups to reach at weaning the adult level of 40 pmol/g wet weight.

Existence of functional VIP receptors in rat brain before and after weaning.

Functional VIP receptors were investigated by measuring VIP-stimulation of adenylate cyclase activity in brain membranes during development. The specific activity of basal, VIP-stimulated, and fluoride-stimulated adenylate cyclase, expressed per mg membrane protein, increased during the first two weeks after birth and reached values characteristic of the adult during the third week (Fig. 2). Lineweaver-Burk plots (Fig. 3) of adenylate cyclase stimulation by

FIG. 2

Effect of development on the specific activity (cyclic AMP produced $\times \text{min}^{-1} \times \text{mg protein}^{-1}$) of brain synaptic membrane adenylate cyclase. The activities were basal ($\square$), stimulated with 10^{-6} M VIP (hatched) or with 10 mM NaF ($\blacksquare$). The number of brain extracts investigated is mentioned. For animals younger than three weeks, several brains were pooled for a single determination as described under Materials and Methods. B = birth; W = end of the considered week; A = 240- to 260-g male rats.

VIP indicate that the apparent activation constant of VIP receptors, estimated by the peptide concentration required for half-maximal stimulation of the enzyme, remained constant during development and was $4 \cdot 10^{-7}$ M in newborn and 1-week-old rats as well as in adult rats.

On the other hand, the 2.5-fold stimulation of basal adenylate cyclase activity at a near maximum VIP concentration (7) observed with brain membranes from newborn and 1- to 2-week-old pups was significantly higher than the 1.7-1.8-fold stimulation observed in brain membranes from 3-4 week-old and adult rats (Fig. 4A).

The efficiency of VIP activation amounted to 60-70 % of that of 10 mM fluoride in brain membranes from newborn and one week-old pups. This relative efficiency was significantly higher than the VIP activation, limited to 30-35 % of that of fluoride, observed in membranes from older rats (Fig. 4B).

Discussion

A neurotransmitter is involved in four biochemical steps (14): 1) synthesis; 2) storage; 3) binding to post-synaptic receptors coupled to an effector; and 4) uptake and/or degradation which terminate the action of the released neurotransmitter. In the case of the candidate neurotransmitter VIP, the second and third steps are amenable to experimentation and it was therefore of interest to explore these parameters during brain development in rat.

FIG. 3

Lineweaver-Burk representation of dose-effect curves of VIP activation of adenylate cyclase in brain membranes from newborn (●), 1 week old (△) or adult (○) rat. Each curve is the mean of, respectively, 5, 8 and 6 experiments. Results are expressed in pmol cyclic AMP formed $\times \text{min}^{-1} \times \text{mg protein}^{-1}$ in excess of the basal unstimulated value. A similar 4.10^{-7} M value was found for the apparent K_a at all ages.

1. VIP levels during brain development.

The radioreceptor used allowed the precise determination of 0.05 to 0.4 pmol VIP per assay (data not shown). The standard curve was established with pure VIP extracted from porcine gut. Different molecular forms of VIP are however present in gut extracts (15) and differences in ionic charges may exist between gut VIP and brain VIP (16). The biological properties of these distinct forms of porcine VIP have not yet been documented. Furthermore, species differences may exist between rat and hog VIP. The present levels of VIP reported for rat brain cannot therefore be considered as absolute levels of rat brain VIP, but as expressed in intestinal porcine VIP equivalents.

The specificity of the assay of VIP depended on that of VIP receptors of guinea pig pancreatic acinar cells. The affinity of these receptors for parent peptides such as secretin, VIP fragments, and secretin fragments is respectively 3000-fold, 5000-fold and 10 000-fold lower than that for VIP (11). Thus, the radioreceptor assay appeared more specific than an immunoassay recently characterized (15).

The level of 40 pmol/g tissue found for VIP in adult rat brain was of the same order of magnitude as that of 155 pmol/g tissue found by radioimmunoassay and by radioreceptor assay (17) and the values of 66, 65, and 24 ng/g tissue reported for, respectively, occipital, frontal and temporal dog cortex, using a radioimmunoassay (18).

At birth, the average VIP concentration attained 42 % of that found in adult rat brain. A two-fold increase in VIP concentration took place between day 10 and day 20 after birth and coincided with the growth of axons and dendrites, the establishment of neuronal connections, and the deposition of myelin sheets

(23). This sudden rise accompanied also increasing levels of acetylcholine (19), gammaaminobutyric acid (20), and the candidate neurotransmitter C-terminal octapeptide of pancreaticozym (21) but contrasted with the progressive linear elevation of dopamine levels observed in rat striatum (22). It is of interest to note that in the rat jejuno-ileum an increase in VIP concentration is also documented between day 13 and day 21 but this first rise is followed by a second one between day 21 and day 42 (24).

FIG. 4

Effect of development on the sensitivity of rat brain membrane adenylate cyclase activity to 10^{-6} M VIP. In A/ the stimulatory effect of VIP was expressed in percent of the basal unstimulated value. In B/ the values observed in presence of 10^{-6} M VIP were expressed in percent of the values observed with 10 mM NaF. For each condition (see Fig. 2) the number of determinations and the standard error of the mean were mentioned. An asterisk indicates a value significantly different ($p < 0.005$) from that observed in adult rats. B = birth; W = end of the considered week.

2. Brain VIP receptors during development.

Although there is not obvious stoichiometry between the number of occupied VIP binding sites and the amount of cyclic AMP formed in the presence of VIP, the adenylate cyclase activity stimulated by VIP can be considered as reflecting the potency and efficiency of functional VIP receptors i.e. of VIP binding sites which are coupled to the adenylate cyclase catalytical subunit when occupied by the peptide (7,8).

The comparison of VIP-stimulated, fluoride-stimulated, and basal activities in preparations of brain membranes demonstrated that: 1/ VIP-stimulated adenylate cyclase was present already at birth; 2/ basal and stimulated adenylate cyclase activities increased during the first two weeks; 3/ at birth and during the first week after birth, VIP could produce 70 % of the maximal stimulation observed with 10 mM sodium fluoride; 4/ the apparent activity constant of the adenylate cyclase system for VIP remained constant during development.

These results are consistent with data on amines. A dopamine-sensitive adenylate cyclase is present in newborn striatum and its activity increases progressively to attain adults levels at 3 weeks after birth (25). Similar findings were found in a particulate norepinephrine-sensitive adenylate cyclase fraction from rat cerebral cortex and in a particulate serotonin-sensitive adenylate cyclase fraction from rat brain subcortex (26).

The development of the dopamine-sensitive adenylate cyclase in the striatum precedes the accumulation and/or synthesis of dopamine (14). Similarly, it appears that VIP-containing neurons were able to establish at birth (or even before) fully efficient connections with neurons possessing VIP receptors coupled to an adenylate cyclase system.

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