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Alterations in vasoactive intestinal polypeptide-related peptides after pentylenetetrazole-induced seizures in rat brain

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The possible involvement of vasoactive intestinal polypeptide-related peptides in pentylenetetrazol (PTZ)-induced seizures in rats was investigated. The chemoconvulsant PTZ was administered (45 mg/kg i.p.) either acutely or chronically for three days. The detailed time course of changes in VIP-(1–28) and VIP-(22–28) was examined in several rat brain areas 5 and 20 min and 24 h after acute treatment and after three days chronic treatment. Ir-VIP levels dramatically decreased in all areas 5 min after PTZ injection, remained low after 20 min and progressively increased back to control values after 24 h and after three days of repeated treatment (except for the cortex). Chromatographic analysis of extracts prepared from PTZ-treated rats revealed a concomitant decrease in VIP-(1–28) and increase in VIP-(22–28). Thus VIP-(22–28) might be a product of the internal cleavage of the precursor VIP-(1–28) after its neuronal release; alternatively, VIP-(22–28) might be generated by post-transcriptional processing of VIP-(1–28), and thus be an 'independent' neuropeptide. The results suggest that VIP-(1–28)/VIP-(22–28)-containing neurons might be involved in PTZ-induced seizures in rat brain, and that VIP-(22–28) might play a role in these experimental seizures.

VIP (vasoactive intestinal polypeptide); VIP-(22–28); Pentylenetetrazole; Seizures; Brain (rat)

1. Introduction

Convulsions produced in laboratory animals by systemically administered convulsant drugs, particularly pentylenetetrazol (PTZ), have been used extensively as experimental models of epilepsy (Nutt et al., 1980; Foote and Gale, 1984; Bandyopadhyay, 1989). These models have also been widely used to investigate the neurochemical mechanisms of seizures, and have provided data concerning the role of various neurotransmitter systems in the processes that initiate, maintain or terminate seizures in the central nervous system (Burley and Ferrendelli, 1984).

Several studies have reported changes in various neuropeptide patterns after seizures induced by either electrical or chemical kindling in the rat brain (Kato et al., 1983; Assouline et al., 1984; Higuchi et al., 1984; Meyer et al., 1986; Marksteiner and Sperk, 1988). There is also evidence that PTZ acts by interfering with neurotransmitters, such as gamma-aminobutyric acid (MacDonald and Barker, 1977). For example, altered levels of somatostatin and neuropeptide Y have been found in the brains of rats with epilepsy, which suggests that these peptides play a role in this pathological condition (Pitkänen et al., 1989).

Vasoactive intestinal polypeptide (VIP) is present in high concentrations in many areas of the rat brain (Said and Rosenberg, 1976; Loren et al., 1979; Morrison et al., 1984) and in nerve terminals (Giachetti et al., 1977), especially cortical interneurons (Eckenstein and Baughman, 1984). There are two major molecular forms of VIP: the complete VIP-(1–28) molecule and the recently identified heptapeptide VIP-(22–28) fragment (Romualdi et al., 1989, 1990).

The present study investigated the possible involvement of VIP-related peptides in epilepsy, i.e., in seizures induced in the rat with PTZ. The VIP-(1–28)/VIP-(22–28) system was monitored to detect possible relative changes in VIP subspecies (i.e., in one specific molecular form compared to the other). We examined in detail the time course of changes in these peptides in several brain areas after single or repeated injections of PTZ. In addition, we used gel filtration

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combined with HPLC to characterize the molecular forms responsible for the VIP-(22–28)-like immunoreactivity (ir-VIP-(22–28)) detected (Romualdi et al., 1989).

2. Materials and methods

2.1. Animals and drugs

Male Sprague-Dawley rats (Nossan, Correzzana, Milan, Italy), weighing 280 ± 20 g, were used in this study. Rats were housed in groups of four per cage, at constant temperature (21 °C), with food and water ad libitum. They were kept on a light/dark cycle with lights on between 7 a.m. and 7 p.m. All experiments were performed during the light cycle.

Pentylenetetrazol (PTZ, Sigma, St. Louis, MO) was dissolved in saline solution and administered intraperitoneally (i.p.) at a dose of 45 mg/kg, either acutely or chronically (once daily) for three days.

Animals were divided into five groups of ten rats each: group A received saline 2 ml/kg i.p.; group B was killed 5 min after a single injection of PTZ; group C was killed 20 min after PTZ; group D was killed 24 h after PTZ; group E was treated with PTZ once daily for three days and killed 20 min after the last PTZ injection.

2.2. Seizure testing and scoring

The dosage of PTZ was chosen to produce convulsive behaviour without causing death. Rats were observed for epileptic behaviour for 20 min (except for rats in group A, which were observed for only 5 min). All rats exhibited epileptic behaviour within 2 min of the PTZ injection. The occurrence of seizures was scored according to the following scale; 0, no seizure activity; 1, myoclonic jerks of the forelimbs; 2, clonic seizures without loss of righting reflex; 3, clonic seizures with loss of righting reflex; 4, tonic extension of the hindlimbs; and 5, tonic extension with death (Czuczwar and Frey, 1986). Each experiment included a control group of rats that received saline 2 ml/kg i.p. and were killed at the end of the observation time.

2.3. Tissue processing

The animals were killed by decapitation 5 and 20 min, and 24 h after PTZ administration in the acute experiment, and 20 min after the last PTZ injection in the three-day experiment. The rat brains were dissected into seven regions: cerebellum, medulla oblongata, hypothalamus, striatum, hippocampus, midbrain and cortex, according to Glowinski and Iversen (1966). The tissues were immediately frozen on dry ice and stored at −70 °C until extraction. They were weighed, homogenized in 10 ml/g of 0.1 M acetic acid at 95 °C and maintained at this temperature for 10 min. Then aliquots were taken for protein assay and the remaining extracts were cooled and centrifuged at 12000 × *g* for 20 min. The supernatants were stored at −20 °C prior to the radioimmunoassay.

2.4. Radioimmunoassay procedures

VIP-(22–28) antiserum (AH78) was prepared as described (Stewart and Young, 1984; Romualdi et al., 1989). The antiserum recognizes the carboxy terminus, VIP-(22–28), and its amino-extended forms. It thus recognizes both VIP-(1–28) and VIP-(22–28) on an equimolar basis, whereas it does not recognize VIP fragments containing only the amino terminus (e.g., VIP-(1–12)) (Romualdi et al., 1989).

The standard VIP-(22–28), H-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2, was synthesized by solution-fragment condensation (4 + 3) and purified by silica gel column chromatography with n-butanol/acetic acid/water (6:2:2) as eluant and by reverse phase HPLC on a C_{18} column with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid as eluant. The homogeneity of the purified product, m.p. 240–242 °C, $[\alpha]_D^{22} + 6.8$ (c 1, DMF), was assessed by thin-layer chromatography and analytical HPLC (purity > 97%). Structure identification was achieved by amino acid analysis (Asp 1.88; Ser 0.94; Ile 1.00; Leu 2.04; Tyr 0.96) and fast atom bombardment mass spectroscopy (FAB-MS = 835). VIP-(22–28) was iodinated with the chloramine-T method (Ghazarossian et al., 1980) and purified by microcolumn Sep-Pak C_{18} reverse phase chromatography. RIA was carried out in 0.15 M phosphate buffer (pH 7.4) and incubations lasted for 18–24 h at 4 °C. The reaction was terminated by the addition of 1.0 ml of charcoal suspension. Bound peptide was then separated by centrifugation and estimated according to Ghazarossian et al. (1980). The final dilution of the VIP-(22–28) antiserum was 1:6000 in an assay volume of 300 μl. The optimal ED_{50} for the radioimmunoassay was calculated as 60 fmol/assay tube.

2.5. G-50 chromatography

Extracts of rat brain regions were concentrated under vacuum and applied to a 1.0 × 100 cm column of Sephadex G-50 (Superfine) equilibrated with 10% (v/v) formic acid (the elution solvent) at 4 °C; 2 ml fractions were collected. Aliquots of each fraction were dried under vacuum and reconstituted in 0.1 M acetic acid, 0.01 M HCl in 50% aqueous methanol before RIA of samples from each fraction. BSA and $CoCl_2$ were used to determine the exclusion volume (V_0) and the total volume (V_t) of the column. Experiments were repeated

three times to confirm the reliability of the profile and the relative content of each peak.

2.6. High-performance liquid chromatography (HPLC)

Fractions from the G-50 column containing peaks of VIP-(22–28) immunoreactivity were dried under vacuum and redissolved in a small volume of 0.1 M acetic acid, 0.01 M HCl in 50% aqueous methanol before injection onto a 30 cm × 3.9 mm μ-Bondapack C_{18} reverse-phase column (Waters Assoc., Milford, MA), pre-equilibrated with 20% acetonitrile in 13 mM trifluoroacetic acid (TFA) in water. Samples were eluted with a linear gradient of 20–50% acetonitrile in 13 mM TFA over a period of 30 min at a flow rate of 1 ml/min (Romualdi et al., 1989); 1 ml fractions were collected, dried under vacuum and subjected to RIA analysis.

2.7. Statistics

The data were compared by using the Newman-Keuls statistical test. The significance level was $P < 0.05$.

3. Results

3.1. PTZ-induced behaviour

All rats treated with PTZ (45 mg/kg i.p.) exhibited epileptic behaviour, reaching score 1 within 2 min; 74% of the animals tested reached score 2 within 4 min and 56% reached score 3 within 10 min. Only 4% of the rats reached score 4 by the end of the observation period and none died during the experiment.

3.2. Ir-VIP-(22–28) levels in rat brain areas after PTZ

As presented in fig. 1, a dramatic decrease in VIP-immunoreactivity was observed 5 min after PTZ injection (group B) in the cortex (78 vs. 135 pmol/g tissue, $P < 0.05$ vs. group A), hypothalamus (10.6 vs. 34.2 pmol/g tissue, $P < 0.01$), hippocampus (35 vs. 57 pmol/g tissue, $P < 0.05$), striatum (7.1 vs. 28.2 pmol/g tissue, $P < 0.01$), midbrain (11.0 vs. 26.8 pmol/g tissue, $P < 0.01$) and medulla (5.7 vs. 19.4 pmol/g tissue, $P < 0.01$).

The levels remained low in group C (20 min) and progressively increased back to control levels in group D (24 h). After the repeated treatment, the ir-VIP-(22–28) levels in comparison with those of group A were: cortex 100.6 vs. 135.0 pmol/g tissue, $P < 0.01$; hypothalamus 43.8 vs. 34.2, $P < 0.05$; hippocampus 50.7 vs. 57.3; striatum 70.4 vs. 28.2, $P < 0.01$; midbrain 30.9 vs. 26.8, and medulla 16.8 vs. 19.4.

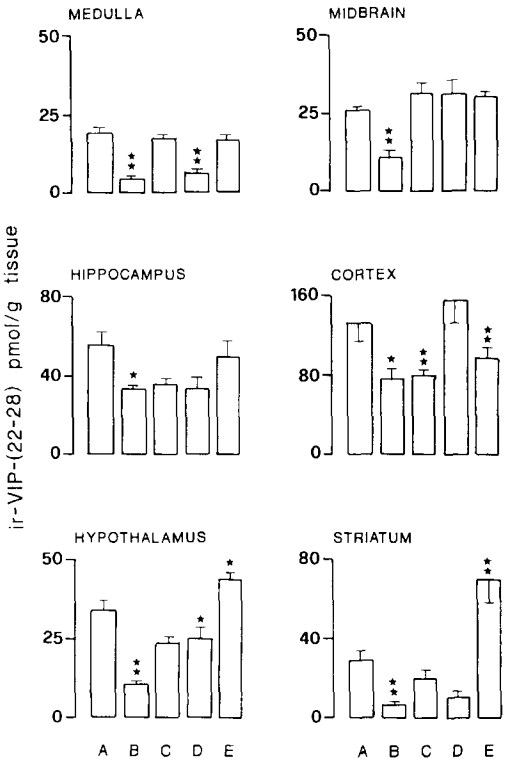

Fig. 1. ir-VIP-(22–28) levels in extracts prepared from different rat brain areas. In each panel histograms represent values for control rats (A) or rats killed 5 min (B), 20 min (C) and 24 h (D) after acute PTZ administration or values for rats treated with PTZ for three days and killed 20 min after the third administration (E). Values are means ± S.E.M. of 10 rats for each group. * $P < 0.05$, ** < 0.01 vs. control (A), Newman-Keuls statistical test.

3.3. Chromatography

The G-50 profile showed that there were two molecular forms of VIP immunoreactivity in all tissue extracts: one consisting of the entire VIP-(1–28) molecule and the other eluting in the same position as VIP-(22–28). As previously ascertained in control rats (Romualdi et al., 1989), VIP-(22–28) accounts for 30% of the total VIP-(1–28) immunoreactivity detected. Chromatographic analysis of the immunoreactivity detected in group B (rats killed 5 min after PTZ injection) revealed a concomitant decrease in the relative content of VIP-(1–28) and an increase in the content of VIP-(22–28). In particular VIP-(22–28) accounted for 47% of the total ir-VIP in the hippocampus, 50% in the striatum, and 55% in the cortex. A representative G-50 profile of a striatal extract from PTZ-treated rats killed 5 min after the injection is shown in fig. 2. In addition, the G-50 analysis of the ir-VIP-(22–28) detected in group E (repeated treatment) showed a decrease in VIP-(1–28) and an increase in VIP-(22–28). VIP-(22–28) accounted for 46% of the total ir-VIP in the hippocampus, 50% in the striatum and 70% in the cortex. A

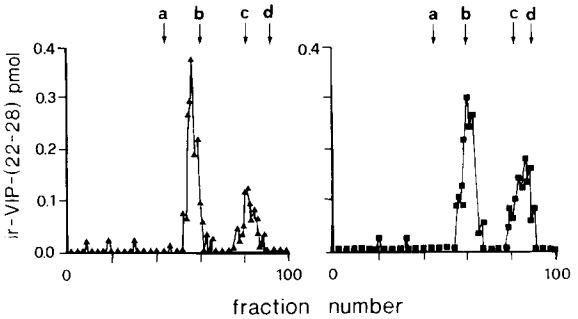

Fig. 2. Chromatographic analysis (G-50) of extracts prepared from brain striatum of control rats (left panel) and PTZ-treated rats killed after 5 min PTZ administration (right panel). A representative experiment is shown. Elution positions of peptide standards or column markers run on the same columns are indicated by the arrows at the top of each panel: a, void volume; b, VIP-(1–28); c, VIP-(22–28); d, total column volume. Details of the chromatographic procedure are given in the Materials and methods section.

representative G-50 profile of a cortex extract from rats treated with PTZ once daily for three days is shown in fig. 3. The HPLC analysis was carried out with pooled fractions containing VIP-(22–28) immunoreactivity after G-50 chromatography. The identity of the immunoreactive material obtained after G-50 chromatography was confirmed in all tissues investigated: the first peak, which eluted as the standard VIP-(1–28), consisted of authentic VIP-(1–28), as determined by HPLC on a C_{18} column; the second peak identified as VIP-(22–28) after G-50 chromatography was confirmed as authentic VIP-(22–28) by HPLC, eluting as three major peaks, one of which corresponded to VIP-(22–28), as reported previously (Romualdi et al., 1989).

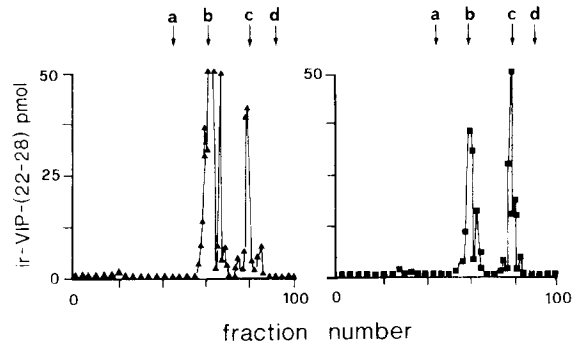

Fig. 3. Chromatographic analysis (G-50) of extracts prepared from brain cortex of control rats (left panel) and rats treated with PTZ for three days (right panel). A representative experiment is shown. Elution positions of peptide standards or column markers run on the same columns are indicated by the arrows at the top of each panel: a, void volume; b, VIP-(1–28); c, VIP-(22–28); d, total column volume. Details of the chromatographic procedure are given in the Materials and methods section.

4. Discussion

The data presented in this paper indicate that systemically administered PTZ in rats produces epileptic behaviour, as rated according to a standardized scale (Czuczwar and Frey, 1986).

VIP-immunoreactivity changed in different ways in the various regions of rat brain during and after PTZ-induced seizures. A detailed analysis of the time course of changes after a single injection of PTZ showed a dramatic decrease in the VIP content of the medulla, hypothalamus, midbrain, hippocampus, striatum and cortex 5 min after PTZ injection. The levels of the peptide in the cortex and hippocampus remained low after 20 min and only started to return to control values 24 h after PTZ, except in the medulla and striatum.

Chronic treatment with PTZ for three days caused VIP levels to increase to control values or significantly higher than control values in the hypothalamus and striatum. Only in the cortex did the ir-VIP content remain lower than in the controls.

Our results are in agreement with data reported by Marksteiner et al. (1989), showing changes in VIP levels in the rat brain after seizures induced by kainic acid. Furthermore, these results are also consistent with other observations of increased amine transmitter turnover and neurochemical changes in other neuropeptides during seizures (Sperk et al., 1983).

The early general decrease in ir-VIP levels observed shortly after convulsions may reflect an increase in peptide release. VIP is possibly released from neurons immediately after the seizures and levels are restored between 20 min and three days afterwards, since VIP levels were then comparable to (or higher than) those of control rats.

In addition, the recovery of peptide levels after 24 h or after chronic treatment for three days suggests that there was only minor reversible damage to the VIP-containing neurons. A significantly lower ir-VIP content after chronic treatment was observed only in the cortex, indicating that major neurochemical changes occurred in this area. Similar observations have been reported by other authors for other neuropeptides, such as somatostatin, NPY and cholecystokinin (Meyer et al., 1986; Sperk et al., 1986; Pitkänen et al., 1989; Marksteiner et al., 1990). However, the events occurring after PTZ-induced seizures may be explained by different mechanisms: increased release of peptide in the period soon after the convulsion, or an increase in the synthesis of peptide at later intervals (24 h and after three days).

Chromatographic characterization made it possible to confirm the presence of two molecular forms of ir-VIP in all tissues: the entire VIP-(1–28) molecule

and the recently identified VIP-(22–28) fragment (Romualdi et al., 1989).

We observed changes in the ratio of these two forms of VIP 5 min after PTZ as well as after the chronic treatment. Interestingly, VIP-(1–28) decreased after seizures whereas VIP-(22–28) increased. As a possible explanation, it can be argued that VIP is released from the neuron and then enzymatically cleaved; thus VIP(22–28) may represent a cleavage product of VIP-(1–28). Alternatively, the increase in VIP-(22–28) as part of the total ir-VIP content after PTZ seizures may result from activation of post-transcriptional processing of its precursor. In this case, the production of VIP-(22–28) in vivo might reflect that it plays a role in the mechanisms underlying the development and continuation of epileptic behaviour. In this respect, two years after the identification of VIP-(22–28), Fahrenkrug (1991) reported a glycine-extended form of VIP, which is produced as an intermediate during enzymatic α-carboxyamidation. It can be hypothesized that this form might represent the immediate precursor of VIP-(22–28) in vivo.

In conclusion, the present study suggests that cerebral neurons containing a VIP-(1–28)/VIP-(22–28) system are involved in PTZ-induced seizures. Further studies are in progress to define the possible role of VIP-related peptides in epilepsy.

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