CYSTEAMINE NORMALIZES CEREBRAL SOMATOSTATIN LEVEL AND
BINDING IN PENTYLENETETRAZOL-KINDLED RATS.

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Summary

Rats were kindled by intraperitoneal injection of pentylenetetrazol (PTZ) (30 mg/Kg) every 48 h. Once kindled, some of the animals received a single injection of cysteamine (200 mg/Kg). Somatostatin-like immunoreactivity (SLI) and ¹²⁵I-Tyr¹¹-somatostatin binding were measured in the frontoparietal cortex and hippocampus of the two experimental groups and the control rats. After PTZ kindling the following was observed: 1) SLI content was increased in the two areas; 2) Somatostatin receptor affinity decreased in the frontoparietal cortex and was unaltered in the hippocampus; 3) The number of somatostatin receptors decreased in the hippocampus and was unaltered in the frontoparietal cortex. Cysteamine, an agent which depletes brain somatostatin and suppresses kindled seizures in PTZ-treated rats, reversed the altered SLI levels and binding in these rats.

"Kindling" refers to an experimental model of epilepsy in which repeated administration of a subconvulsive dose of a drug (pentylenetetrazol, cocaine, carbachol and lidocaine) or electrical current induces a progressive intensification of seizure activity, that culminates with a generalized seizure (1-6). Although the phenomenon of kindling has been extensively investigated, the basic biochemical mechanism underlying the development and maintenance of this effect is still unclear.

It has been suggested that the peptide somatostatin, a possible neurotransmitter or neuromodulator in the central nervous system (7), may play a role in kindling processes (8-11). It was recently reported that the levels of somatostatin-like immunoreactivity (SLI) are decreased in the rat brain, particularly in the striatum, by the injection of a single convulsant dose of pentylenetetrazol (PTZ) (12). Moreover, Assouline et al. (11) have demonstrated that seizures in pentylenetetrazol-kindled rats are suppressed following systemic administration of cysteamine (β -mercaptoethylamine), an agent which causes depletion of brain and gastrointestinal SLI (13). These results suggest that changes in endogenous brain SLI and/or receptors may be critically involved in the kindled state. To test this hypothesis, we used two approaches. First, we determined the effect of the repeated administration of subconvulsant doses of pentylenetetrazol on somatostatin levels and receptor number in rat frontoparietal cortex and hippocampus. Second, the effect of cysteamine on the same parameters in rats which had been subjected to repetitive systemic administration of subconvulsive doses of pentylenetetrazol.

Materials and Methods

Chemicals.

The origin of the chemicals used was the same as in (14). Pentylentetrazol and cysteamine were purchased from Sigma (St. Louis, M.O. U.S.A.). Tyr¹¹-somatostatin was radioiodinated by the chloramine-T method (15). Specific radioactivity of the tracer was about 350 Ci/g. The rabbit antibody used in the radioimmunoassay technique was purchased from the Radiochemical Center (Amersham, U.K.). This antiserum was raised in rabbits against somatostatin-14 conjugated to bovine serum albumin and is specific for somatostatin, but since somatostatin-14 also constitutes the C-terminal portions of somatostatin-25 and somatostatin-28, it does not distinguish between the three forms.

Experimental animals.

Adult Sprague Dawley rats initially weighing approximately 230 g, were divided into three groups. PTZ kindling was achieved through administration of subconvulsant PTZ doses (30 mg/2ml 0.9% NaCl/Kg i.p.) every 48 h. as previously described (16). This schedule avoided drug accumulation because the half-life of PTZ in the rat is less than 4 h (17). The control group was injected with an equivalent volume of saline on a similar schedule. After PTZ injection, the behaviour of the animals was observed for 20-25 min. Behavioural changes were recorded and classified according to a seizure ranking system (16). In all animals, the first PTZ injection did not have any obvious convulsive effect. The severity of subsequent responses increased progressively, and all animals exhibited a fully developed seizure between 7th and 20th PTZ injection as previously described (11). The animals were sacrificed 44 days after the first PTZ or saline injection. In the third group, a single intraperitoneal injection of cysteamine (200 mg/Kg) was administered intraperitoneally, 24 h after the last PTZ injection, and the animals were sacrificed 3 days later.

Tissue extraction and somatostatin radioimmunoassay.

Somatostatin was extracted from both the frontoparietal cortex and hippocampus following the method of Patel and Reichlin (18), and since the yield of the overall extraction procedure was about 85% in the two brain areas studied, the recovery of somatostatin was similar in both brain areas. Protein was determined by the method of Lowry et al. (19). Immunoreactive somatostatin levels were measured by a modified specific radioimmunoassay method (18), with a sensitivity limit of 10 pg/ml. Incubation tubes prepared in triplicate contained 100 μ l samples of unknown or standard solutions of 0-500 pg cyclic somatostatin tetradecapeptide diluted in phosphate buffer (0.05 M, pH 7.2 containing 0.3 % bovine serum albumin, 0.01 M EDTA), 200 μ l appropriately diluted anti-somatostatin serum, 100 μ l freshly prepared ¹²⁵I-Tyr¹¹-somatostatin diluted in buffer to give 6000 cpm (equivalent to 5-10 pg), and enough buffer to give a final volume of 0.8 ml. All reagents and assay tubes were kept chilled in ice before the 48 h incubation at 4°C. Separation of bound and free hormone was accomplished by addition of 1 ml dextran-coated charcoal (dextran 0.2 % w/v; charcoal 2 % w/v). Dilution curves for each brain area were parallel to the standard curve. The intra- and inter-assay variation coefficients were 6.5 % and 8.1 % respectively.

Binding assay on membrane preparations.

Synaptosomal membranes from the frontoparietal cortex and hippocampus were prepared as described by Reubi et al. (20). The specific binding of ¹²⁵I-Tyr¹¹-somatostatin to synaptosomal membranes from frontoparietal cortex

and hippocampus was performed according to the modified method of Czernik and Petrack (21). Briefly, the binding assay was conducted under standard conditions at 30°C in 250 μ l of a medium consisting of 50 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 0.2 % bovine serum albumin and 0.1 mg/ml bacitracin with 250 pM ^{125}I -Tyr¹¹-somatostatin (up to 10 nM). The reaction was initiated by the addition of synaptosomal membranes (1.5 mg protein/ml). After a 60 min incubation, membrane-bound peptide was separated by centrifugation and the radioactivity determined. Nonspecific binding was obtained from the amount of radioactivity bound in the presence of 10^{-7} M somatostatin and represented about 25 % of the binding observed in the absence of unlabelled peptide. This nonspecific component was subtracted from the total bound radioactivity in order to obtain the corresponding specific binding. The inactivation of ^{125}I -Tyr¹¹-somatostatin in the incubation medium after exposure to membranes was studied by observing the ability of the peptide to rebound to fresh membranes (22).

Statistical analysis.

Individual experiments were performed in duplicate. All results are given as the means $\pm$ SEM, and the statistical significance of the differences between groups was calculated by Student's "t" test.

Results

The rate and pattern of PTZ-kindling in the rats were similar to those described previously (11). Table 1 shows that the SLI content in frontoparietal cortex and hippocampus of PTZ-kindled rats is significantly higher, when compared with controls. Cysteamine administration returned the concentration of cortical and hippocampal SLI to control levels in PTZ-kindled rats.

Table 1

Influence of pentylentetrazol (PTZ) and pentylentetrazol plus cysteamine treatment on somatostatin-like immunoreactivity (SLI) levels in the frontoparietal cortex and hippocampus. Results are expressed in ng somatostatin/mg protein as the mean $\pm$ SEM.

Groups	Frontoparietal cortex	Hippocampus	n
Control	6.01 $\pm$ 0.37	5.60 $\pm$ 0.20	5
PTZ	11.15 $\pm$ 1.05(*)	8.48 $\pm$ 0.45(*)	5
PTZ plus cysteamine	7.14 $\pm$ 0.70	5.99 $\pm$ 0.50	5

Statistical significance : * $p < 0.005$ vs control value.

Brain plasma membranes of both control and experimental groups bound ^{125}I -Tyr¹¹-somatostatin in a time-dependent fashion; an apparent equilibrium was observed between 50-180 min at 30°C (data not shown). All subsequent binding studies were, therefore, conducted at 30°C for 60 min. To rule out the possibility of different somatostatin-degrading activities in the membrane preparations that might affect the interpretation of results, the degradation of the peptide was determined (22). Membranes from both brain areas showed a similar peptide degradation capacity (10-12 %) in all cases.

Increasing concentrations of unlabelled somatostatin competitively inhibited the specific binding of ^{125}I -Tyr¹¹-somatostatin to brain membrane preparations from both the experimental groups and the control group (Fig.1,

left panels). However, the specific binding of the tracer on brain membranes in the PTZ-kindled group was significantly lower than that in the control animals in both the absence and presence of unlabelled somatostatin throughout the whole range of concentrations studied.

Fig.1

Influence of pentylene-tetrazol (PTZ) and pentylene-tetrazol plus cysteamine treatment on somatostatin binding to (A) frontoparietal cortex and (B) hippocampal membranes. **Left panel**, membranes (1.5 mg protein/ml) were incubated for 60 min at 30°C in the presence of ^{125}I -Tyr¹¹-somatostatin (^{125}I -Tyr¹¹-SS) and increasing concentrations of native peptide. Values for control (●), PTZ-treated (○) and PTZ-and-cysteamine-treated (△) animals are expressed as the mean $\pm$ SEM of five replicate experiments. **Right panel**, Scatchard analysis of the same data.

To determine whether this decrease in specific binding exhibited by PTZ-kindled rats was due to a change in either the affinity or the number

of receptors, the stoichiometric data were interpreted by the method of Scatchard (23). These studies revealed that there was a decrease in the number of specific somatostatin receptors in the hippocampus and an increase in the dissociation constant in the frontoparietal cortex of PTZ-kindled animals (Fig.1, right panels and Table 2). Scatchard analysis indicated a single class of receptors; the K_d values were in the nanomolar range.

Table 2

The effect of PTZ and PTZ plus Cysteamine treatment on specific somatostatin receptors in rat frontoparietal cortex and hippocampus.

GROUPS	BRAIN AREAS	
	FRONTOPARIETAL CORTEX	HIPPOCAMPUS
CONTROL		
K_d (nM)	0.325 ± 0.02	0.249 ± 0.02
Bmax	248 ± 10	235 ± 12
PTZ		
K_d (nM)	$0.496 \pm 0.04(*)$	0.207 ± 0.03
Bmax	226 ± 9	$161 \pm 9(*)$
PTZ plus cysteamine		
K_d (nM)	0.277 ± 0.04	0.278 ± 0.04
Bmax	216 ± 18	233 ± 19

Binding parameters were obtained by Scatchard (23) analysis of data from right panels in Fig.1. Bmax, Binding capacity (femtomoles of somatostatin bound per mg protein). Values represent the mean $\pm$ SEM of five rats in each group.

* $p < 0.005$ vs. control.

Cysteamine injection led to a suppression of PTZ-induced seizures in PTZ-kindled rats. This treatment completely reversed the increased SLI content and decreased somatostatin binding levels that had been induced by PTZ-kindling (Fig. 1 and Table 2).

Discussion

This study indicates that PTZ-kindling increased SLI levels in the frontoparietal cortex and hippocampus while it decreased the apparent affinity of somatostatin receptors in the frontoparietal cortex and the number of somatostatin receptors in the hippocampus. These effects are reversed by a single dose of cysteamine.

The frontoparietal cortex and hippocampus were selected for analysis because of the evidence that the histopathological changes in PTZ-kindled rats are concentrated in these brain areas (24). In addition, the somatostatin levels and receptor numbers are high in these regions (12,25,26).

The somatostatin levels in both brain areas as well as the binding parameters of brain somatostatin receptors in control rats were similar to those previously reported by others (12,25,26). It should be mentioned that the Scatchard analysis suggested a single receptor population. This feature agrees with some studies (12,21,27), but differs from other previously reported data (28,29). It is conceivable that use of reduced smaller somatostatin analogs (28) or their labelling with very different isotopes (29) might explain this inconsistency.

The reason for the elevated somatostatin levels in the frontoparietal cortex and hippocampus of rats kindled by PTZ remains obscure. It has previously been reported that a single-dose of PTZ did not change the somatostatin content in rat cerebral cortex and hippocampus (12), while in kindling, induced either electrically or pharmacologically, somatostatin was increased in the cortical and subcortical areas of the rat brain (9,10). In addition to somatostatin, changes in the catecholaminergic (30), cholinergic (31) and GABAergic (32) systems have been found after PTZ seizures. Furthermore, enkephalins, dynorphin and thyrotropin-releasing hormone levels, (but not those of β -endorphin), are elevated in the brains of kindled rats (33,34,35).

It might be that in the kindled state there is an increased release of somatostatin in addition to the high content of brain somatostatin. In this regard, Pitkanen et al.(36) have shown that after PTZ-induced generalized convulsion the amount of SLI released into cisternal cerebrospinal fluid was higher in kindled rats than in controls. In general, if the release of ligand increases, the number of receptors for the ligand decreases over time by down regulation and we did detect a decrease of somatostatin receptors in the hippocampus of PTZ-kindled rats. In this regard, recent data has indicated that hypothalamic somatostatin release causes a reduction in the number of somatostatin receptors in rat pituitary membranes (37). It has been also shown that preincubation of pituitary tumor cells with somatostatin results in a marked decrease of the number of somatostatin receptors (38). Thus, regulation of somatostatin receptors appears to be similar to that of other hormone-receptor systems (39-41). However, increasing site occupancy has been shown to be accompanied by decreased affinity in a variety of hormone-receptor systems (42), as was also found in this study at the cortical level.

Higuchi et al.(43) have shown that the number of somatostatin receptors in amygdala kindling are decreased in the hippocampus. Therefore, in the hippocampus both electrically stimulated amygdaloid (43) and PTZ kindling lead to somatostatin receptor changes which result in a decreased ^{125}I -Tyr¹¹-somatostatin binding. Similar changes in this brain structure, known to be highly susceptible to seizure-producing stimuli (1-6), suggest that lowering the number of somatostatin receptors in this brain region may take part in the development of kindled seizures.

The receptor alterations found in the frontoparietal cortex after PTZ kindling in the present study are opposite to the changes found after amygdaloid kindling by Higuchi et al.(43). Who found a significant increase in somatostatin receptor density after amygdaloid kindling. These previous findings together with present results suggest that differing mechanisms operate in response to different seizure-inducing stimuli in the frontoparietal cortex.

The present data together with the lack of significant changes in the number and affinity of somatostatin receptors after the acute seizures induced by Pitkanen et al.(12) suggest that the changes in somatostatin receptor binding after PTZ kindling were not related to seizure sequelae.

It has been shown that cysteamine-induced depletion of brain and stomach somatostatin is associated with up-regulation of the respective cerebrocortical and gastric somatostatin receptors (44,45). We have noted that following intraperitoneal injection of cysteamine, there is complete inhibition of seizures in PTZ-kindled rats. These results are consistent with the previous findings of Assouline et al (11). These authors demonstrated that this effect is actually related to an influence of cysteamine on the "kindled state", and not to an anticonvulsant property of the drug itself.

The time course of the cysteamine influence in PTZ-kindled rats is similar to the time course reported for cysteamine-induced depletion of brain immunoreactive somatostatin (13). On the other hand, cysteamine reversed the changes in the somatostatin levels and binding in PTZ-kindled rats.

These data also provide evidence to support the hypothesis that somatostatin receptors play a role in PTZ-kindled seizure development. If the decreased ¹²⁵I-Tyr¹¹-somatostatin binding found following PTZ kindling is a reflection of a functional subsensitivity, then the present data lend support to the view that a decrease in response to somatostatin, a central nervous system depressing neurotransmitter (46-48), may be a general mechanism involved in the development of increased seizure susceptibility.

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