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Life Sciences, Vol. 24, pp. 1813-1822 1979 (1979) Pergamon Press
Printed in the U.S.A.

SEROTONIN SENSITIVE ADENYLATE CYCLASE IN HORSE BRAIN SYNAPTOSOMAL MEMBRANES

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(Received in final form March 28, 1979)

Summary

In different membranal preparations isolated from horse brain striatum we have shown the existence of an adenylate cyclase system sensitive to serotonin (5-HT). Activation of the adenylate cyclase was determined by measuring cAMP using a radioimmunoassay. This serotoninergic sensitive enzyme is characterized by a high apparent affinity constant (in the nanomolar range), located on synaptosomal membranes. It is inhibited by antiserotoninergic drugs (cyproheptadine, cinanserin, methysergide, LSD), and synergistically activated by GTP. This serotoninergic activation is clearly additive to the activation induced by dopamine. It appears different from the adenylate cyclase system previously described in the literature which is also activated by 5-HT, but which has a low apparent affinity constant (in the micromolar range); the latter is apparently located in non-synaptosomal membranes, and its activation by 5-HT is non-additive to the activation induced by dopamine.

The serotoninergic senstive adenylate cyclase reported in this study, might be related to the serotoninergic binding system which we have previously described which has a similar affinity constant, a similar subcellular distribution and which is inhibited in the same concentration ranges by antiserotoninergic drugs. These two systems might represent a synaptosomal serotoninergic receptor complex.

Different types of molecular events might follow the binding of a neurotransmitter to its receptor system : i.e. the opening of an ionophore as in the case of the nicotinic receptor (1) or the activation of an enzymatic system for the β-adrenergic receptor system (see a review) (2). In the case of a 5-HT receptor system some studies have been realized, mainly in rat brain : von Hungen et al (3) reported the existence of an adenylate cyclase system responsive to 5-HT in superior and inferior colliculi of very young rats; the apparent affinity constant was close to 1 µM.

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0024-3205/79/191813-09$02.00/0

These results have been confirmed and developed in various brain areas in new born rats (4,5). Pagel et al (6) observed in rat brain synaptosomal membranes several populations of activation sites with various apparent affinity constants : 0.5,5,10 and 70 nM. Using horse brain (striatum) we have observed a 5-HT sensitive adenylate cyclase which corresponds to a high apparent affinity (K_A close to 1 nM) and which is localized in synaptosomal membranes as a single population of sites.

Material and Methods

Preparations of the membranal fractions

Horse brains were collected at the slaughter-house 15 to 20 minutes after sacrifice of the animals; they were dissected on ice in order to isolate various brain areas. The results reported herein were obtained using the striatum; each experiment was performed on material prepared from pooled striatum isolated from three brains at each time.

The various membrane preparations were performed as previously described (7,8). Briefly, the tissues were homogenized using a teflon grass homogeneizer and a "crude mitochondrial fraction" was prepared as usual. This fraction was centrifuged on a discontinuous Ficoll density gradient (7-13 %), to obtain the "synaptosomal fraction" (on the 13 % Ficoll layer) and a "microsomal" fraction (on the 7 % Ficoll layer). These fractions were then lysed and subjected to differential centrifugation to obtain the enriched synaptosomal membrane fraction and the microsomal membrane fraction. During the entire procedure, the standard medium used was Tris-HCl buffer 50 mM pH 7.4 at 2° C.

Adenylate cyclase assay

The adenylate cyclase activity was determined by measuring the cAMP production using a specific and sensitive radioimmunoassay (RIA). Samples of 0.5 ml were incubated at 30° C for 2 minutes in a medium (standard medium) consisting of Tris-HCl buffer 50 mM pH 7.4, EDTA 0.5 mM, ATP 0.4 mM, $MgSO_4$ 2 mM, and isobutyl methylxanthine 1 mM. The reaction was stopped by immersion of the tubes in a boiling water bath and the addition of 1 ml ethanol. After centrifugation, the supernatant fluids were collected, lyophilised and resuspended in RIA buffer (sodium acetate 50 mM pH 6,2). The following solutions were added successively into polypropylene test tubes: 0.1 ml of the standard or the sample solutions, 0.1 ml of iodinated tracer (≃ 10,000 dpm) and 0.1 ml of antiserum diluted to give 50 % binding of the labeled antigen in the absence of unlabeled cyclic nucleotide. The tubes were gently vortexed and allowed to equilibrate for 18 to 24 h at 4° C. The free and bound antigens were separated by the addition of 50 µl of 1 % bovine serum albumine and 1 ml of cold ethanol. The tubes were centrifuged at 2,200 g for 15 minutes. The supernatant fluid was discarded and the radioactivity of the pellet (bound fraction) was counted in an NE 1500 gamma spectrometer. All the manipulations were performed at 4° C. The minimum detectable amount of adenosine 3',5'-monophosphate (cAMP) was approximately 1 pmole. Unlabeled cAMP (50 picomoles) reduced the amount of label bound by 50 %. The cross-reactivity of other structurally related nucleotides tested was less than 0.3 % at 50 % displacement. Internal standars (10 and 100 picomoles) of cAMP were added to aliquots of some samples as controls.

Protein assays

The protein determinations were realized using the Method of Lowry et al (9).

Chemicals

5-Hydroxytryptamine creatinine sulfate was purchased from Koch Light, ATP, GTP and other nucleotides from SIGMA company. Rabbit cAMP antibodies (Institut Pasteur Production N° A/79590) were obtained according to procedure described by Steiner et al (10). Succinyl cAMP-Tyrosine methyl ester was iodinated with ^{125}I using the method of Hunter and Greenwood (11) and purified by thin layer chromatography.

Results

Adenylate cyclase activation

The effects of 5-HT on the adenylate cyclase activation have been studied on three different preparations:

Crude mitochondrial fraction

Serotonin tested at concentrations ranging from 0.5 nM to 10 µM enhances the production of cAMP with two distinct apparent affinities, one corresponding to a high apparent affinity close to 1 nM, the other to a low apparent affinity close to 1 µM (fig. 1A). Similar results have been observed for crude striatum.

Enriched synaptosomal membrane preparation

Using a similar concentration range of 5-HT, a single peak of activation of adenylate cyclase corresponding to a high apparent affinity (K_A close to 1 nM) was observed. No additional cAMP production was detectable in the micromolar range (fig. 1B).

Microsomal membrane preparation

Serotonin activates an adenylate cyclase system with two apparent affinities : the highest apparent affinity constant corresponding to a K_A close to 1 nM, the other has a low affinity constant close to 1 µM.

The cAMP productions observed with the different preparations are illustrated in Table I.

Table I

Preparation	cAMP Production in Picomole/mg Protein min^{-1} Corresponding Affinity Constants	
	µM Range	nM Range
Crude mitochondrial fraction	40-50	5-10
Synaptosomal Mb	Not detected	20-60

Specificity

The high affinity constant activation present in enriched synaptosomal membrane preparations is inhibited by antiserotoninergic drugs such as cinanserin, cyproheptadine and methysergide; the observed ID 50 % were 20, 0.6 and 0.2 µM respectively. These

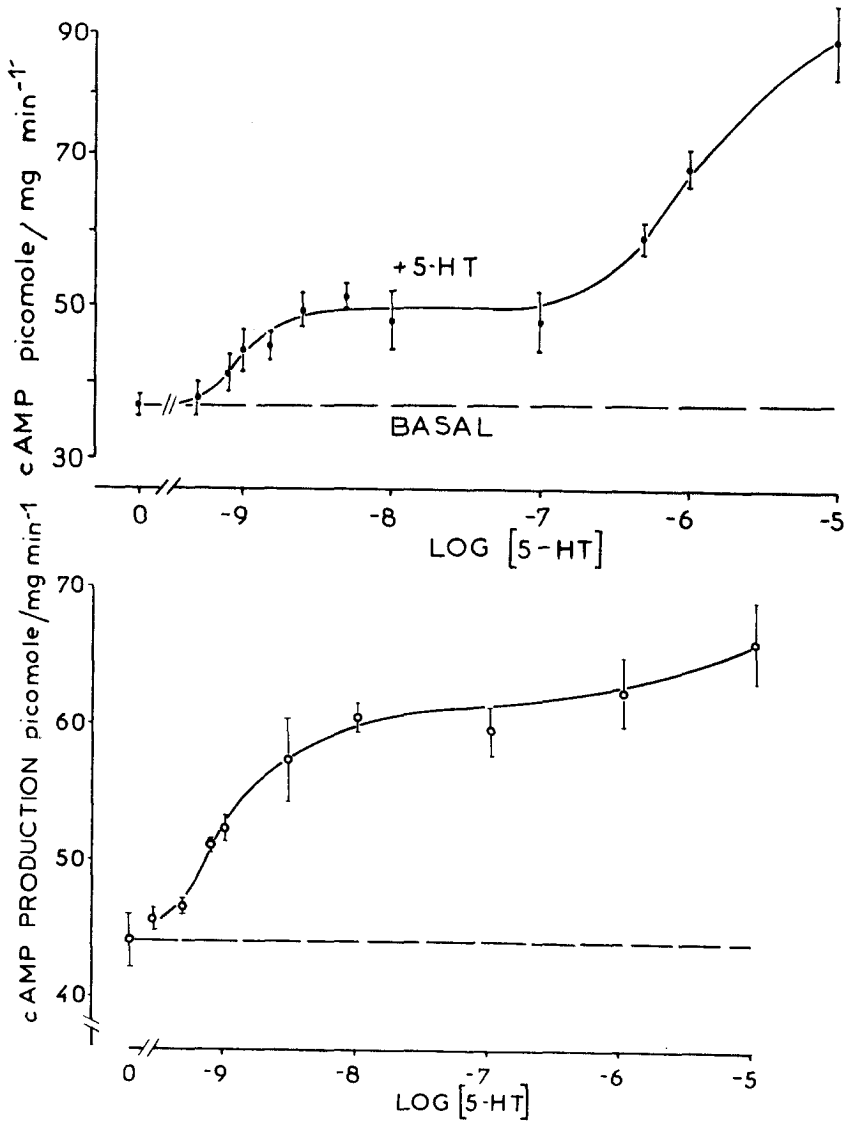

Fig. 1

cAMP production induced by 5-HT in the crude mitochondrial fraction (A. upper panel) and in the synaptosomal membrane fractions (B. lower panel). The membrane fractions were incubated at 30° C for 2 minutes in standard medium in presence or in absence of the indicated concentrations of 5-HT; the reaction was stopped and cAMP determined as described in Material and Methods.
Each point corresponds to the mean value of triplicates ± S.E.
Apparent affinity constants were calculated by Hill plots and corresponded to a value of 1.2 nM in the crude mitochondrial fraction and 1.4 nM in the synaptosomal membrane fraction.
Experiments have been repeated 8 times.

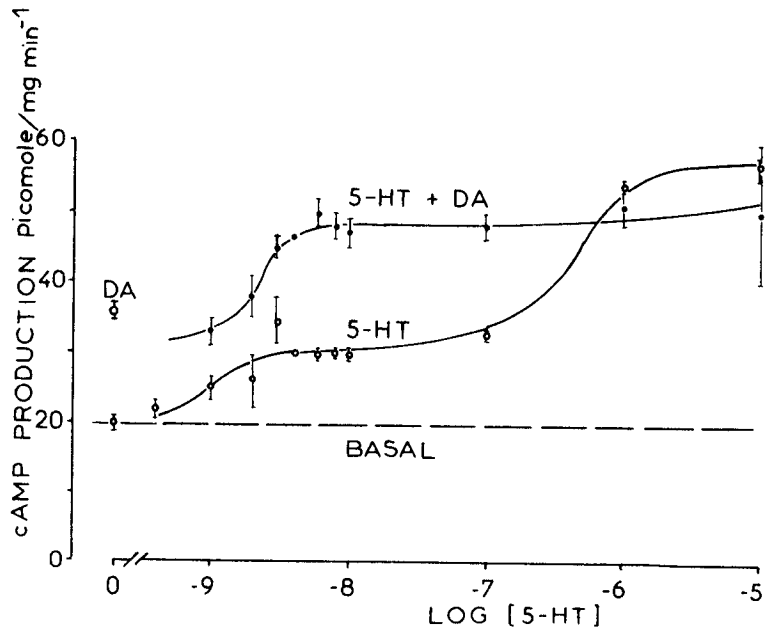

Fig. 2

Additive effects of 5-HT and dopamine (DA) on crude mitochondrial cAMP production.
o—o Control curve of 5-HT induced cAMP production.
●—● The same curve in presence of 1 µM corresponded to an increase of the cAMP production from 20 picomole/mg min^{-1} (basal level) to 36 ± 1 picomole mg protein min^{-1}
Each point corresponds to the mean value of triplicates ± S.E.
The experiments were reproduced twice.
Membrane incubations were performed at 30° C for 2 minutes in the standard medium and in the presence of 1 µM GTP.

substances have no direct effect (cinanserine and methysergide) or a weak effect (cyproheptadine at 10 µM induced an activation corresponding to 30 % of the maximal effect of 5-HT) on the activation of the adenylate cyclase at the concentration ranges used (10 nM to 10 µM).

Lysergic acid diethylamide (LSD) at low concentrations (0.3 nM to 10 nM) has slight stimulating effects on the adenylate cyclase whereas at higher concentrations (10 nM to 1 µM) it markedly increases the production of cAMP. At low concentrations, it inhibits the 5-HT induced adenylate cyclase activation (ID_{50} = 5 to 10 nM).

Dopamine is a well known activator of a striatal adenylate cyclase system with a low apparent affinity constant ($K_A \simeq 1$ µM) (see a review 12). This effect of dopamine is strictly additive to that of 5-HT which corresponds to the high apparent affinity constant (fig. 2).

Effect of GTP

The basal level of cAMP production is increased by GTP (the corresponding affinity constant if close to 0.5 μM). This effect is synergistic with that of 5-HT: in the absence of GTP, 5-HT activates the adenylate cyclase inducing a maximal increase in cAMP production of a few picomoles per mg protein per minute. In the presence of GTP (1 μM) the net maximal increase induced by 5-HT was enhanced to approximately 60 picomoles per mg protein per min. This effect was observed on the crude mitochondrial fraction as well as on the synaptosomal membrane fraction.

In the presence of GTP, the cAMP production is consistently enhanced by low concentrations of 5-HT without any change in the apparent affinity constant; this GTP effect corresponds to an ED 50 close to 0.2 μM (fig. 3). The other nucleotides studied did not appear to have such marked properties.

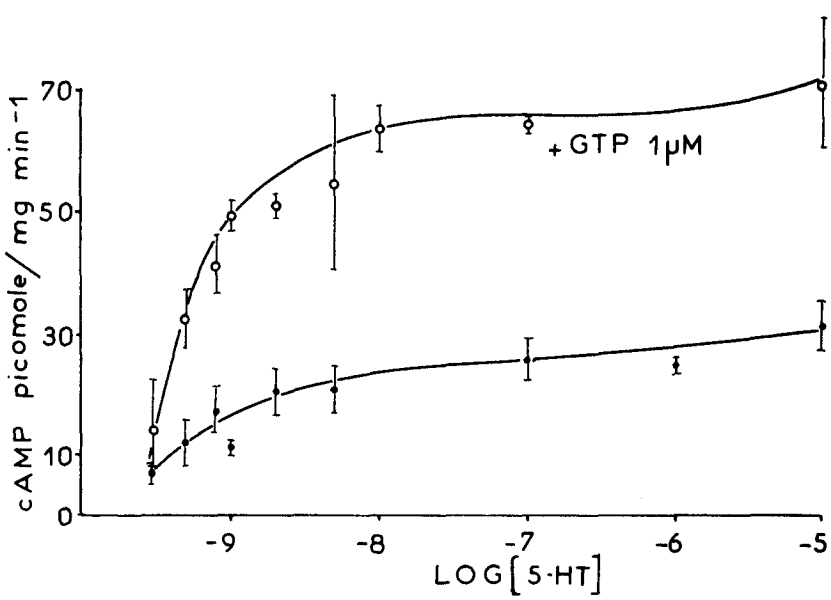

Fig. 3

Synergistic effect of GTP to 5-HT induced cAMP production of synaptosomal membranes.
Incubations (2 minutes at 30° C) of synaptosomal membranes were performed in a standard medium in the presence (o——o) or in the absence (●——●) of 1 μM GTP. The apparent affinity constants were the same for the two curves (0.5 nM). Basal activities were 153 ± 16 and 43 ± 2 picomole/mg protein min^{-1} respectively. Each point corresponds to the mean value of triplicates ± S.E. Experiments were reproduced three times.

Discussion

These results demonstrate the existence of an adenylate cyclase system which is activated by low concentrations of 5-HT. The apparent affinity constant is in the nanomolar range. This activation appears to be located in synaptosomal membranes; it is present in crude striatum homogenates, in the crude mitochondrial fraction, and in synaptosomal membrane enriched preparations. The enrichment of the preparation in synaptosomal membranes corresponds to an increase in cAMP production by 5-HT (from 2 to 10 times depending on the preparations). Experiments using lesions are in progress to confirm these results and to determine if the location of the sites is pre- or postsynaptic. Preliminary results however, indicate that they are located postsynapticly.

These sites are specific for 5-HT. Antiserotoninergic drugs such as cinanserin, cyproheptadine and methysergide inhibit the 5-HT induced activation of the enzyme at concentrations which have no or only a weak stimulating effect on adenylate cyclase. Lysergic acid diethylamide activates the enzyme with two different affinity constants (K_{D1} = 0.2 nM and K_{D2} = 20 to 30 nM). The specificity of these two different activations is studied currently; preliminary results tend to indicate that the enzymatic activation induced by LSD with a high apparent affinity, non-additive with the 5-HT induced activation, is serotoninergic. The activation induced by LSD with a low apparent affinity, additive with that induced by 5-HT, would be dopaminergic. Stimulating effects of LSD on adenylate cyclase have been previously described in new-born rat colliculus with a very low apparent affinity (K_D = 10 µM) (3) and in homogenates of rat brain striatum with a very low (K_D = 10 µM) (13) or a low affinity (K_D = 0.14 µM) (14). These latter activations might have similarities with the low affinity activation we observe in our assays although the affinity constants were not identical; this difference might originate from the fact that we use membranes and not homogenates and that the experimental conditions are not the same.

The 5-HT induced activation of adenylate cyclase is enhanced by GTP. The effect is similar to that previously reported for ß-adrenergic receptors (2-15). This suggests the hypothesis that this serotoninergic receptor system is represented as has been proposed by Pfeuffer (15) for the ß-adrenergic receptor, by a catalytic protein regulated by a protein complex. This site of action of GTP is coupled to the neurotransmitter specific binding site.

The high affinity activation of adenylate cyclase by 5-HT herein described might have some similarities to one of the numerous high affinity activation peaks described by Pagel et al (5). However, our findings differ from their results as we have not observed, in any preparation, such multiple peaks of activation at low 5-HT concentrations. These discrepancies might originate from the specificity of the different methods used to determine cAMP.

The high affinity serotoninergic activation system appears distinct from that previously described for new-born rat brains (2,3,4) and which we have also observed (see fig. 1A). First, the latter system corresponds to an apparent affinity constant which

is 1000 times lower; second, we have observed that the subcellular localization of these two types of activation appear to be different as both sites are present in crude mitochondrial fraction and only the high affinity sites are observed after purification of the synaptosomal membranes. The low affinity sites are found in the microsomal fraction (together with high affinity sites as a result of synaptosomal contamination) indicating that they might be localized in non-synaptosomal membranes (possibly glial). They might also correspond to enzymatic sites (i.e. degradative enzymes). Although the probability of a common location for the two types of sites is weak, we cannot totally exclude the possibility that the differences observed in their subcellular distributions are the result of the preparation process since the microsomal and the synaptosomal membranes are obtained in a different physical environment. Finally, it has been reported (4) that the low affinity 5-HT sensitive adenylate cyclase and the dopamine sensitive adenylate cyclase were activated in a non-additive manner. The serotoninergic activation of adenylcyclase described herein and corresponding to a high affinity constant is clearly additive to that of dopamine. These findings thus, clearly differentiate the high apparent affinity and low apparent affinity adenylate cyclase activation sensitive to 5-HT.

The observation that the stimulating effect of dopamine on adenylate cyclase is strictly additive to that of 5-HT indicates that the two neurotransmitters act on systems which do not appear to be directly related.

We previously observed the existence of two binding site populations for 5-HT in various brain membrane preparations (7,8); on synaptosomal membranes a single population of high affinity binding sites was observed which are specific, reversible, and saturable. It is tempting to suggest that such a population of binding sites is related to that of adenylate cyclase activation sites : both have similar affinity constants in the nanomolar range, and are distributed in parallel in subcellular fractions of the striatum. The serotoninergic specificity of the binding sites seems to correspond to that of the cyclase activation sites as the antiserotoninergic drugs have very similar IC 50 values in both cases (cyproheptadine = 0.6 µM and 2 µM; cinanserine = 20 µM and 9.5 µM; methysergide = 0.2 µM and 1 µM for cyclase activation and binding, respectively).

These results support the hypothesis of the existence of a serotoninergic receptor complex which is localized on synaptosomal membranes and which involves a binding site for 5-HT coupled to an adenylate cyclase and which is sensitive to 5-HT in the nanomolar range. The cyclase unit is synergistically activated by GTP, and its activation by 5-HT involves a process which is different from that involved for dopamine.

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