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EVIDENCE FOR AN ENDOGENOUS FACTOR INVOLVED IN MAINTENANCE OF PIRENZEPINE HIGH-AFFINITY BINDING IN RAT BRAIN STEM

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Sucrose gradient centrifugation was used to isolate membranes enriched in muscarinic receptors from bovine brain stem. Unlike the receptors in crude synaptosomal preparations of this tissue, the enriched preparations displayed only low-affinity pirenzepine binding. Similar results were obtained when purified preparations were preincubated for 1 hr in pH 7.0 buffer at 37°C; however, preincubation in a pH 5.0 buffer partially restored the high-affinity pirenzepine binding. These results suggest that an endogenous factor, which is present in the crude synaptosomes of the brain stem and is removed by sucrose gradient centrifugation, is involved in maintenance of the high-affinity pirenzepine binding of the muscarinic receptors.

It is now well established that the muscarinic receptors in the central and peripheral nervous system exist in subtypes. According to the current working nomenclature, muscarinic M1 receptors display high affinity for the compound pirenzepine whilst those with low affinity for this non-classical muscarinic antagonist are termed M2 receptors (1, 2). Molecular biological studies have revealed that at least three subtypes of M1 receptors exist (3), and the accumulating pharmacological evidence indicates that M2 receptors are not homogeneous either (4, 5, 6).

Activation of muscarinic receptors results in various biochemical and electrophysiological responses and it is believed that the signal transduction is mediated by guanine-nucleotide-binding (G) proteins (7). Purified muscarinic receptors from brain cortex and atrium have been shown to interact with purified G-proteins in reconstituted systems (8, 9). Recent studies in our laboratory strongly suggest that muscarinic receptors in a synaptoneurosomal fraction of brain stem are effectively coupled to sodium channels by G-proteins (10). To facilitate the study of muscarinic receptor interaction with membrane constituents, we partially purified muscarinic receptors from bovine brain stem by sucrose gradient centrifugation. Binding studies carried out with this preparation suggested that factors other than the primary structure of the muscarinic receptor govern the high-affinity binding of pirenzepine.

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MATERIALS AND METHODS

Materials. Scopolamine methyl chloride, [N-methyl-^{3}H] ([^{3}H]NMS; 73.8 Ci/mmole) and propylbenzilylcholine mustard [propyl-2,3-^{3}H] ([^{3}H]-PrBCM; 42.8 Ci/mmol) were purchased from NEN. Pirenzepine hydrochloride was a generous gift from Dr. R. Hammer, Karl Thomae GmbH, Biberach an der Riss, FRG. 3-(2'-Aminobenzhydryloxy) tropane (ABT) was synthesized and coupled to Sepharose according to the procedures of Haga and Haga (11). Digitonin was obtained from Merck. Atropine sulfate and carbachol chloride were from Sigma. To prevent proteolysis, all buffers contained (unless indicated) pepstatin A (1 µg/ml) aprotinin (1 µg/ml, 20 kallikrein units/ml), leupeptin (1 µg/ml) (all from Sigma), 0.1 mM PMSF and 1 mM EDTA.

Membrane fractionation

Crude synaptosomal fraction. Brain stem and hippocampus were homogenized in 50 mM Na-phosphate buffer pH 7.0, using a motor-driven Teflon pestle homogenizer. Homogenates were centrifuged at 30,000 g for 15 min and the pellets resuspended in the fresh buffer and frozen at -80°C.

Enriched synaptosomal fraction. Brain stem and hippocampal membranes were prepared as described by Haga and Haga (11) as a first step in the purification of brain stem muscarinic receptors. Briefly, tissue was homogenized in 10 mM K-phosphate buffer pH 7.0, supplemented with 0.32 M sucrose. The homogenate was centrifuged at 11,000 g for 90 min followed by resuspension of the pellet in fresh buffer and further centrifugation at 11,000 g for 90 min. The pellet was suspended in fresh buffer and 2 M sucrose to achieve a final sucrose concentration of 0.8 M. The suspension was layered on 1.14 M sucrose and centrifuged at 100,000 g for 2 hr. The layer at the interface between 0.8 and 1.14 M sucrose and the 1.14 M sucrose layer were collected, homogenized and stored at -80°C.

Solubilization and purification. The procedures used were as described by Haga and Haga (11), with the following modifications: muscarinic receptors were solubilized by 1% digitonin and eluted from the ABT-agarose affinity column by 0.1 M carbachol. The protease inhibitors were present at every stage except during elution of the receptors from the ABT column.

Binding assay. Frozen membrane suspensions were thawed and centrifuged at 30,000 g for 15 min. The pellets were resuspended in fresh 50 mM Na-phosphate buffer, pH 7.0. Aliquots of 900 µl containing 500 µg/ml protein were added to 50 µl of [^{3}H]NMS in buffer and 50 µl of inhibitor in buffer (or buffer alone), and incubated for 1 hr at 37°C. Incubation was terminated by rapid filtration and washing of samples on Whatman GF/C glass filters with cold phosphate buffer. In some experiments binding assays were preceded by preincubation for 1 hr in 50 mM Na-phosphate buffer pH 7.0 or 50 mM Na-citrate buffer pH 5.0.

Binding activity of the purified bovine brain stem muscarinic receptors was assayed in 50 mM Na-phosphate buffer pH 7.0, 0.1% digitonin in a volume of 2 ml containing 100 µl of the receptor preparation, 100 µl of [^{3}H]NMS and 100 µl of buffer with or without competitor, i.e. atropine or pirenzepine. Incubations were conducted for a period of 24 hr at 4°C to ensure equilibrium of [^{3}H]NMS and pirenzepine binding. Incubations were terminated by ammonium sulfate precipitation on Whatman GF/B filters (12). Specific binding was assumed to be equal to the amount of binding inhibited by inclusion of 10 µM atropine. All binding data were analyzed using the LIGAND computer program (13).

SDS-urea-PAGE. Membranes resuspended in 50 ml of Na-phosphate buffer pH 7.0 were incubated with cyclized [^{3}H]PrBCM for 15 min at 25°C in the absence and presence of 10 µM atropine. Labeling was terminated by dilution with cold buffer and centrifugation at 11,000 g for 8 min, followed by 3 additional washings. Membranes labeled with [^{3}H]PrBCM were resuspended in 50 mM Na-phosphate buffer pH 7.0 and incubated for 1 hr at 37°C. After centrifugation at 30,000 g for 15 min, membranes were resuspended in 60 µl of 12 M urea followed by mixing with 30 µl of sample buffer (30 mM Tris-HCl pH 7.4, 9% SDS, 0.05% bromophenol blue and 15% glycerol). After 1 hr at 25°C samples were run on 7.5% polyacrylamide gel with a 5% stacking gel. Both gels con-

tained 4 M urea. The gels were then stained with Coomassie blue, destained and cut into 2-mm thick slices, digested for 16 hr at 40°C in a mixture of 90% Lipoluma, 9% Lumasolve and 1% H_2O, and subjected to liquid scintillation counting.

Protein assays. Protein concentrations of membrane preparations and purified receptor samples were measured by the Lowry method (14) and the fluorescamine assay (15), respectively.

RESULTS

Table 1 summarizes the profiles of [^{3}H]NMS and pirenzepine binding registered during the various steps of the (partial) purification of bovine brain stem muscarinic receptors. With the initial crude membrane homogenate isolated after a brief period of centrifugation at 30,000 g we obtained a muscarinic receptor preparation of 145 fmol/mg protein. Competition with [^{3}H]NMS binding by pirenzepine revealed that about 40% of the receptors displayed high affinity and 60% displayed low affinity for pirenzepine (Figure 1A). Because of the fairly low concentration of receptors, it was considered worthwhile to isolate the cell membranes by sucrose gradient centrifugation. However, the membrane suspension thus obtained showed only a slightly higher concentration of receptors. More interestingly, it was now possible to analyze the pirenzepine competition curves in terms of a one-binding-site model, which indicated only a low-affinity pirenzepine binding state of the receptor (Figure 1B).

Table 1. Binding parameters of [^{3}H]NMS and pirenzepine in various bovine brain stem receptor preparations

Procedure	[^{3}H]NMS	Pirenzepine
Membranes		
A. Crude synaptosomal fraction (15'; 30,000g)	K_d=272±16 pM B_{max}=145±8 fmol/mg protein	K_H= 53± 18 nM; R_H= (38±15)% K_L=870±340 nM; R_L= (62±15)%
B. Enriched synaptosomal fraction (sucrose gradient centrifugation)	K_d=184±48 pM B_{max}=170±6 fmol/mg protein	K_L=218± 10 nM; R_L=100%
Partially purified	K_d=296±151 pM B_{max}=135±84 pmol/mg protein	K_L=156± 44 nM; R_L=100%

[^{3}H]NMS-pirenzepine competition experiments were performed at a [^{3}H]NMS concentration of approximately 1.0 nM. The proportions of the high (R_H) and low (R_L) affinity binding sites are given as percentages of total [^{3}H]NMS binding. Values shown are means ± S.E.M. of at least two separate experiments done in duplicate. Competition curves were analyzed according to either a one- or a two-binding site model. Preference was given to the latter model when a significant diminution in the residual sum of squares was obtained (F-test, $p<0.05$).

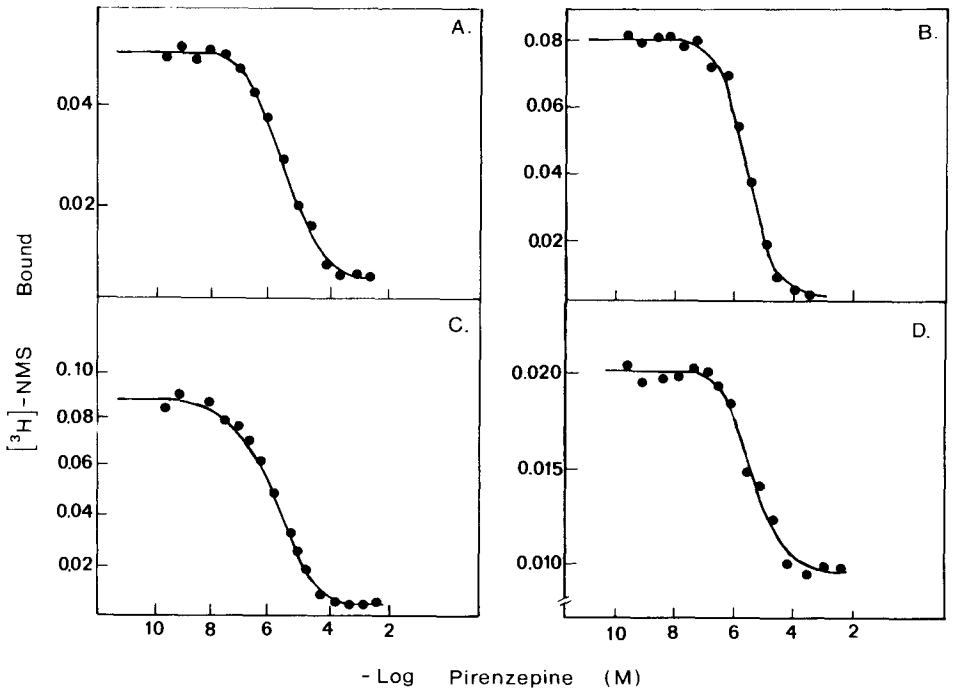

Figure 1

Inhibition of [^{3}H]NMS binding to bovine brain stem receptor preparations by pirenzepine. Each point is the mean of a duplicate measurement. A,B,C and D refer to inhibition experiments with crude synaptosomes, enriched synaptosomes, enriched synaptosomes after preincubation for 1 hr at pH 5.0, and partially purified receptor preparations, respectively. Pirenzepine inhibition curves B and D were adequately fitted by a one-binding-site model. Inhibition curves A and C were significantly better fitted by a two-binding-site model. The amount of [^{3}H]NMS bound is represented as a fraction of total (i.e. bound + free) [^{3}H]NMS.

The disappearance of the high-affinity binding state was not a species-dependent phenomenon: also in rat brain stem, where high-affinity binding sites (K_H=20 nM) were found to account for 17% of the total [^{3}H]NMS binding in the crude synaptosomal fraction (K_L=380 nM), only low-affinity sites (K_L=263 nM) were detectable in membranes obtained by sucrose gradient centrifugation. Unlike the brain stem membranes, bovine hippocampal membranes completely retained their high-affinity pirenzepine binding sites (K_H=10 nM, 76% of total binding sites). Interestingly, preincubation of the bovine brain stem membranes for 1 hr at 37°C in 50 mM Na-citrate buffer pH 5.0 partially restored the high-affinity pirenzepine binding: after subsequent incubation for 1 hr at 37°C in 50 mM Na-phosphate pH 7.0, 21(±2)% of binding sites displayed high affinity for pirenzepine (K_H=24±4 nM; K_L=670±30 nM) (Figure 1C)). In a control experiment similar preincubation in 50 mM Na-phosphate pH 7.0 yielded only the low affinity-pirenzepine binding state of the receptor (K_L=223±4 nM).

SDS-PAGE electrophoresis of [^{3}H]PrBCM-labeled bovine brain stem receptors incubated for 1 hr at 37°C at pH 7.0 or pH 5.0 revealed one band of 78,000 Da (results not shown). The receptor number found after preincubation at pH 5.0 (B_{max}=170±15 fmol/mg protein) equalled the number observed in controls (B_{max}=170±6 fmol/mg protein).

A single round of ABT-affinity chromatography of solubilized receptors yielded a 1000-fold purified receptor preparation with essentially unchanged [^{3}H]NMS and pirenzepine binding profiles (Figure 1D). These results contrast with those of Baumgold et al. (16), who observed conversion of bovine brain stem low-affinity pirenzepine binding sites into sites of higher affinity upon solubilization and purification to a similar degree of purity. The discrepancy might be attributable to different membrane fractionation and/or solubilization procedures or binding assays.

DISCUSSION

Molecular biological data indicate that muscarinic receptors of the M1 and M2 subtypes differ significantly in their amino acid sequences (3). In the present study it was found that pirenzepine high-affinity binding sites ("M1 subtype") in bovine and rat brain stem can disappear on membrane fractionation and be converted into binding sites of low affinity ("M2 subtype") The phenomenon was apparently not due to a selective proteolytic degradation of the M1 receptor, since all procedures were carried out in the presence of a cocktail of five protease inhibitors, and SDS–PAGE electrophoresis revealed a band of 78,000 Da but no evidence of proteolytic degradation products of low molecular weight. Moreover, the similary in the receptor numbers obtained after preincubation of the partially purified membranes at pH 5.0 and at pH 7.0 points to the conversion of pirenzepine high-affinity sites into low-affinity sites rather than a mere degradation.

These results suggest the possibility that an endogenous factor, which is present in crude synaptosomes and is removed during sucrose gradient centrifugation, governs high-affinity pirenzepine binding to muscarinic receptors in the bovine and rat brain stem. This study lends support to recent speculations that factors other than the primary strcuture of the receptor protein may be involved in pirenzepine binding affinity. Studies have shown that solubilization and purification of muscarinic receptors may be accompanied by alterations in their affinity for pirenzepine while nonselective antagonist binding affinity remains unchanged (16, 17). It was suggested that his phenomenon may be due to alterations in the microenvironment of the receptors (16, 17, 18). The present study demonstrates that, in addition to solubilization and purification, the mode of membrane fractionation may affect pirenzepine binding to muscarinic receptors. These findings further

emphasize the need for caution in defining muscarinic receptor subtype classifiction.

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