STUDIES ON NICOTINIC ACETYLCHOLINE RECEPTORS IN MAMMALIAN BRAIN VI. ISOLATION OF A MEMBRANE FRACTION ENRICHED IN RECEPTOR FUNCTION FOR DIFFERENT NEUROTRANSMITTERS*

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Received June 18,1976

SUMMARY

A particulate form of the nicotinic acetylcholine receptor from rat brain cortex appears to be concentrated in membrane fragments with a bouyant density of 1.112 g x ml^{-1}. The same fraction also shows enrichment in binding of agonists or antagonists for other putative neurotransmitters, indicating the presence of muscarinic and β-adrenergic receptors as well as receptors for γ-aminobutyrate, L-glutamate and, perhaps, dopamine. Based on these and other criteria we suggest that these receptors may be located on free postsynaptic membranes concentrated in this fraction.

INTRODUCTION

Receptors for neurotransmitters are membrane-associated entities, usually believed to be proteins with a postsynaptic localization, capable of transducing ligand binding into a change of membrane potential due to rapid and selective changes in cation permeability (1-3). Previous studies from this laboratory (4-8) have identified a membrane-associated protein in mammalian brain capable of interacting with and binding labeled monoiodinated α-bungarotoxin, [^{125}I]αBtx¶. This entity, enriched in the microsomal (P_3) fraction of rat brain homogenates (5), exhibits all the requisite characteristics that define a nicotinic ACh¶ receptor (1,2,9-12).

We now show that the receptor is concentrated in membranes with a bouyant

* For paper V in this series see Ref. 8. Investigation supported by Grant No. NS 08309 from the Institute of Neurological and Communicative Disorders and Stroke. We thank Prof. H David Potter for electron microscopy and helpful discussions.

† Recipient of a Research Career Award KO6 05060 from the Institute of General Medical Sciences.

§ Publication No. 2827.

¶ Non-systematic abbreviations: ACh - acetylcholine; Btx - bungarotoxin; QNB - quinuclidine benzylate

density of 1.112 g x ml^{-1} which can be isolated by isopycnic sedimentation. This observation, together with the measurement of a number of marker activities, suggests that this particular form of the ACh receptor is associated with fragments of free postsynaptic membranes. This inference is strengthened by the demonstration, also documented in this Communication, that the same membrane fraction also appears enriched in receptors for other neurotransmitters, specifically muscarinic receptors for ACh, β-adrenergic receptors, and perhaps receptors for γ-aminobutyrate, L-glutamate and dopamine.

METHODS AND MATERIALS

Subcellular fractionation - The scheme used represents an adaptation of our standard procedure (6): Cerebral cortices from 40 adult (35-40 day old) Sprague-Dawley rats were used per preparation; the crude mitochondrial-synaptosomal (P_2) and microsomal (P_3) fractions were isolated as before, except for the omission of EDTA in the homogenization medium, and the synaptosomal (P_2B) fraction was obtained by sedimentation rather than flotation. The P_3 pellet was suspended in 6 x 9.0 ml of 0.32 M sucrose at a protein concentration of ~13 mg x ml^{-1} and fractionated further, either on continuous (0.8-1.4 M) or a series of two discontinuous sucrose gradients, all in swing-out rotors for 17 h x 95,000 g at 4°. The first discontinuous gradient consisted of 15.0 ml of 0.48 M over 0.96 M sucrose, producing subfractions P_3A, P_3B and P_3C at the 0.32 M/0.48 M, the 0.48 M/0.96 M interfaces and pellet respectively. The second gradient, formed by placing P_3B after pelleting and resuspending in 9 ml of 0.32 M sucrose (~6 mg protein x ml^{-1}) on top of a gradient, consisting of 15 ml of 0.70 M over 15 ml of 0.815 M sucrose, produced subfractions P_3B_1, P_3B_2 and P_3B_3 at the two interfaces and the pellet, as before.

Receptor binding - The centrifugation assay (5) for the binding of [^{125}I]αBtx has been scaled down as follows: two aliquots (A and B) containing 0.4 mg of protein in 0.2 ml of 0.32 M sucrose were placed in siliconized, disposable glass tubes (6 x 50 mm). Sample A was preincubated for 30 min with 3 x 10^{-7} M unlabeled αBtx, followed by incubation of both samples for 30 min with 3 x 10^{-9} M [^{125}I]αBtx (5,6) all at room temperature, followed by the addition of 0.6 ml of Ringer's solution (6). Samples were centrifuged at 10,000 g x 15 min, the pellets resuspended in 0.8 ml of Ringer's and again centrifuged at 10,000 g x 10 min. The surface of each pellet was rinsed with 0.8 ml of Ringer's and the tubes containing the samples placed in plastic vials and counted in a Beckman Biogamma Counter at 75% efficiency. All determinations were in triplicate and specific binding equals B-A.

Assays for specific binding of other ligands were based on the above procedure (always using two sets in 0.32 M sucrose) supplemented with buffers described in the literature: [^{3}H]atropine (10^{-7} M) vs atropine (10^{-4} M) (13), [^{3}H](-)alprenolol (1.5 x 10^{-8} M) vs (-)propanolol (10^{-5} M) in 50 mM Tris pH 8.1, 15 mM $MgCl_2$ (14); [^{3}H]dopamine (5 x 10^{-9} M) vs (+)butaclamol (10^{-5} M) in 50 mM Tris pH 7.4, 5 mM EDTA and 1 mM ascorbate (15,16); [^{3}H]L-Glu (2 x 10^{-6} M) vs L-Glu (5 x 10^{-3} M) in 50 mM Tris pH 7.4 and 0.4 mM $CaCl_2$, or Kainate (2 x 10^{-3} M) in Tris-citrate, pH 7.1 (17,18); [^{3}H]QNB (4 x 10^{-10} M) vs oxotremorine (0.1 mM) in 50 mM P_i, pH 7.5; [^{3}H]GABA (3.0 x 10^{-8} M) vs bicuculline (0.1 mM) in 50 mM Tris pH 7.1 (19). Preincubations and incubations at 37°: 30 min each for atro-

pine, 30 and 60 min for QNB, 20 min each for all others; then 0.6 ml buffer with ligand, or buffer with ligand plus unlabeled competitor, were added and centrifuged for 10,000 g x 15 min at 23°, followed by two rapid (≲1 min) rinses of each with 0.8 ml buffer at 4°. Samples were then dissolved in 1 ml NCS Solubilizer (Amersham-Searle) and counted.

Other assays - Assays for the following enzymes were exactly as described in the references, except where indicated: Acetylcholineasterase (EC 3.1.1.7), choline acetyltransferase (EC 2.3.1.6) and (Na^+-K^+) activated, ouabain sensitive ATPase (EC 3.6.1.4), all as in (6); (Ca^{+2}-Mg^{+2}) activated ATPase (3.6.1.3) as in (20), except with both Ca^{+2} and Mg^{+2} at 1 mM; cyclic nucleotide phosphodiesterase (EC 3.1.4.17) according to (21), acid phosphatase (EC 3.1.3.2) as in (22), NADH:ferricyamide reductase (1.6.99.3) as in (23); NADPH:cytochrome c reductase (EC 1.6.2.4), esterase (EC 3.1.1.3), UDPG galactose-ovalbumin galactosyl transferase (EC 2.4.1), all as in (24), and adenylate cyclase (EC 4.6.1.1) as in (25). Colchicine binding was measured according to (26), RNA according to (27) and protein according to (28).

Materials - Labeled compounds with specific activities indicated were obtained as follows: [^{3}H]dopamine (10 Ci/mmol), [^{3}H]L-Glu (22.7 Ci/mmol), [^{3}H](-)alprenolol (33 Ci/mmol), [^{3}H]GABA (37 Ci/mmol), [^{3}H]3'5'AMP (38 Ci/mmol), [^{14}C]colchicine (20 mCi/mmol), UDP-[U-^{14}C]Gal (270 mCi/mmol), [^{14}C]acetylCoA (55 mCi/mmol) and [α-^{32}P ATP] (22.5 Ci/mmol) all from New England Nuclear; [^{3}H]atropine (350 mCi/mmol); [^{3}H]choline chloride (10 Ci/mmol) and [^{3}H]quinuclidine benzylate (13 Ci/mmol) from Amersham-Searle; [^{125}I]αBtx at a final specific acitivty of 30 Ci/mmol was prepared (5,6) by the use of [^{125}I]Na obtained from New England Nuclear.

The sources of other drugs were: atropine sulfate, Kainate and carbamylcholine (Sigma Chemical Co.), gallamine triethiodide (Flaxedil) (ICN-K and K Laboratories), d-tubocurarine and colchicine (Cal Biochem), decamethonium bromide (Burroughs Wellcome Co.), oxotremorine and scopolamine (Aldrich Chemical Co.), (-)propanolol and (+)butaclamol were kindly provided by Ayerst Laboratories.

RESULTS

Distribution of neurochemical markers in subfractions of P_3 - When P_3 fractions, prepared by several different techniques (5,29,30), are subjected to isopycnic sedimentation in continuous sucrose gradients, maximal binding of αBtx is obtained in a density region of 1.112 ± 0.004 g x ml^{-1}. Its enrichment in specific binding activity relative to the starting homogenate (relative specific activity, RSA) is reproducibly of the order of fourfould, as is the RSA for the membrane marker ouabain sensitive Na^+-K^+ activated ATPase (5,30) and for a Mg^{+2}-Ca^{+2} activated ATPase (31,32). Cyclic phosphodiesterase, a marker for postsynaptic membranes (33-35) and acetylcholinesterase (5) exhibit modest enrichments at this density with RSAs of about 1.5-2 fold, with the last marker showing a peak at a density of 1.090 ± 0.002 g x ml^{-1}. On the other hand, choline acetyltransferase - a marker for intact synaptosomes (5,36,37) - is actually decreased relative to

the homogenate with a RSA = 0.3, but is enriched (RSA = 1.5) in structures sedimenting with densities of 1.125 ± 0.005 and 1.160 ± 0.010 g x ml^{-1}, respectively. This high density region also exhibits peaks in toxin binding, both ATPases, the phosphodiesterase, as well as of adenylcyclase (26). Finally, the RSA for high affinity uptake of choline (37,38) in P_3 and its subfractions is less than one third compared to the homogenate value and less than one tenth compared to that of a P_2B (synaptosomal) fraction.

Isolation of a membrane fraction enriched in receptor sites for various neurotransmitters - Based on the above observations we have developed a fractionation scheme, described in Methods and Materials, for the isolation of these membranes. If - as the data suggest the receptor sites for αBtx are localized on postjunctional membranes (see "Discussion") - they should also exhibit enrichment in receptors for other putative neurotransmitters of the central nervous system (1,3,9,11,12,39,40). The test of this hypothesis is provided by the

TABLE 1

BINDING OF PUTATIVE NEUROTRANSMITTERS AND ANTAGONISTS TO SUBCELLULAR FRACTION OF RAT CORTEX

Receptor	*Ligand*		*Fraction*				
	radioactive (*nM*)	*unlabeled* (μ*M*)	P_2	P_2B	P_3	P_3B	P_3B_2
nicotinic AcCh	αBtx	αBtx			1.3	1.8	3.5
	(3)	(0.3)	1.3	1.3	1.4	2.3	3.3
muscarinic AcCh	atropine	atropine			2.8	7.0	7.4
	(100)	(100)	1.1	0.58	1.1		2.5
β-adrenergic	(-)alprenolol	(-)propanolol			1.5	2.5	3.8
	(15)	(10)	1.5	0.69	1.8		4.1
L-Glu	L-Glu	L-Glu(5000)	1.7	n.d.	1.7		1.3
	(2000)	kainate(2000)	8.0	n.d.	9.0		8.5
GABA	GABA	bicuculline	0.080[a]	0.027[a]	0.027[a]	0.033[a]	0.32[a]
	(32)	(100)					
Dopamine	dopamine	(+)butaclamol			n.d.	n.d.	0.018[a]
	(5)	(10)	5.5	5.5	4.0		14
Protein					0.17	0.084	0.015
			0.33	0.11	0.16	0.074	0.015

Data are specific binding of various ligands in fractions shown (see Methods) relative to whole homogenate set as equal to 1.00. All are means of determinations of triplicate or quadruplicate samples, for two separate preparations where indicated. Protein values are fractional amounts relative to the homogenate set equal to 1.00.
The actual values for specific binding to the homogenate, all in p moles x mg^{-1}, were for preparation 1: [^{125}I]αBtx - 0.048; [^{3}H]atropine - 0.086 and [^{3}H]alprenolol - 0.200; for preparation 2: [^{125}I]αBtx - 0.040; [^{3}H]alprenolol - 0.256: [^{3}H]L-Glu - 3.86; [^{3}H]L-Glu vs kainate - 0.4; GABA 0.011; [^{3}H]dopamine - 0.002; [^{3}H]atropine - 0.230; [^{3}H]QNB vs oxotremorine- 0.270; protein - 138 mg.

[a] no or low specific binding detectable in whole homogenate; values are actual binding data in pmol x mg^{-1} protein in fraction

n.d. - not detectable over non-specific background

data summarized in Table 1, where we used the specific binding of the tritiated ligands indicated to identify and quantitate the muscarinic AcCh (13,41) β-adrenergic (14,42), L-glutamate (17,18,40,46), GABA (19,40,48) and dopamine (15,16) receptors, respectively. Specificity of binding was assured by the use of ligands at the low concentrations shown, in the range where they are known to be physiologically effective, and by the inclusion of either specific pharmacological antagonists, or non-radioactive ligands in large (>10^3 fold) excess (Table 1 and Methods).

It is apparent that all the compounds tested, with the possible exception of dopamine, are bound specifically and effectively, with high affinity, to the membrane fraction P_3B_2 and are enriched in this fraction relative to both the whole homogenate, as well as the crude microsomal (P_3) and synaptosomal (P_2B) fractions.

DISCUSSION

Here we address two questions: i) the nature and specificity of ligand binding and its relevance to transmitter-receptor interactions, and ii) the nature of the entities responsible for this binding. Specificity of binding has been monitored by the use of at least two of the three criteria cited above. The correlation of such binding of monoiodinated αBtx with the levels of the nicotinic AcCh receptor and its use for the quantitation of this molecule has been described and discussed repeatedly in a variety of systems, including the CNS (2,4-8,11,12,43,44) and can by now be regarded as well established. In this case specificity is assured by the use of unlabeled Btx in the assay, as well as the demonstration of effective competition by the nicotinic agonists and antagonists d-tubocurarine, carbamylcholine and decamethonium (8). Quite convincing evidence is also available for the use of the pair (-)alprenolol vs (-)propanolol as a probe for the β-adrenergic receptor (14,42) and, to a somewhat lesser extent, for GABA vs bicuculline (19,39), as well as for dopamine vs (+)butaclamol (15,16). In the absence of additional information the inferences that can be drawn from the kind of binding data

presented here for atropine as a probe for the muscarinic AcCh receptor and of L-glutamate itself as a probe for its receptor are considerably less compelling. Even though these ligands were bound specifically - in contrast to earlier studies with atropine (13) - and with relatively high affinity, the concentrations used in the actual assays had to be higher than those employed for other ligands (Table 1), and no neuropharmacologically *specific* antagonist was included in the case of atropine. Thus binding to other sites capable of binding this molecule with high affinity, but not involved in receptor function, cannot be ruled out *a priori*. However, only specific binding is reduced significantly by the known muscarinic ligands scopolamine and oxytremorine (39), and that is what we find at concentrations (10^{-6} M) at which d-tubocurarine is completely ineffective. Less ambiguous binding data can be obtained by the use of quinuclidine benzylate (QNB) (39,45). Sites for [^{3}H]QNB binding to P_3B_2 are enriched some 2.5 fold over the homogenate and are present at a level 7 x that of nicotinic AChR. Similarly, inhibition by kainate acid can be used as a specific probe for the glutamate receptors (46) and a preliminary experiment with this compound is included. Finally, additional confidence in the inferences drawn here can be derived by comparing our values with those reported in the literature: these correspond closely for the nicotinic AcCh receptor from rat brain subcellular fractions (49), for the muscarinic receptor in such fractions (45) and in P_2B from this source (13,40), for the β-adrenergic receptor in this fraction (50), in various rat brain regions (51) and in synaptic membranes from chick brain (52). Agreement is also satisfactory for values of the GABA receptor in rat brain P_2 (19), for the dopamine receptor in this fraction from rat corpus striatum (15) and for glutamate receptors in synaptic membranes from rat brain determined by means of kainate (46). The variety and specificity of the ligands, the binding of which is enriched in P_3B_2, make it unlikely that this effect is due to some nonspecific electrostatic interaction.

Conclusions regarding the nature of the membranes responsible can be derived from their bouyant densities, the presence or absence of characteristic marker

enzymes and electron microscopic examination. As usual, the most convincing evidence is provided by what is probably absent, rather than what is present. Intact, small synaptosomes with receptor sites on their - presumably post-junctional membranes can be ruled out on the basis of all three criteria: bouyant density (30), the absence of a high affinity uptake system for choline (37,38,52-54) and of choline acetyltransferase (5,35,37,54,55), and their physical absence in electron micrographs. The first - with even greater force - and third criteria, also render unlikely major contributions by junctional complexes or postjunctional membrane fragments derived from synapses containing postsynaptic densities (Gray's Type I) (34,56-59). These considerations leave the Golgi apparatus, smooth endoplasmic reticulum, lysosomes, plasma membranes derived from synaptosomes or glia, and postsynaptic membranes proper, as the most likely organelles to have contributed the membranes in P_3B_2. Synaptosomal plasma membranes are unlikely candidates on the basis of their bouyant densities and relatively high level of choline acetyltransferase (5,30,35,60,60) [and the pattern of their membrane polypeptides (32,62) to be described elsewhere (63)], and perhaps - based on bouyant density (30,64) - so are glial membranes. Although membranes derived from Golgi, smooth ER and lysosomes are almost certainly present as contaminants, they are probably not sites of receptor functions. A number of markers for endoplasmic reticulum, namely RNA (30,63,65), esterase (24), NADH:ferricyanide reductase (23,30) and NADPH:cytochrome c reductase (24,30,37,62) are actually decreased in P_3B_2 relative to P_3 or P_3B, while the lysosomal marker acid phosphatase (24,30,62) remains unchanged. Thus, the organelles under study may be postsynaptic membranes, relatively free of postjunctional attachments either intrinsically or in consequence of their detachment in the course of homogenization and isolation. The enrichment in the general membrane markers Na^+-K^+ ATPase (5,22,30,62,65-67) and Ca^{+2}-Mg^{+2} ATPase (20,31,32,67), in parallel with an increase in the content of receptor sites for a number of transmitters and an increase in the specific activity of cyclic nucleotide phosphodiesterase

(33,35) - all of which are believed to be concentrated on the postjunctional membrane - provide strong support for this supposition.

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