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PRELIMINARY COMMUNICATIONS

BINDING OF MUSCIMOL AND GABA IN SUB-FRACTIONS OF A CRUDE MEMBRANE FRACTION OF RAT BRAIN

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The binding of [^{3}H]γ-aminobutyric acid ([^{3}H]GABA) and [^{3}H]muscimol to subcellular particles of brain, which occurs in the absence of added Na^+ (i.e., "Na^+-independent binding"), has been used to estimate synaptic GABA-receptors (1-3). This binding appears to be most enriched in crude synaptic membrane fractions of brain (4-6), but all of the binding sites do not appear to be localized to synaptic membranes (e.g., 2,7,8). Recent studies have revealed further that the highest-affinity process for [^{3}H]muscimol binding to subcellular particles of rat brain has a higher capacity than that of [^{3}H]GABA (9-12). Herein, the binding of these ligands to sub-fractions of a crude membrane fraction of rat brain are compared.

MATERIALS AND METHODS

Adult male Wistar rats (170-240 g) were used to prepare a crude synaptic membrane fraction (4). A portion of this preparation was used for binding studies after it had been frozen and thawed and thoroughly washed (see below). Another portion was fractionated. In this case, the crude membrane fraction was re-suspended in 9.5 ml of 1.0 M sucrose solution and layered between 0.8 M (10 ml), and 1.2 M (12 ml) sucrose solution (Fig. 1). These gradients were centrifuged at 53,000 g (SW-27 rotor) for 2 hrs using a Beckman Model L-5-65 ultracentrifuge. Five fractions (A,B,C,D,E) were collected (Fig. 1), pelletted by centrifugation at 100,000 g, 60 min (Type-30 rotor) and then stored at -25° C for 5-8 days. Each fraction was then re-suspended in 20 ml water, allowed to stand at 23°C for 20 min and then centrifuged at 50,000g, 30 min ; this washing cycle was repeated twice more. Pellets were stored at -25°C for 1-4 days.

For binding assays, frozen pellets were re-suspended in small volumes (1-3 ml) of Na^+-free, Tris-citrate buffer (50 mM ; pH 7.1) and homogenized ; all further operations were conducted at 0°-4°C using Na^+-free Tris-citrate medium. Aliquots (100 µl) of tissue suspension (representing about 0.1 - 0.3 mg protein) plus 100 µl of medium, either free of added substance or containing a final concentration of 10^{-4} M unlabelled GABA, were mixed in small centrifuge tubes and left to stand for 10 min ; then, 250 µl of medium, providing a final concentration of 6.2 nM for both γ-[2,3-^{3}H(N)]-aminobutyric acid (36.12 Ci/m-mole) and [methylene-^{3}H(N)]-3-hydroxy-5-aminomethylisoxazole ([^{3}H]muscimol ; 13.68 Ci/m-mole and 125 nM for [^{14}C(U)]sucrose (673 mCi/m-mole), were added. Samples were mixed, left to stand for 20 min, and then centrifuged at 79,000 g (Type-25 rotor) for 5 min. Under these conditions, both radio-ligands were maximally bound and were maximally displaced by 10^{-4} M unlabelled GABA. Radioactive products were purchased from New England Nuclear.

Determination of radioactivity due to ^{3}H and ^{14}C, protein assays, and electron microscopy were performed as previously described (13,14,15). Results are expressed as mole ligand bound/g pellet, corrected for [^{14}C]sucrose space (13).

RESULTS

A diagram of the sub-fractionation procedure with corresponding electron micrographs is shown in Figure 1. Pellet weights, pellet protein contents, and [^{14}C]sucrose distribution ratios of the various fractions were not altered by the presence of 10^{-4} M unlabelled GABA (data not shown). "Specific" binding (i.e., those amounts of radio-ligand sensitive to 10^{-4} M unlabelled GABA) to the crude membrane fraction occurred to a greater extent for [^{3}H]muscimol (49 ± 5 p-mole/g pellet ; n = 6) than for [^{3}H]GABA (17 ± 6 p-mole/g pellet ; n = 6) ; means ± S.D. ; n = number of samples. The binding of both radio-ligands to the various sub-fractions is presented in Table 1. Note that very little or no binding of [^{3}H]GABA or [^{3}H] muscimol occurred in sub-fractions A (which contained mainly myelin) and B (which was enriched in low-density membrane fragments), and that both ligands were bound mainly to sub-fractions C (enriched in dense membranes and heavy myelin), D (enriched in heavy membranes) and E (enriched in mitochondria). [^{3}H]Muscimol was bound to a greater extent than [^{3}H]GABA in all sub-fractions.

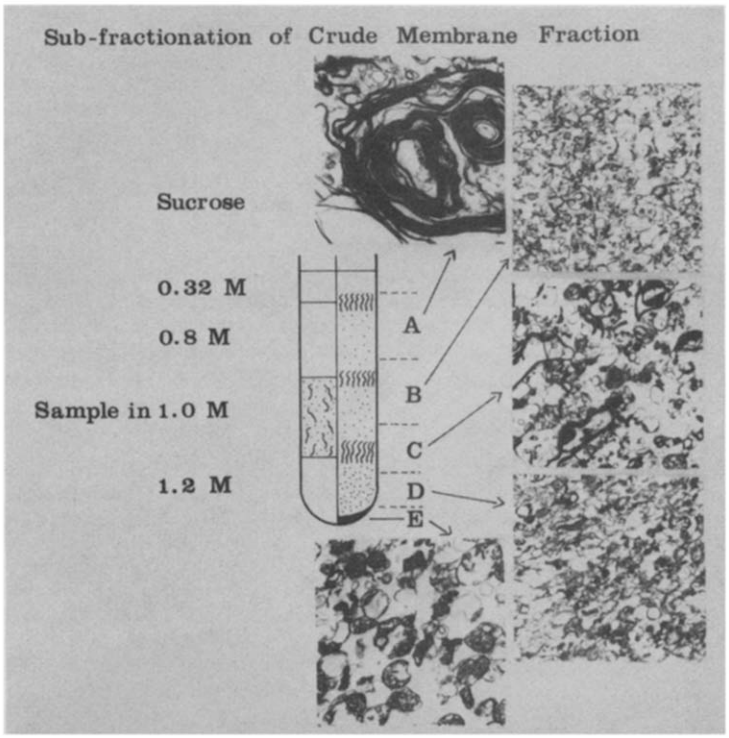

Fig. 1. Sub-fractionation of a crude synaptic membrane fraction of rat whole brain on a discontinuous sucrose gradient using a sedimentation-flotation method. The crude membrane fraction was re-suspended in 1.0 M sucrose solution and layered between 0.8 M and 1.2 M sucrose (left side). After centrifugation for 2 hrs at 53,000 g, gradient fractions A,B,C,D, and E (right side) were collected using a syringe, pelletted at 100,000 g for 60 minutes, and stored at -25°C. For binding assays, frozen pellets were thoroughly washed with water and then re-suspended in Na^+-free, Tris-citrate buffer. Electron micrographs corresponding to the various sub-fractions are also shown.

DISCUSSION

In accord with previous studies which revealed that CNS subcellular preparations possess more binding sites for [^{3}H]muscimol than for [^{3}H]GABA (9-12), the present results show that greater amounts of [^{3}H]muscimol than of [^{3}H]GABA were bound to five sub-fractions of a crude synaptic membrane fraction of rat brain. Both radio-ligands were bound to a greater extent in fractions containing heavy membranes and mitochondria than in those enriched in myelin or low-density membranes. These results indicate a marked heterogeneity of "Na^+-independent" binding sites for both GABA and muscimol with respect to subcellular particles of brain. Therefore, Na^+-independent binding of [^{3}H]GABA cannot be used as a specific probe for synaptic GABA-receptors. The pronounced binding of both ligands (especially [^{3}H]muscimol) to the mitochondrial pellet indicates that some GABA binding sites are not localized to plasma membranes. More recent studies in our laboratory, conducted with more purified mitochondrial preparations, have revealed that about three times more muscimol sites than GABA sites exist at a radio-ligand concentration of 17 nM. Thus far, the purities of our fractions have been tested only by electron microscopy. In order to determine more accurately the degree of binding of [^{3}H]GABA and [^{3}H]muscimol to mitochondria, we are now using positive and negative chemical markers to identify the mitochondria and autoradiography to visualize the binding sites.

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Table 1. Comparison of the binding of [^{3}H]GABA and [^{3}H]muscimol to sub-fractions of a crude synaptic membrane fraction of rat whole brain

Radio-ligand (6.2 nM)	Fraction	Amount in pellet p-mole/g pellet[1] Control	+ 10^{-4} M GABA	"Specific" binding[2]
[^{3}H]GABA	A	5.9 ± 0.4	6.2 ± 1.0	0
	B	5.2 ± 0.3	4.0 ± 0.2*	1.2
	C	7.0 ± 0.9	3.0 ± 0.4***	4.0
	D	8.1 ± 1.1	3.4 ± 0.5***	4.7
	E	6.7 ± 0.9	4.0 ± 0.9*	2.7
[^{3}H]Muscimol	A	3.7 ± 0.5	2.2 ± 0.4*	1.5
	B	6.0 ± 0.9	3.3 ± 1.1***	2.7
	C	39.0 ± 2.5	2.7 ± 0.7***	36.3
	D	26.8 ± 3.6	2.8 ± 0.7***	24.0
	E	29.5 ± 3.3	3.1 ± 0.8***	26.4

Sub-fractions were prepared as described in Methods ; see Fig. 1. Means ± S.E.M. of 5 or 6 samples in all cases ; *, ** and *** indicate, respectively, $p < 0.05$, $p < 0.01$ and $p < 0.001$ with respect to corresponding control values ; Student's t-test (two-tailed).

[1] These values were corrected for [^{3}H]ligand that was present in trapped supernatant fluid of the pellet using [^{14}C]sucrose distribution ratios (13).

[2] "Specific" binding refers to [^{3}H]ligand binding that is sensitive to 10^{-4} M unlabelled GABA.

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