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Partial purification of dihydrotetrabenazine binding activity from bovine adrenal

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The catecholamine depleting agent tetrabenazine and its analog dihydrotetrabenazine are thought to inhibit storage of monoamines by interacting with a subunit of the monoamine transporter that is distinct from that carrying the binding site for reserpine (see Ref. 1 for recent review). We have reported recently that immobilized lectins bind soluble dihydrotetrabenazine binding activity from bovine striatum [2]. We now describe the use of this interaction for enrichment of binding activity from frozen bovine adrenal medulla.

Materials and Methods

Preparation of a fraction enriched in chromaffin granule membranes from frozen tissue was accomplished by a procedure based on the difference in densities of chromaffin cell plasma and granule membranes observed previously by others [3]. Frozen bovine adrenal glands (PelFreeze, Rogers, AK) were dissected while frozen, and medullary tissue (~3 g) was homogenized in 12.5 mL of 0.3 M sucrose, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6, for 15 sec with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) at half speed. The homogenate was diluted with 3 vol. of the same buffer, filtered through six layers of gauze, and the gauze was washed with an additional 4 vol. buffer. The filtrate was centrifuged for 10 min (750 g_{max}) and the supernatant fraction was centrifuged further for 1 hr (245,000 g_{max}). The resulting pellet was homogenized in 20 mL of 45% (w/w) sucrose, 10 mM HEPES, pH 7.6, and 10-mL portions were placed in centrifuge tubes. Each portion was overlaid with 10 mL of 28% sucrose and 5 mL of 10% sucrose in the same buffer and centrifuged for 3 hr (148,000 g_{max}). Each layer was collected with a pasteur pipette, diluted with 4 vol. of 10 mM HEPES, 150 mM KCl, pH 7.6, and centrifuged for 1 hr (245,000 g_{max}). The final pellet was resuspended in HEPES/KCl and stored frozen (−20°) for up to 2 months before use. All centrifugations were performed in a Beckman 50.2 Ti rotor at 4°.

Membranes from the 28% layer were thawed and diluted with HEPES/KCl to a final protein concentration of approximately 0.5 mg/mL. A detergent mixture containing 10% (w/v) sodium cholate and 2% (w/v) soybean lecithin (>95%, Avanti Polar Lipids, Pelham, AL) in the same buffer was added dropwise to the membranes to give a final concentration of 1% cholate. The mixture was stirred slowly at room temperature for 30 min followed by centrifugation (60 min, 220,000 g_{ave}) at 4° using a Beckman 75 Ti rotor.

Columns for affinity chromatography were prepared by washing 2 mL of packed wheat germ lectin Sepharose 6MB (Sigma Chemical Co., St. Louis, MO) with 10 vol. of HEPES/KCl followed by 2 vol. of 1% cholate, 0.2% lecithin in the same buffer. The disposable polypropylene columns were drained of excess liquid in a low speed desk top centrifuge. Soluble protein (7.0 mL) was added, and the sealed columns were rocked for 1 hr at 4°. The flow-through was collected, and the columns were washed twice with 7.0 mL of the cholate/lecithin buffer. Elution was accomplished by incubation for 15 min with 7.0 mL of the 10 mM *N*-acetylglucosamine in the same buffer.

Binding assays contained 15 nM [^{3}H]dihydrotetrabenazine and were conducted essentially as in Ref. 2, with the exception that binding to gradient fractions was assayed using untreated Whatman GF/C glass fiber filters. Ketanserin tartrate (Research Biochemicals Inc., Wayland, MA) was dissolved in dimethyl sulfoxide (DMSO); the solvent concentration in the assay did not exceed 0.33%. The apparent K_i for ketanserin inhibition was calculated from the Cheng–Prusoff equation using IC_{50} values obtained from logit analysis [4]. Binding parameters were determined as described in Ref. 5. Protein was measured by the method of Peterson [6].

Results and Discussion

Examination of dihydrotetrabenazine binding activity in fractions from the sucrose gradient revealed that a 6.9-fold

Table 1. Preparation of membranes enriched in dihydrotetrabenazine binding activity

Fraction	Total protein (mg)	Recovery (% protein)	Total binding (pmol)	Recovery (% binding)	Specific activity (pmol/mg)
Homogenate	230		1320		5.7
		100		100	
P1	32.9		100		3.0
S1	188		1220		6.5
		96		100	
P2	80.2		948		11.8
S2	91.4		19.3		0.2
		91		79	
10% Sucrose supernatant	0.16		0.55		3.4
10% Sucrose pellet	0.44		14.3		33
28% Sucrose supernatant	2.06		1.00		0.5
28% Sucrose pellet	9.14		366		40.0
45% Sucrose supernatant	16.1		3.20		0.2
45% Sucrose pellet	47.2		655		13.9
		94		110	

Each value for total protein and total binding is the median of three determinations. Recoveries were calculated as percent starting material for each step recovered in all fractions from that step.

Table 2. Solubilization and lectin affinity chromatography of dihydrotetrabenazine binding activity

Fraction	Total protein (mg)	Total binding (pmol)	Specific activity (pmol/mg)
Starting material	3.11	107	34.4
Cholate supernatant	2.19	72.6	33.2
Cholate pellet	0.882	13.9	15.8
Flow through	1.70	13.0	7.6
Wash 1	0.143	2.92	20.4
Wash 2	0.029	1.82	62.9
Eluate 1	0.075	22.8	304
Eluate 2	0.098	25.2	257

Cholate supernatant was applied to the lectin column, washed twice, then eluted with 10 mM *N*-acetylglucosamine for 15 min (eluate 1), followed by 200 mM *N*-acetylglucosamine overnight (eluate 2). Each value for total protein and total binding is the median of three determinations.

enrichment in specific activity was obtained within the 28% sucrose fraction, with a yield of 28% of the original binding activity (Table 1). Scatchard analysis of this fraction gave a K_d of 6.5 nM and a binding site density of 57 pmol/mg protein. For comparison, K_d values of 3.1 and 2.5 nM, and site densities of 62 and 15 pmol/mg were reported for chromaffin granule membranes purified from fresh adrenals [7] and striatal synaptic vesicles [8] respectively. Another characteristic of the dihydrotetrabenazine binding site is a high affinity for ketanserin, a structurally unrelated drug [9]. Its K_i for inhibition of [^{3}H]dihydrotetrabenazine binding to membranes from the 28% layer, 59 nM (not shown), agrees well with the value of 55 nm reported for the conventional preparation [9].

Binding activity was purified further by cholate solubilization and lectin affinity chromatography (Table 2). More than 80% of the applied soluble binding was retained by a wheat germ lectin column, and elution with 10 mM hapten sugar for 15 min gave binding activity with a 9-fold increase in specific activity over the soluble starting material. For two other experiments increases from 5- to 18-fold were observed. Additional binding activity, with a lower specific activity, could be eluted by overnight incubation with 200 mM hapten.

We have described previously a lower affinity binding activity present in brain, liver, and other tissues which is probably not associated with monoamine transport because it is absent from purified synaptic vesicles [5]. This binding activity was undetectable in homogenates and other fractions of adrenal medulla. Scatchard analysis of dihydrotetrabenazine binding to material purified in the experiment described in Table 2 revealed a single binding site with an apparent dissociation constant of 4.6 nM and site density of 463 pmol/mg protein. The apparent K_i for ketanserin inhibition of binding to this fraction was 77 nM. Using a molecular mass of 70,000 Da [10, 11] and assuming one binding site per molecule, we estimate a further 31-fold purification will be required to achieve homogeneity.

Results presented here demonstrate the feasibility of using commercially available frozen bovine adrenals as starting material for preparation of a particulate fraction enriched in dihydrotetrabenzine binding activity. Further purification of solubilized binding activity was accomplished by wheat germ lectin affinity chromatography. The latter finding suggests that dihydrotetrabenazine binding is associated with a chromaffin granule component that is glycosylated.

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Interactions between phencyclidine and nifedipine at $^{45}Ca^{2+}$-uptake sites on mouse brain neurons

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The effects of phencyclidine (PCP*) on neuronal calcium channels have not been fully evaluated, while in peripheral tissues they remain equivocal. Both blockade and stimulation of calcium channels have been invoked as mechanisms to explain the pharmacologic effects of PCP in cardiac and vascular smooth muscles [1, 2]. The ability of PCP to both enhance and block neurotransmitter release [3] also suggests that facilitation and inhibition of neuronal calcium currents by PCP may exist at the level of the neuron. However, all such studies are complicated by the potent inhibitory effects of PCP on sodium, potassium and *N*-methyl-D-aspartate (NMDA) gated channels [4].

Dihydropyridine (DHP) calcium antagonists interact with specific neuronal binding sites that regulate functionally relevant 'L'-type voltage-dependent calcium channels [5]. We have demonstrated previously that PCP and the acylating derivatives 1-[1-(3-isothiocyanatophenyl)cyclohexyl]piperidine (METAPHIT, a specific acylator of neuronal PCP binding sites [6]) and 4-isothiocyanato-1-[1-phenylcyclohexyl] piperidine (FOURPHIT) produce specific irreversible changes in neuronal DHP binding sites by interacting with them both directly and in an allosteric manner [7].

We have extended the study of the effects of PCP on voltage-dependent calcium channels by investigating the effects of PCP, FOURPHIT and METAPHIT on K^+-stimulated $^{45}Ca^{2+}$-uptake into a preparation of mouse brain neurons in the absence and presence of the DHP calcium antagonist nifedipine.

Methods

$^{45}CaCl_2$ (1 mCi) was obtained from New England Nuclear-Dupont, Boston, MA. PCP was obtained from the Bureau of Dangerous Drugs, Department of Health and Welfare, Ottawa, Canada. FOURPHIT (hydrochloride salt) and METAPHIT (hydrochloride salt) were synthesized and supplied by Dr. R. Lessor, Department of Chemistry, NIDDK, NIH, Bethesda, MD. Mesh screen (Nitex 210 and 130) were purchased from Tetko, Elmsford, NY. All other reagents were obtained from standard commercial sources and were of the highest purity possible.

Male mice (18–22 g, Charles River, St. Constant, Quebec) were used for all studies. Mouse brain neurons were prepared according to the method used by Skattebol and Triggle [8] for preparation of rat brain neurons. Briefly, mice were killed by cervical dislocation. The brain was rapidly removed and placed into an ice-cold physiologic solution of the following composition (mM): NaCl, 138; KCl, 5.4; Na_2HPO_4, 0.17; KH_2PO_4, 0.22; glucose, 5.5; sucrose, 46.9; and PMSF, 0.1; the pH was adjusted to 7.35 with 1 M NaOH. The brains were then first sieved through a 210 μM mesh screen (Nitex 210) and subsequently twice through a finer 130 μM mesh screen (Nitex 130). All subsequent steps were carried out as described by Skattebol and Triggle [8]. Mouse brain neurons prepared in this manner displayed a heterogenous morphology with many rounded cellular-like structures dispersed between larger aggregates of neuronal tissue. The viability of all components identified as measured using trypan blue was 80–90%. Protein was determined using the modified Lowry method of Miller [9].

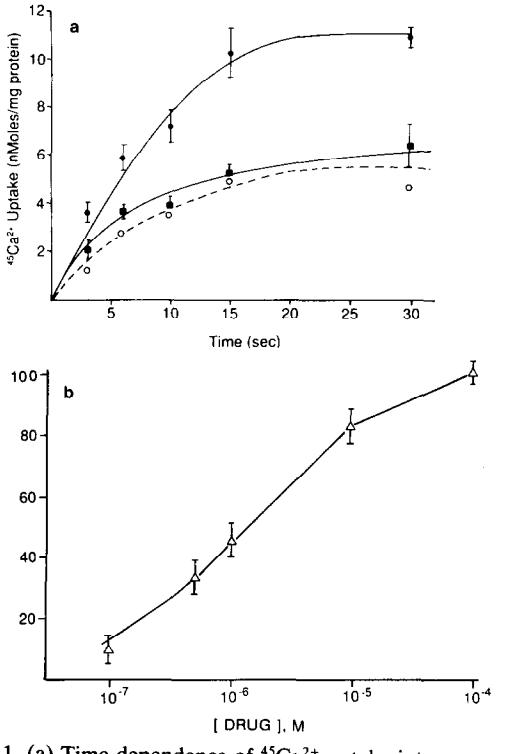

Fig. 1. (a) Time dependence of $^{45}Ca^{2+}$ uptake into mouse brain neurons. Stimulated (●), resting (■) and net (○) $^{45}Ca^{2+}$ uptake in response to 53 mM K^+ are illustrated. Each data point is the mean ± SE of three experiments. The curves through the points were hand drawn. Concentration dependence for PCP inhibition of $^{45}Ca^{2+}$ uptake into mouse brain neurons. Each data point is the mean ± SE of three to six experiments. Net $^{45}Ca^{2+}$ uptake: 2.81 ± 0.3 nmol/mg protein.

* Abbreviations: DHP, dihydropyridine; PCP, phencyclidine; FOURPHIT, 4-isothiocyanato-1-[1-phenylcyclohexyl] piperidine; METAPHIT, 1-[1-(3-isothiocyanatophenyl)cyclohexyl] piperidine; NMDA, *N*-methyl-D-aspartate; and PMSF, phenylmethylsulfonyl fluoride.