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REGIONAL DISTRIBUTION OF CALCIUM CHANNEL LIGAND (1,4-DIHYDROPYRIDINE) BINDING SITES AND $^{45}Ca^{2+}$ UPTAKE PROCESSES IN RAT BRAIN

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Abstract—The binding of nimodipine, a 1,4-dihydropyridine Ca^{2+} channel antagonist, and of Bay K 8644, a Ca^{2+} channel activator, was measured in several regions of rat brain and compared to the distribution of K^+ depolarization-induced $^{45}Ca^{2+}$ uptake into synaptosomes. The maximum binding densities (B_{max}) of [^{3}H]nimodipine and [^{3}H]Bay K 8644 were not significantly different one from the other, but differed according to brain region with binding being highest in the olfactory bulb and hippocampus, intermediate in the caudate nucleus and cerebral cortex (various regions), and lowest in the cerebellum [563 to 107 fmol/mg protein (mean)]. The K_D values, [^{3}H]nimodipine = 1.8×10^{-10} M (mean) and [^{3}H]Bay K 8644 = 1.4×10^{-9} M (mean), did not differ according to region. Depolarization-induced $^{45}Ca^{2+}$ uptake in synaptosomes occurred as fast (1 sec) and slow (10 sec) components distinguished by their selective occurrence in choline-containing and pre-depolarized preparations respectively. Distribution of the fast component of uptake paralleled that of [^{3}H]nimodipine binding, being least in the cerebellum and greatest in the hippocampus and cortex, but the magnitude of the slow phase of $^{45}Ca^{2+}$ uptake did not vary in the three brain regions studied.

Ca^{2+} influx through voltage-dependent Ca^{2+} channels [1–3] is important to excitation–contraction coupling in cardiac and smooth muscle [4, 5] and to communication and transmitter release in neuronal systems [6, 7]. The Ca^{2+} channel antagonists, including verapamil, diltiazem and nifedipine, and more recently the Ca^{2+} channel activators, such as Bay K 8644, have been shown to be potent modulators of Ca^{2+} channel function in smooth and cardiac muscle [for reviews see Refs. 4, 5 and 8–10]. The identification of specific binding sites for these ligands in smooth and cardiac muscle and the general accord of binding and pharmacologic properties suggest that these sites are components of, or are closely associated with, the Ca^{2+} channel [11–13].

Binding sites of high affinity for these ligands, particularly for 1,4-dihydropyridines including [^{3}H]nitrendipine [ethyl, methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3, 5-dicarboxylate], [^{3}H]nimodipine [isopropyl(2-methoxyethyl)-1, 4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate], and [^{3}H]Bay K 8644 [methyl-1,4-dihydro-2, 6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate], have been found in neural preparations and have properties very similar to those seen in cardiac and smooth muscle [14–16; for reviews see Refs. 12 and 17]. However, unlike the situation in smooth muscle where the Ca^{2+} channel antagonists and activators are generally potently effective [9, 11, 12], it is well recognized that neuronal preparations vary considerably in their sensitivities to these same ligands [17, 18]. Thus, $^{45}Ca^{2+}$ uptake in synaptosomes has been reported by several groups to be insensitive to the organic channel ligands [19–21], although Turner and Goldin [22] have reported that a fraction of synaptosomal $^{45}Ca^{2+}$ uptake is antagonist sensitive. In contrast, neurotransmitter release from brain slices is sensitive to the 1,4-dihydropyridine Ca^{2+} channel activators and antagonists [23, 24]. Similarly, contrasting situations may be found in other neuronal systems where $^{45}Ca^{2+}$ uptake in primary cultures of brain neurons is sensitive to both activator and antagonist ligands [25] and where uptake in cultured sensory and sympathetic neurons is modulated by these ligands, but where depolarization-induced norepinephrine release is insensitive [26].

To probe further the apparent paradox of high affinity binding sites without obvious pharmacologic action in neuronal tissue, we measured the regional distribution of [^{3}H]1,4-dihydropyridine antagonist and activator binding sites in rat brain and compared this distribution to that of the fast and slow phases of $^{45}Ca^{2+}$ uptake in depolarized synaptosomes.

MATERIALS AND METHODS

Male Holtzman rats (200–300 g) obtained from Charles River were used in both ligand binding and Ca^{2+} uptake studies. Rats were killed by decapitation, and the brains were quickly removed and placed in ice-cold 50 mM Tris-hydroxymethylaminomethane (Tris) buffer, pH 7.4. The olfactory bulb, hippocampus, caudate nucleus, cerebral cortex and

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cerebellum were dissected and homogenized individually in 10 vol. of Tris buffer using a Tri-R homogenizer (Tri-R Instruments, Inc.) at setting 3.5–4. The homogenates were centrifuged at 48,000 *g* for 10 min, and the pellet was frozen at −80°. Frozen pellets were used within 10 days, and control experiments showed that this period of freezing was without effect on the properties of radioligand binding. Frozen pellets were thawed at 18°, resuspended in Tris buffer, and centrifuged at 48,000 *g* for 10 min. The final pellet was then resuspended in Tris buffer for radioligand binding. Tissue preparations were employed within 90 min of final suspension.

[^{3}H]Nimodipine and [^{3}H]Bay K K 8644 binding assays were performed by slight modifications of existing procedures [11, 21]. Binding of both ligands was performed on samples of the same tissue preparation. In brief, membrane proteins (100–300 μg) were incubated at 15° in 50 mM Tris buffer (5.0 ml for [^{3}H]nimodipine and 2.5 ml for [^{3}H]Bay K 8644) for 60 min, with the appropriate radioligand. Control experiments indicated that this incubation time was adequate for equilibrium binding. Membranes were filtered through a Brandel cell harvester (model M-24-R, Gaithersberg, MD) over GF/B filters and washed twice with a volume of incubation medium. Nonspecific binding was defined by the presence of 5×10^{-7} M unlabeled nimodipine or 10^{-6} M unlabeled Bay K 8644 for [^{3}H]nimodipine and [^{3}H]Bay K 8644 assays respectively. Radioactivity was determined by liquid scintillation spectrometry. Protein was determined by the method of Bradford [27].

Synaptosomes were prepared by the method of Hajos [28] from hippocampus, cerebral cortex and cerebellum. The synaptosome-enriched material in the 0.8 M sucrose layer was equilibrated with 2.5 vol. of a resting buffer of the following composition (mM): NCl, 132; KCl, 5; $MgCl_2$, 1.3; $CaCl_2$, 1.2; glucose, 10; Tris, 25; pH 7.4. The suspension was centrifuged at 10,000 *g* for 5 min, and the resulting pellet was then gently resuspended in the appropriate volume of resting buffer.

To determine fast $^{45}Ca^{2+}$ uptake, synaptosomes were equilibrated in resting buffer with equimolar substitution of NaCl by choline chloride [22, 29, 30]. Synaptosomes were incubated at 30° for 12 min, and 50-μl aliquots containing 200–800 μg of protein were added to incubation medium at 30° containing $^{45}Ca^{2+}$ (1.2 μCi/μmol Ca^{2+}), 5 mM K^+ (resting buffer) or 68.5 mM K^+ (isotonic substitution of choline chloride by KCl) in a final volume of 0.5 ml and were incubated for 1 sec. $^{45}Ca^{2+}$ uptake was terminated by the addition of 4 ml of ice-cold Ca^{2+}-free resting buffer containing 3 mM ethyleneglycolbis(amino-ethyl-ether)tetra-acetate (EGTA). This solution was filtered over Whatman GF/B filters and washed four times with portions of ice-cold resting buffer.

The slow phase of $^{45}Ca^{2+}$ uptake was determined using synaptosomes suspended in normal resting buffer. A prepulse procedure was employed [29–31] in which 100-μl aliquots of synaptosomes containing 300–500 μg protein and incubated at 30° for 12 min were added to 100 μl of resting buffer containing 132 mM KCl (isotonic substitution for NaCl) or 5 mM KCl. After 15-sec incubation, 150 μl of this suspension was added to incubation medium at 30° containing $^{45}Ca^{2+}$ (1.2 μCi $^{45}Ca^{2+}$/μmol Ca^{2+}), 5 mM K^+ (resting buffer), or 68.5 mM K^+ (isotonic substitution for NaCl) in a final volume of 0.5 ml and incubated for 10 sec. $^{45}Ca^{2+}$ uptake was terminated as described, the synaptosomes were filtered and washed on GF/B filters, and radioactivity was determined by liquid scintillation spectrometry. Net $^{45}Ca^{2+}$ uptake was determined [19, 30] as the difference between the uptake in resting buffer (5 mM K^+) and that in depolarizing buffer (68.5 mM K^+).

$^{45}CaCl_2$ (4–50 Ci/g) was purchased from New England Nuclear (Boston, MA). [^{3}H]Nimodipine (130.0 Ci/mmol), [^{3}H]Bay K 8644 (70 Ci/mmol), nimodipine and Bay K 8644 were gifts from Dr. A. Scriabine (Miles Institute for Preclinical Pharmacology, New Haven, CT). Stock solutions of drugs (10^{-3} M) in ethanol were kept refrigerated and protected from light. Other chemicals were obtained from Fisher Scientific or Sigma and were of the highest purity routinely available.

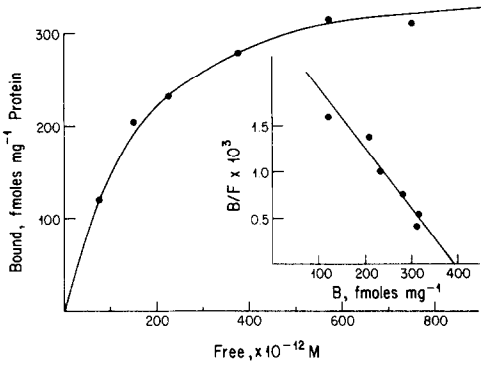

Fig. 1. Specific binding of [^{3}H]nimodipine to rat cerebral cortex membranes. Inset: Scatchard analysis of the specific binding data. The experiment was repeated six to eight times yielding similar data (Table 1).

RESULTS

Figures 1 and 2 show the specific binding of [^{3}H]nimodipine and [^{3}H]Bay K 8644 to membranes of rat cerebral cortex. Binding was saturable and for [^{3}H]nimodipine, $K_D = 1.8 \pm 0.8 \times 10^{-10}$ M and $B_{max} = 363 \pm 29$ fmol/mg protein, and for [^{3}H]Bay

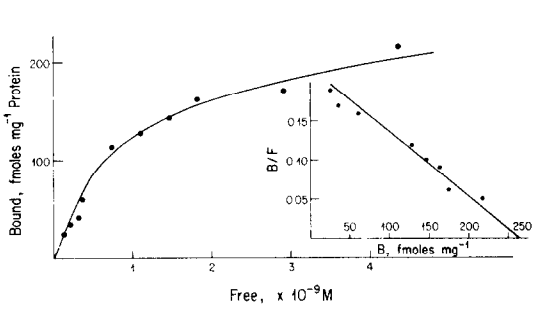

Fig. 2. Specific binding of [^{3}H]Bay K 8644 to rat cerebral cortex membranes. Inset: Scatchard analysis of the specific binding data. The experiment was repeated six to eight times yielding similar data (Table 1).

Table 1. Binding of [^{3}H]nimodipine and [^{3}H]Bay K 8644 in rat brain regions

Brain region	[^{3}H]Nimodipine*		[^{3}H]Bay K 8644*	
	K_D $\times 10^{-10}$ M	B_{max} (fmoles/mg)	K_D $\times 10^{-9}$ M	B_{max} (fmoles/mg)
Olfactory bulb	1.70 ± 0.20	563 ± 62†	1.05 ± 0.11	423 ± 49†
Hippocampus	1.90 ± 0.20	454 ± 59‡	1.34 ± 0.10	359 ± 45‡
Caudate nucleus	1.85 ± 0.05	358 ± 38§	1.40 ± 0.11	292 ± 15§
Cortex:				
Whole	1.84 ± 0.18	363 ± 29§	1.47 ± 0.14	291 ± 15§
Prefrontal	2.32 ± 0.12	456 ± 45‖		
Frontal	2.51 ± 0.20	386 ± 33‖		
Parietal	2.29 ± 0.07	398 ± 48‖		
Occipital	2.24 ± 0.60	440 ± 42‖		
Cerebellum	1.87 ± 0.28	107 ± 17	1.54 ± 0.20	109 ± 6

* Mean values ± SEM (N = 6–8).
† Significantly higher ($P < 0.05$) than other brain regions, except hippocampus.
‡ Significantly higher ($P < 0.05$) than other brain regions, except olfactory bulb.
§ Significantly higher ($P < 0.05$) than cerebellum.
‖ Not significantly different than values of whole cortex.

K 8644, $K_D = 1.5 \pm 0.14 \times 10^{-9}$ M and $B_{max} = 291 \pm 15$ fmol/mg^{-1} protein. The B_{max} values for [^{3}H]nimodipine and [^{3}H]Bay K 8644 were not significantly different. Similar comparisons were made in four other brain regions: olfactory bulb, hippocampus, caudate nucleus and cerebellum. There were no significant variations of K_D values for either [^{3}H]nimodipine or [^{3}H]Bay K 8644 binding between brain regions (Table 1). Significant regional differences existed, however, in maximum binding capacities for both ligands between regions with binding being highest in the olfactory bulb and hippocampus, intermediate in the caudate nucleus and cerebral cortex, and lowest in the cerebellum. There were no significant differences between the maximum binding densities of [^{3}H]nimodipine and [^{3}H]Bay K 8644. Comparison of binding of [^{3}H]nimodipine in four discrete regions (frontal, prefrontal, parietal and occipital) of the cerebral cortex did not reveal any significant differences in either K_D or B_{max} values.

$^{45}Ca^{2+}$ uptake into synaptosomes following K^+ depolarization was studied in three brain regions. In accord with previous reports [29–31], uptake was composed of two components. A slow component of uptake was abolished when K^+ depolarization was carried out in medium where choline chloride had been substituted for NaCl, and the fast component of uptake was abolished by the predepolarization protocol. This biphasic component of K^+ depolarization-induced $^{45}Ca^{2+}$ uptake has also been demonstrated in an ontogenic study of rat brain cerebral cortex from 2 to 140 days [30]. $^{45}Ca^{2+}$ uptake at 10 sec was greater than at 1 sec in all three brain regions. The fast (1 sec) component of uptake was greater in cortex and hippocampus than in cerebellum, but the slow phase (10 sec) component of uptake did not differ between the three brain regions (Fig. 3). The binding of [^{3}H]nimodipine to membranes from these brain areas showed regional variation paralleling that for the fast component of $^{45}Ca^{2+}$ uptake (Fig. 3). Neither the fast (1 sec) nor the slow (10 sec) component of uptake was sensitive to nimodipine or Bay K 8644 (to 10^{-6} M) in the cerebral cortex or hippocampus (data not shown).

DISCUSSION

The occurrence of high affinity binding sites for Ca^{2+} channel ligands in neuronal preparations suggests, because of similarities to peripheral binding sites, a localization of voltage-dependent Ca^{2+} channels. Several previous studies have described the heterogeneous distribution of radiolabeled 1,4-dihydropyridine antagonist binding sites in brain tissue employing either binding in dissected regions [15, 30, 32] or autoradiographic localization [33–35]. The highest densities of binding sites were found in cortex, hippocampus, striatum, and thalamus with lower binding densities elsewhere: distribution accords neither with blood vessel localization nor with regional blood flow [29, 33, 35, 36]. Distribution does appear, however, to be preferentially associated with certain areas rich in synaptic contacts.

The present data, though comparatively limited in scope, are confirmatory of previous analyses of regional binding. Thus, of the five regions studied,

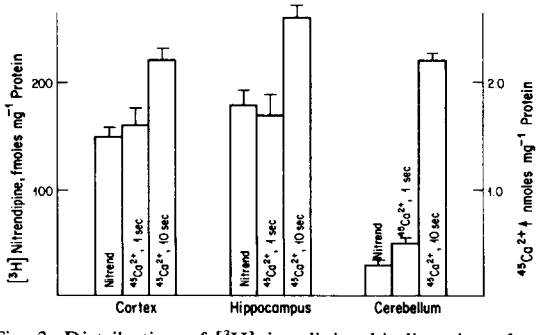

Fig. 3. Distribution of [^{3}H]nimodipine binding sites, fast phase (1 sec) $^{45}Ca^{2+}$ uptake and slow phase (10 sec) $^{45}Ca^{2+}$ uptake in synaptosomes from different regions of rat brain. Each value is the mean of four to eight separate determinations. Bars indicate mean ± SEM.

the cerebellum has the lowest binding density of [^{3}H]nimodipine-labeled sites. Although the 1,4-dihydropyridine activator Bay K 8644 is reportedly without effect in brain synaptosomes [this work and 21], its distribution of binding sites parallels that of the antagonist nimodipine, and there is, in fact, no significant difference in binding site density between these ligands in any given region. This is consistent with activator and antagonist 1,4-dihydropyridines sharing a common site [13, 21, 36, 37].

Fast and slow components of $^{45}Ca^{2+}$ uptake in K^+ depolarized rat brain synaptosomes were distinguished by Nachshen and Blaustein [31] as representing separate pathways of Ca^{2+} entry. The fast phase was the more sensitive to La^{3+} and could be eliminated by a predepolarization protocol. Our data provide a further distinction between the two phases of uptake. The distribution of the fast phase of K^+ depolarization-induced $^{45}Ca^{2+}$ uptake parallels that for the high affinity 1,4-dihydropyridine binding sites. This conclusion is consistent with the results of our ontogeny study in rat brain showing parallel development of high affinity [^{3}H]nitrendipine binding and fast phase $^{45}Ca^{2+}$ uptake [30]. In neither case, however, was this phase of $^{45}Ca^{2+}$ uptake sensitive to 1,4-dihydropyridine modulators. The slow phase of uptake, which has frequently not been distinguished from the fast phase and may represent a $Na^+:Ca^{2+}$ exchange process rather than a Ca^{2+} channel [22, 29, 30], enjoys a different ontogeny [30] and, from the present study of limited scope, a different distribution. Despite the apparent associations between synaptosomal high affinity 1,4-dihydropyridine binding sites and depolarization-induced $^{45}Ca^{2+}$ uptake apparent from this and a previous ontogeny study, most reports indicate that the uptake process is, in fact, insensitive to the 1,4-dihydropyridine Ca^{2+} channel ligands. Several reasons likely underlie this discrepancy. The binding sites may represent the Ca^{2+} channel, but they are present in large excess or are physiclly or biochemically uncoupled from functional channels. The latter may well constitute other classes of Ca^{2+} channels with different electrophysiologic and pharmacologic properties [38–40], but our limited data presented here and previously suggest similar distribution and development profiles. A comprehensive comparison of the distribution and ontogeny of the different classes of neuronal Ca^{2+} channels is a project of some importance since it is clear that 1,4-dihydropyridine sensitive sites are widely distributed [41].

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