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Novel interactions of cations with dihydropyridine calcium antagonist binding sites in brain

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1 The effects of monovalent (Na^+, Li^+, K^+, Rb^+) and divalent (Ca^{2+}, Mg^{2+}, Mn^{2+}) cations on dihydropyridine calcium antagonist binding sites in brain and cardiac membranes were investigated using a low ionic strength buffer (5 mM Tris-HCl, pH 7.4), and the dihydropyridine, [^{3}H]-nitrendipine.

2 At 25°C, the monovalent cations Na^+, Li^+, and K^+ (100 mM) but not Rb^+ significantly decreased the apparent dissociation constant (K_D) but had no effect on the maximum binding site capacity (B_{max}) of [^{3}H]-nitrendipine in brain. The divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} (2 mM) significantly increased the B_{max}, but did not affect the K_D of [^{3}H]-nitrendipine. The effects of cations were concentration-dependent (EC_{50} monovalent cations 10–25 mM; EC_{50} divalent cations 50–200 μM) and demonstrated brain region selectivity. The effect of Ca^{2+}, but not Mg^{2+} or Mn^{2+} on [^{3}H]-nitrendipine binding was described by a two-site model.

3 At 25°C, neither mono- nor divalent cations altered the characteristics of [^{3}H]-nitrendipine binding to rat cardiac membranes.

4 At 37°C, Na^+ (100 mM) but not K^+ (100 mM) significantly increased the B_{max} of [^{3}H]-nitrendipine in rat brain membranes. Ca^{2+} (2 mM) significantly increased the B_{max} of [^{3}H]-nitrendipine binding to rat brain membranes to a greater extent than at 25°C. Both Na^+ and K^+ had no effect on [^{3}H]-nitrendipine binding to cardiac membranes, while Ca^{2+} (2 mM) significantly decreased the K_D of [^{3}H]-nitrendipine.

5 It is suggested that the selective effects of mono- and divalent cations on [^{3}H]-nitrendipine binding to rat brain and cardiac membranes may be associated with differences in the calcium current blocking activity of dihydropyridine calcium antagonists in brain and cardiac tissues.

Introduction

High affinity binding sites for dihydropyridine calcium channel antagonists (DHPCAs), have been detected in cardiac, smooth and skeletal muscles and brain (Belleman *et al.*, 1981; Janis *et al.*, 1982; Bolger *et al.*, 1982; 1983; Ferry & Glossmann, 1982; Murphy & Snyder, 1982; Ehlert *et al.*, 1982; Fosset *et al.*, 1983). Biochemical and electrophysiological evidence suggests that the ability of DHPCAs to inhibit calcium currents in cardiac and smooth muscles (Fleckenstein, 1977; Rosenberger & Triggle, 1978; Triggle & Swamy, 1980; Triggle, 1981) is due to an interaction of the DHPCA with a binding site that constitutes a 'regulatory' component of calcium channels (Venter *et al.*, 1983; Goll *et al.*, 1983; Horne *et al.*, 1984; Triggle & Janis, 1984b; Rengasamy *et al.*, 1985). In contrast to their effects in cardiac and smooth muscles, DHPCAs possess very low potencies or are ineffective at inhibiting calcium-dependent processes (e.g. $^{45}Ca^{2+}$-uptake, neurotransmitter release) in brain (Toll, 1982; Daniell *et al.*, 1983; Freedman *et al.*, 1984; Starke *et al.*, 1984; Ogura & Takahasi, 1984; Rampe *et al.*, 1984) despite the presence of high affinity brain DHPCA binding sites (Murphy *et al.*, 1982; Cortes *et al.*, 1983; 1984). Several hypotheses have been advanced to provide an explanation for discrepancies in the calcium current blocking activity of DHPCAs between tissues (Triggle & Janis, 1984a, b; Bean, 1984). There is, however, little direct biochemical evidence to support such large differential tissue activities for DHPCAs. Nonetheless, a marked similarity in the properties of DHPCA binding sites in brain and cardiac tissues (e.g. binding site affinity, structure-activity relationships, ion-dependence, interactions of other non-DHP calcium antagonists) (Triggle & Janis, 1984a) implies that the brain DHPCA binding site may serve an analogous function in regulating calcium-dependent processes as in pheripheral tissues. Both behavioural (Hoffmeister *et al.*, 1982; Mendelson *et al.*, 1984; Bolger *et al.*,

1985a) and biochemical (Turner & Goldin, 1985; Middlemiss & Spedding, 1985; Kendall & Nahorski, 1985) observations indicate that DHPCAs can regulate neuronal processes in the central nervous system (CNS).

[^{3}H]-nitrendipine binding to a partially purified membrane perparation from guinea-pig cardiac tissue was shown to be dependent on the ionic strength of the assay buffer, being increased by mono- and divalent cations and anions (Glossmann & Ferry, 1983). Recently, it was found that Na^+ could increase the K_D and B_{max} of [^{3}H]-nitrendipine binding to rat cardiac tissue but not brain (Schwartz & Velly, 1985), providing evidence for differences in the biochemical characteristics of DHPCA binding sites in these tissues. We recently observed that phencyclidine could increase the apparent affinity of the DHPCA, [^{3}H]-nitrendipine, in rat brain but not cardiac tissue using a low ionic strength (5 mM Tris-HCl pH 7.4) buffer (Bolger *et al.*, 1985a, b). Subsequently, we have investigated the effects of mono- and divalent cations on [^{3}H]-nitrendipine binding to rat brain and cardiac membranes under similar incubation conditions and now show that low concentrations of mono- (Na^+, Li^+, K^+, Rb^+) and divalent (Ca^{2+}, Mg^{2+}, Mn^+) cations differentially affect [^{3}H]-nitrendipine binding to brain and cardiac membranes. The selective effects of mono- and divalent cations on DHPCA binding support the contention that DHP binding sites in brain and cardiac tissues may be differentially regulated. Such differences may provide a biochemical basis for the marked discrepancies in the biochemical and physiological activity of DHPCAs in these tissues.

Methods

Preparation of membranes

Male Sprague-Dawley rats (150–175 g, Taconic Farms, Germantown, NY) were killed by decapitation. Tissues were rapidly removed and placed in ice-cold 5 mM Tris-HCl buffer pH 7.4. Brain regions (isolated by blunt dissection) or forebrain (defined as that region isolated by making an oblique cut from the superior colliculus on the dorsal surface to the mammilary bodies on the ventral surface) were disrupted in 50 volumes (w/v) of 5 mM Tris-HCl using a Brinkman Polytron (10 s, speed setting 6–7). The homogenate was centrifuged at 24,000 *g* for 15 min, the supernatant discarded and the membrane pellet resuspended with a polytron (5 s, speed setting 6–7) in 100 volumes of 5 mM Tris-HCl buffer. Rat heart ventricular tissue was separated from atrial tissue and minced with scissors prior to disruption in 30 volumes of 5 mM Tris-HCl buffer with a polytron (20 s, speed setting 6–7). The homogenate was centrifuged for 10 min at 1,000 *g*, the pellet discarded and the supernatant centrifuged at 24,000 *g* for 20 min. The membrane pellet from this centrifugation was resuspended by polytron (5 s, speed setting 6–7) in 30 volumes of 5 mM Tris-HCl.

[^{3}H]-nitrendipine binding

[^{3}H]-nitrendipine (specific activity, 81.3 Ci mmol^{-1}, New England Nuclear, Boston, MA) binding was measured in a total incubation volume of 2 ml consisting of 0.5 ml of membrane suspension (brain membranes 0.3–0.5 mg protein; cardiac membranes 0.1–0.3 mg protein), 0.1 ml radioligand and 1.4 ml of 5 mM Tris-HCl buffer. Inorganic cations were dissolved in 5 mM Tris-HCl. Binding assays were initiated by addition of membrane suspension and incubated for 60 min at 25°C or 45 min at 37°C before termination by rapid filtration under vacuum through Whatman GF/B glass fibre filters followed by two 5 ml washes with ice-cold 5 mM Tris-HCl using a Brandel M-24R Cell Harvester (Brandel instruments, Gaithersburg, MD). Unless otherwise stated, incubations were conducted at 25°C. The filters were incubated overnight in 8 ml of ReadySolv MP (Beckman Instruments, Fullerton, CA) and the radioactivity determined in a Beckman liquid scintillation counter (Model LS 250, counting efficiency 41%). Specific [^{3}H]-nitrendipine bound was defined as the difference between [^{3}H]-nitrendipine binding in the absence and in the presence of 10^{-6} M nifedipine (Pfizer Co., Groton, CT). The effects of cations on [^{3}H]-nitrendipine binding to three or more different membrane preparations were studied on the same day to account for the inherent daily variability in the characteristics of the [^{3}H]-nitrendipine binding assay (Bolger *et al.*, 1983). Tris base and all inorganic salts (as chlorides) were obtained from standard commercial sources. Protein determinations were made by the Miller modification (Miller, 1959) of the Lowry technique (Lowry *et al.*, 1951).

Data analysis

Computer analysis of binding data was done using the non-linear regression/statistical computer package, BMDPAR (University of California, Los Angeles, 1977), fitting binding data to the general multisite binding equation of Birdsall *et al.* (1978):

$$B = \sum_{1}^{N} \frac{B_N L}{K_N + L}$$

Where B is the total ligand bound, B_N is the maximum binding site capacity of site N with an apparent dissociation constant K_N. L is the free concentration of ligand.

Results

Effects of monovalent cations

[3H]-nitrendipine binding to rat brain and cardiac membranes at 25°C yielded K_D (120–200 pM) and B_{max} (forebrain, 150–250 fmol mg^{-1} protein; cardiac, 400–500 fmol mg^{-1} protein) values in agreement with previous findings (Triggle & Janis, 1984a). Na^+, Li^+, and K^+, but not Rb^+, increased specific [3H]-nitrendipine binding to brain membranes in a concentration-dependent manner (Figure 1a). The EC_{50} values for these ions were in the range of 10–25 mM; maximum increases in binding were observed at 80–120 mM. Scatchard analyses of the effects of Na^+, Li^+ and K^+ revealed that these ions increased [3H]-nitrendipine binding by reducing the K_D (25% $P < 0.05$), without affecting the B_{max} (Figure 2b, Table 1). The Na^+ (100 mM) mediated increase in [3H]-nitrendipine binding to brain membranes demonstrated brain region specificity. The greatest increases in binding were observed in the cortex, striatum and hippocampus, with smaller increases in the cerebellum and brain stem (Figure 3).

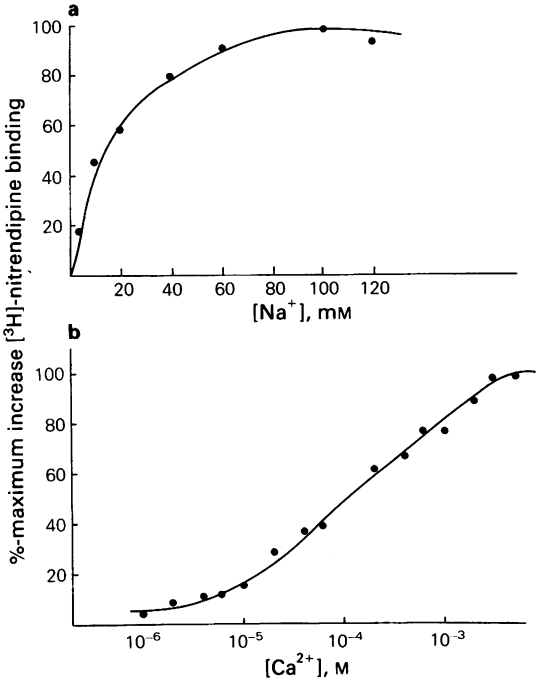


Figure 1 Concentration-dependent effects of Na^+ and Ca^{2+} on [3H]-nitrendipine binding to rat forebrain membranes. The concentration-dependence of cation mediated increases in [3H]-nitrendipine binding for (a) Na^+ and (b) Ca^{2+}. Similar results as in (a) were obtained with the monovalent cations K^+ and Li^+. Na^+, K^+, Li^+ produced equal maximal increases in [3H]-nitrendipine binding with EC_{50} values (mM) of 15.8 ± 3.2, 23.1 ± 3.9, 11.0 ± 4.0 for Na^+, K^+ and Li^+ respectively. Rb^+ did not produce a significant change in [3H]-nitrendipine binding. In (b) the Hill coefficient (n_H) for the Ca^{2+} mediated increase in [3H]-nitrendipine binding was 0.72 ± 0.04; EC_{50} of 111.2 ± 16.1 μM. Computer analysis demonstrated a significantly better fit ($0.02 > P > 0.002$) to a two-site model with affinities (and fractional occupancies) of 15.8 ± 5.7 μM (0.44 ± 0.08) and 441.3 ± 119.5 μM (0.56 ± 0.08), respectively. For Mg^{2+} and Mn^{2+}, the n_H and EC_{50} values were 1.88 ± 0.54, 223.4 ± 77.5 μM and 1.43 ± 0.31, 126.2 ± 40.1 μM respectively. All divalent cations produced equivalent maximum increases in [3H]-nitrendipine binding. The results are expressed as a % of the maximum increase in [3H]-nitrendipine binding (25°C) produced by the cation and are the mean of 5–6 experiments. The concentration of [3H]-nitrendipine in the binding assay was 165 pM.

At 37°C, differences were observed in the effects of monovalent cations on [3H]-nitrendipine binding to brain membranes. In the absence of additional cations, the K_D of [3H]-nitrendipine was increased approximately 5 fold compared to that at 25°C (Tables 1 and 2). Na^+ and K^+ did not significantly affect the K_D of [3H]-nitrendipine (Table 2). However, Na^+ produced a significant increase in the B_{max} (45%) of [3H]-nitrendipine (Table 2).

[3H]-nitrendipine binding to cardiac membranes was unaffected by Na^+, Li^+, K^+, and Rb^+ at either 25°C or 37°C at concentrations up to 120 mM (Figure 2 and Table 3).

Effects of divalent cations

The divalent cations Ca^{2+}, Mg^{2+}, and Mn^{2+} increased [3H]-nitrendipine binding to brain membranes in a concentration-dependent manner (Figure 1b). The maximum increase in [3H]-nitrendipine binding occurred at 1–2 mM with EC_{50} values of 50–200 μM. Scatchard analysis of the effects of Ca^{2+}, Mg^{2+}, and Mn^{2+} revealed an increase in the B_{max} (25%), with no change in the K_D of [3H]-nitrendipine (Figure 4b and Table 1). The effect of Ca^{2+} (2 mM) on [3H]-nitrendipine binding to brain membranes demonstrated brain regon specificity (Figure 3), with the greatest effect in the striatum.

The Ca^{2+}-dependent increase in [3H]-nitrendipine binding to brain membranes (Figure 1b) was described by a Hill coefficient (n_H) of 0.72, which is consistent with a two site interaction for Ca^{2+}. Computer assisted non-linear regression analysis revealed a significantly better fit ($0.02 > P > 0.002$) to a two-site model than a one-site model, with apparent dissociation constants (and fractional occupancies) of 15.8 μM (0.43) and 441.3 μM (0.57) respectively (Figure 1b). Similar analyses with Mg^{2+} and Mn^{2+} yielded n_H values > 1 consistent with a positive co-operative

interaction of these cations at the [^{3}H]-nitrendipine binding site (Figure 1 legend). In the presence of Ca^{2+} (2 mM), Na^+ (100 mM) did not produce a decrease in the K_D of [^{3}H]-nitrendipine. Na^+ did not affect the Ca^{2+}-mediated increase in the B_{max} of [^{3}H]-nitrendipine (K_D and B_{max} values for control, 191.4 ± 24.0 pM, 156.3 ± 13.5 fmol mg^{-1} protein and in the presence of Ca^{2+} (2 mM), plus Na (100 mM), 199.4 ± 9.7 pM, 201.7 ± 23.2 fmol mg^{-1}* protein respectively; means ± s.e.mean of 4 experiments; *significantly different from control, $0.05 > P > 0.01$, unpaired t test). At 37°C, Ca^{2+} evoked a greater increase (68%) in the B_{max} of [^{3}H]-nitrendipine (Table 2) when compared to its effects at 25°C.

Divalent cations did not produce a significant change in [^{3}H]-nitrendipine binding to cardiac membranes at 25°C (Figure 4a and Table 3). However, at 37°C, Ca^{2+} produced a significant increase (80%) in the K_D of [^{3}H]-nitrendipine (Table 3).

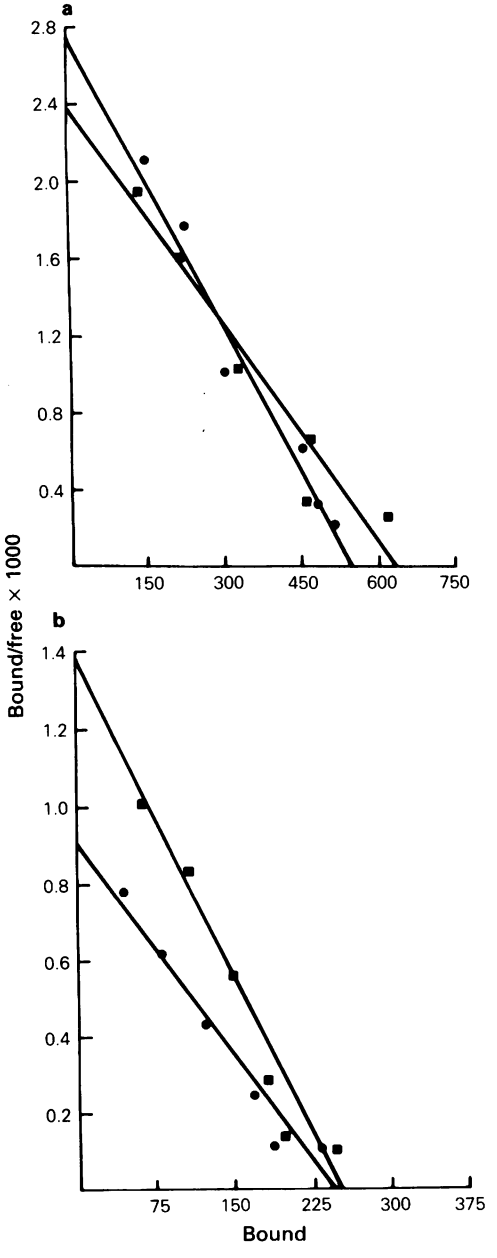


Figure 2 The effects of Na^+ on [^{3}H]-nitrendipine binding to rat forebrain and cardiac membranes. Scatchard analysis of [^{3}H]-nitrendipine binding to (a) rat cardiac membranes and (b) rat forebrain membranes. Binding (25°C) was determined in the absence (●) and presence (■) of Na^+ (100 mM) using [^{3}H]-nitrendipine in a concentration range of 25–1200 pM. The K_D and B_{max} values were for cardiac and brain membranes respectively, control: 196.9 pM, 531.8 fmol mg^{-1} protein; + Na^+, 100 mM: 267.0 pM, 640.9 fmol mg^{-1} protein; control; 278.6 pM, 253.5 fmol mg^{-1} protein; + Na^+, 100 mM: 174.7 pM, 242.9 fmol mg^{-1} protein. The results are from a representative experiment that was repeated four times with similar results (Tables 1 and 3).

Effects of Tris cation

Since most studies characterizing the effects of ions on DHP binding sites have been conducted in a 50 mM Tris-HCl buffer (Triggle *et al.*, 1984a), the effects of increasing concentrations of Tris-HCl were investigated on [^{3}H]-nitrendipine binding to brain and cardiac membranes. Tris-HCl, pH 7.4, in a concentration range 10–52 mM increased [^{3}H]-nitrendipine binding to brain membranes and to a lesser extent cardiac membranes (Figure 5). In the presence of either 100 mM Na^+ or 2 mM Ca^{2+}, the concentration-dependent increase of [^{3}H]-nitrendipine binding by Tris-HCl to brain membranes was abolished. Neither Na^+ nor Ca^{2+} increased [^{3}H]-nitrendipine binding when measured in the presence of 50 mM Tris-HCl (Figure 5). Scatchard analysis of the effects of 50 mM Tris HCl indicated a significant decrease in the K_D of [^{3}H]-nitrendipine (K_D and B_{max} values for 5 mM Tris-HCl were 183.4 ± 12.6 pM, 163.2 ± 12.9 fmol mg^{-1} protein, and for 50 mM Tris-HCl, and for 50 mM Tris-HCl, 126.8 ± 14.7 pM, 171.9 ± 10.5 fmol mg^{-1}* protein, 126.8 ± 14.7 pM, 171.9 ± 10.5 fmol mg^{-1}* protein, respectively; means ± s.e.mean of 4 experiments; *significantly different from control, $0.05 > P > 0.01$, unpaired t test).

Table 1 The effect of mono- and divalent cations on [^{3}H]-nitrendipine binding to rat forebrain membranes

	[*Cation*]], mM	K_D (pM)	B_{max} (fmol mg^{-1} protein)
Monovalent cations			
None		161.1 ± 13.5	194.4 ± 9.7
Na^+	100	120.3 ± 6.9*	202.7 ± 9.9
K^+	100	128.2 ± 12.2*	200.3 ± 9.5
Li^+	100	118.8 ± 17.8*	209.7 ± 13.0
Rb^+	100	161.5 ± 9.7	196.9 ± 14.7
Divalent cations			
Ca^{2+}	2	145.4 ± 13.8	237.0 ± 10.5*
Mg^{2+}	2	185.9 ± 7.3	247.9 ± 12.9*
Mn^{2+}	2	198.2 ± 22.3	245.7 ± 17.7*

Values represent the mean ± s.e.mean of 4–5 experiments. [^{3}H]-nitrendipine binding was measured in a concentration range of 25–1200 pM. *Significantly different ($0.05 > P > 0.01$, unpaired t test) from [^{3}H]-nitrendipine binding (25°C) in the absence of cations.

Discussion

Large differences have been described in the potencies of DHPCAs to block calcium currents in cardiac and neuronal tissues. DHPCAs inhibit cardiac calcium currents in the concentration range 10^{-9}–10^{-6} M (Fleckenstein, 1977; Rosenberger & Triggle, 1978; Triggle & Swamy, 1980; Triggle, 1981). In contrast, DHPCA concentrations of $> 10^{-6}$ M produce only marginal effects on total synaptosomal calcium uptake (Toll, 1982; Daniell *et al.*, 1983; Ogura & Takahashi, 1984; Rampe *et al.*, 1984) or calcium-dependent neurotransmitter release (Freedman *et al.*, 1984; Ogura & Takahashi, 1984). Until recently, major differences in the binding characteristics of brain and cardiac DHPCA binding sites (Triggle & Janis, 1984a) had not been previously described. However, Schwartz & Velly (1985) found a different sensitivity of brain and cardiac DHPCA binding sites to Na^+, thus providing biochemical evidence to support the distinct regulatory properties of these sites.

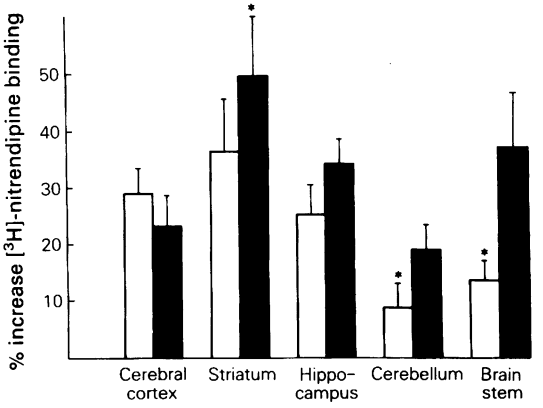


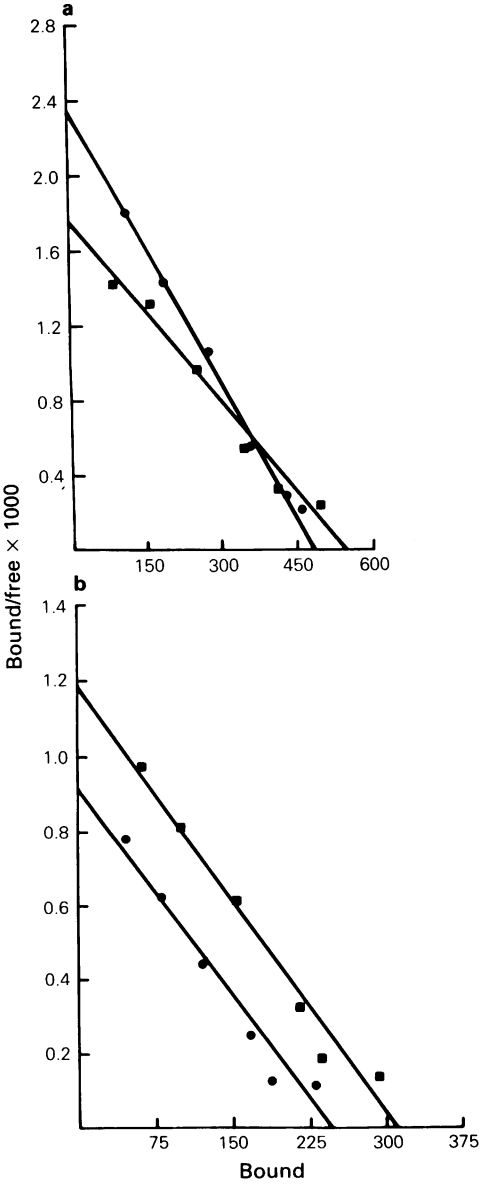
Figure 3 Brain region-dependence of cation effects on [^{3}H]-nitrendipine binding. The ability of Na^+ (100 mM) (open columns) and Ca^{2+} (2 mM) (solid columns) to increase [^{3}H]-nitrendipine binding to brain membranes was investigated in different brain regions. The results are presented as a % increase in [^{3}H]-nitrendipine binding in the presence, compared to that in the absence of cations. Values are presented as the mean ± s.e.mean of 5–6 experiments. The concentration of [^{3}H]-nitrendipine in the binding assay was 165 pM. Significantly different from cortical, striatal and hippocampal [^{3}H]-nitrendipine binding ($P < 0.05$); **significantly different from cortical, hippocampal, cerebellar, and brain stem [^{3}H]-nitrendipine binding ($P < 0.05$). [^{3}H]-nitrendipine binding (25°C) in the presence of cations was significantly different, $P < 0.005$, from control binding.

We observed that low concentrations (with respect to extracellular concentrations) of monovalent (Na^+, K^+, Li^+) and divalent (Ca^{2+}, Mg^{2+}, Mn^{2+}) cations increased brain [^{3}H]-nitrendipine binding in a low ionic strength buffer (5 mM Tris-HCl). Similar effects at brain DHPCA binding sites have not previously been reported, since most studies used 50 mM Tris-HCl to characterize the effects of both mono- and divalent cations on [^{3}H]-nitrendipine binding (Triggle & Janis, 1984a). Tris-HCl (50 mM) significantly increased [^{3}H]-nitrendipine binding to brain membranes (decreased K_D) and inhibited the effects of mono- and divalent cations when compared to 5 mM Tris-HCl. The ability of Tris-HCl to interfere with cation-dependent effects on radioligand binding is not unique, since a similar phenomenon was found for sodium-dependent [^{3}H]-cocaine binding (Reith *et al.*, 1984). Furthermore, Tris-HCl was found to alter the phospholipid binding, conformation, and aggregation of membrane proteins (Yeagle *et al.*, 1986). Thus, it appears that 50 mM Tris-HCl in DHPCA binding assays has

Table 2 The effect of mono- and divalent cations on [^{3}H]-nitrendipine binding to rat forebrain membranes at 37°C

Cation	[*Cation*]], mM	K_D (pM)	B_{max} (fmol mg^{-1} protein)
None		789.9 ± 126.2	182.0 ± 10.0
Na^+	100	693.9 ± 57.5	264.7 ± 9.1*
K^+	100	606.0 ± 66.6	196.2 ± 16.8
Ca^{2+}	2	619.9 ± 29.9	306.4 ± 6.0*

Values represent the mean ± s.e.mean of 4–5 experiments. [^{3}H]-nitrendipine binding was measured in a concentration range of 25–1200 pM. *Significantly different ($P < 0.005$, unpaired t test) from [^{3}H]-nitrendipine binding in the absence of cations.



masked the subtle regulatory effects of cations at brain DHPCA binding sites. Regulation of DHPCA binding by changing the ionic milieu of the binding assay was also found for guinea-pig cardiac tissue (Glossmann & Ferry, 1983), and supports a critical role for cations in the regulation of DHPCA binding sites (Glossmann & Ferry, 1983; Triggle & Janis, 1984a).

The K_D of [^{3}H]-nitrendipine binding to rat brain membranes at 25°C was reduced by monovalent cations (Na^+, K^+ Li^+) (EC_{50} 10–25 mM) with no change in the B_{max}. This action of monovalent cations was selective and cannot be attributed to changes in ionic strength, since Rb^+ was ineffective. Thus, the apparent ion selectivity, concentrations needed, temperature-dependence, and brain region selectivity for affecting [^{3}H]-nitrendipine binding suggests a specific effect. Na^+ increased [^{3}H]-nitrendipine binding to the greatest extent in brain regions containing high DHPCA binding site densities (cortex, striatum, and hippocampus) and to a lesser extent in brain regions low in DHPCA binding sites (cerebellum and brainstem) (Marangos *et al.*, 1982; Murphy *et al.*, 1982; Cortes *et al.*, 1984). This suggests, as have previous studies (Cortes *et al.*, 1982; Bolger *et al.*, 1985a), that DHPCA binding sites possess a pharmacological diversity in the CNS.

It appears that monovalent cations decrease the K_D of the DHPCA binding site in brain membranes through an interaction with a distinct site(s). Can-

Figure 4 The effects of Ca^{2+} on [^{3}H]-nitrendipine binding to rat forebrain and cardiac membranes. Scatchard analysis of [^{3}H]-nitrendipine binding to (a) rat cardiac membranes and (b) rat forebrain membranes. Binding (25°C) was determined in the absence (●) and presence (■) of Ca^{2+} (2 mM) using [^{3}H]-nitrendipine in a concentration range of 25–1200 pM. The K_D and B_{max} values were for cardiac and brain membranes, respectively: control: 196.9 pM, 531.8 fmol mg^{-1} protein, + Ca^{2+}, 2 mM: 234.1 pM, 545.5 fmol mg^{-1} protein; control: 278.6 pM, 253.5 fmol mg^{-1} protein; + Ca^{2+}, 2 mM: 257.8 pM, 306.8 fmol mg^{-1} protein. The results are from a representative experiment that was repeated four times with similar results (Tables 1 and 3).

Table 3 The effect of mono- and divalent cations on [^{3}H]-nitrendipine binding to rat cardiac membranes

Cation	[*Cation*], mM	K_D (pM)	B_{max} (fmol mg^{-1} protein)
Binding at 25°C			
None		100.7 ± 10.9	428.3 ± 26.2
Na^+	100	85.2 ± 5.9	429.7 ± 7.1
K^+	100	111.9 ± 9.6	478.9 ± 73.3
Ca^{2+}	2	112.2 ± 4.2	471.2 ± 25.2
Binding at 37°C			
None		659.8 ± 111.7	311.4 ± 8.1
Na^+	100	658.7 ± 71.5	307.7 ± 46.0
K^+	100	637.5 ± 141.6	381.9 ± 45.8
Ca^{2+}	2	1,1951.3 ± 46.3*	353.0 ± 32.5

Values represent the mean ± s.e.mean of 4–5 experiments. [^{3}H]-nitrendipine binding was measured in a concentration range of 25–1200 pM. *Significantly different ($0.05 > P > 0.01$, unpaired t test) from [^{3}H]-nitrendipine binding in the absence of cations.

didates for this site(s) include synaptic sodium and potassium channels since: (1) cation channel perturbing agents such as phencyclidine and histrionicotoxin (Bolger *et al.*, 1985a, b) and veratridine (Bolger *et al.*, 1983) have a modulatory activity at DHPCA binding sites in brain and smooth muscle respectively, and (2) DHPCAs produce a small, but significant inhibition of $^{24}Na^+$ uptake into rat cortex synaptosomes (Harris *et al.*, 1985). Alternatively, Na^+/K^+-ATPase might be a target for the action of monovalent cations, given the similar effects of Na^+ and K^+ and the observation that DHPCAs (nimodipine and nitrendipine) are potent (10^{-10}–10^{-6} M) stimulators of this enzyme (Pan & Janis, 1983).

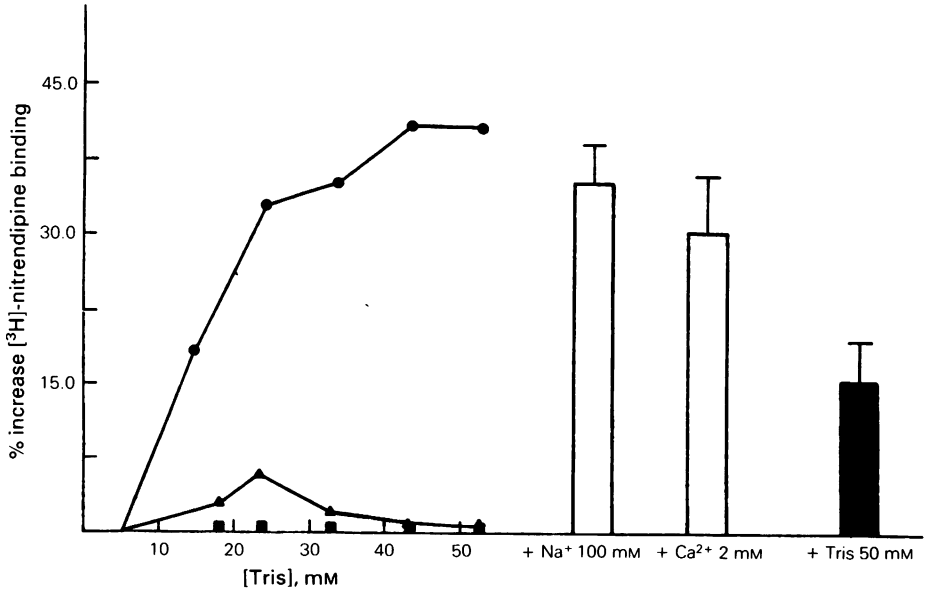


Figure 5 The effect of Tris cation of [^{3}H]-nitrendipine binding to rat forebrain and cardiac membranes. The effects of Tris HCl (5–52.5 mM) on [^{3}H]-nitrendipine binding (25°C) were measured in the absence of additional cations (●) in the presence of 100 mM Na^+ (▲) and in the presence of 2 mM Ca^{2+} (■). The EC_{50} for the enhancement of [^{3}H]-nitrendipine binding by Tris HCl is 15.6 ± 2.8 mM. Open and closed columns represent cation mediated increases in [^{3}H]-nitrendipine binding to forebrain and cardiac membranes, respectively. The results are presented as the % increase in [^{3}H]-nitrendipine binding compared to that in 5 mM Tris-HCl and are the mean ± s.e.mean of four experiments. The concentration of [^{3}H]-nitrendipine in the binding assay was 165 pM.

These findings suggest that the brain DHPCA binding site is allosterically regulated by monovalent cations. Modulation of the DHPCA binding site by Na^+ may underlie facilitation of the inhibitory activity of DHPCAs on the rapid phase of calcium uptake into synaptosomes by removal of extra-synaptic Na^+ (Turner & Goldin, 1985). Monovalent cation activity at the DHPCA binding site may also be linked to modulation of intrasynaptic terminal levels of Na^+ and perhaps K^+, changes in intrasynaptic terminal levels of Na^+ playing an important role in neurotransmitter release (Sandoval *et al.*, 1985). Although highly speculatory, the implication of such a non-calcium channel function for DHPCA binding sites in neuronal tissue would seem appropriate, given (1) the lack of a potent calcium current blocking activity for DHPCAs in neuronal tissue, and (2) the effect of the DHP calcium agonist Bay K 8644 (Schramm *et al.*, 1983; Janis *et al.*, 1984) at enhancing neurotransmitter release and depolarization-dependent phosphoinositol turnover in rat brain slices (Middlemiss & Spedding, 1985; Kendall & Nahorski, 1985) in an apparently Ca^{2+}-independent (Rampe *et al.*, 1985), but DHPCA-sensitive manner (Middlemiss & Spedding, 1985).

In contrast to monovalent cations, low concentrations (EC_{50} 200–300 μM) of divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) increased the B_{max} of [^{3}H]-nitrendipine in rat brain membranes, but had no significant effect on the K_D. The low concentrations of divalent cations required to increase the B_{max} of [^{3}H]-nitrendipine together with both the temperature sensitivity and brain region selectivity of their action, suggests that divalent cations interact with the neuronal DHPCA binding site in a specific manner.

Although Ca^{2+}, Mg^{2+} and Mn^{2+} increased [^{3}H]-nitrendipine binding to rat brain membranes with similar efficacies, some differences in their mechanism of action were noted. Ca^{2+} increased [^{3}H]-nitrendipine binding through interaction with two sites, described by affinities and fractional occupancies of 15.8 μM, 0.44 and 441.3 μM, 0.56, respectively. In contrast, Mg^{2+} and Mn^{2+} both demonstrated positive cooperativity ($n_H > 1$) at the DHPCA binding site and interaction with a single set of sites. Ca^{2+}, Mg^{2+} and Mn^{2+} have previously been shown to increase DHPCA binding to EDTA-treated guinea-pig brain membranes with n_H values > 1 (Glossmann & Ferry, 1983). The apparent differences in action of Mg^{2+} or Mn^{2+} and Ca^{2+} may be related to the calcium antagonistic properties of the former cations (Rosenberger & Triggle, 1978). The high affinity binding site for Ca^{2+} may represent a calcium binding protein such as calmodulin. Calmodulin, present in large quantities in the brain (Wallace *et al.*, 1978; Sandoval *et al.*, 1985) is activated fully by calcium concentrations in the range of 10^{-6}–10^{-4} M. The finding that Mg^{2+} and Mn^{2+} did not possess a high affinity interaction site agrees well with their inability to activate fully calmodulin (Chao *et al.*, 1984), further suggesting the association of a calcium binding protein with the high affinity Ca^{2+} binding site.

Higher concentrations of Ca^{2+}, Mg^{2+} and Mn^{2+} (10^{-4}–10^{-3} M) may expose previously inaccessible DHPCA binding sites on brain membranes through binding to a divalent cation membrane binding site (Rosenberger & Triggle, 1978). It is unlikely, however, that this divalent cation binding site or the high affinity Ca^{2+} binding site are associated with that which modulates the DHPCA binding site at submicromolar concentrations of Ca^{2+}, since contaminant concentrations of divalent cations in the binding assay ($\sim$1 μM) are adequate to fulfil its requirements (Gould *et al.*, 1982; Luchowski *et al.*, 1984; Triggle & Janis, 1984a).

Clearly, mono- and divalent cations can increase [^{3}H]-nitrendipine binding by different mechanisms. Nonetheless, the observation that Ca^{2+} could inhibit the Na^+ mediated decrease in the K_D of [^{3}H]-nitrendipine, indicates that they can influence each others activity. Previously it was demonstrated that low concentrations of Ca^{2+} (10–100 μM) could inhibit veratridine stimulated synaptosomal $^{22}Na^+$ uptake (Matthews *et al.*, 1981). This evidence coupled with the former observation, strengthens speculation that the neuronal DHPCA binding site may be an important regulator of monovalent cation activity at the synapse.

Specific modulatory effects of cations on [^{3}H]-nitrendipine binding to cardiac membranes were only evident for Ca^{2+} at 37°C. This is in direct contrast to the modulatory effects of both mono- and divalent cations on [^{3}H]-nitrendipine binding to brain membranes and implies that DHPCA binding sites exist in a different membrane microenvironment/effector-coupled state within these two tissues. These differences may provide a biochemical basis for the large differences in the calcium current blocking activity of DHPCAs in cardiac and brain tissues. DHPCAs can potently modulate Na^+/K^+-ATPase (Pan & Janis, 1984) and Ca^{2+}-ATPase (David-Dufilho *et al.*, 1984) activities and adenosine uptake (Marangos *et al.*, 1984) in brain and peripheral tissues. While the relationship of DHPCA binding sites to these events remains to be determined (Triggle, 1984), such findings indicate that a number of effector systems may mediate the actions of DHPCAs. The findings presented here may provide new insights into the approaches used to evaluate the regulatory function(s) and the associated effector(s) of the brain DHPCA binding site.

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