# MODERN METHODS IN PHARMACOLOGY

## Volume 2

*Editors*

Sydney Spector

Nathan Back

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# Preface

Advances in the field of pharmacology, as is true for all scientific disciplines, are very much dependent on the development and introduction of new methods.

A citation list of instruments used in pharmacologic studies would contain enormous numbers of citations for such routinely used instruments as the spectrophotometer, spectrophotofluorometer, or the beta or gamma counter. As advances are made in instrumentation, investigators generate procedures to study biological phenomena utilizing these often more sophisticated and sensitive new instruments.

Pharmacologists have come a long way from the not too-distant "smoked drum" era, although we do not minimize the contributions made by investigators who have used or still use the "smoked drum" technique or modifications thereof. However, within the past few years the introduction and application of new methods have become increasingly apparent and have provided compelling reasons to assemble information about new biochemical pharmacologic techniques and their application in one reference source. We also felt that a book series is the best and most convenient format for disseminating this current information and that such a series publication would provide the stimulus for future books on the subject.

As editors we express our sincere appreciation to those authors whose contributions appear in the series for their efforts, and for sharing our belief in the timeliness of such a publication.

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# Calcium Channel Antagonists: New Perspectives From the Radioligand Binding Assay

D.J. Triggle and R.A. Janis

## INTRODUCTION

The group of compounds variously referred to as the $Ca^{2+}$ channel antagonists, $Ca^{2+}$ channel blockers, $Ca^{2+}$ entry blockers, or slow channel blockers has assumed considerable pharmacologic and therapeutic importance [1–5]. This chemically heterogeneous group of compounds, which includes the clinically available verapamil, nifedipine, and diltiazem (Fig. 1), is currently used in the treatment of angina, supraventricular tachycardias, and hypertension, but the potential spectrum of therapeutic use is far wider and extends generally to situations in which excessive smooth muscle tone (vascular and nonvascular) may be present [5–8].

Although much remains to be learned of the sites and mechanisms of action of the $Ca^{2+}$ channel antagonists, it is generally agreed that they serve as potent and selective inhibitors of plasmalemmal $Ca^{2+}$ entry through voltage-dependent $Ca^{++}$ channels. This evidence derives from observations that these agents inhibit the $Ca^{2+}$ carried inward current and the plateau component of the cardiac action potential [9–11] and that they inhibit stimulated, but not resting, $Ca^{2+}$ uptake [12–14], both effects being observed at concentrations which impair excitation-contraction coupling.

Evidence for a specific, rather than a nonspecific, mode of action is also found in the existence of the structure-activity relationships, including stereoselectivity, that have been described for the major classes of agents [2,5,15,16]. However, the chemical and pharmacological heterogeneity of these agents and the apparent absence of an all-encompassing structure-activity relationship sug-

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gests that they exert their channel antagonism at different sites by different mechanisms. Thus, nifedipine and other 1,4-dihydropyridines are significantly more effective as vasodilators than as cardiac depressants, whereas verapamil and diltiazem are appoximately equipotent in this respect [1,5–8]. Additionally, verapamil and diltiazem both show the phenomenon of use- or frequency-dependence whereby potency is enhanced with increasing stimulus rate. In contrast, nifedipine, nitrendipine, and nisoldipine exhibit this phenomenon to a lesser extent [11,17,18].

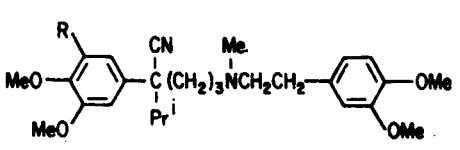
A number of important questions may thus be raised concerning the $Ca^{2+}$ channel antagonists. Among them are the following: 1) Are there discrete, specific sites of action? 2) Are the sites different for each antagonist class and what is their mutual interaction? 3) How do such sites relate to modulation of $Ca^{2+}$ channel function? 4) What is the relationship of $Ca^{2+}$ antagonists to $Ca^{2+}$ and to the process of $Ca^{2+}$ permeation? 5) What is the basis for the apparent tissue selectivity of action?

The recent development of radiolabeled $Ca^{2+}$ channel antagonists and activators of high specific activity has permitted an approach to these questions that complements the existing physiologic and pharmacologic approaches.

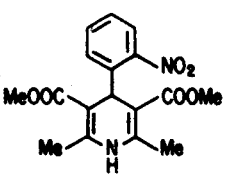
## THE RADIOLIGAND BINDING ASSAY

### Experimental Considerations and Specificity of Binding

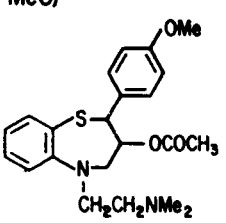
Six [$^3$H]-labeled 1,4-dihydropyridines, five antagonists (nifedipine, nitrendipine, nimodipine, nisoldipine, and PN 200 110), and one agonist (BAY K 8644)




Verapamil (R=H)
D600 (R=MeO)




Nifedipine



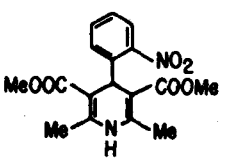

Diltiazem

Fig. 1. Structural formulae of calcium channel antagonists.

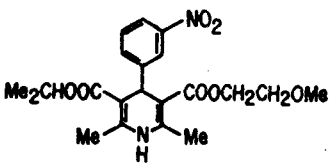
(Fig. 2) have been used in the radioligand binding assay [19–56, 59, 64–70]. Additionally, two reports have appeared of the use of [$^3$H]verapamil [57,58a], and one report of successful binding of [$^3$H]diltiazem has appeared [58b]. Specific activities range from 2.0 to 160 Ci/mmole, the lower activities being those for [$^3$H]verapamil and PN 200 110. The majority of studies have employed [$^3$H]nitrendipine, which is the only ligand currently commercially available.

The basic technique thus far employed has been the rapid filtration assay through glass fiber filters. The majority of studies published to date has employed a 50-mM Tris buffer, pH 7.0–7.7 and temperatures of 25 or 37°C using a dilute membrane suspension (10–50 $\mu$g/ml) with definition of nonspecific binding by $10^{-5}$–$10^{-7}$ M unlabeled dihydropyridine (usually nifedipine or nitrendipine).

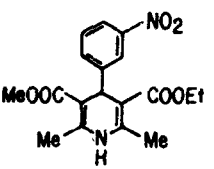
Certain general conditions must be established in any radioligand binding assay to ensure that significant data are obtained [61–63]. For equilibrium binding studies in general, it is necessary to ensure that binding is reversible, that it has attained equilibrium at the time of measurement, that the binding site and the ligand are stable under the conditions of the assay, that the concentration of



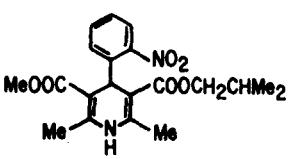

Nifedipine



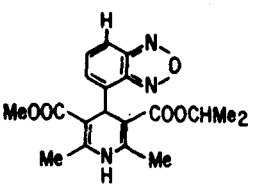

Nimodipine



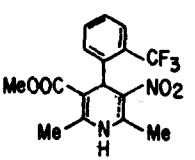

Nitrendipine



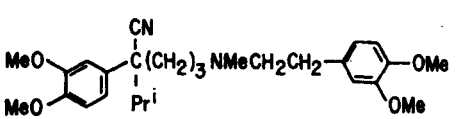

Nisoldipine



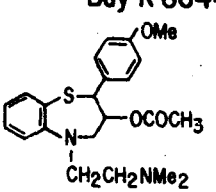

PN 200 110




Bay K 8644




Verapamil




Diltiazem

Fig. 2. Structural formulae of 1,4-dihydropyridine $Ca^{2+}$ channel ligands employed in the [$^3$H]radioligand binding assay.

ligand binding sites relative to ligand affinity is appropriate to avoid excessive ligand depletion, that possible interactions of the ligand with glass or plastic materials be measured, and that an adequate estimate of nonspecific binding can be obtained. Additionally, it is desirable to show that the binding is pharmacologically appropriate. For the [$^3$H]1,4-dihydropyridine binding assay in particular it is necessary to work in subdued or dim light because of the light sensitivity of some of the compounds. This problem is much more serious with o-$NO_2$ derivatives such as nisoldipine and nifedipine. Because of the poor aqueous solubility of most of the 1,4-dihydropyridine, organic solvents, usually alcohol must be used. It must be determined that these solvents do not interfere with binding in the concentrations employed. Reports have also appeared of the binding or uptake of 1,4-dihydropyridines by plastic [19,45]. Most workers appear to employ sodium light or subdued lighting during the course of the binding experiments.

Many of the papers dealing with [$^3$H]1,4-dihydropyridine binding do not give sufficient experimental detail to fully evaluate the results in terms of the previously mentioned limitations. However, the published data that are summarized in Table I do show a remarkably high degree of agreement.

[$^3$H]Nitrendipine binding reveals generally very similar binding to smooth muscle, cardiac muscle and neuronal tissue. Binding at 25°C is of high affinity ($K_D$ = 1 - 3 × $10^{-10}$ M) and in most laboratories evidence for only a single major class of binding sites is found ($n_H \approx 1.0$). The few exceptions to this appear to come from the studies in which high concentrations of protein were used in the binding assay. This can be seen in several entries listed in Table I. The lower affinity of [$^3$H]nitrendipine reported in these experiments can arise from the use of a high binding site concentration relative to $K_D$ for the radioligand or from large amounts of nonspecific binding not detected in the assay [35,50b,61]. However, the greater receptor density reported for [$^3$H]nisoldipine [31] and [$^3$H]PN 200 110 [41b] relative to that for other [$^3$H]1,4-dihydropyridines, suggests that these ligands may convert low affinity sites to high affinity binding sites. In addition, low affinity binding sites ($K_D \approx$ 60nM) have been reported for [$^3$H]nitrendipine in isolated brain and heart membranes when bound and free ligands were separated by centrifugation [70]. Low affinity binding sites for [$^3$H]nitrendipine have also been reported in intact cells [65]. Finally, a higher binding site density is found in rabbit ventricle membranes for the $Ca^{2+}$ agonist Bay K 8644 than for the antagonist [$^3$H]nitrendipine [59]. Binding of Bay K 8644 to cardiac, smooth muscle and brain membranes demonstrates both high and low affinity components [59], but it remains to be determined whether these represent distinct sites or interconvertible states. [$^3$H]Nitrendipine binding to skeletal muscle preparations from rat and rabbit is consistently of lower affinity (1.5 - 8 × $10^{-9}$ M), even when measured at 10°C. Skeletal muscle thus differs

**TABLE I. Binding Characteristics of Radiolabelled Direct Acting $Ca^{2+}$ Channel Modulating Drugs**

| | $K_d$ (nM) | Temp. (C°), incubation time, (min) and pH[a] | μg protein $ml^{-1}$ and assay vol. (ml) | $B_{max}$ (fmole $mg^{-1}$) | Type of prep.[b] | Ref. |
|---|---|---|---|---|---|---|
| A. [$^3$H]Nitrendipine | | | | | | |
| 1. Smooth muscle | | | | | | |
| Guinea pig ileum | 0.16 | 25°, 90, 7.0 | 10–20 (5) | 1,130 | micros. | [19] |
| Rat ileum | 0.26 | 25°, 90, 7.4 | 10[c](2) | 25 | tot. mem. | [20] |
| Bovine aorta | 2.1 | 37°, 12, 7.5 | 3,000 (0.14) | 40–60 | tot. mem. | [21] |
| | 0.16 | 25°, 90, 7.4 | 20 (5) | 80 | pur. mem. | [22] |
| Canine aorta | 0.3; 4.4 | 25°, 60, 7.4 | 72 (5) | 20; 178 | pur. mem. | [23] |
| Rabbit aorta | 1 | --- | ---- | 54 | Light micros. | [24] |
| Pig coronary artery | 1.6 | 37°, 30, 7.4 | 2,000 (0.1) | 35 | | [25] |
| Canine mesenteric artery | 0.25; 1.5 | 25°, 60, 7.4 | 42 (5) | 25; 62 | Frozen tissue pur. mem. | [23] |
| Rat mesenteric artery | 0.10 | 25°, 60, 7.4 | 34 (5) | 18 | pur. mem. | [23] |
| Rat myometrium | 0.18 | 25°, 90, 7.4 | 20 (5) | 220 | micros. | [26a] |
| | 0.14 | 25°, 60, 7.4 | 20 (5) | 720 | Sarcolemma | [26b] |
| Rabbit myometrium | 0.70 | 4°, -- | -- -- | 116 | micros. | [26c] |
| Rat vas deferens | 0.20 | 25°, 90, 7.4 | 20 (5) | 230 | micros. | [26a] |
| Rat stomach | 0.15 | 25°, 90, 7.4 | 20 (5) | 90 | micros. | [26a] |
| Rat fundus | 0.13 | 25°, 60, 7.4 | 20 (5) | 430 | Sarcolemma | [26b] |
| Rat bladder | 0.20 | 25°, 90, 7.4 | 20 (5) | 85 | micros. | [26a] |
| Guinea pig bladder | 0.11 | 25°, 60, 7.0 | 9 (5) | 480 | micros. | [26a] |
| Guinea pig lung | 0.18 | 25°, 60, 7.0 | (5) | 40 | micros. | [26a] |
| 2. Cardiac muscle | | | | | | |
| Rabbit ventricle | 0.16 | 25°, 90, 7.4 | 20 (5) | 300 | micros. | [26a] |
| Rat ventricle | 0.18 | 25°, 90, 7.4 | 16 (5) | 400 | micros. | [27] |
| | 0.3 | 25°, 30, 7.7 | 500 (2) | 88 | Post 1,000 xg membranes | [28] |
| | 0.23 | 25°, 90, 7.4 | 5[c](2) | 120 | tot. mem. | [20] |
| | 0.24 | 25°, 90, 7.4 | 5[c](2) | 6.7[d] | tot. mem. | [29] |
| | 0.21 | 22°, 60, 7.4 | 1,000 (0.5) | 110 | micros. | [30] |
| | 0.11 | 25°, 90, 7.4 | 28 (5) | 400 | micros. | [31] |
| | 0.33 | 37°, 15, 7.4 | 28 (5) | 350 | micros. | [31] |

*(continued)*

**TABLE I. Binding Characteristics of Radiolabelled Direct Acting $Ca^{2+}$ Channel Modulating Drugs (Continued)**

| | $K_d$ (nM) | Temp. (C°), incubation time, (min) and pH[a] | $\mu$g protein $ml^{-1}$ and assay vol. (ml) | $B_{max}$ (fmole $mg^{-1}$) | Type of prep.[b] | Ref. |
|---|---|---|---|---|---|---|
| Guinea pig ventricle | 0.14; 67 | 37°, 60, 7.4 | 100–300 (0.25) | 300<br>35,000 | micros. | [32] |
| Dog atria | 0.14 | 25°, 90, 7.4 | 16 (5) | 170 | micros. | [33] |
| Dog ventricle | 0.13 | 25°, 90, 7.4 | 16 (5) | 190 | micros. | [33] |
| | 0.16 | 25°, 90, 7.4 | 2 (5) | 1,000 | Sarcolemma | [34] |
| | --- | 25°, 90, 7.4 | 20 (5) | < 10 | Mitochondrial | [34] |
| | 0.11 | 30°, 30, 7.4 | 250 (0.1) | 230 | Sarcolemma | [25] |
| | 6.0 | 37°, 15, 7.5 | 3,000 (0.14) | --- | tot. mem. | [21] |
| | 0.30 | 37°, 20, 7.5 | 25 (3.0) | 1,500 | sarc. ret. | [35] |
| Fetal rat heart | 0.3 | 25°, 90, 7.4 | 20 (5) | 80 | micros. | [36] |
| Rat heart WKY | 0.08 | 25°, 90, 7.4 | 25 (2) | 187 | Homogenate | [37] |
| SHR | 0.08 | 25°, 90, 7.4 | 25 (2) | 218 | Homogenate | [37] |
| 3. Skeletal muscle | | | | | | |
| Rabbit hindlimb | 1.5 | 10°, 40, 7.5 | 10–300 (–) | 1,800 | Sarcolemma | [38] |
| | 1.8 | 10°, 40, 7.5 | 10–300 (–) | 800 | Homogenate | [38] |
| | --- | 10°, 40, 7.5 | 10–300 | < 0.01 | Light SR | [38] |
| | 1.8 | 10°, 40, 7.5 | 10–300 | 50,000 | t-tubules | [38] |
| | 1.5 | 25°, 30, 7.4 | 80 (2) | 1,590 | Light SR | [39] |
| | 2.5 | 25°, 30, 7.4 | 20 (2) | 6,720 | Heavy SR | [39] |
| | 2.0 | 25°, 60, 7.4 | --- | 2,000 | Heavy SR | [40] |
| Guinea pig leg | 2.5 | 25°, 60, 7.7 | (2) | | | [41a] |
| | 3.6 | 37°, 30, 7.4 | 20–60 (0.25) | 7,000 | micros. | [41b] |
| Rat hindlimb | 8.5 | 10°, 60, 7.4 | --- | 6,200 | micros. | [42] |
| Rat diaphragm | 4.6 | 10°, 60, 7.4 | --- | 8,580 | micros. | [42] |
| Frog hindlimb | 0.5 | 10°, 40, 7.4 | 100–300 | 20,000 | t-tubules | [38] |
| 4. Nerve | | | | | | |
| Rat cerebral cortex | 1.0 | 25°, 30, 7.7 | 500 (2) | 103 | post 1,000 | [28] |
| Rat cerebal cortex | 0.16 | 0°, 90, 7.4 | 5[c](2) | 7.3[d] | × g mem. | [29] |
| | 0.11 | 25°, 90, 7.4 | 10[c](5) | 102 | tot. mem. | [20] |

| | | | | | | |
|---|---|---|---|---|---|---|
| Rat forebrain | 0.16 | 22°, 60, 7.4 | 500 (0.5) | 92 | tot. mem. | [30] |
| Fetal rat brain | 0.2 | 25°, 90, 7.4 | 20 (5) | 126 | micros. | [36] |
| Rat pheochromacytoma cell line, PC12 | 1.1 | 25°, 30, 7.7 | 750 (0.25) | 27.5 | tot. mem. | [43] |
| PC12 | 0.15 | 25°, 75, 7.3 | --- (2) | 6.5 | Homogenate | [44] |
| Rat brain | | | | | | |
| cerebral cortex | 0.11 | 24°, 45, 7.7 | 4 mg[c](2) | 13[d] | Homogenate | [45] |
| hippocampus | 0.13 | | | 14[d] | | |
| olfactory bulb | 0.11 | | | 12[d] | | |
| striatum | 0.13 | | | 11[d] | | |
| thalamus/ hypothalamus | 0.12 | | | 6[d] | | |
| cerebellum | 0.09 | | | 4[d] | | |
| midbrain | ---- | | | < 1[d] | | |
| brainstem | ---- | | | < 1[d] | | |
| Rat brain | 0.17 | 25°, —, 7.4 | --- (2) | 114 | Synaptosomes | [46, 47] |
| | 0.01 | 0°, 180, 7.4 | 240 (2) | 160 | Synaptosomes | [47] |
| Rat brain | 0.69 | 37°, 10, 7.4 | 240 (2) | 167 | Synaptosomes | [47] |
| Rat brain WKY | 0.12 | 25°, 90, 7.4 | 25 (2) | 68 | Synaptosomes | [37] |
| SHR | 0.12 | 25°, 90, 7.4 | | 107 | Synaptosomes | [37] |
| B. [$^3$H]Nifedipine | | | | | | |
| 1. Cardiac muscle | | | | | | |
| Rabbit ventricle | 1.8 | 25°, 25, 7.4 | 2,500 (0.3) | 72 | Homogenate | [48, 49] |
| Rat ventricle | 0.45 | 25°, 90, 7.4 | 28 (5) | 390 | Microsomes | [31] |
| | 1.3 | 37°, 30, 7.4 | 28 (5) | 350 | Microsomes | [31] |
| 2. Skeletal muscle | | | | | | |
| Guinea pig leg | 4.9 | 37°, 30, 7.4 | 20–60 (0.25) | 3,900 | Microsomes | [41b] |
| C. [$^3$H]Nimodipine | | | | | | |
| 1. Smooth muscle | | | | | | |
| Guinea pig ileum | 0.12 | 25°, 90, 7.0 | 9 (5) | 750 | micros. | [27] |
| Rat vas deferens | 0.15 | 25°, 90, 7.0 | 30 (5) | 200 | micros. | [36] |
| Pig mesenteric artery | 1.9 | 37°, 90, 70 | (0.16) | 200 | micros. | [49] |

*(continued)*

**TABLE I. Binding Characteristics of Radiolabelled Direct Acting $Ca^{2+}$ Channel Modulating Drugs (Continued)**

| | $K_d$ (nM) | Temp. (C°), incubation time, (min) and pH[a] | $\mu$g protein $ml^{-1}$ and assay vol. (ml) | $B_{max}$ (fmole $mg^{-1}$) | Type of prep.[b] | Ref. |
|---|---|---|---|---|---|---|
| Pig coronary | 1.5 | 37°, 90, 7.0 | (0.16) | 100 | micros. | [49] |
| 2. Cardiac muscle | | | | | | |
| Rat ventricle | 0.24 | 25°, 90, 7.0 | 20 (5) | 400 | micros. | [27] |
| | 0.59 | 37°, 15, 7.4 | 28 (5) | 420 | micros. | [31] |
| Guinea pig vent. | 0.26 | 37°, ---, 7.4 | (0.3) | 333 | micros. | [50a] |
| Canine ventricle | 4.0 | 30°, 30, 7.4 | 260 (1) | --- | Sarcolemma | [50b] |
| | 0.18 | 30°, 30, 7.4 | 7.5 (1) | 1,100 | Sarcolemma | [50b] |
| Bovine ventricle | 0.25 | 37°, 45, 7.4 | 11.5 (0.25) | 350–900 | Sarcolemma | [50c] |
| 3. Skeletal muscle | | | | | | |
| Guinea pig | 1.3 | 37°, 30, 7.4 | 40 (0.25) | 18,100 | micros. | [50d] |
| hind limb | 3.6 | 37°, 30, 7.4 | 20–600 (0.25) | 8,000 | micros. | [41b] |
| Pig | 10.0 | 37°, 30, 7.4 | (0.25) | 800 | micros. | [49] |
| 4. Nerve | | | | | | |
| Rat brain | 1.11 | 37°, 30, 7.4 | 300 (5) | 500 | micros. | [52a] |
| Guinea pig brain | 0.3–0.6 | 37°, 45, 7.4 | 67–700 (0.25) | 300–750 | micros. | [52b, 53] |
| | 0.4–0.8 | 37°, -- 7.4 | 100–300 (0.25) | 600 | micros. | [54a, 55] |
| Human brain | 0.27 | 25°, 60, 7.0 | (1) | 5.8[d] | micros. | [55b] |
| Pig cerebral cortex | 1.4 | 37°, --, 7.4 | (0.25) | 160 | micros. | [49] |
| Pig cerebellum | 1.4 | 37°, --, 7.4 | (0.25) | 40 | micros. | [49] |
| Pig adrenal gland | 1.6 | 37°, --, 7.4 | ------ (0.25) | 100 | micros. | [49] |
| D. [$^3$H]Nisoldipine | | | | | | |
| 1. Cardiac muscle | | | | | | |
| Rat ventricle | 0.14 | 25°, 90, 7.4 | 20 (5) | 530 | micros. | [31] |
| Rat ventricle | 0.04 | 37°, 15, 7.4 | 28 (5) | 430 | micros. | [31] |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2. Skeletal muscle | | | | | | |
| Rabbit hindlimb | 2.2 | 25°, 60, 7.4 | 20 (5) | 8,250 | Heavy sarc. ret. | [40] |
| E. [$^3$H]-PN 200 110 | | | | | | |
| 1. Skeletal muscle | 1.4 | 37°, 15°, 7.4° | 20–60 (1) | 20,600 | micros. | [41b] |
| F. [$^3$H]Verapamil | | | | | | |
| 1. Cardiac muscle | | | | | | |
| Frog heart | 4.2 | 25°, 30°, 7.4° | 750 (1) | 50 | micros. | [57] |
| Dog heart | 500.0 | --- | --- | --- | micros. | [58] |
| G. [$^3$H]Diltiazem | | | | | | |
| 1. Skeletal muscle | | | | | | |
| Guinea pig handlimb | 39 | 2°, 240°, 7.4° | 100–300 (0.25) | 11,000 | micros. | [58b] |
| | 37 | 37°, 30°, 7.4° | 100–300 (0.25) | 2,900 | micros. | [58b] |
| H. [$^3$H]Bay K 8644 | | | | | | |
| 1. Cardiac muscle | | | | | | |
| Rabbit ventricle | 4[e] | 25°, 60, 7.4 | 40 (5) | 259 | micros. | [59] |
| 2. Nerve | | | | | | |
| Guinea pig | 3 | 15°, 60, 7.4 | 40 (5) | 808 | Synaptosomes | [59] |
| cerebral cortex | 6 | 37°, 15, 7.4 | (2) | 190 | micros. | [60] |

[a]Most studies were carried out in 50 mM Tris HCl buffer. No marked differences in binding characteristices were reported when HEPES [29] or phosphate buffers [19, 46, 47] were used, or when monovalent cations were present. Stimulatory effects of certain anions were reported [50c, 52b]. As discussed in the text, calcium and magnesium chelators, heavy metals, and lanthanides decrease binding. Little or no difference in binding is seen between pH 7.0 to 7.7.

[b]Abbreviations for type of preparation: micros., microsomal membranes isolated by differential centrifugations; tot. mem., total or nearly total membrane preparations; in some studies a slow-speed centrifugation was used to remove nuclear membranes; pur. mem., additional purification steps were used, such as density gradient centrifugation, to yield various degrees of enrichment, usually of plasma membrane; sarc. retic., purified sarcoplasmic reticular membranes.

[c]Milligrams original wet weight tissue per milliliter assay volume.

[d]fmole $mg^{-1}$ weight tissue.

[e]An additional lower affinity binding site is also observed.

significantly in its binding behaviour from that of smooth and cardiac muscle and brain tissue. A basically similar pattern of observations has been reported, but on a less extensive scale, for [$^3$H]nimodipine and [$^3$H]nisoldipine (Table I).

The specificity of [$^3$H]1,4-dihydropyridine binding has been examined by a number of workers [19,28,30,32,45,51]. Quite generally, specific binding is insensitive or weakly sensitive to a wide number of ligands specific for receptors, including adenosine, adrenergic ($\alpha$- and $\beta$-), cholinergic (muscarinic and nicotinic), histamine ($H_1$ and $H_2$), phencyclidine, opiate, GABA, benzodiazepine, and opiate, and is similarly insensitive to such agents as tetrodotoxin ($Na^+$ channel), 4-aminopyridine ($K^+$ channel), antiarrhythmics (quinidine), and intracellular $Ca^{++}$ antagonists (TMB-8).

Although reversibility of [$^3$H]1,4-dihydropyridine binding has been demonstrated, detailed rate measurements have not always been determined. When available (Table II), they are usually consistent with single association and dissociation processes and give quite good agreement with binding constants derived from equilibrium studies. However, [$^3$H]nisoldipine exhibits a complex dissociation process [31] and evidence for isomerization of the 1,4-dihydropyridine receptor between high and low affinity states has been presented [20,47].

Only two reports describing [$^3$H]verapamil binding have appeared [57,58]. In frog heart membranes a low capacity (50 fmol/mg protein) high affinity ($K_D$ = $4.25 \times 10^{-9}$ M) was described that was sensitive to inhibition by nitrendipine ($IC_{50}$, $7 \times 10^{-9}$ M). However, comparable [$^3$H]1,4-dihydropyridine binding data were not reported and the relationship between [$^3$H]verapamil binding and the nitrendipine binding site remains to be determined.

Saturable, specific binding of d-cis-[$^3$H]diltiazem to skeletal muscle microsomes has been reported [58b]. Binding is reversible, stereoselective, d -(cis)- > l -(cis)-diltiazem, and the $B_{MAX}$ is temperature-dependent, being significantly greater at 2° (11 pmol/mg protein) than at 37° (2.9 pmol/mg protein).

## Competition Studies With $Ca^{2+}$ Channel Antagonists

The preceding studies, together with demonstrations of the sensitivity of binding to proteolytic- and phospholipid-degrading enzymes [19,38,54], indicate that [$^3$H]1,4-dihydropyridine binding is to a cellular site that does not represent a known neurotransmitter or hormone receptor system. Powerful supporting evidence comes from the many studies demonstrating that binding is sensitive to the several structural categories of $Ca^{2+}$ channel antagonist.

Competition of [$^3$H]1,4-dihydropyridine with 1,4-dihydropyridines is consistent with competitive antagonism. Complete displacement of specific binding is observed with Hill coefficients of close to unity. Competition studies with extensive series of 1,4-dihydropyridines have been reported by Bolger et al [19],

**TABLE II. Rate Constants for [$^3$H]1,4-dihydropyridine Binding**

| Ligand | System | Rate constant[a] | Ref. |
|---|---|---|---|
| [$^3$H]Nitrendipine | Bovine aorta, 25° | $k_1$ 1.84 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.018 $min^{-1}$ | [22] |
| [$^3$H]Nitrendipine | Rabbit heart, 25° | $k_1$ 4.4 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.048 $min^{-1}$ | [26] |
| [$^3$H]Nitrendipine | Dog heart, 25° | $k_1$ 3.44 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.055 $min^{-1}$ | [26] |
| [$^3$H]Nitrendipine | Rat heart, 37° | $k_1$ 6.5 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.333 $min^{-1}$ | [31] |
| [$^3$H]Nitrendipine | Guinea pig heart, 37° | $k_1$ 1.0–1.5 × $10^7 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.0074–0.028 $min^{-1}$ | [32] |
| [$^3$H]Nitrendipine | Rat brain, 25° | $k_1$ 6.5 × $10^8 M^{-1}$<br>$k_{-1}$ 0.036 $min^{-1}$ | [46] |
| [$^3$H]Nitrendipine | Guinea pig ileum, 25° | $k_1$ 6.0 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.06 $min^{-1}$ | [19] |
| [$^3$H]Nitrendipine | Rabbit skeletal muscle, 10° | $k_1$ 1.1 × $10^7 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.11 $min^{-1}$ | [41] |
| [$^3$H]Nimodipine | Rat heart, 37° | $k_1$ 3.7 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.23 $min^{-1}$ | [31] |
| [$^3$H]Nimodipine | Guinea pig brain, 37° | $k_1$ 3.2 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.18 $min^{-1}$ | [53] |
| [$^3$H]Nimodipine | Guinea pig hind limb, 37° | $k_1$-----<br>$k_{-1}$ 1.4 $min^{-1}$ | [51] |
| [$^3$H]Nimodipine | Rat brain, 37° | $k_1$-----<br>$k_{-1}$ 0.099 $min^{-1}$ | [52] |
| [$^3$H]Nifedipine | Rabbit heart, 25° | $k_1$ 7.7 × $10^7 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.052 $min^{-1}$ | [48] |
| | Rat heart, 37° | $k_1$ 3.1 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.49 $min^{-1}$ | [31] |
| [$^3$H]Nisoldipine | Rat heart, 37° | $k_1$ 6.7 × $10^8 M^{-1}$ $min^{-1}$<br>$k_{-1}$ 0.04 $min^{-1}$ | [31] |

[a] $k_1$, association rate; $k_{-1}$, dissociation rate.

Janis et al [27] and by Bellemann et al [52a] (Table III). Very good correlations were obtained between the abilities of these 1,4-dihydropyridines to compete with [$^3$H]nitrendipine or [$^3$H]nimodipine binding and to inhibit smooth muscle contraction (Fig. 3). A comparison of the sensitivities in several systems of [$^3$H]nitrendipine or [$^3$H]nimodipine binding to displacement by nifedipine, nitrendipine, nimodipine, and nisoldipine is presented in Table IV. Additional support for the pharmacologic specificity of [$^3$H]1,4-dihydropyridine binding is provided by the stereoselectivity of inhibition of binding shown by several enantiomeric pairs of 1,4-dihydropyridines (Table V).

Extensive analyses of the inhibition of [$^3$H]1,4-dihydropyridine binding by other structural categories of $Ca^{++}$ channel antagonist have also been carried

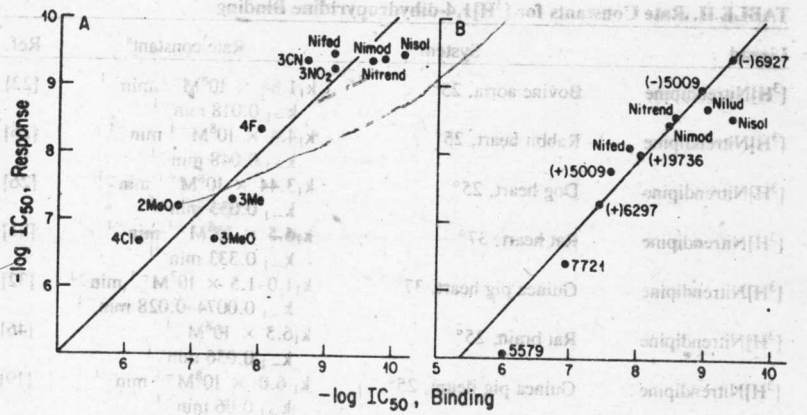
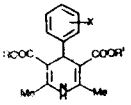



Fig. 3. Correlations between 1,4-dihydropyridine binding and inhibition of smooth muscle mechanical response ($K^+$-induced contracture). A. Guinea pig ileal longitudinal smooth muscle; substituent designations refer to 2,6-dimethyl-3,5-dicarbomethoxy-4-(substituted phenyl)-1,4-dihydropyridine (data from Bolger et al [19]). B. Rabbit aortic smooth muscle. (−) and (+)-5009 are the optical isomers of 2,6-dimethyl-3-carbomethoxy-5-carboisopropoxy-4-(3-nitrophenyl)-1,4-dihydropyridine; (+) and (−) 9736 are the optical isomers of 2,6-dimethyl-3-carboxymethyl-5-carboisopropoxy-4-(3-nitro)phenyl-1,4-dihydropyridine. (Data from Bellemann et al [52a]). Nifed, nifedipine; Nimod, nimodipine; Nisol, nisoldipine; nitrend, nitrendipine.

out. Despite earlier negative results [28,32], it is now generally agreed that agents including verapamil, D600, diltiazem, cinnarizine, flunarizine, bepridil, and inorganic ions do modulate [$^3$H]1,4-dihydropyridine binding [19,20,25,29,45,46,50,52–54]. However, unlike the dihydropyridine competition, the kinetics of interaction of the other categories of agent are complex and have been subject to several descriptions.

Thus, verapamil and D600 have been reported to function as stereoselective ((−) −> (+)− ) inhibitors of [$^3$H]1,4-dihydropyridine binding in smooth and cardiac muscle and brain (Table VI). However, in skeletal muscle from guinea pig, (+)-D600 was reported to be a more effective displacer of [$^3$H]nimodipine than was (−)-D600, although (−)-D600 was more effective than (+)-D600 in blocking diltiazem-potentiated [$^3$H]nimodipine binding in this tissue [50b]. This distinguishes skeletal muscle from other tissues. Complex interactions have been reported. Thus, verapamil and D600 have been reported as partial competitive inhibitors producing a concentration-limited reduction in $K_D$ without change in $B_{MAX}$, [19,20], to produce a reduction in $B_{MAX}$ without change in $K_D$ [30], or to act at both high and low affinity sites to affect [$^3$H]1,4-dihydropyridine binding

TABLE I.1. Activities of 1,4-Dihydropyridines as Inhibitors of [$^3$H]1,4-Dihydropyridine Binding



| X | R | R′ | System: [$^3$H]nitrendipine — Guinea pig ileal long. $IC_{50}$, nM [19] | [$^3$H]nitrendipine — Rabbit heart $IC_{50}$, nM [36] | [$^3$H]nimodipine — Rat heart $IC_{50}$, nM [27] | [$^3$H]nimodipine — Rat brain $K_I$, nM [52a,b] |
|---|---|---|---|---|---|---|
| 2-$NO_2$ | Me | Me (Nifedipine) | 0.83 | 0.23 | 0.18 | 7.0 |
| 3-$NO_2$ | Me | Et (Nitrendipine) | 0.20 | 0.23 | 0.27 | 0.93 |
| 3-$NO_2$ | $CHMe_2$ | $CH_2CH_2OMe$ (Nimodipine) | 0.13 | 0.39 | 0.24 | 1.44 |
| 2-$NO_2$ | Me | $CH_2CHMe_2$ (Nisoldipine) | 0.61 | | | 0.24 |
| 3-$NO_2$ | R = R′ | = $CH_2CH_2OC_3H_7$ (Niludipine) | | | | 0.33 |
| 3-$NO_2$ | Me | $CHMe_2$ | | 2.0 | | 0.22 |
| 2-CN | Me | Me | 0.71 | 1.51 | 1.50 | |
| 2-Me | Me | Me | 1.94 | 1.58 | --- | |
| 2-Cl | Me | Me | 0.20 | 0.42 | --- | |
| 2-MeO | Me | Me | 13.5 | --- | --- | |
| 3-$NO_2$ | Me | Me | 0.10 | 1.20 | --- | |
| 3-MeO | Me | Me | 54.0 | --- | --- | |
| 3-CN | Me | Me | 2.1 | 1.70 | --- | |
| 3-Me | Me | Me | 52.4 | 3.39 | 21.4 | |
| 3-Cl | Me | Me | 0.50 | --- | --- | |
| 4-Cl | Me | Me | 602 | 316 | 288 | |
| 4-Me | Me | Me | 66 | --- | --- | |
| 4-F | Me | Me | 35 | --- | --- | |
| 4-$NO_2$ | Me | Me | 302 | 380 | 450 | |
| H | Me | Me | 14.0 | 5.3 | 36 | |
| $F_5$ | Me | Me | 0.40 | 0.17 | --- | |
| 4-MeO | Me | Me | --- | 3630 | --- | |