*Proc. Natl. Acad. Sci. USA*
Vol. 81, pp. 1549–1552, March 1984
Medical Sciences

# Interaction of calcium channel blockers with non-neuronal benzodiazepine binding sites*

**(nitrendipine/nifedipine/Ro5-4864)**

E. H. CANTOR, A. KENESSEY, G. SEMENUK, AND S. SPECTOR

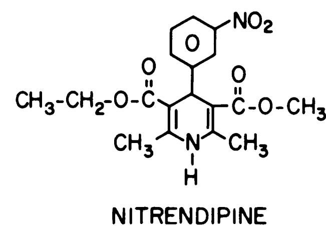
Department of Physiological Chemistry and Pharmacology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

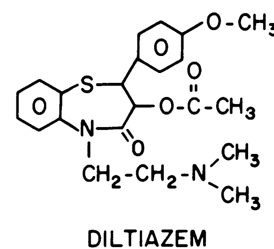
*Communicated by John J. Burns, November 18, 1983*

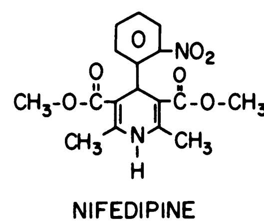
**ABSTRACT The ability of calcium channel blockers to displace the binding of benzodiazepine ligands was investigated in rat heart, kidney, and brain. The dihydropyridine calcium channel blockers nifedipine and nitrendipine displaced the binding of the non-neuronal-site ligand [$^3$H]Ro5-4864, but not that of the neuronal-site ligands [$^3$H]flurazepam or [$^3$H]clonazepam. The inhibition was competitive, with $K_i$ values in the micromolar range. Other calcium channel blockers—i.e., verapamil and diltiazem—were inactive at both sites. Thus, non-neuronal benzodiazepines bind to a class of sites that also binds dihydropyridines. This implies a role for benzodiazepines in the mediation of calcium-dependent phenomena.**

Binding sites for benzodiazepines can be divided into two major categories. Those on neuronal membranes are generally influenced by γ-aminobutyric acid (1–3), are linked to a chloride channel (4), and are probably responsible for the anxiolytic and anticonvulsant activities of the benzodiazepines (4). Benzodiazepines also bind to membranes from a variety of other tissues including kidney (5, 6), heart (6, 7), lung (5, 6), liver (5, 6), mast cells (6), platelets (8), intestinal smooth muscle (9), several cell lines (10, 11), and central nervous system non-neuronal elements (12–15). The sites on these latter cell types represent a population of binding sites pharmacologically distinct from the neuronal type because of the different structural requirements for their respective ligands (unpublished data). Furthermore, the non-neuronal site is not modified by γ-aminobutyric acid and does not bind other neuronal-type ligands such as the β-carbolines (14). Although a specific function has not yet been ascribed to the non-neuronal sites, recent evidence suggests that agents acting at these sites may interfere with calcium-dependent activities *in vitro* (9, 11, 16).

Calcium channel blockers (17) provide useful tools for investigating the potential interaction of non-neuronal benzodiazepines with membrane sites regulating calcium movement. In this study, we used ligand binding techniques to examine the relationship between a number of calcium channel blockers (Fig. 1) and the binding of the benzodiazepine Ro5-4864 (7-chloro-1,3-dihydro-1-methyl-5-(*p*-chlorophenyl)-2H-1,4-benzodiazepin-2-one), which is specific for the non-neuronal site (5, 6, 12, 14).



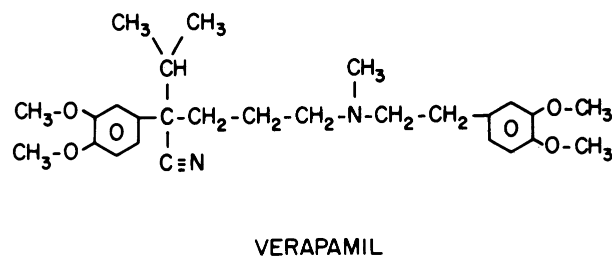




FIG. 1. Structural formulas of the calcium channel blockers used in this study.

## MATERIALS AND METHODS

Male Sprague–Dawley rats (250–300 g, Charles River Breeding Laboratories) were killed by decapitation, and brain, heart, and kidneys were removed. Crude membrane fractions were prepared by homogenizing the tissues in 50 mM Na/K phosphate buffer (pH 7.4). The homogenates were centrifuged at 20,000 × *g* for 15 min. The pellets were washed and then suspended in phosphate buffer for binding assays. Membranes (0.25–0.50 mg of protein) were incubated at 4°C for 15 min either with the non-neuronal benzodiazepine-site ligand [$^3$H]Ro5-4864 or with [$^3$H]flurazepam or [$^3$H]clonazepam, neuronal-site-specific ligands, at the indicated concentrations in total volume of 0.5 ml of phosphate buffer. Nonspecific binding was determined by the addition of diazepam (10 μM). Calcium channel blockers were prepared as fresh dilutions from ethanol stock solutions and

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*A preliminary report of these data was presented to the American Society of Pharmacology and Experimental Therapeutics, Philadelphia, August 7–11, 1983.

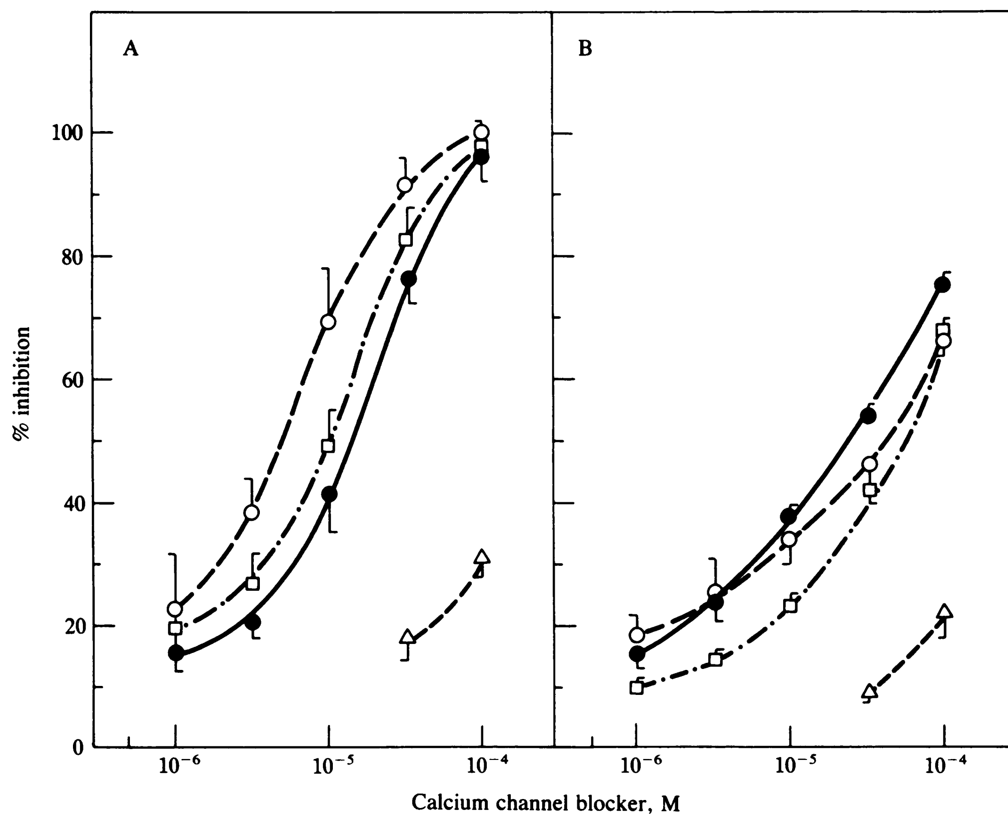



FIG. 2. Displacement of benzodiazepine ligand binding by calcium channel blockers. Crude membrane preparations were incubated with [$^3$H]Ro5-4864 (5.4 mM) or [$^3$H]flurazepam (20 nM) in the presence of increasing concentrations of nitrendipine (*A*) or nifedipine (*B*). Control specific binding (in the absence of calcium channel blocker) was 411 ± 30 fmol per mg of protein ($n$ = 3) for [$^3$H]flurazepam (△) and is given in Table 1 for [$^3$H]Ro5-4864 (●, ○, □). Nonspecific binding was determined by addition of 10 $\mu$M unlabeled diazepam. Data are mean ± SEM of three separate experiments carried out in triplicate, ● and △, Brain; ○, kidney; □, heart.

were included in the reactions as indicated. The final concentration of ethanol was maintained at <1% in the reaction and did not interfere with binding (data not shown). All experiments were carried out in yellow lighting conditions appropriate for the stability of the dihydropyridine derivatives. Reactions were terminated by dilution with 3 ml of cold buffer and rapid vacuum filtration through Millipore AP40 glass fiber filters. The filters were washed with an additional 6 ml of buffer, and bound radioactivity was measured in a liquid scintillation counter.

Protein was determined by the method of Lowry *et al.* (18) using bovine serum albumin as the standard.

Diazepam and [$^3$H]clonazepam (43.5 Ci/mmol; 1 Ci = 37 GBq) were provided by Hoffmann-La Roche. Diltiazem, nifedipine, nitrendipine, and verapamil were generous gifts from Marion Laboratories, Pfizer, Miles, and Searle (Skokie, IL), respectively. [$^3$H]Ro5-4864 (70–90 Ci/mmol) and [$^3$H]flurazepam (>40 Ci/mmol) were purchased from New England Nuclear. All other reagents were obtained from standard commercial sources.

Table 1. $K_i$ values for inhibition of binding of [$^3$H]Ro5-4864 by calcium channel blockers

| Tissue | Control binding, pmol per mg of protein | $K_i$ Nitrendipine | $K_i$ Nifedipine |
|---|---|---|---|
| Brain | 0.19 ± 0.01 | 5.0 ± 0.7 | 9.8 ± 1.0 |
| Kidney | 3.40 ± 0.57 | 2.3 ± 0.9 | 16 ± 3.3 |
| Heart | 2.49 ± 1.50 | 3.4 ± 0.7 | 18 ± 1.5 |

$K_i$ values were calculated from the $IC_{50}$ values obtained by linear-regression analysis of the data shown in Fig. 1 according to the formula $K_i = IC_{50}/[1 + (C/K_d)]$ (19). Control specific binding was determined in the absence of inhibitor using 5.4 nM ligand. Values are mean ± SEM of three experiments with triplicate determinations at each point.

## RESULTS

The ability of various calcium channel blockers to displace the binding of [$^3$H]Ro5-4864 (5.4 nM) or [$^3$H]flurazepam (20 nM) was investigated. These concentrations of the ligands were chosen to approximate the $K_d$ values previously determined in our laboratory. Two dihydropyridine derivatives, nitrendipine and nifedipine, displaced the binding of the non-neuronal-site ligand [$^3$H]Ro5-4864 in a concentration-dependent fashion in all tissues studied (Fig. 2). These agents were inactive against the binding of the central-specific ligand

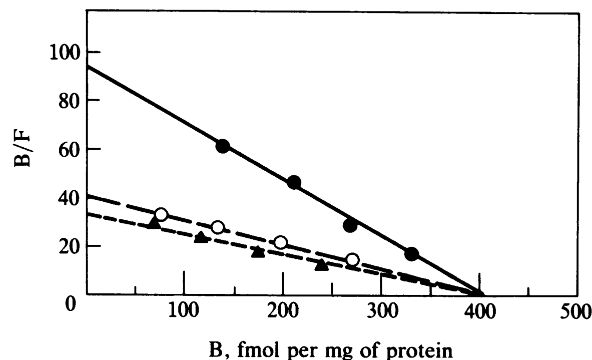



FIG. 3. Binding of [$^3$H]Ro5-4864 (2–20 nM) was measured in the presence or absence of calcium channel blockers and the data were analyzed by the method of Scatchard (20). ●, Control; ○, 10 $\mu$M nitrendipine; ▲, 30 $\mu$M nitrendipine. $B_{max}$ and $K_d$ values are given in Table 2. B, bound; B/F, bound-to-free ratio.

Table 2. Summary of kinetic data for interaction of calcium channel blockers with non-neuronal benzodiazepine binding sites

| Tissue | $B_{max}$, pmol per mg of protein | | | $K_d$, nM | | |
|---|---|---|---|---|---|---|
| | Control | Nitrendipine | Nifedipine | Control | Nitrendipine | Nifedipine |
| Brain | 0.41 ± 0.02 (4) | 0.43 ± 0.05 (4) | 0.43 ± 0.05 (4) | 5.14 ± 1.50 (4) | 11.2 ± 1.03* (4) | 14.2 ± 3.39* (4) |
| Kidney | 8.16 ± 0.93 (4) | 8.48 ± 1.48 (3) | 8.44 ± 0.99 (3) | 4.63 ± 0.35 (4) | 15.7 ± 2.66† (3) | 21.4 ± 3.56† (3) |
| Heart | 7.53 ± 0.36 (3) | 7.47 ± 0.68 (3) | 7.91 ± 0.27 (3) | 3.78 ± 0.04 (3) | 18.7 ± 0.58† (3) | 13.4 ± 1.19† (3) |

$B_{max}$ and $K_d$ values were calculated by Scatchard analysis of saturation isotherms for the binding of [$^3$H]Ro5-4864 using computer-assisted least-squares linear regression. The concentrations of nitrendipine were 10 $\mu$M for brain and heart and 5 $\mu$M for kidney. Nifedipine was present at 30 $\mu$M in brain and heart and 40 $\mu$M in kidney. Values are mean ± SEM of the number of experiments shown in parentheses, each carried out in triplicate.
*$P < 0.05$ vs. control.
†$P < 0.01$ vs. control.

[$^3$H]flurazepam in brain (Fig. 2), while two other calcium channel blockers, diltiazam and verapamil, were without effect in either system at concentrations as high as 100 $\mu$M. The testing of higher concentrations was precluded by the relative insolubility of these compounds. The calcium channel blockers were also inactive against a second neuronal-site-specific ligand [$^3$H]clonazepam (2–20 nM) (data not shown).

$IC_{50}$ values (data not shown) for nitrendipine and nifedipine were calculated by linear-regression analysis of the log-probability plots of the displacement data shown in Fig. 1. $K_i$ values (Table 1) were calculated from the $IC_{50}$ values according to the Cheng and Prusoff correction (19), from which nitrendipine was found to be slightly more potent than nifedipine against the binding of [$^3$H]Ro5-4864, with $K_i$ values of 2–5 × $10^{-6}$ M and 10–18 × $10^{-6}$ M, respectively.

The nature of the interaction with the non-neuronal benzodiazepine site was evaluated in the presence or absence of the two active calcium channel blockers. Maximum binding ($B_{max}$) and apparent dissociation constants ($K_d$) were calculated by the method of Scatchard (20) from saturation isotherms and the data summarized in Table 2. A representative Scatchard plot is shown in Fig. 3. In all three tissues, $B_{max}$ was unaffected by the addition of calcium channel blockers to the binding assay. The $K_d$, on the other hand, was significantly increased 2- to 5-fold in the presence of nifedipine or nitrendipine, indicating an apparent decrease in affinity due to the calcium channel blocker. These data suggest a competitive interaction between the dihydropyridines and the non-neuronal benzodiazepine binding site.

## DISCUSSION

The physiological significance of the non-neuronal benzodiazepine binding site is as yet unknown despite the presence of a high density of such sites in a wide variety of tissues (5–15). Recently, however, several pharmacological effects have been ascribed to agents acting at these sites, and a common mechanism of action, that of calcium antagonism, has been suggested by these findings. For instance, non-neuronal benzodiazepines were found to inhibit calcium-induced contractions in guinea pig ileum in a competitive and reversible manner (9). In a mouse thymoma cell line, non-neuronal-type benzodiazepines inhibit cell proliferation (11), whereas calcium stimulates the proliferation of thymocytes both *in vivo* (21) and *in vitro* (22). Non-neuronal-type benzodiazepines also inhibit calcium-dependent uptake of serotonin by platelets (unpublished data) as well as calcium–calmodulin-stimulated membrane protein phosphorylation (16). All of the above effects required micromolar concentrations of benzodiazepine.

Based on these observations, we examined the interaction between benzodiazepines and recognized calcium channel blockers in an *in vitro* ligand-binding system. Rat kidney and heart were chosen as tissues expressing only the non-neuronal-type site (5–7), and rat brain was selected because it contains both neuronal and non-neuronal binding sites (22) that could be differentiated using site-specific ligands. The calcium channel blockers used in this study (verapamil, diltiazem, and two dihydropyridine derivatives, nitrendipine and nifedipine) represent three classes within this structurally diverse group of agents. These agents inhibit the slow inward potential-dependent calcium current (23), each apparently acting at its own unique receptor site (24, 25).

The dihydropyridine derivatives were active only against the non-neuronal-type benzodiazepine ligand. Verapamil was inactive. This is consistent with the report that this structural analogue of D600 acts from within the cell (26). Diltiazem, despite its close structural similarity to the benzodiazepines, was also without effect, implying that the benzodiazepine binding site is highly selective. The inhibition by the dihydropyridines was competitive as there was no change in the number of binding sites, but the apparent affinity for [$^3$H]Ro5-4864 was decreased.

It is important to note that the concentrations of dihydropyridines required to displace nanomolar concentrations of benzodiazepine fell in the micromolar range, whereas the affinity of these calcium channel blockers for their previously defined receptor site is subnanomolar (23, 24, 27, 28). Conversely, Bolger *et al.* (23) recently reported that 10 $\mu$M diazepam displaced ≈50% of the binding of 55 pM [$^3$H]nitrendipine in guinea pig ileum, whereas the affinity of the benzodiazepine for its own binding site in that tissue is 50 nM (9).

These data point to at least two binding interactions for benzodiazepines and dihydropyridines. At nanomolar concentrations, each is capable of binding with high affinity to its own specific binding site. At micromolar concentrations, however, the dihydropyridines and non-neuronal-type benzodiazepines are each capable of binding with low affinity to the primary site for the other group of drugs. These low-affinity sites for the benzodiazepines may be similar to the "micromolar" benzodiazepine receptors previously described (16, 29).

Although these findings do not shed light on the function of the high-affinity non-neuronal benzodiazepine binding site, they do point to an important action of these compounds at a second site. Thus, the ability of non-neuronal-type benzodiazepines to bind to a site that also binds calcium channel blockers may provide an explanation for the apparent calcium antagonist activity of the benzodiazepines in various *in vitro* systems (9, 11) as well as for previously unexplained reports of systemic and coronary vasodilation by diazepam *in vivo* (30–33).


1. Gallager, D. W. (1978) *Eur. J. Pharmacol.* **49**, 133–143.
2. Tallman, J. F., Thomas, J. W. & Gallager, D. W. (1978) *Nature (London)* **274**, 383–385.
3. Marangos, P. J. & Martino, A. M. (1981) *Mol. Pharmacol.* **20**, 16–21.
4. Paul, S. M., Marangos, P. J. & Skolnick, P. (1981) *Biol. Psychiatry* **16**, 213–229.
5. Braestrup, C. & Squires, R. F. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3805–3809.

6. Taniguchi, T., Wang, J. K. T. & Spector, S. (1980) *Life Sci.* **27,** 171–178.
7. Davies, L. P. & Huston, V. (1981) *Eur. J. Pharmacol.* **73,** 209–211.
8. Wang, J. K. T., Taniguchi, T. & Spector, S. (1980) *Life Sci.* **27,** 1881–1888.
9. Hullihan, J. P., Spector, S., Taniguchi, T. & Wang, J. K. T. (1983) *Br. J. Pharmacol.* **78,** 321–327.
10. Syapin, P. J. & Skolnick, P. (1979) *J. Neurochem.* **32,** 1047–1051.
11. Wang, J. K. T., Morgan, J. I. & Spector, S. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41,** 1328 (abstr.).
12. Gallager, D. W., Mallorga, P., Oertel, W., Henneberry, R. & Tallman, J. (1981) *J. Neurosoci.* **1,** 218–225.
13. Schoemaker, H., Bliss, M. & Yamamura, H. I. (1981) *Eur. J. Pharmacol.* **71,** 173–175.
14. Marangos, P. J., Patel, J., Boulenger, J.-P. & Clark-Rosenberg, R. (1982) *Mol. Pharmacol.* **22,** 26–32.
15. Del Zompo, M., Post, R. M. & Tallman, J. F. (1983) *Neuropharmacology* **22,** 115–118.
16. DeLorenzo, R. J., Burdette, S. & Holderness, J. (1981) *Science* **213,** 546–549.
17. Fleckenstein, A. (1977) *Annu. Rev. Pharmacol. Toxicol.* **17,** 149–166.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
19. Cheng, Y. C. & Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22,** 3099–3108.
20. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51,** 660–672.
21. Perris, A. D. (1971) in *Cellular Mechanisms for Calcium Transfer and Homeostasis*, eds. Nichols, G., Jr., & Wasserman, R. H. (Academic, New York), pp. 101–131.
22. Morgan, J. I. & Perris, A. D. (1974) *J. Cell. Physiol.* **83,** 287–296.
23. Bolger, G. T., Gengo, P., Klockowski, R., Luchowski, E., Siegel, H., Janis, R. A., Triggle, A. M. & Triggle, D. J. (1983) *J. Pharmacol. Exp. Ther.* **225,** 291–309.
24. Marangos, P. J., Patel, J., Miller, C. & Martino, A. M. (1982) *Life Sci.* **31,** 1575–1585.
25. Glossmann, H., Ferry, D. R., Lubbecke, F., Mewes, R. P. & Hofmann, F. (1983) *J. Receptor Res.* **3,** 177–190.
26. Hescheler, J., Pelzer, D., Trube, G. & Trautwein, W. (1982) *Pflügers Arch.* **393,** 287–291.
27. Ehlert, F. J., Itoga, E., Roeske, W. R. & Yamamura, H. I. (1982) *Biochem. Biophys. Res. Commun.* **104,** 937–943.
28. De Pover, A., Matlib, M. A., Lee, S. W., Dube, G. P., Grupp, I. L., Grupp, G. & Schwartz, A. (1982) *Biochem. Biophys. Res. Commun.* **108,** 110–117.
29. Bowling, A. C. & DeLorenzo, R. J. (1982) *Science* **216,** 1247–1250.
30. Chai, C. Y. & Wang, S. C. (1966) *J. Pharmacol. Exp. Ther.* **154,** 271–280.
31. Abel, R. M., Reis, R. L. & Staroscik, R. N. (1970) *Br. J. Pharmacol.* **38,** 620–631.
32. Abel, R. M., Staroscik, R. N. & Reis, R. L. (1970) *J. Pharmacol. Exp. Ther.* **173,** 364–370.
33. Daniell, H. B. (1975) *Eur. J. Pharmacol.* **32,** 58–65.