REFERENCES

1. C. A. Wagenvoort and N. Wagenvoort, *Pathology of Pulmonary Hypertension*. John Wiley, New York (1977).
2. A. E. Pegg and P. P. McCann, *Am. J. Physiol.* **243**, C212 (1982).
3. B. Meyrick and L. Reid, *Lab Invest.* **38**, 188 (1978).
4. B. Meyrick and L. Reid, *Am. J. Path.* **96**, 51 (1979).
5. C. Niedenzu, K. Grasedyck, N. F. Voelkel, S. Bittmann and J. Lindner, *Int. Archs occup. environ. Hlth* **48**, 185 (1981).
6. S. S. Sobin, H. M. Tremer, J. D. Hardy and H. P. Chiodi, *J. appl. Physiol.* **55**, 1445 (1983).
7. J. W. Olson, R. J. Altiere and M. N. Gillespie, *Biochem. Pharmac.* **33**, 3633 (1984).
8. J. W. Olson, A. D. Hacker, R. J. Altiere and M. N. Gillespie, *Am. J. Physiol.* **247**, H682 (1984).
9. J. W. Olson, J. E. Atkinson, A. D. Hacken, R. J. Altiere and M. N. Gillespie, *Tox. Appl. Pharmac.* **81**, (1985).
10. A. D. Hacker, D. F. Tierney and T. K. O'Brien, *Biochem. biophys. Res. Commun.* **113**, 491 (1983).
11. B. G. Erwin, D. R. Bethell and A. E. Pegg, *Am. J. Physiol.* **246**, C293 (1984).
12. M. N. Gillespie, C. N. Reinsel, J. W. Olson and R. J. Altiere, *Fedn Proc.* **43**, 881 (1984).

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Stereoselective interactions of 2-[(2′,6′-dimethoxyphenoxyethyl)aminomethyl]-1,4-benzodioxane (WB-4101) with the calcium channel

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There have been numerous reports in the literature regarding the α-adrenergic blocking activity of various calcium channel blockers [1–4]. Several investigators have suggested that some of the clinical effects of verapamil, a calcium channel blocker, may be due to its α_1-adrenergic blocking effects [2–4]. Conversely, it appears that certain adrenergic blocking agents may have calcium channel blocking activity [5]. A study by Atlas and Adler [5] suggests that the low affinity binding sites of the ^{3}H-labeled α_1-adrenergic antagonist 2-[(2′,-dimethoxyphenoxyethyl)-aminomethyl]-1,4-benzodioxane ([^{3}H]WB-4101) may actually represent binding sites on the calcium channel.

To determine more directly whether WB-4101 interacts with binding sites closely linked to calcium channels, we examined its interaction with the receptor recently identified for the dihydropyridine calcium channel blockers [6–8]. We used enantiomeric pairs of WB-4101 to aid in differentiating specific from non-specific interactions. Our results suggest that, at higher concentrations than those needed for adrenergic blocking activity, WB-4101 interacts specifically with the dihydropyridine receptor and that these higher concentrations may be associated with calcium channel blocking properties.

Methods

Compounds. [^{3}H]Nitrendipine (71 Ci/mmole) was obtained from New England Nuclear (Boston, MA). The enantiomers of WB-4101 were synthesized by previously reported methods [9, 10].

Binding studies. Male Sprague–Dawley rats (200–300 g) were killed by cervical dislocation. After removal, the heart was rapidly perfused with 10 ml of ice-cold buffer (50 mM Tris–HCl, pH 7.4) to remove red cells. The perfused heart was stripped of great vessels and extraneous connective tissue and placed in ice-cold buffer to produce a concentration of 100 mg tissue/ml buffer. The tissue was homogenized on a Brinkmann Polytron homogenizer using three 5-sec bursts at 80% of the maximum speed. The resultant homogenate was centrifuged at 48,000 *g* for 15 min, and the supernatant fraction was discarded. This procedure was repeated twice. The pellet was resuspended in buffer to a final protein concentration of 0.1 mg/ml.

Incubations were carried out at 25° for 90 min. Saturation experiments were performed with concentrations of [^{3}H]-nitrendipine ranging from 0.02 to 1.0 nM. For displacement experiments, the concentration of [^{3}H]nitrendipine in the incubates was 0.05 nM. Concentrations of antagonists ranging from 1×10^{-7} M to 3.2×10^{-4} were present. Concentrations greater than 3.2×10^{-4} M were not employed because of limited quantities of the enantiomers. Incubations were terminated by rapid vacuum filtration over filters (Whatman GF/B) that were subsequently rinsed with 12 ml of ice-cold buffer. Bound radioactivity was determined by liquid scintillation counting of the filters. Counting efficiency ranged from 38 to 42%. Non-specific binding was determined as the amount of [^{3}H]nitrendipine bound in the presence of 10^{-6} M nifedipine and routinely accounted for 40–50% of total binding.

Pharmacologic studies. Male Sprague–Dawley rats (200–300 g) were killed by cervical dislocation. Longitudinal strips of the right ventricle (~5 mm in length, 2 mm in width) were obtained and placed in a 100 ml organ bath at 37° containing isotonic Krebs–Henseleit buffer (pH 7.4) and aerated with 95% O_2 and 5% CO_2. Muscle strips were mounted and attached to a Kistler–Morse model dSC-6 force displacement transducer. Contractions were induced by field stimulation at a rate of 2.0 Hz. After an equilibration period of 60 min at 1.5 g tension, the contraction signals were amplified and recorded (Gould model ES1000 Physiologic Recorder) at a tension of 1 g. Cumulative concentration–response curves for inhibition of isometric contractions by racemic WB-4101 were obtained. Because of limited quantities of the enantiomers, we could not carry out pharmacologic studies with these compounds.

Data analysis. The data from individual saturation experiments were expressed as amount bound and plotted against concentration of [^{3}H]nitrendipine. To obtain the K_d and B_{max} (maximum number of binding sites) of nitrendipine the data were fit by digital computer (FIT FUNCTION on the PROPHET SYSTEM) to the following equation:

$$\text{Amount bound} = \frac{B_{max} \times C}{K_d + C} + K_{ns} \times C$$

where C is the total concentration of nitrendipine in the incubation mixture and K_{ns} is the non-specific binding constant. Since the amount bound to the membrane particulates routinely accounted for less than 10% of the added amount of [^{3}H]nitrendipine, total approximated free concentrations.

The data from the displacement experiments were expressed as a percentage of specifically bound [^{3}H]nitrendipine and plotted against the log concentration of the displacing agent. The data ranging from 20 to 80% specifically bound [^{3}H]nitrendipine were fit by log-linear least squares regression analysis. The IC_{50} was obtained by calculating the concentration at which the specifically bound [^{3}H]nitrendipine was 50%. Data were handled in this way instead of with traditional 3–4 parameter logistic equations because maximum displacement of [^{3}H]nitrendipine was not obtained.

A similar approach was used to fit the data obtained in the pharmacologic studies except that instead of percent specifically bound, the fit was for percent of maximum contraction versus the logarithm of the concentration of WB-4101.

Experiments to test for differences between *R*- and *S*-WB-4101 were carried out as paired studies: studies with both *R*- and *S*-WB-4101 were carried out in a single membrane particulate preparation. This experiment was repeated six times. Statistical analysis to test for differences between the IC_{50} values was carried out by a Wilcoxon Signed Rank test for paired data.

Results

A representative saturation isotherm of [^{3}H]nitrendipine in cardiac membrane particulates is shown in Fig. 1. The K_d (mean ± S.D.) was 0.33 ± 0.09 nM and the B_{max} was 199 ± 13 fmoles/mg protein (N = 3).

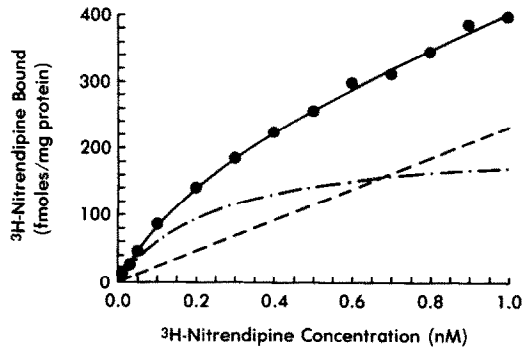

Fig. 1. A representative saturation isotherm (25°) of [^{3}H]-nitrendipine in rat myocardial membrane particulates. Shown is the computer-generated fit of the data together with the computer-generated curves for the non-specific and saturable binding processes. The K_d for this experiment was 0.25 nM and the B_{max} was 214 fmoles/mg protein.

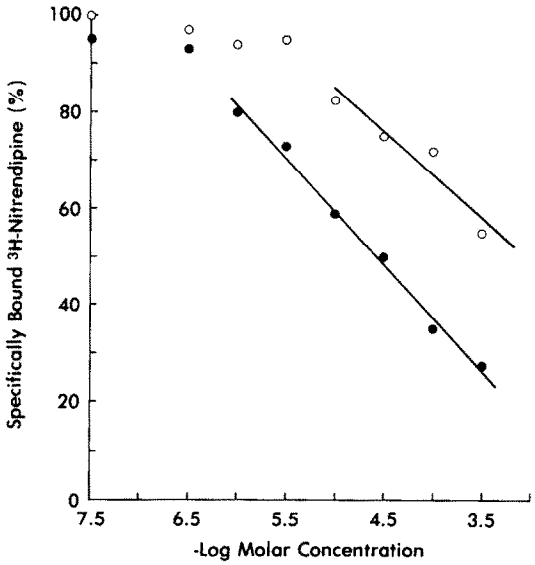

Fig. 2. Representative displacement curves of [^{3}H]nitrendipine from its binding sites on rat myocardial membrane particulates by *R* (○) and *S*- (●) WB-4101. Each point is the mean of triplicate determinations. The lines represent the best fit obtained by log-linear least squares regression analysis.

Representative displacement curves of bound [^{3}H]nitrendipine by the enantiomers of WB-4101 are shown in Fig. 2. Both the *R*- and *S*-enantiomers of WB-4101 displaced [^{3}H]nitrendipine in a concentration-dependent fashion; however, complete inhibition of specifically bound [^{3}H]-nitrendipine was not obtained even at the highest concentrations studied (3.2×10^{-4} M). The results of the paired studies with *R*- and *S*-WB-4101 are shown in Table 1. *S*-WB-4101 was substantially more potent than *R*-WB-4101 in displacing bound [^{3}H]nitrendipine ($P < 0.05$). The IC_{50} (mean ± S.D.) for *S*-WB-4101 was $1.22 \times 10^{-4} \pm 0.66 \times 10^{-4}$ M. In three of the six paired experiments, *R*-WB-4101 at concentrations of 3.2×10^{-4} M displaced less than 20% of the specifically bound [^{3}H]nitrendipine and the IC_{50}, although not computed, was simply expressed as $>3.2 \times 10^{-4}$ M. The IC_{50} (mean ± S.D.) of racemic WB-4101 was $7.27 \pm 4.44 \times 10^{-5}$ M (N = 3).

To determine whether WB-4101 was affecting non-specifically bound [^{3}H]nitrendipine, we carried out three displacement experiments of [^{3}H]nitrendipine in the presence of 10^{-6} M nifedipine. The results in all three experiments demonstrated that, even at high concentrations (3.2×10^{-4} M), racemic WB-4101 did not affect non-specifically bound [^{3}H]nitrendipine.

With respect to pharmacologic potency, the IC_{50} (mean ± S.D.) for racemic WB-4101 obtained from three experiments was $8.98 \pm 2.81 \times 10^{-5}$ M.

Discussion

Recently, Atlas and Adler [5] demonstrated that the α_1-adrenergic blocking agent WB-4101 appears to be a calcium channel blocker at higher concentrations. The evidence presented in their study was obtained primarily in neuroblastoma-glioma hybrid cells which are devoid of α_1-adrenergic receptors. A major question unanswered by the study was whether the calcium channel blocking effects were due to a specific receptor interaction or to non-specific membrane interactions present at higher concentrations. Arguments presented in favor of specific interactions at the calcium channel included the fact that the low affinity binding capacity of [^{3}H]WB-4101 is the same order of magnitude as computed densities of ion channels.

To specifically address the questions raised in the study of Atlas and Adler [5], we examined the interaction of WB-4101 with the recently identified receptor for the dihydropyridine calcium channel blockers [6–8]. We employed the enantiomers of WB-4101 to aid in differentiating specific from non-specific effects. Our results demonstrated that

Table 1. Relative potencies of *R*- and *S*-WB-4101 at the putative dihydropyridine receptor

	IC_{50}*($\times 10^4$ M)	
Expt. No.	*R*-WB-4101	*S*-WB-4101
1	>3.2†	2.45
2	2.34	1.00
3	>3.2	1.38
4	5.75	0.91
5	3.02	0.55
6	>3.2	1.00

Experiments were carried out in particulates prepared from rat myocardium using 0.05 nM [^{3}H]nitrendipine to specifically label the receptor.

* Values were obtained by fitting the data between 20 and 80% specifically bound [^{3}H]nitrendipine to a log-linear equation and calculating the concentration of WB-4101 enantiomer needed to displace 50% of the specifically bound [^{3}H]nitrendipine.

† Greater than 80% of the [^{3}H]nitrendipine still bound at the highest concentration studied (3.2×10^{-4} M).

WB-4101 does indeed interact in a concentration-dependent fashion with the dihydropyridine receptor in myocardial tissue. This interaction occurred with specifically rather than non-specifically bound [^{3}H]nitrendipine as suggested by the stereoselectivity of WB-4101 and the experiments demonstrating that racemic WB-4101 did not alter non-specifically bound [^{3}H]nitrendipine. Concentrations of WB-4101 ranging between 10^{-5} and 10^{-4} M are approximately 100-fold higher than needed to evoke α_1-adrenergic blocking effects [5, 9, 11]. However, the concentrations are similar to those observed by Atlas and Adler [5] to displace [^{3}H]WB-4101 from its binding sites in neuroblastoma glioma cells, suggesting that the low affinity binding site of [^{3}H]WB-4101 in these cells may represent the dihydropyridine receptor.

Because of the high concentrations needed and the limited amounts of compounds, the data allow us to conclude that specific rather than non-specific binding is being altered, but do not allow us to infer whether the mechanism is competitive or allosteric. Nevertheless, the interaction of WB-4101 with the [^{3}H]nitrendipine binding site may be related to the calcium channel blocking effects of this compound as is suggested by the similar pharmacologic and binding IC_{50} values (0.9×10^{-4} M compared to 0.73×10^{-4} M). However, other mechanisms for the pharmacologic response of WB-4101 in these experiments cannot be excluded.

In conclusion, our results demonstrate that WB-4101, a potent α_1-adrenergic blocking agent, also interacted specifically with the dihydropyridine calcium channel receptor. The potency of WB-4101 at the dihydropyridine receptor correlated with its pharmacologic potency as a calcium channel blocker. Because of the high concentrations required for calcium channel blocking activity, the implications of these findings to studies performed at concentrations required to elicit α_1-adrenergic blocking effects are not important. However, the observations may be important for understanding the toxicities of WB-4101 and perhaps other α-adrenergic blocking agents. In addition, the study has important implications for pharmacologic studies employing adrenergic blocking agents. In particular, α_1-adrenergic blocking agents such as WB-4101 may be used in pharmacologic studies to "block" adrenergic receptors. In these situations, it is important to understand that at high concentrations α_1-adrenergic blocking agents may also inhibit calcium channels. Thus, the interpretation of the pharmacologic specificity of α_1-adrenergic blocking agents at higher concentrations should be made with the knowledge that these concentrations may also invoke calcium channel blocking effects.

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REFERENCES

1. A. S. Fairhurst, M. L. Whittaker and F. J. Ehlert, *Biochem. Pharmac.* **29**, 155 (1980).
2. J. S. Karliner, H. J. Motulsky, J. Dunlap, J. H. Brown and P. A. Insel, *J. cardiovasc. Pharmac.* **4**, 515 (1982).
3. W. G. Nayler, J. E. Thompson and B. Jarrott, *J. molec. cell. Cardiol.* **14**, 185 (1982).
4. H. J. Motulsky, M. D. Snavely, R. J. Hughes and P. A. Insel, *Circulation Res.* **52**, 226 (1983).
5. D. Atlas and M. Adler, *Proc. natn. Acad. Sci. U.S.A.* **78**, 1237 (1981).
6. K. M. M. Murphy and S. H. Snyder, *Eur. J. Pharmac.* **77**, 201 (1982).
7. L. T. Williams and P. Tremble, *J. clin. Invest.* **70**, 209 (1982).
8. G. T. Bolger, P. Gengo, R. Klockowski, E. Luchowski, H. Siegel, R. A. Janis, A. M. Triggle and D. J. Triggle, *J. Pharmac. exp. Ther.* **225**, 291 (1983).
9. W. L. Nelson, J. E. Wennerstrom, D. C. Dyer and M. Engel, *J. med. Chem.* **20**, 880 (1977).
10. W. L. Nelson, M. L. Powell and D. C. Dyer, *J. med. Chem.* **22**, 1125 (1979).
11. R. R. Ruffolo, W. L. Nelson and E. L. Yaden, *Naunyn-Schmiedeberg's Archs Pharmac.* **322**, 93 (1983).

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An example of P-450 catalytic activities not correlated with corresponding P-450 mRNA concentrations

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Enzyme induction is usually associated with elevated levels of messenger RNA [reviewed in Refs. 1 and 2]. Increased mRNA concentrations can be the result of *de novo* synthesis (transcriptional activation of the gene), enhanced mRNA stabilization, or some combination of both. Likewise, increased catalytic activity can be the result of *de novo* protein synthesis, enhanced stabilization of the active protein, or some combination of both.

The induction of P-450 by phenobarbital [3–6] and polycyclic aromatic compounds such as 3-methylcholanthrene or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [7–10] has been shown to be principally due to transcriptional activation of the corresponding genes. At maximally induced P_1-450 mRNA levels in mouse liver, inducible aryl hydrocarbon hydroxylase activity appears to reach a plateau, however, suggesting that translational processes may be involved in limiting the expression of the enzyme activity [11]. To our knowledge this study is the only reported example [11] in which P-450 mRNA levels have been shown not always to be correlated with their corresponding catalytic activity. The purpose of this report is to illustrate another example.

In this study, high intraperitoneal doses of isosafrole were given to the C57BL/6N mouse. Hepatic P_1-450 and P_3-450 mRNA concentrations were elevated markedly, while their corresponding catalytic activities (P_1-450 = aryl