0026-895X/92/030535-07$03.00/0

MOLECULAR PHARMACOLOGY, 41:535-541

Pharmacologic and Radioligand Binding Studies of 1,4-Dihydropyridines in Rat Cardiac and Vascular Preparations: Stereoselectivity and Voltage Dependence of Antagonist and Activator Interactions

WEI ZHENG, JUERGEN STOLTEFUSS, SIEGFRIED GOLDMANN, and DAVID J. TRIGGLE

Department of Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo, Buffalo, New York 14260 (W.Z., D.J.T.), and Chem. Wiss. Lab. Pharmaceutical Research Center, Bayer AG, 5600 Wuppertal, FRG (J.S., S.G.)

Received August 5, 1991; Accepted December 10, 1991

SUMMARY

The pharmacologic and radioligand-binding properties of 1,4-dihydropyridines in an activator (Bay K 8644) and an antagonist (nifedipine) series were studied in rat tail artery, heart membrane, and neonatal rat ventricular myocytes. The *S*-enantiomers of the activator series contracted rat tail artery in the presence of 15 mM K^+ (EC_{50} values of 10^{-8} to 10^{-5} M). (*S*)-Bay K 8644 (I) and its *o*-difluoromethoxy analog (III) were the most potent members of the activator series examined. The abilities of the activators to stimulate maximum tension response of the artery differed with structure; thus, the efficacy of (*S*)-Bay K 8644 was 70% that of the analog lacking the 3-carbomethoxy group. The *R*-enantiomers of the activator series and a series of achiral nifedipine analogs were inhibitory in the same tissue. The intact-cell binding assay revealed the binding affinities of 1,4-dihydropyridine antagonists in depolarized cells (50 mM K^+) to be higher than those in polarized cells (5 mM K^+). The ratio K_D (polarized)/K_D (depolarized) was 77 for nifedipine ($IC_{50} = 5.4 \times 10^{-9}$ M) but was only 2.9 for the weak 3-methoxy nifedipine analog ($IC_{50} = 4.8 \times 10^{-6}$ M); an approximately linear relationship exists between this ratio and the antagonist potency. In marked contrast, and in confirmation of previous work [*Mol. Pharmacol.* 35:541-552 (1989)], the binding affinities of activators were not significantly affected by membrane potential, regardless of potency. We conclude that the *S*-enantiomers of Bay K 8644 analogs are activators with different potency and efficacy and that the *R*-enantiomers are antagonists, that the binding of 1,4-dihydropyridine antagonists is voltage dependent, whereas binding of the activators is not, and that the voltage-dependence of binding of the antagonists is correlated with the potency of the antagonist.

Compounds with the 1,4-dihydropyridine nucleus include both potent antagonists and activators for the L class of voltage-gated Ca^{2+} channels. Only small structural differences distinguish activators and antagonists (1, 2); in particular, the presence of a C_5 nitro group, the major structural distinction between nifedipine and Bay K 8644, confers potent activator properties (3). Several studies suggest that antagonists and activators in the 1,4-dihydropyridine series may interact at a common receptor site (reviewed in Refs. 2, 4, and 5).

The binding properties of this site have been characterized by several structure-activity studies (6-9), but these refer to antagonists, and corresponding studies for activators are rare (10). Interpretation of the structure-activity relationships of 1,4-dihydropyridine activities at Ca^{2+} channels is complicated by the voltage dependence of action of both antagonists and activators (11-17), in which the expression of antagonist or activator activity is quantitatively or qualitatively dependent, respectively, upon membrane potential. Additionally, the enantiomeric selectivity, whereby for a number of agents, including Bay K 8644, activator and antagonist activity is associated with *S*- and *R*-enantiomers, respectively (5, 18, 19), adds an additional complicating factor. Such complexities underlie a number of suggestions that 1,4-dihydropyridine activators may interact at discrete activator and antagonist sites (5).

Our previous structure-activity study of 1,4-dihydropyridine activators was confined to the available racemic forms (10). It appeared of interest to examine the pharmacologic and radioligand binding properties, including voltage-dependent interactions, of a series of 1,4-dihydropyridine enantiomers, to permit further elucidation of structural factors controlling activator and antagonist activities in the 1,4-dihydropyridines.

Experimental Procedures

Tissue preparation and recording of mechanical response. Rat tail artery strips were prepared as described by Su *et al.* (20). Male

This work was supported by a grant from the National Institutes of Health (HL 16003).

ABBREVIATION: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Holtzman rats of age 15–17 weeks (weight, 250–290 g; Hilltop Farms, Scottdale, PA) were killed by decapitation, and the median tail artery was removed and placed in a physiologic salt solution (aerated with 95% O_2/5% CO_2 at 37°) of the following composition (in mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; $MgCl_2$, 1.18; KH_2PO_4, 1.18; $NaHCO_3$, 2.5; dextrose, 5.5. Arterial strips of 1-cm length, cut at an angle of 45°, were suspended in glass organ baths containing 10 ml of aerated physiologic salt solution at 37°, connected to a force-displacement transducer (Grass FT03), and equilibrated for 2 hr under a resting tension of 600 mg, with a solution change every 15 min. Isometric contractions were recorded on a Grass polygraph (model 7D). Before determination of responses, tissues were stimulated three times with 80 mM KCl, to initiate a steady level of response (20). The tissue was then pretreated with phentolamine (1×10^{-6} M) to block the effect of any norepinephrine released from sympathetic nerve terminals. After an additional 30-min equilibrium period, two additional responses to 80 mM KCl depolarization were recorded and the second, measured after 30 min of re-equilibration, was taken as a control response. To determine antagonist activity, the contractile response to 80 mM KCl depolarization was recorded again after the tissue was incubated with the antagonist for 30 min. Only one concentration of antagonist was used in each individual tissue. Cumulative dose-response curves for calcium channel activators in rat tail artery were constructed by stepwise addition of the drugs in the presence of 15 mM KCl, because at that K^+ concentration the activator produced maximum response (20). The concentration of activator was increased only after the response to the previous addition had attained a maximum steady level.

Radioligand binding assay in rat heart membrane preparation. A microsomal membrane preparation (21) was suspended in cold buffer of the following composition (in mM): NaCl, 127; KCl, 5.36; $CaCl_2$, 1.26; $MgCl_2$, 0.98; KH_2PO_4, 0.44; $NaHCO_3$, 4.16; Na_2HPO_4, 0.63; glucose, 5.56; HEPES, 20; pH 7.4. Protein concentration was determined by the method of Bradford (22), using bovine serum albumin as standard.

The competition binding assay of calcium activators and antagonists with (+)-[^{3}H]PN 200-110 was carried out by using the methods established previously in our laboratory (10, 21, 23). Membrane protein (70–100 μg) was incubated with (+)-[^{3}H]PN 200-110 (4.5×10^{-11} M) and various concentrations of the drugs for 2 hr, in a 5-ml incubation volume. After incubation, the samples were filtered over Whatman GF/B filters and washed twice with 5 ml of ice-cold buffer, using a cell harvester (model M-24R; Brandel Instruments, Gaithersburg, MD). Nonspecific binding was defined by addition of 10^{-6} M unlabeled PN 200-110. Binding experiments were carried out in glass tubes and in subdued light. The radioactivity trapped on the filter was measured by liquid scintillation counting, at an efficiency of approximately 50%.

Radioligand binding assay in cultured neonatal rat ventricle myocytes. Cells were cultured by the methods described by Wei *et al.* (17). The whole-cell binding assay was performed with the cells attached to the culture dishes under polarized (resting) and depolarized conditions (17). Our previous work indicated that the conditions of 5.8 mM KCl and 50 mM KCl represented polarized and depolarized conditions, respectively, as determined by lipophilic cation (tetraphenylphosphonium) distribution (17). The dishes were washed once with a buffer containing 5.8 mM K^+, of the following composition (in mM): NaCl, 127; KCl, 5.36; $CaCl_2$, 1.26; $MgCl_2$, 0.98; KH_2PO_4, 0.44; $NaHCO_3$, 4.16; Na_2HPO_4, 0.63; glucose, 5.56; HEPES, 20; pH 7.4. Buffer containing 5.8 mM K^+ (resting) or 50 mM K^+ (depolarized) was then added (17). The cells were then incubated with (+)-[^{3}H]200-110 (0.45 nM in resting cells and 0.15 nM in depolarized cells) and various concentrations of 1,4-dihydropyridines at 37° for 90 min. After incubation, the binding buffer was aspired under vacuum and the dish was rapidly washed three times in 15 sec with ice-cold buffer. Cells were extracted overnight with 1 ml of 0.5 M NaOH, and the radioactivity was determined by liquid scintillation counting. Nonspecific binding was routinely determined in the presence of 10^{-6} M unlabeled PN 200-110.

Materials. (+)-[^{3}H]PN 200-110 (71.5 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston, MA). Tissue culture medium, L-glutamine, serum, and antibiotics were obtained from GIBCO (Grand Island, NY). The 1,4-dihydropyridines (Fig. 1) were prepared as 10^{-2} M stock solutions in ethanol and were stored refrigerated and protected from light.

Data analysis and statistics. All data were processed using an IBM personal computer. Pharmacologic data were analyzed using a standard set of pharmacologic programs (24). Significance of difference was accepted at the 0.05 level. Radioligand binding data were analyzed with the program LIGAND (25).

Results

Contractile responses of 1,4-dihydropyridine activators and antagonists. The contractile activities of 1,4-dihy-

TABLE 1

Pharmacologic effects of and inhibition of [^{3}H]PN 200-110 binding by nifedipine and (S)-Bay K 8644 analogs in rat tail artery and heart preparations

Drugs	EC_{50}	IC_{50}	K_i	n_H
	M	M	M	
Nifedipine series				
−$2NO_2$ (nifedipine)		$5.41 \pm 0.30 \times 10^{-9}$	$1.30 \pm 0.14 \times 10^{-9}$	1.01 ± 0.05
−3CN		$1.74 \pm 0.33 \times 10^{-8}$	$4.06 \pm 0.42 \times 10^{-9}$	0.91 ± 0.03
−H		$1.50 \pm 0.11 \times 10^{-7}$	$2.22 \pm 0.19 \times 10^{-8}$	1.02 ± 0.06
−4Cl		$1.30 \pm 0.21 \times 10^{-6}$	$1.08 \pm 0.03 \times 10^{-7}$	1.05 ± 0.02
−3MeO		$4.81 \pm 0.79 \times 10^{-6}$	$1.80 \pm 0.26 \times 10^{-6}$	1.02 ± 0.06
Bay K 8644 series				
I	$1.38 \pm 0.15 \times 10^{-8}$		$3.03 \pm 0.28 \times 10^{-9}$	1.14 ± 0.05
II		$3.35 \pm 0.26 \times 10^{-8}$	$9.41 \pm 1.18 \times 10^{-9}$	0.99 ± 0.05
V	$3.72 \pm 0.50 \times 10^{-5}$		$2.03 \pm 0.48 \times 10^{-5}$	1.28 ± 0.23
VI	No activity at 10^{-4}		$3.64 \pm 0.36 \times 10^{-5}$	1.45 ± 0.06
X	$3.24 \pm 0.29 \times 10^{-6}$		$4.94 \pm 0.64 \times 10^{-6}$	1.17 ± 0.01
IX		$1.04 \pm 0.11 \times 10^{-5}$	$2.69 \pm 0.52 \times 10^{-6}$	1.05 ± 0.09
III	$1.37 \pm 0.17 \times 10^{-8}$		$9.22 \pm 0.56 \times 10^{-9}$	0.93 ± 0.04
IV		$5.03 \pm 0.48 \times 10^{-8}$	$5.60 \pm 0.90 \times 18^{-8}$	0.84 ± 0.06
VII	$2.71 \pm 0.32 \times 10^{-5}$		No displacement at 10^{-4}	
VIII		$4.74 \pm 0.35 \times 10^{-5}$	$6.35 \pm 0.36 \times 10^{-6}$	1.53 ± 0.10
XII	$2.90 \pm 0.62 \times 10^{-6}$		$2.86 \pm 0.52 \times 10^{-6}$	1.01 ± 0.48
XI		$4.24 \pm 0.38 \times 10^{-5}$	$6.58 \pm 0.30 \times 10^{-6}$	1.02 ± 0.07
XIII	$1.76 \pm 0.28 \times 10^{-7}$		$2.63 \pm 0.38 \times 10^{-7}$	1.08 ± 0.10
XIV	$1.40 \pm 0.27 \times 10^{-7}$		$2.94 \pm 0.24 \times 10^{-7}$	0.98 ± 0.07

TABLE 2

Maximum tension responses of 1,4-dihydropyridine activators relative to 80 mM K^+ response (as 100% control) in rat tail artery

Activator	Maximal response[a]	Efficacy[b]
	% of 80 mM K^+ response	
I [(S)-Bay K 8644]	77.0 ± 12.1	0.68
V	14.8 ± 0.8	0.13
X	112.7 ± 7.9	1.00
III	36.5 ± 5.6	0.32
VII	12.1 ± 5.6	0.10
XII	104.0 ± 7.2	0.92
XIII	29.2 ± 5.7	0.25
XIV	18.3 ± 3.8	0.16

[a] % maximum response = maximum response/80 mM K^+ response.
[b] Efficacy = maximum response of a drug/maximum response of X.

dropyridine Ca^{2+} channel activators were measured in rat tail artery strips in the presence of 15 mM KCl, which was optimum for the responses to the activators, as previously reported by Su *et al.* (20). These activators contracted the tissue dose dependently and at higher concentrations produced inhibitory effects, with IC_{50} values approximately 50–100-fold higher than the EC_{50} values (data not shown). The EC_{50} values are summarized in Table 1. Among the activators, (−)-(*S*)-Bay K 8644 (I) and the ortho-difluoromethoxy-substituted derivative (III) were the most potent. The presence of the free 5-carboxy group in V and VII, substituted for the 5-carbomethoxy group of I and III, respectively, decreased the potency by some 200-fold. A dramatic decrease of activity (some 2000-fold) was observed with the replacement of the 5-carbomethoxy group by hydrogen (X and XII).

The maximum contractile responses to these activators in rat tail artery are summarized in Table 2. (*S*)-Bay K 8644 (I) induced a maximum response (tension = 472 ± 27 mg) that was 77% of the response to 80 mM K^+ depolarization, consistent with a previous report (20). Both EC_{50} values and maximum tension responses were dependent upon structure, but the same sequence was not followed. Thus, X and XII were less potent than (*S*)-Bay K 8644 (I), but their maximum tension responses were 20–30% higher than that of (*S*)-Bay K 8644. However, in V and VII potency and efficacy were both decreased relative to (*S*)-Bay K 8644 (Table 2), and XIII and XIV, which are nonchiral 1,4-dihydropyridines, showed intermediate activator potency with very weak efficacy.

The inhibitory activities of calcium channel antagonists were determined against the single response to a maximally effective concentration of K^+ (80 mM) in rat tail arterial strips. The antagonism reached equilibrium after preincubation of the drugs with the arterial strips for 30 min. The IC_{50} values for the 1,4-dihydropyridine series studied are listed in Table 1. The results for the nifedipine series are in good accord with those previously published by us for smooth muscle and cardiac membrane preparations (21, 22), with activity reduced by phenyl ring substituents in the 4-position or by substituents with electron-releasing characteristics (9).

Competition binding of (+)-[^{3}H]PN 200-110 in rat heart membrane preparation. Specific binding of (+)-[^{3}H]PN 200-110 in rat heart membrane preparations was inhibited competitively by 1,4-dihydropyridine activators and antagonists. The K_I values and pseudo-Hill coefficients are presented in Table 1. The affinities of the nifedipine series decreased with phenyl ring substitutions, paralleling the results in the tail artery preparation (Table 1).

The binding affinities of Bay-K 8644 analogs exhibited stereoselectivity, and the activator *S*-enantiomers were generally more potent than the antagonist *R*-enantiomers. These binding affinities correlated well with the pharmacologic activities (EC_{50} and IC_{50} values) of these compounds (see Discussion).

Competition binding of (+)-[^{3}H]PN 200-110 in resting and depolarized heart cells. Competition binding in cultured cardiac cells was carried out in 5.8 and 50 mM K^+ buffer, in an effort to determine binding properties of 1,4-dihydropyridines under polarized and depolarized conditions, respectively (17). Concentration-dependent inhibition of (+)-[^{3}H]PN-200 110 binding was observed under polarized and depolarized conditions, and the data are summarized in Table 3. The extent to which binding affinity shifted between polarized and depolarized media varied according to 1,4-dihydropyridine structure and function. In general, activator 1,4-dihydropyridines showed little shift, whereas antagonist 1,4-dihydropyridines showed

TABLE 3

Inhibition of [^{3}H]PN-200-110 binding by nifedipine and Bay K 8644 series in polarized (5.8 mM K^+) and depolarized (50 mM K^+) neonatal rat heart cells

Drugs	K_I		Ratio
	Cells in 50 mM K^+	Cells in 5 mM K^+	
	M		
Nifedipine series			
−$2NO_2$	$5.12 \pm 1.05 \times 10^{-10}$	$3.97 \pm 0.46 \times 10^{-8}$	77.5
−3CN	$2.93 \pm 0.43 \times 10^{-9}$	$1.44 \pm 0.37 \times 10^{-7}$	49.1
−H	$4.79 \pm 1.57 \times 10^{-8}$	$9.05 \pm 1.85 \times 10^{-7}$	18.9
−4Cl	$4.82 \pm 1.05 \times 10^{-7}$	$1.64 \pm 0.55 \times 10^{-6}$	3.4
−3MeO	$1.09 \pm 0.22 \times 10^{-6}$	$3.09 \pm 1.21 \times 10^{-6}$	2.8
Bay K 8644 series			
I	$3.17 \pm 0.87 \times 10^{-8}$	$1.45 \pm 1.32 \times 10^{-7}$	4.5
II	$5.54 \pm 1.14 \times 10^{-8}$	$6.26 \pm 1.40 \times 10^{-7}$	11.2
X	$3.51 \pm 1.37 \times 10^{-6}$	$6.46 \pm 1.03 \times 10^{-6}$	1.8
IX	$4.17 \pm 1.71 \times 10^{-6}$	$1.23 \pm 0.38 \times 10^{-5}$	2.9
III	$4.16 \pm 1.32 \times 10^{-8}$	$1.80 \pm 0.06 \times 10^{-7}$	4.3
IV	$3.59 \pm 1.07 \times 10^{-8}$	$2.96 \pm 0.86 \times 10^{-7}$	8.2
XII	$3.27 \pm 1.72 \times 10^{-6}$	$5.88 \pm 2.22 \times 10^{-6}$	1.8
XI	$1.47 \pm 0.07 \times 10^{-6}$	$2.89 \pm 0.65 \times 10^{-6}$	2.0
XIII	$3.80 \pm 0.36 \times 10^{-7}$	$1.52 \pm 1.59 \times 10^{-6}$	4.0
XIV	$7.89 \pm 0.19 \times 10^{-7}$	$3.10 \pm 0.64 \times 10^{-6}$	3.9

increased affinity in depolarizing medium, approximately according to their affinity. These observations parallel, in more extended fashion, those made previously by us (17).

Discussion

There were two principal objectives to our work. We wished to know whether the activator/antagonist discrimination revealed for the *S*- and *R*-enantiomers of Bay K 8644 extended to analogs in this 1,4-dihydropyridine series. Additionally, we wished to know whether the voltage-dependent and voltage-independent binding of antagonists and activators, respectively, previously reported by us for potent 1,4-dihydropyridines in cardiac cells extended to a larger series containing both chiral and nonchiral members (12, 17).

Bay K 8644 and nifedipine served as standard 1,4-dihydropyridines in this investigation. Their properties are well established and, in particular, the pharmacologic and radioligand-binding characteristics of the enantiomers of Bay K 8644 are well described (10, 17, 18, 26). The pharmacologic and radioligand binding data for nifedipine and Bay K 8644 in rat tail artery and cardiac microsomes are in good accord with those previously published by us (17, 20, 22, 26). (*S*)- and (*R*)-Bay K 8644 (**I** and **II**; Fig. 1) behaved as activator and antagonist, respectively, in the tail artery exposed to 15 mM KCl. In confirmation of previous reports (20, 26), a moderate elevation of KCl concentration was necessary to demonstrate activator properties, and this was found also for other members of this activator series. Additionally, (*S*)-Bay K 8644 yielded a bell-shaped dose-response curve in the tail artery preparation, consistent with Ca^{2+} channel antagonism at higher concentrations (20, 26). This was also observed with **III** but was not observed with the other, and less potent, activators in this series, presumably because limitations of solubility prevented attainment of the high concentrations necessary.

		R	X
I	(-)	CF_3	COOMe
III	(-)	OCF_2H	COOMe
V	(+)	CF_3	COOH
VII	(+)	OCF_2H	COOH
IX	(+)	CF_3	H
XI	(+)	OCF_2H	H

		R	X
II	(+)	CF_3	COOMe
IV	(+)	OCF_2H	COOMe
VI	(-)	CF_3	COOH
VIII	(-)	OCF_2H	COOH
X	(-)	CF_3	H
XII	(-)	OCF_2H	H

	R	X
XIII	CF_3	NO_2
XIV	OCF_2H	NO_2

Fig. 1. Structural formulae of 1,4-dihydropyridines series that serve as activator/antagonist enantiomeric pairs. I and II represent (−)-(*S*)-Bay K 8644 and (+)-(*R*)-Bay K 8644, respectively.

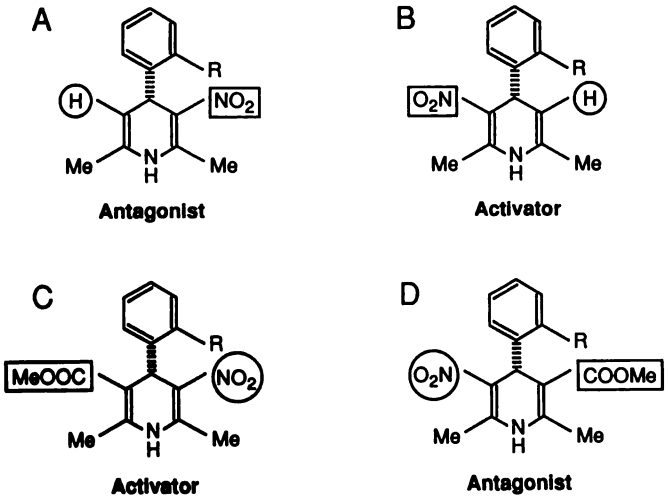

Fig. 2. Comparison of the structural orientation requirement for 1,4-dihydropyridine activator/antagonist pairs A and B or C and D at the C-3 and C-5 positions; the smaller substituent is depicted. The model (see text for details) suggests that differential hydrogen bonding occurs with the substituents and that activator properties are associated with the right-side orientation of the smaller substituent and with a hydrogen-bonding group on the left.

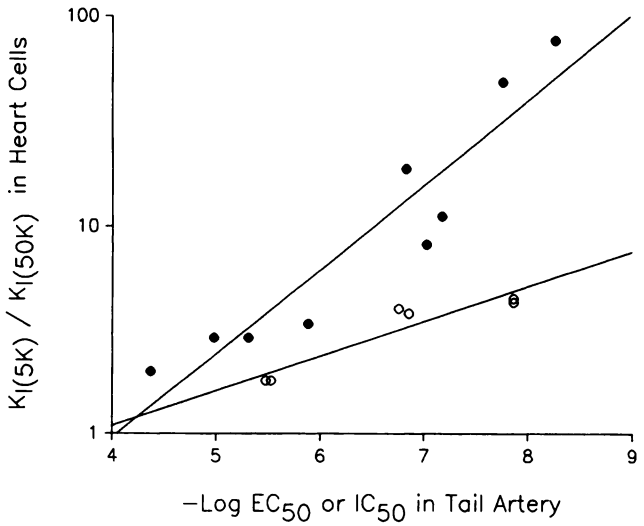

Fig. 3. Relationship of the ratios of radioligand binding affinities of 1,4-dihydropyridine activators (○) and antagonists (●) in polarized (5 mM K^+)/depolarized (50 mM K^+) neonatal rat heart cells and the pharmacologic potencies (EC_{50} or IC_{50}) in rat tail artery.

The presence of the 3-carbomethoxy group is necessary for both activator and antagonist potency, because its replacement by the carboxy group (**V/VI** and **VII/VIII**) or its replacement by hydrogen (**IX/X** and **XI/XII**) reduced these activities dramatically. Of interest are the findings that replacement of the C-3 ester group by a second nitro substituent (**XIII** and **XIV**) also reduced activity, although these agents still demonstrated activator affinity. These changes in pharmacologic activities were accompanied by similar changes in radioligand binding

Replacement of the 2-trifluoromethyl group in **I** and **II** by the 2-difluoromethoxy group, to give **III** and **IV**, respectively, did not affect enantiomeric selectivity or agonist or antagonist potency. However, the efficacy of **III**, measured by the maximum tension response in rat tail artery, was <50% of that of **I**, and the significantly less potent **X** generated a larger tension response than did **I**. We also observed the absence of any obvious relationship between potency and efficacy of these compounds. This confirms a conclusion previously drawn by us for a series of racemic 1,4-dihydropyridine activators (10).

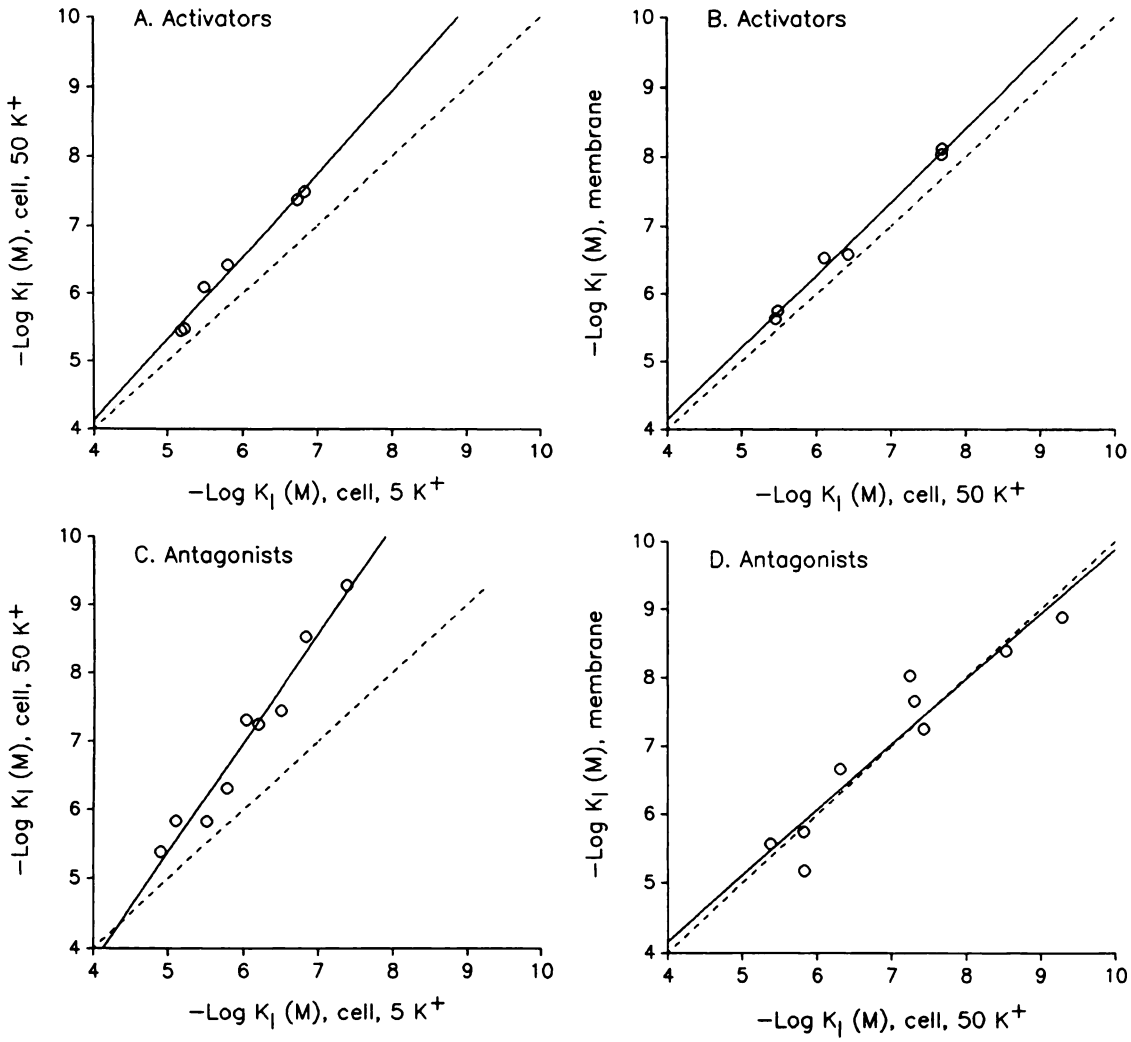

Fig. 4. Correlations between radioligand binding affinities (K_i) in depolarized (50 mM K^+) and polarized (5 mM K^+) neonatal rat heart cells. A, Activators in a series of Bay K 8644 analogs; C, antagonists in a series of nifedipine and Bay K 8644 analogs. B and D, correlations between radioligand binding affinities (K_i) of activators in a series of Bay K 8644 analogs (B) and antagonists in nifedipine and Bay K 8644 series (D) in rat heart membranes and in depolarized (50 K^+) neonatal rat heart cells. – – –, Theoretical 1:1 linear regression; ——, actual linear regression. The slope of the actual linear regression in C is significantly different from the 1:1 theoretical regression ($p < 0.05$), but the regressions in A, B, and D are not significantly different ($p > 0.05$).

affinities in cardiac membrane preparations (see Fig. 4, B and D). The significance of these correlations is discussed below.

Of particular interest in the structure-activity data are the inversions in enantiomeric selectivity, whereby the replacement of the 3-carbomethoxy or the 3-carboxy group by hydrogen results in chiral switching of activator/antagonist properties. Chirality is an important property of drug-receptor interactions, and chiral inversions contain, in principle, data important to the definition of the drug binding site (27, 28). It should be noted, however, that such replacement of the 3-carbomethoxy substituent results in substantial decreases in both activator and antagonist activities (Table 1).

One interpretation of these observations, which retains a single 1,4-dihydropyridine binding site, is that there are differential requirements for electron-withdrawing, hydrogen-bonding groups at the C-3 and C-5 positions of the 1,4-dihydropyridine ring; optimum binding requires both interactions. These sites have different steric and electronic characteristics, accommodating observations that the stereoselectivity of 1,4-dihydropyridine interactions increases with progressive difference between the C-3 and C-5 substituents (2). If we assume that the smaller substituent (or hydrogen) is always preferentially oriented as shown in Fig. 2 for activator interactions, with the requisite hydrogen bonding occurring at the opposite face, then the inversion in stereoselectivity of activator/antagonist properties becomes clear. The model also predicts similar inversions in activator/antagonist stereoselectivity for other mono-substituted 1,4-dihydropyridines, including H 160/51 [2-methyl-6-amino-3-carboethoxy 4-(2-chlorophenyl)-1,4-dihydropyridine] (29), and for lactone activators such as CGP 28392 (30). Confirmation of this, or of other models that may involve two or more binding sites (31), must await studies on the sequence localization of 1,4-dihydropyridine binding sites. Such studies are under way (32).

Our second objective was to extend to a larger series our previous work on the voltage-dependent binding behavior of activator and antagonist 1,4-dihydropyridines (17). To achieve this, we have compared binding ratios in polarized and depo-

larized cardiac cells with functional measurements of activity in vascular smooth muscle. Clear confirmation is provided of our previous conclusion, drawn from direct and competition radioligand binding studies (12, 17), that the affinities of activator 1,4-dihydropyridines are little affected by membrane potential in this cardiac cell preparation. The ratios of binding affinity constants in 5.8 and 50 mM KCl for the activator structures vary only from 1.8 to 4.5, and these ratios are essentially structure independent. In marked contrast, the binding behavior of 1,4-dihydropyridine antagonists is voltage dependent and the extent of this is structure dependent, increasing with the increase of antagonist activity, with ratios of binding constants in 5 and 50 mM KCl varying from 2 to 77 (Table 3; Fig. 3). Because cardiac and vascular smooth muscle tissues are being compared, these conclusions need further and direct validation by electrophysiologic techniques.

We have previously suggested that the voltage-dependent binding of 1,4-dihydropyridine antagonists was, consistent with many electrophysiologic data (11, 14, 15, 33), attributable to a selective interaction with inactivated channel states favored or promoted by depolarization. The new data are consistent with the thesis that the structure-activity relationship for 1,4-dihydropyridine antagonists differs quantitatively between channel states and that those structural features that enhance activity do so by increasing interactions with the cardiac Ca^{2+} channel state favored by depolarization. This is revealed in the correlations of binding affinities for the nifedipine series of antagonists depicted in Fig. 4. A 1:1 correlation of binding affinities is seen with cardiac membranes and depolarized cardiac cells (Fig. 4D) and, although the same rank orders of activities are seen when comparisons are made between polarized and depolarized cells (Fig. 4C), these correlations differ significantly from the 1:1 relationship. The antagonist enantiomers of the Bay K 8644 series behave in fundamentally similar fashion. These findings are consistent with previous observations of nonlinear correlations between cardiac pharmacologic and membrane binding affinities for a 1,4-dihydropyridine antagonist series, in which the difference in affinities decreases with decreasing 1,4-dihydropyridine activity and, with the least potent agents, there is essentially 1:1 agreement between the two affinity values (1, 10, 22). Similar observations have been made by us in smooth muscle preparations (21). In contrast, the new data for 1,4-dihydropyridine activator affinities, showing little dependence upon membrane potential (Fig. 4, A and B), are consistent with our previous observations and the interpretation that 1,4-dihydropyridine activator interactions are only modestly voltage dependent and that activators may, accordingly, promote or block channel function according to the dominant channel population (13, 26, 34). Such data are also consistent with the lack of activator selectivity between smooth and cardiac muscle (10).

These data are important to the interpretation of structure-activity relationships of 1,4-dihydropyridines. For the antagonist agents investigated, the increases of activity are associated with structural changes that enhance the affinity for the depolarized state, relative to the polarized state. For the activator series, changes in activities are not associated with major changes in selectivity. In principle, 1,4-dihydropyridines should exist for which different dependencies of activity upon membrane potential generate different spectra of pharmacologic activities.

References

1. Janis, R. A., P. Silver, and D. J. Triggle. Drug action and cellular calcium regulation. *Adv. Drug Res.* **16**:309–591 (1987).
2. Triggle, D. J., D. A. Langs, and R. A. Janis. Calcium channel ligands: structure-function relationships of the 1,4-dihydropyridines. *Med. Res. Rev.* **9**:123–180 (1989).
3. Schramm, M., G. Thomas, R. Towart, and G. Franckowiak. Novel dihydropyridines with inotropic action through activation of calcium channels. *Nature (Lond.)* **303**:535–537 (1983).
4. Triggle, D. J., and R. A. Janis. Calcium channel ligands. *Annu. Rev. Pharmacol. Toxicol.* **27**:347–369 (1987).
5. Triggle, D. J., and D. Rampe. 1,4-Dihydropyridine activators and antagonists: structural and functional distinctions. *Trends Pharmacol. Sci.* **10**:507–511 (1989).
6. Loev, B., M. M. Goodman, K. M. Snader, R. Tedesche, and E. Macko. 'Hantzsch-type' dihydropyridine hypotensive agents. *J. Med. Chem.* **17**:956–965 (1974).
7. Rodenkirchen, R., R. Bayer, R. Steiner, F. Bossert, H. Meyer, and E. Moller. Structure-activity studies on nifedipine in isolated cardiac muscle. *Naunyn-Schmiedebergs Arch. Pharmacol.* **310**:69–78 (1979).
8. Mannhold, R., R. Rodenkirchen, and R. Bayer. Qualitative and quantitative structure-activity relationships of specific Ca^{2+} antagonists. *Prog. Pharmacol.* **5**:25–52 (1982).
9. Coburn, R. A., M. Wierzba, M. J. Suto, A. J. Solo, A. M. Triggle, and D. J. Triggle. 1,4-Dihydropyridine antagonist activities at the calcium channel: a quantitative structure-activity relationship approach. *J. Med. Chem.* **31**:2103–2107 (1988).
10. Kwon, Y. W., G. Franckowiak, D. A. Langs, M. Hawthorn, A. Joslyn, and D. J. Triggle. Pharmacologic and radioligand binding analysis of the action of 1,4-dihydropyridine activators related to Bay K 8644 in smooth muscle, cardiac muscle and neuronal preparations. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339**:19–30 (1989).
11. Bean, B. P. Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. *Proc. Natl. Acad. Sci. USA* **81**:6388–6392 (1984).
12. Ferrante, J., E. Luchowski, A. Rutledge, and D. J. Triggle. Binding of a 1,4-dihydropyridine calcium channel activator, (−)*S*-Bay K 8644, to cardiac preparations. *Biochem. Biophy. Res. Commun.* **158**:149–154 (1989).
13. Kass, R. S. Voltage-dependent modulation of cardiac calcium channel current by optical isomers of Bay K 8644: implications for channel gating. *Circ. Res.* **61** (Suppl. I):I1–I15 (1987).
14. Kokubun, S., B. Prod'hom, C. Becker, H. Porzig, and H. Reuter. Studies on Ca^{2+} channels in intact cardiac cells: voltage-dependent effects and cooperative interactions of dihydropyridine enantiomers. *Mol. Pharmacol.* **30**:571–584 (1986).
15. Sanguinetti, M. C., and R. S. Kass. Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circ. Res.* **55**:336–348 (1984).
16. Sanguinetti, M. C., and R. S. Kass. Regulation of cardiac calcium channel current and contractile activity by the dihydropyridine Bay K 8644 is voltage-dependent. *Mol. Cell. Cardiol.* **16**:667–670 (1984).
17. Wei, X.-Y., A. Rutledge, and D. J. Triggle. Voltage-dependent binding of 1,4-dihydropyridine Ca^{2+} channel antagonists and activators in cultured neonatal rat ventricular myocytes. *Mol. Pharmacol.* **35**:541–552 (1989).
18. Franckowiak, G., M. Bechem, M. Schramm, and G. Thomas. The optical isomers of the 1,4-dihydropyridine Bay K 8644 show opposite effects on Ca^{2+} channels. *Eur. J. Pharmacol.* **114**:223–226 (1985).
19. Hof, R. P., U. T. Ruegg, and A. Vogel. Stereoselectivity at the calcium channel: opposite actions of the enantiomers of a 1,4-dihydropyridine. *J. Cardiovasc. Pharmacol.* **7**:689–693 (1985).
20. Su, C. M., V. C. Swamy, and D. J. Triggle. Calcium channel activation in vascular smooth muscle by Bay K 8644. *Can. J. Physiol. Pharmacol.* **62**:1401–1410 (1984).
21. Bolger, G. T., P. Gengo, R. Klockowski, E. Luchowski, H. Siegel, R. A. Janis, A. M. Triggle, and D. J. Triggle. Characterization of the binding of the Ca^{2+} channel antagonist, [^{3}H]nitrendipine, to guinea pig ileal smooth muscle. *J. Pharmacol. Exp. Ther.* **225**:291–309 (1983).
22. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
23. Janis, R. A., J. G. Sarmiento, S. C. Maurer, G. T. Bolger, and D. J. Triggle. Characteristics of the binding of [^{3}H]nitrendipine to rabbit ventricular membranes: modification by other Ca^{2+} channel antagonists and by the Ca^{2+} channel agonist Bay K 8644. *J. Pharmacol. Exp. Ther.* **231**:8–15 (1984).
24. Tallarida, R. J., and R. B. Murray. *Manual of Pharmacologic Calculations.* Springer-Verlag, New York (1981).
25. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1981).
26. Wei, X.-Y., E. M. Luchowski, A. Rutledge, C. M. Su, and D. J. Triggle. Pharmacologic and radioligand binding analysis of the actions of 1,4-dihydropyridine activator-antagonist pairs in smooth muscle. *J. Pharmacol. Exp. Ther.* **239**:144–153 (1986).
27. Ariens, E. J., W. Soudijn, and P. B. M. W. M. Timmermans, eds. *Stereochem-*

istry and Biological Activity of Drugs. Blackwell Scientific Publications, London (1983).
28. Kwon, Y. W., and D. J. Triggle. Chiral aspects of drug action at ion channels: a commentary on the stereoselectivity of drug action at voltage-gated ion channels with particular reference to verapamil actions at Ca^{2+} channels. *Chirality*, in press.
29. Holtje, H. D., and S. Marrer. A molecular graphics study on structure-action relationships of calcium-antagonistic and agonistic 1,4-dihydropyridines. *J. Computer Aided Mol. Design* **1**:23–20 (1987).
30. Erne, P., E. Burgisser, F. R. Buhler, B. Dubach, H. Kuhnis, M. Merier, and H. Rogg. Enhancement of calcium influx in human platelets by CGP 28392, a novel dihydropyridine. *Biochem. Biophys. Res. Commun.* **118**:842–847 (1984).
31. Langs, D. A., Y. W. Kwon, P. D. Strong, and D. J. Triggle. Molecular level model for the activator and antagonist selectivity of the 1,4-dihydropyridine calcium channel receptor. *J. Computer Aided Mol. Design* **5**:95–106 (1991).
32. Regulla, S., T. Schneider, W. Nastainczyk, H. E. Meyer, and F. Hofmann. Identification of the site of interaction of the dihydropyridine channel blockers nitrendipine and azidopine with the calcium channel α_1 subunit. *EMBO J.* **10**:45–49 (1991).
33. Porzig, H., and C. Becker. Binding of dihydropyridine Ca-channel ligands to living cardiac cells at different membrane potentials. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **329**:R47 (1985).
34. Hadley, R. W., and J. R. Hume. Calcium channel antagonist properties of Bay K 8644 in single guinea pig ventricular cells. *Circ. Res.* **62**:97–104 (1988).

Send reprint requests to: Dr. David J. Triggle, 126 Cooke Hall, State University of New York at Buffalo, Buffalo, NY 14260.