0026-895X/93/020293-09$03.00/0

MOLECULAR PHARMACOLOGY, 43:293-301



# Heterogeneity of L-Type Calcium Channel $\alpha_1$ Subunits: Stereoselective Discrimination of Different Populations by the Novel 1,4-Dihydropyridine B 874–67

MARION LAKITSCH, HANS-GÜNTHER KNAUS, GERDA TOPAR, CHRISTOPH ROMANIN, RAINER BOER, DIETER FLOCKERZI, JÖRG STRIESSNIG, HANSGEORG SCHINDLER, HANS-DIETER HOELTJE, and H. GLOSSMANN

*Institute for Biochemical Pharmacology, University of Innsbruck, A-6020 Innsbruck, Austria (M.L., H.-G.K., G.T., J.S., H.G.), Institute for Biophysics, University of Linz, A-4040 Linz, Austria (C.R., H.S.), BYK Gulden-Lomberg GmbH., D-7750 Konstanz, Germany (R.B., D.F.), and Department of Pharmacy, Free University of Berlin, D-1000 Berlin, Germany (H.-D.H.)*

Received September 8, 1992; Accepted November 5, 1992

## SUMMARY

The basic (p$K_a$ = 8.49) 1,4-dihydropyridine B 874–67 [[3-(C1*R*,2*S*)-2-methylamino-1-phenylpropyl]-5-methyl-1,4-dihydro-2,6-dimethyl-(4*R*)-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate hydrochloride] has unique properties; it can discriminate two populations of $\alpha_1$ subunits in 1,4-dihydropyridine-sensitive calcium channels labeled with the neutral 1,4-dihydropyridine (+)-[$^3$H]PN 200–110. The two populations, which occur in proportions of ≈2:1 in rabbit skeletal muscle membranes and highly purified calcium channel preparations, differ ≈20-fold in their affinity. The corresponding diastereomer, B 874–66, and other 1,4-dihydropyridines (neutral, basic, or permanently charged) do not share this property. The two populations were observed at 2°, 22°, and 37° in similar proportions. Heterogeneity was also observed for guinea pig heart membrane calcium channels labeled with (+)-[$^3$H]PN 200–110. Heterotropic allosteric regulators, $Ca^{2+}$, and $Mg^{2+}$, but not $Ba^{2+}$ and $Ni^{2+}$, abolished the discriminatory activity of B 874–67 at 2° and 22°, regardless of whether binding of the neutral 1,4-dihydropyridine was stimulated or inhibited. It is proposed that the two $\alpha_1$ subunit populations differ with respect to their 1,4-dihydropyridine binding domain. The structural basis for the two populations is unclear but may relate to the functional heterogeneity of membrane-bound and highly purified calcium channel preparations previously observed by others.

Of all the different chemical classes of L-type calcium channel ligands, the 1,4-dihydropyridines are the most well characterized (1). These drugs bind with very high affinity to the pore-forming $\alpha_1$ subunit, which occurs in mammalian skeletal muscle in a complex associated with $\alpha_2$-$\delta$, $\beta$, and $\gamma$ subunits (1, 2). Expression of the $\alpha_1$ subunit alone, e.g., in L fibroblasts, is sufficient for high affinity 1,4-dihydropyridine binding (3). We recently investigated a new series of 1,4-dihydropyridines that exhibited biphasic displacement curves in radioligand binding assays. Because pure enantiomers or diastereomers were used, chemical heterogeneity of the ligands could be excluded. We initiated these studies because of the possibility that the calcium channel $\alpha_1$ subunits were heterogeneous. In this work we report on a pair of diastereomers, of which one (B 874–67) has discriminatory properties and the other (B 874–66) behaves like all other 1,4-dihydropyridines investigated. The B 874–67 diastereomer is a unique probe that reveals heterogeneity as well as other previously unrecognized features of the $\alpha_1$ subunit of L-type calcium channels.

This work was supported by grants from Fonds zur Förderung der Wissenschaftlichen Forschung (S-45/01 and S-45/03) to H.G. and H.S.

## Experimental Procedures

### Materials

(+)-[$^3$H]PN 200–110 (isradipine) (75–85 Ci/mmol) was from Amersham (Vienna, Austria). (*R*)-(+)-, (*S*)-(−)-, and (*RS*)-(±)-amlodipine (p$K_a$ = 8.7), as well as racemic UK-118,434-05 [4-(2-chlorophenyl)-3-ethoxycarbonyl-5-methoxymethyl-6-methyl-2-(2-trimethylammonio)ethoxymethyl-1,4-dihydropyridine)], were gifts from Pfizer (Sandwich, Kent, UK). The absolute configuration of the amlodipine enantiomers has been corrected recently; the active enantiomer is *S*-(−), formerly designated *R*-(−). (±)-Isradipine, (+)-isradipine, and (±)-SDZ 207–180 [(±)-10-[[[4-(4-benzofurazanyl)-1,4-dihydro-2,6-dimethyl-5-[(1-methylethoxy)carbonyl]-3-pyridinyl]carbonyl]oxy]-*N*,*N*,*N*-trimethyl-1-decanaminium iodide] were gifts from Sandoz Ltd. (Basle, Switzerland). The enantiomers of BM 20.1140 [ethyl-2,2-diphenyl-4-1(1-pyrrolidion)-5-(2-picolyl)oxyvalerate] were gifts from Boehringer Manheim (FRG). The sources of the other chemicals and unlabeled drugs are given elsewhere (4, 5).

### Membrane Preparation and Calcium Channel Purification

Partially purified rabbit skeletal muscle T-tubule and guinea pig heart membranes were prepared according to previously published procedures (4). Membranes were finally suspended in 50 mM Tris·HCl,

**ABBREVIATIONS:** PMSF, phenylmethylsulfonyl fluoride; $I_{Ca}$, L-type calcium currents; DMSO, dimethylsulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

pH 7.4, 0.1 mM PMSF, and stored up to 2 months in liquid nitrogen until use. Purification of rabbit skeletal muscle L-type calcium channels was performed up to the wheat germ agglutinin-Sepharose column step as described. These preparations have a density of ≈300 pmol/mg of protein (6) of 1,4-dihydropyridine binding sites [labeled with (+)-[$^3$H] PN 200–110].

### Synthesis of B 874–66 and B 874–67

B 874–66 [3-(C1*R*,2*S*)-2-methylamino-1-phenylpropyl-5-methyl-1,4-dihydro-2,6-dimethyl-(4*S*)-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate hydrochloride; $[\alpha]_D^{22} = +202.6°$ ($c$ = 1, 10 mg/ml, methanol)] and B 874–67 [3-(C1*R*,2*S*)-2-methylamino-1-phenylpropyl-5-methyl-1,4-dihydro-2,6-dimethyl-(4*R*)-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate hydrochloride; $[\alpha]_D^{22} = +88.8°$ ($c$ = 1, 10 mg/ml, methanol)] were synthesized as described (7). The p$K_a$ value was 8.49 ± 0.06 for B 874–66 and B 874–67. The structure is shown in Fig. 1.

### Calcium Channel Labeling Studies

All binding studies with rabbit skeletal muscle membranes were carried out in 50 mM Tris·HCl, pH 7.4, 0.1 mM PMSF, in a final volume of 0.505 ml. Serial dilutions of unlabeled compounds were in glass tubes and in DMSO to prevent adsorption of the lipophilic drugs to plastic material (8). DMSO-diluted drugs were added to assays in 5-μl aliquots. The final DMSO concentration never exceeded 1% (v/v) of the assay volume, which had no effect on radioligand binding. Labeling of 1,4-dihydropyridine receptors in skeletal muscle membranes with (+)-[$^3$H]PN 200–110 was for 40 min at 37°, 60 min at 22°, and 720 min at 2° under sodium vaporlight. For guinea pig heart membranes labeling was at 37° in a 1.05-ml assay volume for 60 min; the conditions are given in the legends to the figures. Nonspecific binding was determined in the presence of 1 μM (±)-PN200–110 and was subtracted from total binding to yield specific binding. Bound and free ligand were separated after the given incubation times by rapid filtration of the incubation mixture through GF/C Whatman filters, which were immediately washed twice with ice-cold 10% (w/v) polyethylene glycol 6000, 10 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$.

Labeling of 1,4-dihydropyridine receptors with (+)-[$^3$H]PN 200–110 in purified rabbit skeletal muscle calcium channel preparations was at 22° for 60 min, in a final assay volume of 0.505 ml. Nonspecific binding was defined with 1 μM (+)-PN 200–110. The assay buffer was 50 mM Tris·HCl, 0.2 mg/ml bovine serum albumin, 0.1 mM PMSF, 0.1% (w/v) digitonin, pH 7.4. Receptor-bound radioactivity was precipitated by addition of ice-cold 10% polyethylene glycol 6000, 10 mM Tris·HCl, pH 7.4, 10 mM $MgCl_2$, in the presence of 0.1 ml of bovine serum albumin/γ-globulin (5 mg/ml each), 3 min before filtration as described above. The conditions (radioligand and protein concentrations) were standardized for skeletal muscle 1,4-dihydropyridine receptor labeling over a period of 2 years and are given in the legends to the tables. Experiments were always in duplicate; examples are shown in the figures and mean values of the binding constants are given in the tables. The quality of our membranes and of the purified preparations were routinely monitored for $\alpha_1$ subunit integrity in Western blots or by photoaffinity labeling (9), where we find >90% in the truncated form designated $\alpha_{175}$ (10), which has 194 kDa by Ferguson analysis (11).

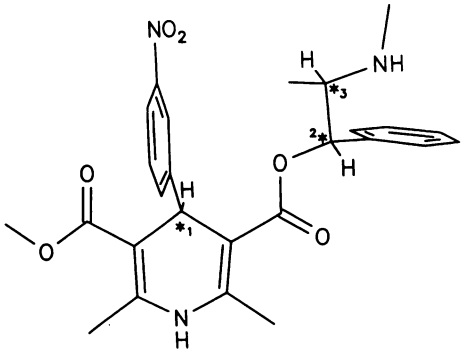


**Fig. 1.** Structure of the B 874–66 and B 874–67 diastereomers. *, Chiral carbon atoms are indicated on the minimal energy conformation used in Fig. 6.

### Protein Determination

Determination of protein concentrations of particulate membranes (12) and of purified skeletal muscle L-type calcium channels (13) was performed according to the cited procedures. Bovine serum albumin was used as a standard.

### Data Analysis

Binding inhibition data were parameter optimized by nonlinear methods using GraphPAD InPlot version 3.14 (GraphPAD Software, Inc.). We determined the $IC_{50}$ value (total concentration of added drug that inhibits specific radioligand binding by 50%) and the apparent Hill slope ($n_H$). If these $n_H$ values were <0.80 a two-site fit was tried, and the $IC_{50}$ (high and low affinity) values as well as percentages of sites that interacted with high and low affinity are presented.

### Electrophysiological Experiments

**Cell isolation.** Single ventricular myocytes from guinea pigs were obtained by enzymatic disaggregation as described (14). In brief, after a 3-min perfusion of the excised heart with calcium-free Tyrode solution, enzymatic digestion was initiated by addition of 125 units/ml collagenase (type CLS; Worthington) and 42 μM $CaCl_2$. After 25 min the heart was rinsed with ice-cold KB medium (see below). Some pieces of ventricular tissues were dissected and stored in KB medium at 4° for up to 20 hr.

**Solutions.** The calcium-free Tyrode solution contained (in mM) 137 NaCl, 5.4 KCl, 11.9 $NaHCO_3$, 0.4 $NaH_2PO_4$, 2 $MgCl_2$, 5 HEPES/$Na^+$, and 5 glucose, pH 7.4. The KB medium consisted of (in mM) 10 taurine, 10 oxalic acid, 10 glutamic acid, 25 KCl, 10 $KH_2PO_4$, 0.5 EGTA, 10 HEPES/$K^+$, and 11 glucose, pH 7.2.

**Electrophysiology.** Guinea pig ventricular myocytes were bathed in external solution containing (in mM) 140 NaCl, 5 CsCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose, and 10 HEPES/$Na^+$, pH 7.4. The bath chamber (0.08 ml) was continuously perfused with external solution at a flow rate of 0.33–0.5 ml/min. Drugs were added to the external solution. The temperature in all experiments was 20–22°. Membrane currents were measured by the patch-clamp technique in whole-cell recording configuration (15), using a List L/M EPC5 amplifier, and were stored on magnetic tape. Soft glass pipettes (Microhematocrit; Assistent) with a resistance of 1.5–3 MΩ were used. The pipette solution contained (in mM) 120 aspartic acid, 30 CsCl, 1 $MgCl_2$, 0.1 EGTA, 2 ATP/Mg, and 5 HEPES/CsOH, pH 7.3. Cells were clamped between −45 and −40 mV and depolarizing voltage pulses of 0.2 sec were applied at a rate of 0.25 Hz. Leak and capacity currents were not corrected.

### Computer Modeling

A detailed description of hardware and software requirements are given elsewhere (16). The molecular structures (cartesian coordinates) of B 874–67 and B 874–66 were constructed by using the fragment library of SYBIL (Tripos Associates Inc., St. Louis, MO). Conformational analyses for both diastereomers were performed during the SYSYL search routine as well as with the program GROSCON (E. Maurhofer and H.-D. Höltje, Free University, Berlin). Volume evaluations and distance measurements were done with the bioactive conformers of the diastereomers, based on earlier results (17). Program GRID (Molecular Discovery, Oxford, UK) was used for the determination of the molecular binding capacities (molecular field analysis).

## Results

**B 874–67 and B 874–66 are L-type $Ca^{2+}$ channel blockers in guinea pig heart cells.** For isolation of $I_{Ca}$, outward currents were suppressed by substituting $Cs^+$ for $K^+$

in both pipette and external solutions. Under these conditions the cells initially showed a resting potential of −69 ± 17 mV (four experiments, mean ± standard deviation), which dropped to −28 ± 12 mV (10 experiments, mean ± standard deviation) after about 20 min. Fast $Na^+$ and T-type calcium currents were inactivated by clamping of the cells at holding potentials of −45 to −40 mV, and depolarizations were performed to evoke $I_{Ca}$. We investigated the effects of B 874–66 and B 874–67 on $I_{Ca}$. Application of 10 μM B 874–66 inhibited $I_{Ca}$ by 99 ± 3% within 2–2.5 min (four experiments) (Fig. 2). The block was not reversible upon wash-out (at least 60-fold solution exchange). Current-voltage relationships for $I_{Ca}$ usually exhibited a maximum between 0 and 10 mV and reversal potentials of about +50 mV. B 874–66 at 10 μM abolished the amplitude of $I_{Ca}$ at all voltages examined. The effects of 10 μM B 874–67 on $I_{Ca}$ were clearly different, because inhibition ocurred more slowly (see Fig. 2C) and to a lesser extent [reduction of $I_{Ca}$ to 65 ± 6% (four experiments) and to 21 ± 2% (three experiments) after 2–3 min (1 ml perfused) and 4–5 min (2 ml perfused), respectively]. Current-voltage relationships for $I_{Ca}$ exhibited a decrease of $Ca^{2+}$ current amplitudes at all voltages with 10 μM B 874–67, whereas no shift in the maximum was observed (Fig. 2B). Similarily to B 874–66, inhibition of $I_{Ca}$ could not be reversed by wash-out. From these studies we concluded that both 1,4-dihydropyridines were L-type calcium channel blockers and that B 874–67 was most likely the less active diastereomer.

**B 874–67 but not B 874–66 discriminates between two different populations of the 1,4-dihydropyridine receptors in muscle membranes.** The two diastereomers were investigated with respect to their interaction with 1,4-dihydropyridine receptors in different muscle tissues, namely

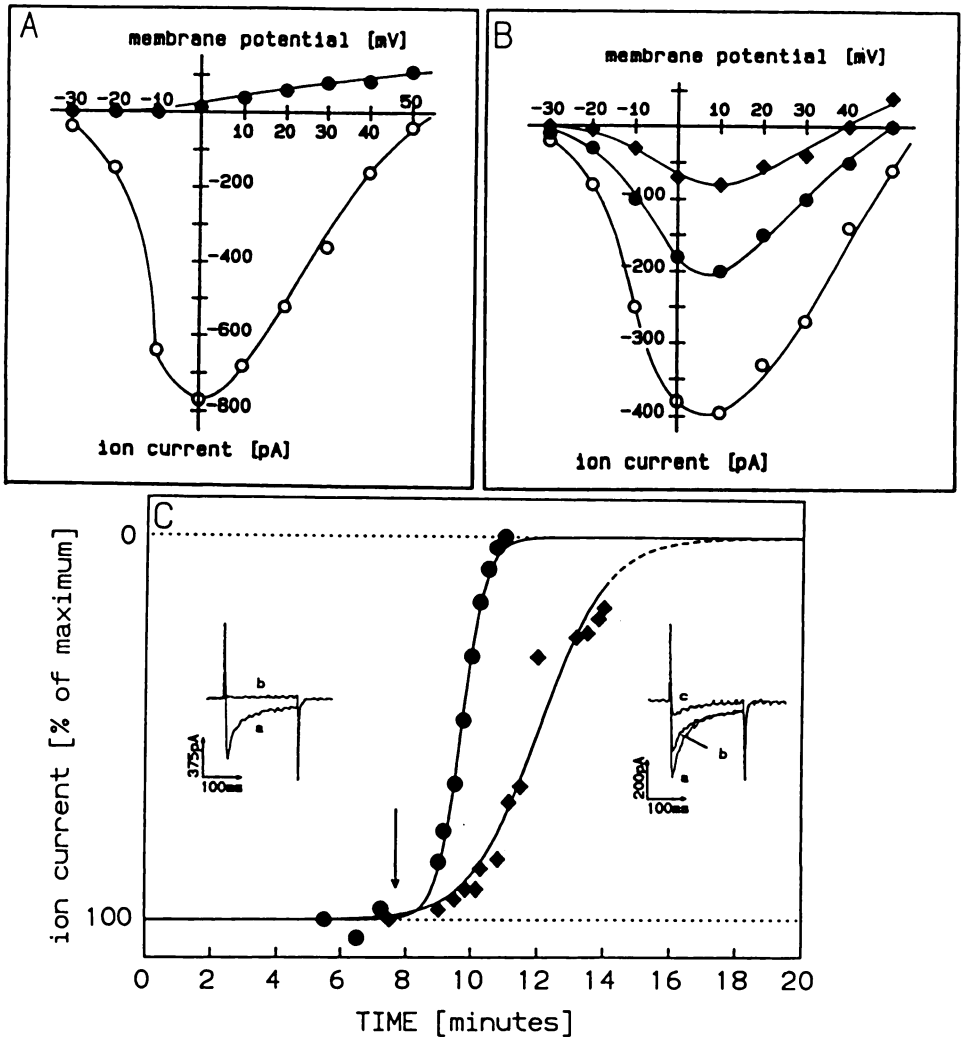


**Fig. 2.** Inhibition of $I_{Ca}$ in isolated guinea pig heart ventricular cells by B 874–66 and B 874–67. A, Current-voltage relationships for $I_{Ca}$ under control conditions (○) (recorded at 6.5 min) and in the presence of 10 μM B 874–66 (●) (recorded at 11.5 min). B, As in A, but with 10 μM B 874–67 (○, control, recorded at 6.5 min; ●, 10.8 min after addition of B 874–67; ♦, 12.8 min after addition of B 874–67). C, Time course of inhibition of $I_{Ca}$ by B 874–66 (●) and B 874–67 (♦), each added (*arrow*) at 10 μM. $I_{Ca}$ was evoked by depolarization from −45 mV to 0 mV and peak inward currents are plotted as a function of time. *Left inset*, records for $I_{Ca}$ under control conditions (*a* at 9 min) and in the presence of B 874–66 (*b* at 11 min). · · · ·, Zero current. *Right inset*, records for $I_{Ca}$ under control conditions (*a* at 8 min) and in the presence of B 874–67 (*b* at 10 min and *c* at 12.3 min). Note the slow onset of inhibition by B 874–67 (to −250 pA at 2 min, to −95 pA after 4 min), in comparison with B 874–66. The initial current in the experiment with B 874–66 was −700 pA at 5.5 min and showed a run-down to −600 pA at 9.0 min. The initial current for the experiment with B 874–67 was −380 pA at 6 min and exhibited a run-down to −370 pA at 7.5 min.

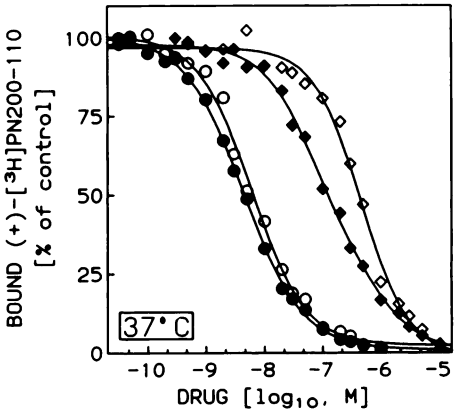


**Fig. 3.** Interaction of the two 1,4-dihydropyridine diastereomers with guinea pig heart membranes. Guinea pig heart membranes (0.035 mg of protein/ml) were incubated with 0.057 nM (+)-[$^3$H]PN 200–110, as described in Experimental Procedures, in the absence (*closed symbols*) or presence (*open symbols*) of 3 μM (+)-(*cis*)-diltiazem. Equilibrium-bound radioligand was 7.5 ± 0.2 pM in the presence and 9.0 ± 0.4 pM in the absence of the allosteric modulator (control binding = 100%). The following best-fit parameters (*drawn lines*) were calculated; B 874–66: ●, $IC_{50}$ = 4.5 nM, $n_H$ = 0.87; ○, $IC_{50}$ = 6.0 nM, $n_H$ = 0.93; B 874–67: ♦, $IC_{50high}$ = 47.4 nM (60%), $IC_{50low}$ = 631 nM (40%); ◇, $IC_{50}$ = 468 nM, $n_H$ = 1.07.

in guinea pig heart and rabbit skeletal muscle membranes (Figs. 3 and 4). In the myocardial plasma membranes the $\alpha_1$ subunit serves solely as an ionic pore, whereas in skeletal muscle a structually distinct $\alpha_1$ subunit (18) has a dual role, namely as a voltage sensor (to trigger $Ca^{2+}$ release from sarcoplamatic reticulum) and as an ionic pore (19). Much to our surprise, the B 874–67 1,4-dihydropyridine binding-inhibition data exhibited a pseudo-Hill slope significantly smaller than unity, whereas the diastereomer B 874–66 did not. An example with guinea pig heart membranes is shown in Fig. 3. The data for B 874–67 could be better fitted with a two-site model where ≈66% of the sites interacted with high affinity and ≈34% interacted with low affinity. In the presence of (+)-(*cis*)-diltiazem (an allosteric regulator of 1,4-dihydropyridine binding) the inhibition curve exhibited a pseudo-Hill slope close to unity, indicating that a uniform population of $\alpha_1$ subunits was recognized by B 874–67 with lower affinity.

The L-type calcium channels in skeletal muscle are biochemically well characterized, the signal-to-noise ratio in partially purified T-tubule membranes is excellent, and the latter are a convenient source for isolation and purification of stable $Ca^{2+}$ channel complexes by a one-step lectin-affinity column chromatography step (6). We, therefore, decided to perform all subsequent experiments with skeletal muscle L-type $Ca^{2+}$ channels.

**Discrimination of high and low affinity populations is a unique feature of B 874–67 and can be abolished by allosteric regulators.** We have performed experiments with skeletal muscle membranes at three different temperatures (2°, 22°, and 37°), and pseudo-Hill slopes of <0.8 for B 874–67 (but not for B 874–66) were observed in every case (Fig. 4). At 22° the mean $n_H$ value for B 874–67 was 0.71 ± 0.04 (11 experiments) and at 2° it was 0.71 ± 0.04 (12 experiments). In Table 1 we have classified the 1,4-dihydropyridines into three groups, namely uncharged compounds [(+)-PN 200–110], those with $pK_a$ values of ≈8.5, and permanently charged quaternary compounds. From the electrophysiological experiments (see above)

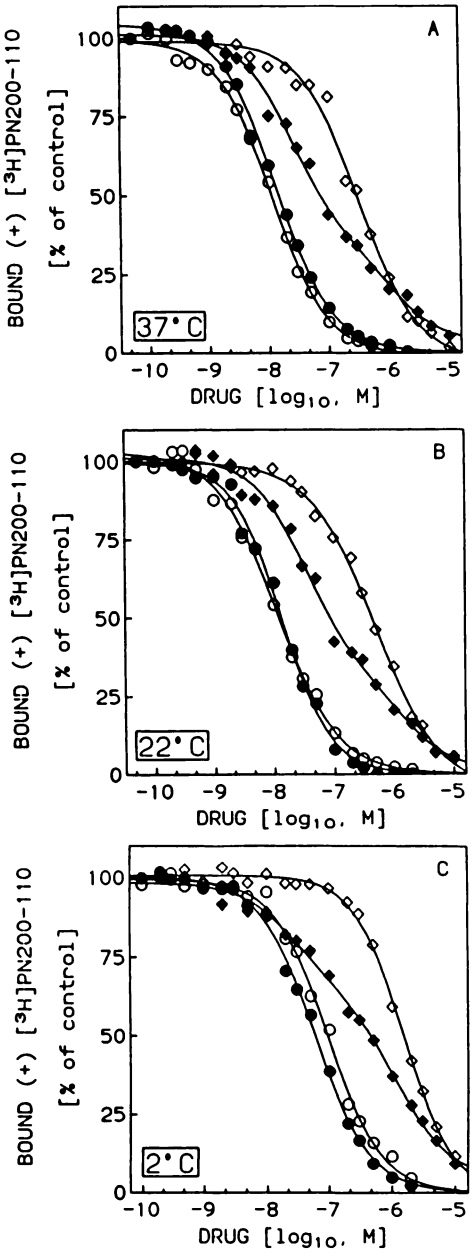


**Fig. 4.** Binding-inhibition profiles of B 874–66 and B 874–67 at three different temperatures and effects of the allosteric regulator (+)-(*cis*)-diltiazem. A, Experiments were performed at 37° with 0.02 mg/ml rabbit skeletal muscle protein and 0.185 nM (+)-[$^3$H]PN 200–110 in the absence (*closed symbols*) or presence (*open symbols*) of 3 μM (+)-(*cis*)-diltiazem. In the absence of the allosteric regulator 30 ± 4 pM and in its presence 44.2 ± 3 pM radioligand was specifically bound (control binding = 100%). Binding-inhibition data for B 874–66 were computer-fitted (*drawn lines*) with the following best-fit parameters: absence of allosteric regulator (●), $IC_{50}$ = 13.8 nM, $n_H$ = 0.94; presence of allosteric regulator (○), $IC_{50}$ = 11.7 nM, $n_H$ = 0.953; B 874–67: ♦, $IC_{50high}$ = 23.9 nM (65.9%), $IC_{50low}$ = 1047 nM (34.1%); ◇, $IC_{50}$ = 331 nM, $n_H$ = 0.96. B, As in A (symbols are identical) but at 22° with 0.007 mg/ml membrane protein and 0.196 nM radioligand. The specifically bound radioligand in the absence of allosteric regulator was 24 ± 2 pM and in the presence of regulator was 29.2 ± 3 pM. ●, $IC_{50}$ = 14.1 nM, $n_H$ = 1.03; ○, $IC_{50}$ = 11.5 nM, $n_H$ = 0.87; ♦, $IC_{50high}$ = 33.8 nM (67.9%), $IC_{50low}$ = 1399 nM (32.1%); ◇, $IC_{50}$ = 503 nM, $n_H$ = 0.81. C, As in A but at 2° with 0.023 mg/ml protein and 0.25 nM radioligand. The specifically bound radioligand was 108 ± 14 pM in the absence of allosteric regulators and was 65 ± 18 pM in their presence. ●, $IC_{50}$ = 60.2 nM, $n_H$ = 0.99; ○, $IC_{50}$ = 95.5 nM, $n_H$ = 1.0; ♦, $IC_{50high}$ = 26.6 nM (35%), $IC_{50low}$ = 1150 nM (65%); ◇, $IC_{50}$ = 1548 nM; $n_H$ = 1.1.

TABLE 1

**B 874-67 discriminates two populations of rabbit skeletal muscle 1,4-dihydropyridine receptors**

Experiments were performed with an average membrane protein concentration of 9.5 ± 2.3 μg/ml and an average radioligand concentration of 292 ± 35 pM at 22°. Under these conditions the specifically bound receptor concentration was 60 ± 13 pM. In cases where a one-site fit yielded pseudo-Hill slopes of <0.8, a two-site fit procedure was tried and the percentage of sites corresponding to the respective $IC_{50}$ ("high" or "low") value is given in parentheses; *n* is the number of experiments. Data are presented as means ± standard deviations. $IC_{50}$ values are in nM.

| Group | 1,4-Dihydropyridine | Binding inhibition parameters | | *n* |
|---|---|---|---|---|
| | | $IC_{50}$ or $IC_{50\,high}$ | $n_H$ or $IC_{50\,low}$ | |
| A | (+)-PN 200–110 | 0.85 ± 0.18 | 0.98 ± 0.05 | 4 |
| B | (*S*)-(−)-Amlodipine | 19.5 ± 7.7 | 1.05 ± 0.06 | 3 |
| | (*R*)-(+)-Amlodipine | 417 | 0.94 | 1 |
| | (*RS*)-(±)-Amlodipine | 32.9 | 0.90 | 1 |
| | B 874-66 | 15.7 ± 4.2 | 0.92 ± 0.06 | 9 |
| | B 874-67 | 73.7 ± 15.4 (67.2 ± 5.2) | 1440 ± 300 (32.8 ± 5.2) | 11 |
| C | (±)-SDZ 207-180 | 125.5 ± 40 | 0.94 ± 0.2 | 3 |
| | (*RS*)-UK 118,434-05 | 4690 ± 770 | 0.91 ± 0.09 | 2 |

and allosteric inhibition of phenylalkylamine or (+)-(*cis*)-[$^3$H] diltiazem binding (data not shown), B 874–66 was the more potent diastereomer, i.e., the eutomer. We initially suspected that the distomer of positively charged, chiral 1,4-dihydropyridines could always discriminate between the two populations. This was clearly not the case, because (*R*)-(+)-amlodipine inhibited (+)-[$^3$H]PN200-MO binding with a $n_H$ value close to unity. Furthermore, the two permanently charged 1,4-dihydropyridines were not discriminatory (Table 1).

Based on the observations with (+)-(*cis*)-diltiazem exemplified in Fig. 3, we next studied in greater detail the effects of heterotropic allosteric regulators. All of the regulators in Table 2 have previously been shown to change the kinetics ($k_{-1}$ and $k_{+1}$) of the 1,4-dihydropyridines in a complex, temperature-dependent manner (20). The net result can be an increase, decrease, or no change (null effect) of the $K_d$ value. When conditions are chosen such that the net effect is an increase in affinity for 1,4-dihydropyridines [reflected by a stimulation of, for example, (+)-[$^3$H]PN 200–110 binding at nonsaturating free concentrations], the allosteric regulators increase the affinity for an $\alpha_1$ subunit-bound $Ca^{2+}$ ion (20). The first finding was that the regulators converted the L-type $Ca^{2+}$ channel into a conformation where the discriminatory properties of B 874–67 were lost, independently of the temperature and regardless whether the net result on the equilibrium binding of the neutral 1,4-dihydropyridine (+)-[$^3$H]PN 200–110 was stimulatory [e.g., (+)-tetrandrine and fostedil at 22°] or inhibitory [e.g., (*trans*)-diclofurim at 2°].

The second finding was that the conversion into a uniform binding population was stereoselective for the diltiazem diastereomers [(−)-(*cis*)-diltiazem was ineffective] but not for the BM 20.1140 enantiomers. Interestingly, both BM 20.1140 enantiomers have identical (stimulatory) effects on the association rate constant ($k_{+1}$) but are distinguished by their differential effects on the dissociation rate constant ($k_{-1}$) for the 1,4-dihydropyridine (+)-PN 200–110 (20).

**Two populations are discriminated by B 874–67 in purified calcium channel preparations.** Table 3 and Fig. 5 show that the purified calcium channel preparation exhibits properties with respect to B 874–67 similar to those of the membrane-bound $\alpha_1$ subunits. Remarkably, the $IC_{50}$ values for the high and low affinity interaction of B 874–67 were similar to or even lower than those for the membrane-bound channel. Thus, the characteristic loss of affinity (4–10-fold; compare data in Tables 1, 2, and 3 and see Ref. 21) for other 1,4-dihydropyridines is not shared by B 874–67. These experiments also exclude the remote possibility that sidedness problems of the vesicular membrane preparation were the basis of the biphasic inhibition curves for B 874–67. Again, as for particulate preparations, both BM 20.1140 enantiomers converted the $\alpha_1$ subunits into a conformation that did not allow the dicrimination, although (−)-BM 20.1140, in agreement with previous studies (20), stimulated and (+)-BM 20.1140 inhibited the equilibrium binding of the radioligand. In other experiments we purified calcium channels to near-homogeneity (6) and still observed the biphasic B 874–67 inhibition profile (data not shown).

**Divalent cations can mimic allosteric regulators.** Decreasing the free $Ca^{2+}$ concentration to very low values (≈1 nM) by addition of EDTA (see Table 2) or EGTA (data not shown) had no effect on the behavior of B 874–67 with respect to its characteristic biphasic inhibition profile. Likewise, addition of monovalent cations ($Na^+$ or $K^+$) at 1–100 mM was ineffective (data not shown). However, $Ca^{2+}$ and $Mg^{2+}$ but not $Ba^{2+}$ were able to mimic (in the millimolar range) the action of micromolar concentrations of the allosteric regulators at both temperatures, i.e., 2° and 22°. We investigated the concentration dependence for added $Ca^{2+}$ (as $CaCl_2$) in more detail (data not shown). These data suggested that saturation of divalent cation sites with $Ca^{2+}$ occurred around 1 mM and half-maximal effects occurred at ≈50–100 μM $Ca^{2+}$. We have, however, routinely used 10 mM for added divalent cations. In Table 2 we included the divalent cation $Ni^{2+}$, which is a potent L-type calcium channel blocker (22). $Ni^{2+}$ strongly inhibited (+)-[$^3$H] PN 200–110 binding but was not able to change the proportion of $\alpha_1$ subunits that differentially interacted with B 874–67. When the channel was liganded with 3 μM (+)-(*cis*)-diltiazem the B 874–67 inhibition profile was virtually unchanged (i.e., remained monophasic) in the presence of 10 mM EDTA (data not shown), assuring us that this allosteric regulator had no requirement for free $Ca^{2+}$ at less than ≈10 nM. Likewise, the effects of 10 mM $Ca^{2+}$ and 3 μM (+)-(*cis*)-diltiazem combined were identical to those observed for each added separately.

**B 874–66 and B 874–67 can interact with different regions of the $\alpha_1$ subunit outside the core of the dihydropyridine-binding region.** We first performed a conformational analysis of the diastereomers to ensure that the conformers for all the subsequent calculations were energetically allowed. Then we calculated molecular interactions with simple chemical probes that were moved around each diastereomer in

TABLE 2

**Effects of allosteric regulators and cations on the discrimination of 1,4-dihydropyridine receptors by B 874-67**

Experiments were performed under standard conditions (see Table 1) at 22° and 2°. The mean concentration of specifically liganded 1,4-dihydropyridine receptors was 60 ± 13 pM at 22° (see Table 1) and 69.2 ± 13 pM at 2°. Relative specific binding (RSB) is defined as specifically bound radioligand in the absence of allosteric regulators or ions/specifically bound radioligand in the presence of allosteric regulators or ions. Ions were always added as chloride salts. Data in the absence of allosteric regulators or added cations and at 22° are taken from Table 1. $IC_{50}$ values are in nM.

| | Concentration | Binding inhibition Parameters | | RSB | *n* |
|---|---|---|---|---|---|
| | | $IC_{50}$ or $IC_{50high}$ | $n_H$ or $IC_{50low}$ | | |
| | μM | | | | |
| 22° experiments | | | | | |
| Allosteric regulator | | | | | |
| None | | 73.7 ± 15.4 (67.2 ± 5.2) | 1440 ± 300 (32.8 ± 5) | 1.00 | |
| (+)-(*cis*)-Diltiazem | 3 | 422 ± 70 | 0.89 ± 0.11 | 0.90 | 5 |
| (−)-(*cis*)-Diltiazem | 3 | 87.8 ± 24.7 (64.4 ± 5.9) | 1380 ± 140 (35.7 ± 5.9) | 0.82 | 2 |
| (+)-Tetrandrine | 1 | 303 ± 75 | 0.93 ± 0.07 | 1.33 | 2 |
| (*trans*)-Diclofurim | 1 | 689 ± 31 | 0.98 ± 0.09 | 0.72 | 2 |
| Fostedil | 1 | 554 ± 211 | 0.86 ± 0.09 | 1.30 | 2 |
| (+)-BM 20.1140 | 1 | 886 ± 274 | 0.88 ± 0.05 | 0.90 | 2 |
| (−)-BM 20.1140 | 1 | 1047 ± 357 | 1.08 ± 0.06 | 0.97 | 2 |
| Ions/chelator | mM | | | | |
| EDTA | 10 | 82.5 ± 20 (62.7 ± 4) | 1138 ± 294 (36.9 ± 4) | 0.84 | 4 |
| $Ca^{2+}$ | 10 | 340 ± 41 | 0.82 ± 0.05 | 1.00 | 3 |
| $Mg^{2+}$ | 10 | 348 ± 44 | 0.87 ± 0.06 | 0.83 | 2 |
| $Ba^{2+}$ | 10 | 49.2 ± 7 (42.6 ± 7) | 751 ± 120 (58 ± 6) | 0.80 | 2 |
| $Ni^{2+}$ | 10 | 200 ± 78 (59.4 ± 4) | 5485 ± 1960 (41 ± 4) | 0.26 | 2 |
| 2° experiments | | | | | |
| Allosteric regulator | μM | | | | |
| None | | 57.2 ± 32 (53.6 ± 12.6) | 1297 ± 567 (46.3 ± 12.6) | 1.00 | 12 |
| (+)-(*cis*)-Diltiazem | 3 | 889 ± 571 | 0.97 ± 0.13 | 0.87 | 3 |
| (−)-(*cis*)-Diltiazem | 3 | 97.7 ± 71 (44 ± 10) | 1111 ± 267 (56 ± 10) | 0.84 | 2 |
| (+)-Tetrandrine | 1 | 326 ± 79 | 0.85 ± 0.02 | 0.95 | 2 |
| *trans*-Diclofurim | 1 | 1137 ± 113 | 0.94 ± 0.02 | 0.55 | 2 |
| Fostedil | 1 | 817 ± 379 | 0.86 ± 0.11 | 1.11 | 2 |
| (+)-BM 20.1140 | 1 | 1179 ± 172 | 0.98 ± 0.14 | 0.89 | 2 |
| (−)-BM 20.1140 | 1 | 1502 ± 290 | 1.17 ± 0.01 | 0.92 | 2 |
| Ions/chelator | mM | | | | |
| EDTA | 10 | 70.7 ± 43 (39.5 ± 14) | 1122 ± 142 (59.8 ± 13.6) | 0.70 | 4 |
| $Ca^{2+}$ | 10 | 395 ± 12 | 0.83 ± 0.03 | 0.90 | 2 |
| $Mg^{2+}$ | 10 | 454 ± 153 | 0.81 ± 0.05 | 0.86 | 2 |
| $Ba^{2+}$ | 10 | 99.2 ± 38 (38.3 ± 6) | 1030 ± 300 (61.7 ± 14) | 0.60 | 2 |
| $Ni^{2+}$ | 10 | 226 ± 98 (67 ± 8.4) | 6510 ± 240 (32.2 ± 4) | 0.26 | 2 |

TABLE 3

**B 874-67 discriminates two populations of 1,4-dihydropyridine receptors in purified calcium channel preparations**

Experiments were performed with an average total concentration of 1.05 ± 0.47 nM (+)-[$^3$H]PN 200-110 and 3.8 ± 0.2 μg/ml purified calcium channel protein. Under these incubation conditions the average concentration of specifically labeled 1,4-dihydropyridine receptors was 54 ± 17 pM. The relative specific binding (RSB) is defined as specifically bound radioligand in the presence of the allosteric regulator/specifically bound radioligand in its absence. The percentage of sites having high and low affinity is given in parentheses. $IC_{50}$ values are in nM.

| Compound | Allosteric regulator | Binding inhibition parameters | | RSB | *n* |
|---|---|---|---|---|---|
| | | $IC_{50}$ or $IC_{50high}$ | $n_H$ or $IC_{50low}$ | | |
| (+)-PN 200-110 | None | 3.03 ± 1.0 | 1.00 ± 0.01 | 1.00 | 2 |
| BYK 874-67 | None | 45 ± 4 (60.4 ± 5) | 1238 ± 323 (39.6 ± 5) | 1.00 | 4 |
| BYK 874-67 | (+)-BM 20.1140, 3 μM | 837 ± 514 | 0.94 ± 0.02 | 0.54 | 2 |
| BYK 874-67 | (−)-BM 20.1140, 3 μM | 826 ± 245 | 0.95 ± 0.01 | 1.42 | 2 |
| BYK 874-67 | (+)-Tetrandrine, 1 μM | 307 ± 160 | 0.90 ± 0.04 | 1.61 | 2 |

three-dimensional space, in order to determine potential binding contacts between the compounds and the corresponding receptor protein. Both compounds can utilize identical hydrophobic interactions as well as identical polar hydrogen-bonding contacts (data not shown).

The most significant distinguishing feature was found in the spatial arrangement of the center of mass of the chiral side chains of the two dihydropyridines with respect to the center of the 1,4-dihydropyridine ring system. This is made visible in Fig. 6 by calculation of the differential volume. It is evident that the two diastereomers, when the 1,4-dihydropyridine-phenyl moiety is assumed to be fixed in its domain, occupy different regions in space. Estimations of the distance from the center of the 1,4-dihydropyridine ring show that this differential volume is located in opposite directions at a distance of 6.1 Å from the assumed center of the binding domain.

## Discussion

In this communication we present evidence for a heterogeneity of $\alpha_1$ subunits of mammalian muscle L-type $Ca^{2+}$ channels, as revealed by a novel basic 1,4-dihydropyridine. One diastereomer, B 874–67, inhibited the binding of the standard radioligand (+)-[$^3$H]PN200–110 in a clearly biphasic manner,

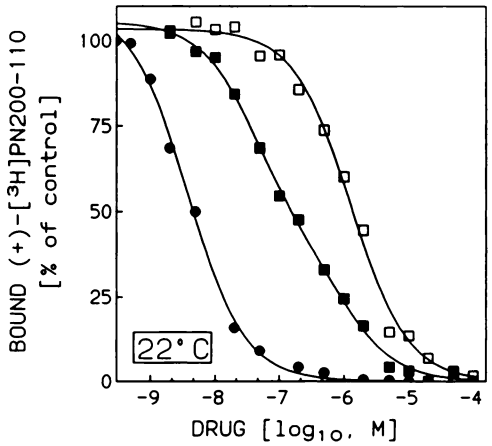


**Fig. 5.** Interaction of B 874–67 and (+)-PN 200–110 with purified calcium channel 1,4-dihydropyridine sites. Purified calcium channels (0.0027 mg/ml) were labeled with 0.7 nM (+)-[$^3$H]PN 200–110 in the absence and presence of 3 $\mu$M (+)-BM 20.1140 and in the presence of increasing concentrations of (+)-PN 200–110 or B 874–67. The data for (+)-PN 200–110 (●) (in the absence of allosteric modulators) were fitted with following best parameters (*drawn lines*): $IC_{50}$ = 3.76 nM, $n_H$ = 0.97; for B 874–67: in the absence of allosteric modulator (■), $IC_{50high}$ = 39.9 nM (59%), $IC_{50low}$ = 954 nM (41%); in the presence of (+)-BM 20.1140 (□), $IC_{50}$ = 1280 nM, $n_H$ = 0.98.

whereas the more potent diastereomer, B 874–66, did not. Likewise, other basic or permanently charged 1,4-dihydropyridines, as well as the neutral unlabeled 1,4-dihydropyridine (+)-PN 200–110, inhibited binding with pseudo-Hill slopes close to unity. This indicates that these 1,4-dihydropyridines bind to a uniform population of $\alpha_1$ subunits or, alternatively, interact nonselectively with two populations. The binding-inhibition data for B 874–67 were better fitted assuming two sites (having low and high affinity), compared with a one-site binding model. Our interest in B 874–67 was raised considerably when we found that (+)-(*cis*)-diltiazem occupation of the benzothiazepine binding domain (which is allostericly coupled to the 1,4-dihydropyridine site) (1) converted the myocardial $\alpha_1$ subunits into a uniform (low affinity) population with respect to B 874–67. Because this is a formal analogy to guanine nucleotide-binding protein-coupled receptors (where agonists have high affinity for receptors liganded with the nucleotide-free trimeric $\alpha\beta\gamma$ form of guanine nucleotide-binding protein and low affinity for free receptors) (23), we investigated this phenomenon in more detail and studied other allosteric regulators as well.

Skeletal muscle calcium channels were selected for all further studies because they are biochemically well characterized (10) and can be easily purified and postlabeled with radioligands (6). Furthermore, the regions within the primary amino acid sequence of the skeletal muscle $\alpha_1$ subunit that form the 1,4-dihydropyridine antagonist binding domain have been identified (24–26). In the membrane-bound state 67% and 56.2%, respectively, of the calcium channel $\alpha_1$ subunits exhibited high affinity ($IC_{50}$ values of 73.7 and 57.2 nM) for B 874–67 at 22° and 2° (Table 1). Purified $\alpha_1$ subunits displayed 60.4% high affinity binding. Thus, there is no evidence that an equilibrium between two populations of $\alpha_1$ subunits exists that is affected by changing the temperature between 2° and 22° or by solubilization and purification.

In contrast, allosteric regulators that belong to completely different chemical classes converted the $\alpha_1$ subunits into a uniform population with low affinity for B 874–67. This was observed at 2°, 22°, and 37° and was completely independent from the effects of the regulators on the kinetic constants of the neutral 1,4-dihydropyridine [$^3$H]PN 200–110. These differential changes in the kinetic constants can result in stimulation, inhibition, or an apparent null effect at nonsaturating concentrations of radioligand, as were used here and previously (4, 20). All of these regulators have been recently demonstrated to change the apparent affinity of a high affinity $Ca^{2+}$ binding site (20), which was later designated $Ca^{2+}$ site I (5). These regulators include the (−)-BM 20.1140 enantiomer, which can act as a positive heterotropic regulator of PN 200–110 binding and increases the affinity of $Ca^{2+}$ site I, as well as (+)-BM 20.1140, which can act as a negative heterotropic allosteric regulator and decreases the affinity of $Ca^{2+}$ site I (20). Thus, the common feature of the allosteric regulators is to induce conformational changes of the 1,4-dihydropyridine binding domain and the interdependent $Ca^{2+}$ site I. For the B 874–67 diastereomer these changes result in a ≈4–25-fold loss of affinity, consistent with the breakage of, for example, one hydrogen bond (2–3 kcal/mol). The active enantiomers of the photoaffinity ligands [$^3$H]azidopine and [$^3$H]diazipine (23, 24) label what were termed secondary sites and subsites of the 1,4-dihydropyridine binding domain (25) on the extracellular end of transmembrane helix IVS6 and the extracellular side of IIIS6, whereas (+)-[$^3$H]PN 200–110 photoincorporated into the binding domain core in transmembrane helix IIIS6 (26). In current models of voltage-

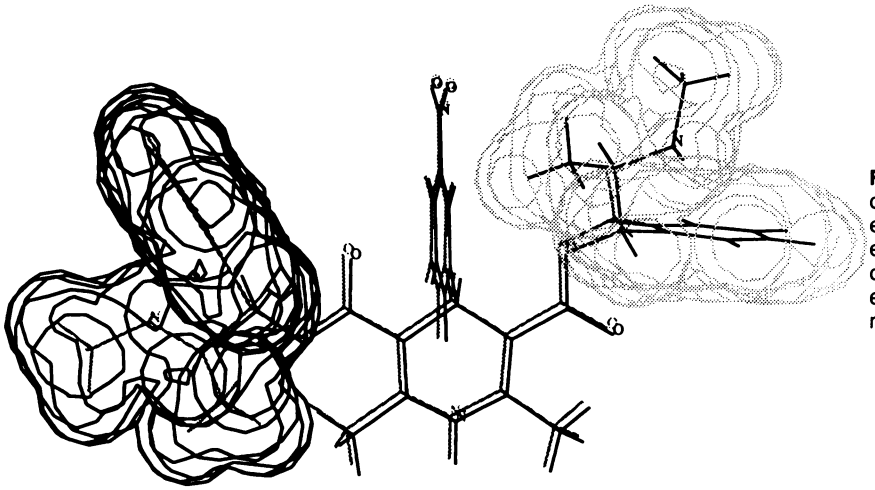

**Fig. 6.** Different steric volumes of the 1,4-dihydropyridines B 874–67 and B 874–66. Volumes of space that each diastereomer occupies are shown as the differential volumes. *Shaded in black*, volume of the *S*-(+)-diastereomer minus the volume of the *R*-(−)-diastereomer; *shaded in gray*, volume of the *R*-(−)-diastereomer minus the volume of the *S*-(+)-diastereomer.

gated cation channels these regions form the outer mouth and the pore lining (27).

The different steric volume occupied by B 874–67, in comparison with B 874–66, locates the amino acid participating in the formation of a bond (that is interrupted by the allosteric regulators or $Ca^{2+}$ or $Mg^{2+}$) as being within 6.1 Å from the 1,4-dihydropyridine binding domain core. This amino acid may be at a different location than the secondary site and subsite regions, reached by the flexible side chains of the arylazide photoligands. Just as surprising as the effects of the allosteric regulators, which [as demonstrated for (+)-(*cis*)-diltiazem] had no requirement for free calcium, were the actions of divalent cations. The most straightforward interpretation is that a low affinity divalent cation binding site exists on the $\alpha_1$ subunits and induces the same conformational changes as do the allosteric regulators. Consequently, there must be communication between this site (termed here $Ca^{2+}$ site II) and $Ca^{2+}$ site I, which is interdependent with the 1,4-dihydropyridine binding domain (20). $Ca^{2+}$ site II had rather stringent requirements that only $Ca^{2+}$ and $Mg^{2+}$ and not $Ba^{2+}$ or $Ni^{2+}$ could fulfill. Calcium binding sites have not yet been structurally mapped within the $\alpha_1$ subunit. An EF hand motif (putative $Ca^{2+}$ binding domain) 25 amino acids downstream of the IVS6 region was, however, recently predicted by the Tufty-Kretsinger test and suggested to participate in channel inactivation (28).

Calcium channel inactivation can indeed occur rapidly by an intracellular, $Ca^{2+}$-dependent mechanism that is not triggered by $Ba^{2+}$ and by a slower, voltage-dependent process that is accelerated by $Mg^{2+}$ (29). When myocardial cells are perfused internally with free concentrations of 0.3–3 mM $Mg^{2+}$, the voltage-dependent inactivation of L-type calcium channels is markedly increased both in the rate and in the steady state level (29, 30). Taken together these data suggest internally oriented $Mg^{2+}$ (or $Ca^{2+}$) binding sites of the $\alpha_1$ subunit that participate in inactivation. The location of $Ca^{2+}$ site II, which mimics the effects of the allosteric regulators, is not known but this site is perhaps identical to the sites postulated to participate in channel inactivation from functional studies (29, 30) and predicted by the Tufty-Kretsinger test (28).

We explain our findings with two populations of $\alpha_1$ subunits that are distinguished perhaps by a subtle structural difference and occur in relative proportions of 65% ($\alpha_1$*) and 35% ($\alpha_1$**) in skeletal muscle membranes. The majority ($\alpha_1$*) have a ≈10–20-fold higher affinity for B 874–67, by either increased association or decreased dissociation rate or a combination of both. Any perturbation of the 1,4-dihydropyridine binding domain in the $\alpha_1$* population (via $Ca^{2+}$ site II or upon binding of allosteric regulators) removes the constraints that are requirements for a high affinity interaction. Consequently B 874–67 now binds with lower (but perhaps not identical) affinity to $\alpha_1$*, as to $\alpha_1$**. Evidence for a kinetic heterogeneity of the $\alpha_1$ subunits already exists. (i) (+)-[$^{35}$S]Sadopine has 10-fold higher association and dissociation rate constants for skeletal muscle $\alpha_1$ subunits than does (−)-[$^{35}$S]sadopine. The $K_d$ value is identical. (+)-[$^{35}$S] Sadopine maximally labeled 1.31 pmol/mg of protein but (−)-[$^{35}$S]sadopine labeled 2.21 pmol/mg of protein (31). (+)-[$^{3}$H] PN 200–110 labeled at least 5–10 pmol/mg of protein. If each of the two diastereomers exclusively labeled one of the two postulated $\alpha_1$ subunit populations, the relative fractions of $\alpha_1$* and $\alpha_1$** are 37 and 63%, respectively. (ii) Between 67.7 and 61.8% of the skeletal muscle calcium channel drug binding sites were converted (by emptying $Ca^{2+}$ site I) into a very low binding affinity state with rate constants of 0.044 and 0.041 $min^{-1}$, whereas 32.3 and 38.2% were converted with rate constants of 1.49 and 1.37 $min^{-1}$ (5). (iii) We recently developed assays with fluorescent ligands that allow high resolution kinetic studies (32). Preliminary data indicate that two populations of $\alpha_1$ subunits (with relative proportions of ≈65 and 35%) exist in purified calcium channel preparations and exhibit a ≈10-fold difference in their dissociation rate constants. (iv) Two different classes of L-type $Ca^{2+}$ channels, which occur in an approximately 30/70% proportion and have different kinetics of inactivation, are observed in rat myoballs (33). (v) Heterogeneity of membrane-bound and highly purified rabbit skeletal muscle calcium channels is also consistently observed in functional reconstitution experiments (34, 35). Based on the two conductance levels the high conductance channel had properties of the classical L-type, whereas the low conductance channel was suggested to be similar to a T-type channel (34). Unfortunately there are, at present, no biochemical tools to characterize calcium channels that exhibit T-type currents and are blocked by some but not all 1,4-dihydropyridines (36). The hypothesis that the two $\alpha_1$ subunit populations discriminated by B 874–67 represent the distinct calcium channels that have to be postulated from the findings described above is attractive but must remain speculative until the structural basis for the heterogeneity (different amino acid sequences, post-translational modifications) has been clarified.

In summary, the 1,4-dihydropyridine B 874–67 is the prototype of a new class of calcium channel blockers than can discriminate (by means of different affinity) two populations of $\alpha_1$ subunits in muscle membranes and purified channel preparations. The two populations occur in a ratio of ≈2:1 and are postulated to differ with respect to the 1,4-dihydropyridine binding domain.

**References**


1. Glossmann, H., and J. Striessnig. Molecular properties of calcium channels. *Rev. Physiol. Biochem. Pharmacol.* **114**:1–105 (1990).
2. Catterall, W. A. Functional subunit structure of voltage-gated calcium channels. *Science (Washington D. C.)* **253**:1499–1500 (1991).
3. Bosse, E., R. Bottlender, T. Kleppisch, J. Hescheler, A. Welling, F. Hofmann, and V. Flockerzi. Stable and functional expression of the calcium-channel *alpha*1 subunit from smooth muscle in somatic cell lines. *EMBO J.* **11**:2033–2038 (1992).
4. Glossmann, H., and D. R. Ferry. Assay for calcium channels. *Methods Enzymol.* **109**:513–550 (1985).
5. Knaus, H. G., T. Moshammer, H. C. Kang, R. P. Haugland, and H. Glossmann. A unique fluorescent phenylalkylamine probe for L-type $Ca^{2+}$ channels. *J. Biol. Chem.* **267**:2179–2189 (1992).
6. Striessnig, J., and H. Glossmann. Purification of L-type calcium channel drug receptors. *Methods Neurosci.* **4**:210–229 (1991).
7. Flockerzi, D., W. R. Ulrich, K. Sanders, K. D. Beller, R. Boer, M. Eltze, and K. Klemm. International Patent Application WO 89/08644 (1989).
8. Boer, R., A. Grassegger, C. Schudt, and H. Glossmann. (+)-Niguldipine binds with very high affinity to $Ca^{2+}$ channels and to a subtype of $\alpha_1$-adrenoceptors. *Eur. J. Pharmacol.* **172**:131–145 (1989).
9. Grabner, M., K. Friedrich, H. G. Knaus, J. Striessnig, F. Scheffauer, R. Staudinger, W. J. Koch, A. Schwartz, and H. Glossmann. Calcium channels from *Cyprinus carpio* skeletal muscle. *Proc. Natl. Acad. Sci. USA* **88**:727–731 (1991).
10. De Jongh, K. S., D. K. Merrick, and W. A. Catterall. Subunits of purified calcium channels: a 212-kDa form of $\alpha$1 and partial amino acid sequence of a phosphorylation site of an independent $\beta$ subunit. *Proc. Natl. Acad. Sci. USA* **86**:8585–8589 (1989).
11. Glossmann, H., J. Striessnig, L. Hymel, G. Zernig, H. G. Knaus, and H. Schindler. The structure of the $Ca^{2+}$ channel: photoaffinity labeling and tissue distribution, in *The Calcium Channel: Structure, Function and Implications* (M. Morad, W. Nayler, M. Schramm, and S. Kazda, eds.). Springer, Berlin, 168–191 (1988).
12. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).

13. 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).
14. Romanin, C., P. Grösswagen, and H. Schindler. Calpastatin and nucleotides stabilize cardiac calcium channel activity in excised patches. *Pflüger's Arch.* **418:**86–92 (1991).
15. Hamill, O. P., A. Marry, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch-clamp technique for high resolution current recording from cell and cell-free membrane patches. *Pflüger's Arch.* **391:**85–100 (1981).
16. Höltje, H. D., and S. Marrer. A molecular graphics study on structure activity relationships of calcium-antagonistic and agonistic 1,4-dihydropyridines. *J. Comput. Aided Mol. Design* **1:**23–30 (1987).
17. Höltje, H. D., and S. Marrer. Qualitative and quantitative structure-activity relationships for calcium-channel modulating 1,4-dihydropyridine derivatives: a hypothetical receptor model. *Quant. Struct.-Activity Relat. (QSAR)* **7:**174–178 (1988).
18. Tsien, R. W., P. T. Ellinor, and W. A. Horne. Molecular diversity of voltage-dependent calcium channels. *Trends Pharmacol. Sci.* **12:**349–354 (1991).
19. Catterall, W. A. Excitation contraction coupling in skeletal muscle: a tale of two calcium channels. *Cell* **64:**871–874 (1991).
20. Staudinger, R., H. G. Knaus, and H. Glossmann. Positive heterotropic allosteric regulators of dihydropyridine binding increase the $Ca^{2+}$ affinity of the L-type calcium channel. *J. Biol. Chem.* **266:**10787–10795 (1991).
21. Striessnig, J., K. Moosburger, A. Goll, D. R. Ferry, and H. Glossmann. Stereoselective photoaffinity labelling of the purified 1,4-dihydropyridine receptor of the voltage-dependent calcium channel. *Eur. J. Biochem.* **161:**603–609 (1986).
22. Hess, P., J. B. Lansman, A. P. Fox, M. C. Nowycky, B. Nilius, E. W. McCleskey, and R. W. Tsien. Calcium channels: mechanisms of modulation and ion permeation. *J. Gen. Physiol.* **86:**5–27 (1985).
23. Freissmuth, M., P. J. Casey, and A. G. Gilman. G-proteins control diverse pathways of transmembrane signaling. *FASEB J.* **3:**2125–2131 (1989).
24. Nakayama, H., M. Taki, J. Striessnig, H. Glossmann, W. A. Catterall, and Y. Kanaoka. Identification of 1,4-dihydropyridine binding regions within the *alpha*1 subunit of skeletal muscle calcium channels by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA* **88:**9203–9207 (1991).
25. Striessnig, J., B. J. Murphy, and W. A. Catterall. Dihydropyridine receptor of L-type $Ca^{2+}$-channels: identification of binding domains for [$^3$H](+)-PN200–110 and [$^3$H]azidopine within the *alpha*1 subunit. *Proc. Natl. Acad. Sci. USA* **88:**10769–10773 (1991).
26. Catterall, W. A., and J. Striessnig. Receptor sites for $Ca^{2+}$-channel antagonists. *Trends Pharmacol. Sci.* **13:**256–262 (1992).
27. Durell, S. R., and H. R. Guy. Atomic scale structure and functional models of voltage-gated potassium channels. *Biophys. J.* **62:**238–250 (1992).
28. Babitch, J. A. Channel hands (Letter). *Nature (Lond.)* **346:**321–322 (1990).
29. Hartzell, H. C., and R. E. White. Effects of magnesium on inactivation of the voltage-gated calcium current in cardiac myocytes. *J. Gen. Physiol.* **94:**745–767 (1989).
30. Rourke, B. O'., P. H. Backx, and E. Marban. Phosphorylation-independent modulation of L-type calcium channels by magnesium-nucleotide complexes. *Science (Washington D. C.)* **257:**245–248 (1992).
31. Knaus, H. G., T. Moshammer, H. C. Kang, R. P. Haugland, and H. Glossmann. A unique fluorescent phenylalkylamine probe for L-type $Ca^{2+}$ channels. *J. Biol. Chem.* **267:**2179–2189 (1992).
32. Berger, W., K. Friedrich, T. Moshammer, J. Striessnig, and H. Glossmann. Direct identification of drug binding to L-type $Ca^{+}$ channels by means of fluorescent energy-transfer (Abstract). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **346:**R7 (1992).
33. Cognard, C., M. Lazdunski, and G. Romey. Different types of $Ca^{2+}$ channels in mammalian skeletal muscle cells in culture. *Proc. Natl. Acad. Sci. USA* **83:**517–521 (1986).
34. Pelzer, D., A. Cavalie, V. Flockerzi, F. Hofmann, and W. Trautwein. Reconstitution of solubilized and purified dihydropyridine receptor from skeletal muscle microsomes as two single calcium channel conductances with different functional properties, in *The Calcium Channel: Structure, Function and Implications* (M. Morad, W. Nayler, M. Schramm, and S. Kazda, eds.). Springer, Berlin, 217–230 (1988).
35. Affolter, H., and R. Coronado. Agonists Bay K 8644 and CGP-28392 open calcium channels reconstituted from skeletal muscle transverse tubules. *Biophys. J.* **48:**341–347 (1985).
36. Romanin, C., K. Seydl, H. Glossmann, and H. Schindler. The dihydropyridine niguldipine inhibits T-type $Ca^{2+}$ currents in atrial myocytes. *Pflüger's Arch.* **420:**410–412 (1992).

**Send reprint requests to:** Prof. H. Glossmann, Institute for Biochemical Pharmacology, University of Innsbruck, Peter-Mayr-Straβe 1, A-6020 Innsbruck, Austria.