Permanently Charged Chiral 1,4-Dihydropyridines: Molecular Probes of L-Type Calcium Channels. Synthesis and Pharmacological Characterization of Methyl (ω-Trimethylalkylammonium) 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate Iodide, Calcium Channel Antagonists

Ravikumar Peri,[‡,#] Seetharamaiyer Padmanabhan,[†,#] Aleta Rutledge, Satpal Singh, and David J. Triggle*

Department of Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo, Buffalo, New York 14260

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We report the synthesis of the single enantiomers of permanently charged dihydropyridine derivatives (DHPs with alkyl linker lengths of two and eight carbon atoms) and their activities on cardiac and neuronal L-type calcium channels. Permanently charged chiral 1,4-dihydropyridines and methyl (ω-trimethylalkylammonium) 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate iodides were synthesized in high optical purities from (*R*)-(−) and (*S*)-(+)-1,4-dihydro-2,6-dimethyl-5-methoxycarbonyl-4-(3-nitrophenyl)-3-pyridinecarboxylic acid, obtained by resolution of racemic 1,4-dihydro-2,6-dimethyl-5-methoxycarbonyl-4-(3-nitrophenyl)-3-pyridinecarboxylic acid. Competition binding experiments with radioligand [^{3}H]-(+)-PN200-110 and the block of whole cell barium currents through L-type calcium channels in GH_4C_1 cells show that the compounds with the eight-carbon alkyl linker optimally block the L-type Ca^{2+} channels, and that the *S*-enantiomer is more potent than the *R*-enantiomer.

Introduction

L-type Ca^{2+} channels play a crucial role in many physiologic functions, including muscle contraction, neurosecretion, etc. These heteromultimeric proteins are comprised of α_1, β, and $\alpha_2\delta$ subunits: additionally, skeletal muscle L-type Ca^{2+} channels and some neuronal Ca^{2+} channels may uniquely express a γ subunit (for review, see ref 1). The pore forming α_1 subunit confers basic functionality to the channel and comprises four homologous domains (I, II, III, and IV) of six putative transmembrane segments (S1−S6) and two short segments SS1−SS2 between segment five and six. Segments S5 and S6 form the pore lining and intracellular mouth of the channel, and segments SS1−SS2 form the extracellular mouth of the pore. Three different gene products and their splice variants code for the α_1 subunit of the L-type calcium channel: these are the skeletal muscle type α_{1S},[2] the cardiac type α_{1C},[3] and the neuronal/neuroendocrine type α_{1D}.[4,5]

The L-type Ca^{2+} channel represents a complex pharmacophore with sites of interaction for drugs of three different chemical categories—1,4-dihydropyridines (DHP) (e.g., nifedipine), phenylalkylamines (e.g., verapamil), and the benzothiazepines (e.g., diltiazem).[6−11] These drugs are used in the treatment of cardiovascular disorders, including hypertension, arrhythmias, angina, and cerebral and peripheral vascular disorders. They are known to bind to three different, but allosterically linked, sites on the α_1 subunit of the calcium channel. Molecular characterization of the binding sites of these drugs is therefore of importance to understand their mode of action.

DHPs act both as L-type calcium channel activators and antagonists at the DHP receptor. The differentiation of the activity derives from the nature of the functional groups substituted on the 1,4-dihydropyridine moiety, the stereochemistry at C-4, and the membrane potential (voltage dependence of interaction). The location of the DHP binding site within the α_1 subunit of L-type calcium channel has been studied by three different approaches, including the use of charged DHP analogues,[12−14] photoaffinity labeling studies,[15,16] and molecular biology approaches with chimeric channels and site directed mutagenesis. Different approaches have, however, localized several different regions on the channel as sites for DHP binding. Studies using chimeric channels reveal the determinants of DHP binding in the domain IIIS6 and in domain IV between segments IVS5 and IVS6 and in segment IVS6.[17−19] Site-directed mutagenesis studies have also identified several amino acid residues that are critical for DHP binding.[20] These studies indicate that a concerted allosteric action at different residues is important for DHP binding.

The DHP binding site in the cardiac L-type calcium channel has also been characterized using permanently charged 1,4-dihydropyridine probes with the DHP pharmacophore linked to a permanently charged quaternary ammonium ion through a polymethylene chain of variable length. The charged quaternary ammonium ion anchors either at the cell surface or at a negatively charged residue in the channel in the aqueous environment and permits the 1,4-DHP moiety to diffuse into

* Corresponding author address: Office of the Provost, 562 Capen Hall, State University of NewYork at Buffalo, Buffalo, NY 14260.
‡ Current address: Rammelkamp Center for Education and Research, Room R303, 2500 Metro Health Drive, Cleveland, OH 44109.
† Current address: Cambridge Neuroscience, Inc., One Kendall Square, Bldg. #700, Cambridge, MA 02139.
# These individuals contributed dominantly and equally to this work.

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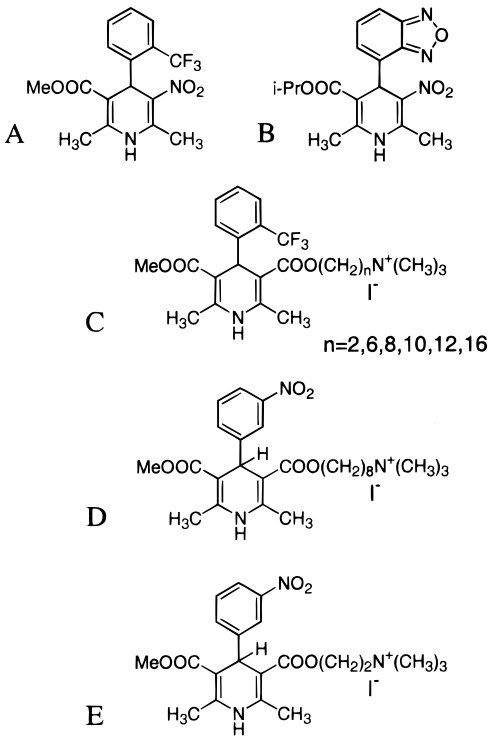

Figure 1. Structures of 1,4-dihydropyridines (1,4 DHP). (A) Bay K 8644, (B) PN 202-791, (C) racemic permanently charged 1,4 DHP analogues used in earlier experiments, (D and E) optically pure permanently charged 1,4 DHP enantiomers used in the present study.

the lipophilic lipid bilayer to an extent depending on the length of the polymethylene linker. With compounds having tether lengths of 2−16 carbon atoms, the eight-carbon tether had optimal activity.[12] Patch clamp studies in guinea pig ventricular myocytes revealed that these compounds access the binding site from the extracellular surface and inhibit the channel activity depending on the length of the alkyl tether.[13] These studies suggest the DHP binding site to be 11−14 Å from the extracellular surface of the membrane.[12] Neutral DHPs show access to the binding site that is essentially independent of the length of the carbon tether. The activity of charged and neutral DHPs on cardiac α_{1C} channel, and various chimeric channels having mutations in the putative DHP binding regions has been reported recently.[14] All of these earlier studies were, however, carried out with racemic nifedipine analogues on cardiac L-type calcium channels only. These did not address the effects of permanently charged DHPs on other L-type calcium channels, nor did they address the stereoselectivity of interaction.

Stereoselectivity plays an important role in determining the activity of a number of drugs including DHPs.[8,11,21,22] On the basis of the stereochemistry at C-4, DHPs can exhibit both qualitative and quantitative differences in activity. Drugs such as BAY K-8644 [Figure 1A] exhibit qualitative differences in activity; one enantiomer is an activator of the channel and the other is an antagonist.[23−25] The stereoisomers of some DHPs such as PN 202-791, on the other hand, show a more conventional quantitative difference in activity (100-fold difference in antagonist potencies).[24,26−28] It is therefore of interest to examine the stereoselectivity of permanently charged DHPs at the binding site.

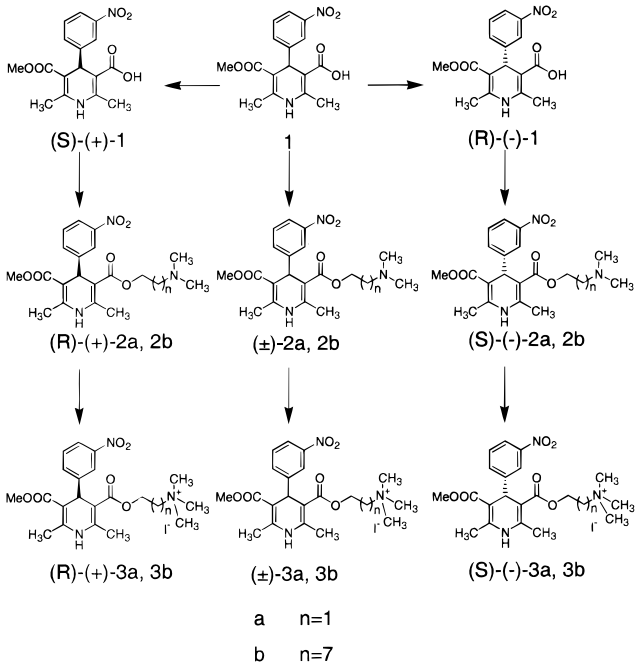

Figure 2. Scheme of synthesis of optically pure permanently charged 1,4 DHP enantiomers used in the present study.

The present study reports the synthesis of single enantiomers of permanently charged DHPs. Using these as probes we (a) verified the depth of DHP binding site in the α_{1D} subunit of L-type calcium channel in pituitary GH_4C_1 cells and membranes, the α_{1C}-type calcium channels in heart membranes, and the neuronal α_{1D}- and α_{1C}-type channels in brain membranes; and (b) investigated the stereoselectivity of 1,4-DHP action at the binding site. The permanently charged 1,4-DHPs [Figure 1] were synthesized as pure enantiomers and were evaluated for their effect on L-type calcium channels in GH_4C_1 cells, cardiac tissue, and neural tissue. Clonal pituitary GH_4C_1 cells exhibit both L- and T-type calcium channels,[29,30] heart expresses cardiac α_{1C}-type calcium channels, and brain expresses two L-type calcium channels, namely the α_{1D} and neuronal α_{1C}.[31]

Results

Chemistry: Synthesis of the Permanently Charged Racemic and Enantiomeric Dihydropyridines. Synthesis of the permanently charged racemic and enantiomeric nifedipine analogues with a variable carbon chain alkyl tether between the quaternary ammonium ion and the DHP is illustrated in the scheme shown in [Figure 2]. It involves the preparation of the key intermediate, dihydropyridinecarboxylic acid (**1**),[32] which was esterified through the acid chloride with dimethylaminoethanol and *ω*-dimethylaminooctanol to give the corresponding esters **(±) 2a** and **2b**. Subsequent treatment with methyl iodide gave the final racemic quarternized salts **(±) 3a** and **3b**.

Synthesis of chiral compounds of the DHP class can be achieved either through an asymmetric synthesis[33] or through the classical method of resolution of racemic mixtures. Optically active 1,4-dihydropyridine derivatives have been synthesized from the enantiomers of the 1-ethoxymethylated carboxylic acid followed by removal of the protecting group.[34−36] The absolute configurations of the carboxylic acid derivatives have been previously

determined by X-ray crystallography.[34,37] It has been established that (+)- and (−)- have *S*- and *R*-configurations at C-4, respectively. We chose the resolution protocol of the unprotected racemic acid **1**[32,38] using quinidine and cinchonidine as chiral bases to give the carboxylic acids **(*S*)-(+)-1** and **(*R*)-(−)-1** respectively. The resulting enantiomeric carboxylic acids **(*S*)-(+)-1** and **(*R*)-(−)-1** were separately esterified with both dimethylaminoethanol and (*ω*-dimethylamionooctanol) via the acid chlorides. Subsequent quarternization of the chiral esters with methyl iodide led to the chiral permanently charged compounds.

Biochemical Experiments. Charged DHPs were evaluated for their ability to compete with [^{3}H]-(+)-PN200-110 in membrane binding assays and in whole cell binding assays in normal resting and depolarized conditions. These compounds were also evaluated for their ability to block L-type calcium channels in electrophysiological assays.

Binding Studies: Competition Binding of [^{3}H]-(+)-PN200-110 with Permanently Charged 1,4-Dihydropyridine Antagonists (DHP) in Tissue Membranes. Specific binding of [^{3}H]-(+)-PN200-110 in rat cerebral cortex membranes [Figure 3A], heart membranes [Figure 3B], and in GH_4C_1 membranes [Figure 3C] was inhibited competitively by 1,4-dihydropyridine antagonists. The K_I values and the pseudo Hill coefficients are presented in Table 1. The affinity of charged DHP antagonists for the receptor depends on the length of the alkyl linker and is greater for the eight-carbon alkyl linker relative to the two-carbon alkyl linker by a 15−170-fold factor depending on the preparation and the stereochemistry of the DHP. These results indicate the higher accessibility of the compounds with the longer chain length to the binding site as opposed to compounds with chain length of two carbons. These observations parallel those made previously for binding of charged DHP analogues to ventricular myocytes.[12] The binding affinity also exhibits stereoselectivity and the **(4*S*)(+)3b** enantiomer is more potent than the **(4*R*)-(−)3b** enantiomer in all the tissues with a ratio of 37 in GH_4C_1 membranes, 29.2 in cerebral cortex membranes, and 62.2 in heart membranes. Similarly, the ratios of DHP **(4*S*)-(+)3a** and DHP **(4*R*)-(−)3a** were 24.8 times in GH_4C_1 membranes, 11.2 in cerebral cortex membranes, and 13.7 in heart membranes. The potency of the racemic compound lies between the *S*- and the *R*-enantiomers and is closer to the *S*-enantiomer. The DHP **(4*S*)-(+)3b** had the highest affinity for the heart membranes followed by GH_4C_1 membranes and cerebral cortex membranes. These differences are statistically significant at $p = 0.05$ in one-way Anova test. Even though the DHP **(4*R*)-(−)3b** seems to have the highest affinity for GH_4C_1 membranes followed by the heart membranes and cerebral cortex membranes, the results are not statistically significant. Figure 3D gives a schematic representation of the dissociation constants for the binding of the charged DHPs to GH_4C_1 membranes, rat cerebral cortex membranes, and rat heart membranes. It shows an approximate 15−170-fold difference between the dissociation constants of DHPs with eight- and two-carbon linkers depending on the preparation, and an approximate 10−60-fold difference between the *S*- and *R*-enantiomers. The lower limits of the ratios (15 and 10, respectively) may be obscured by the fact that the K_I values of **(4*R*)-(−)3a** are extrapolated values as the IC_{50} value was very high and could not be reached in the actual experiment.

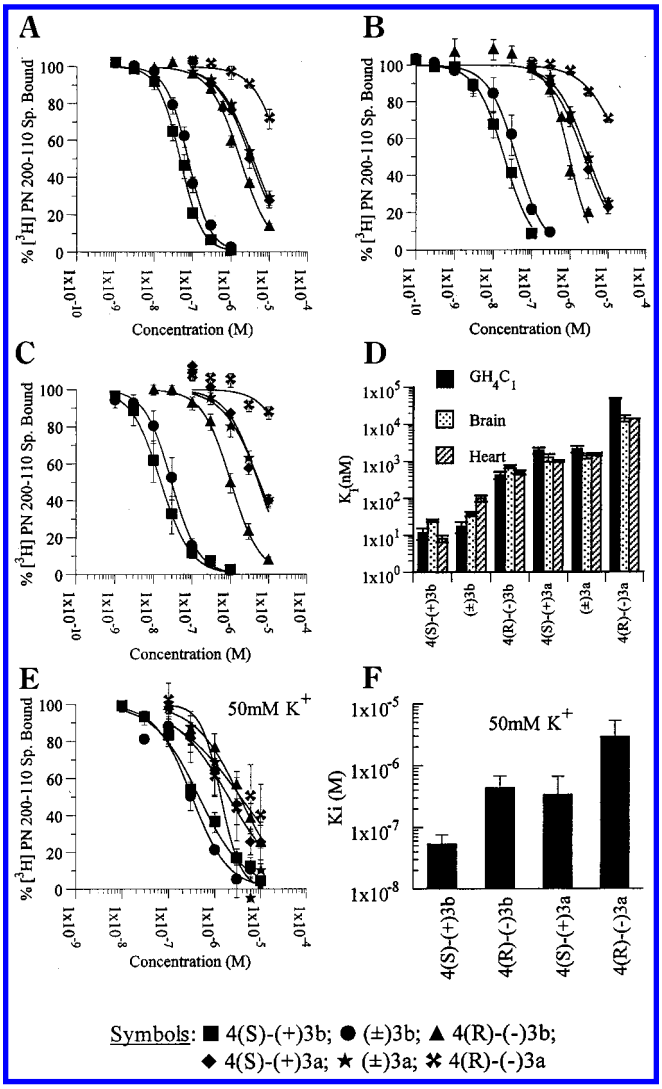

Figure 3. Competitive inhibition of [^{3}H]-(+)-PN200-110 binding by permanently charged 1,4-dihydropyridine analogues (A) to rat cerebral cortex membranes, (B) to rat heart membranes, (C) to GH_4C_1 membranes, and (E) to intact GH_4C_1 cells under depolarized condition (50 mM K^+). (D) Comparison of dissociation constants (K_I) for the charged DHP binding to the three different membranes. (E) Comparison of dissociation constants (K_I) for the charged DHP binding to intact GH_4C_1 cells under depolarized condition (50 mM K^+). ($n = 4-6$ experiments, error bars represent SEM).

Competition Binding of [^{3}H]-(+)-PN200-110 with Permanently Charged 1,4-Dihydropyridine (DHP) Antagonists in Cultured GH_4C_1 Cells under Depolarized (50 mM K^+) and Resting Conditions (5.8 mM K^+). Competition binding of [^{3}H]-(+)-PN200-110 by permanently charged 1,4-dihydropyridine antagonists in GH_4C_1 cells was carried out in 5.8 mM K^+ and 50 mM K^+ buffer to determine the binding properties under resting and depolarized conditions. The K_I values and the pseudo Hill coefficients are presented in Table 2. As was described for membrane binding, the affinity of the charged DHPs used in the experiment depends on the length of the alkyl linker and the stereochemistry

Table 1. Comparative Analysis of Inhibition of [^{3}H]-(+)-PN200-110 Binding to Different Membrane Proteins by Permanently Charged 1,4-Dihydropyridine Analogues

	GH_4C_1		cerebral cortex		heart	
drug	K_I (nM)	n_H	K_I (nM)	n_H	K_I (nM)	n_H
(4*S*)-(+)3b	11.4 ± 3.9	1.3 ± 0.2	24.6 ± 2.0	1.6 ± 0.2	8.0 ± 1.7	1.2 ± 0.1
(4*R*)-(−)3b	420 ± 93	1.2 ± 0.0	717.8 ± 75.4	1.0 ± 0.0	497 ± 63.4	1.1 ± 0.0
(±)3b	16.8 ± 5.7	1.5 ± 0.1	37.2 ± 5.3	1.4 ± 0.1	99.2 ± 19.2	1.1 ± 0.2
(4*S*)-(+)3a	1940 ± 373	0.9 ± 0.1	1246 ± 293	0.9 ± 0.1	999 ± 59	1.0 ± 0.1
(4*R*)-(−)3a	48120 ± 1485	0.6 ± 0.1	14013 ± 2743	0.8 ± 0.0	13720 ± 95.5	0.9 ± 0.1
(±)3a	2111 ± 435	0.9 ± 0.1	1372 ± 226	1.0 ± 0.1	1512 ± 139	1.0 ± 0.1

Table 2. Comparative Analysis of Inhibition of [^{3}H]-(+)-PN200-110 Binding to GH_4C_1 Cells under Depolarized Conditions by Permanently Charged 1,4-Dihydropyridine Analogues[a]

	50 K^+	
drug	K_I (nM)	n_H
(4*S*)-(+)3b	129.2 ± 27.8	1.1 ± 0.1
(4*R*)-(−)3b	895 ± 336	1.0 ± 0.1
(±)3b	68.6 ± 3.6	1.3 ± 0.1
(4*S*)-(+)3a	651 ± 343	0.9 ± 0.2
(4*R*)-(−)3a	3988 ± 1752	0.5 ± 0.0
(±)3a	248.5 ± 50.5	1.7 ± 0.7

[a] The compounds had a very low affinity under resting conditions and no significant dose-dependent inhibition was observed. However, there was significant inhibition at a concentration of 10 μM.

at C-4. The *S*-enantiomer has a higher affinity than the *R*-enantiomer. Under depolarized conditions the DHP **(4*S*)-(+)3b** was 5 times more potent than the DHP **(4*S*)-(+)3a** and 6.9 times more potent than DHP **(4*R*)-(−)-3b** in inhibiting [^{3}H]-(+)-PN200-110 binding to intact cells [Figure 3E]. This shows the requirement for optimal access to the binding site reflected by the length of the linker, as well as stereochemistry at C-4, to be important determinants in the binding of DHPs to the site on receptor. The compounds had a very low affinity under resting conditions, and no dose response curves could be constructed in 5.8 mM K^+ buffer. However, under resting conditions (5.8 K^+), a concentration of 10^{-5} M of the DHP with the eight-carbon linker showed a significant inhibition of [^{3}H]-(+)-PN200-110 binding.

Electrophysiologic Studies: Inhibition of Barium Currents in Cultured GH_4C_1 Cells by Permanently Charged 1,4-Dihydropyridine Antagonists (DHP). The permanently charged DHPs were also evaluated for their functional activity in an electrophysiological assay. The currents were elicited by either a 200 or 50 ms test pulse to +10 mV from a holding potential of −40 mV, and instantaneous dose response curves (DRC) of the DHP effects were evaluated after a brief equilibration of the cell with a given dose of DHP for 30 s. Longer equilibration times were not used to avoid the interference in response due to channel run down. Figure 4A shows the effect of increasing concentrations of charged DHPs **(4*S*)-(+)3b**, **(4*R*)-(−)3b**, and **(±)3b**, respectively, on the barium currents in GH_4C_1 cells. Figure 4B shows the effect of increasing concentrations of charged DHPs **(4*S*)-(+)3a**, **(4*R*)-(−)3a**, and **(±)3a**, respectively, on the barium currents. The DHP with the eight-carbon alkyl linker is a more potent inhibitor of the channel activity than the DHP with the two-carbon linker. Hill coefficient signifies the stoichiometry of ligand receptor interaction in both binding experiments as well as functional studies. $n = 1$ indicates a 1:1 interaction under equilibrium conditions. The compounds **(4*S*)-(+)-3b**, **(4*R*)-(−)3b**, and **(±)3b** having an eight-carbon linker exhibit a Hill coefficients close to 1, indicating optimal 1:1 interaction at the DHP binding site. Shallow numbers of less than 1 for compounds of **(4*S*)-(+)3a**, **(4*R*)-(−)3a**, **(±)3a** indicate that the small tether length of two carbons is preventing these compounds from establishing an optimal interaction at the binding site. It is also evident that both the *S* and *R* charged DHP enantiomers act as neuronal/neuroendocrine L-type calcium channel antagonists. However, the **(4*S*)-(+)3b** enantiomer is 3.6 times more potent than the **(4*R*)-(−)3b** enantiomer. The relative potencies of these charged DHP antagonists on barium currents in GH_4C_1 cells are depicted in Table 3. The racemic compounds seemed to be slightly more potent than the *S*-enantiomer, but the difference is not statistically significant. The block was rapid, and no use dependence of block

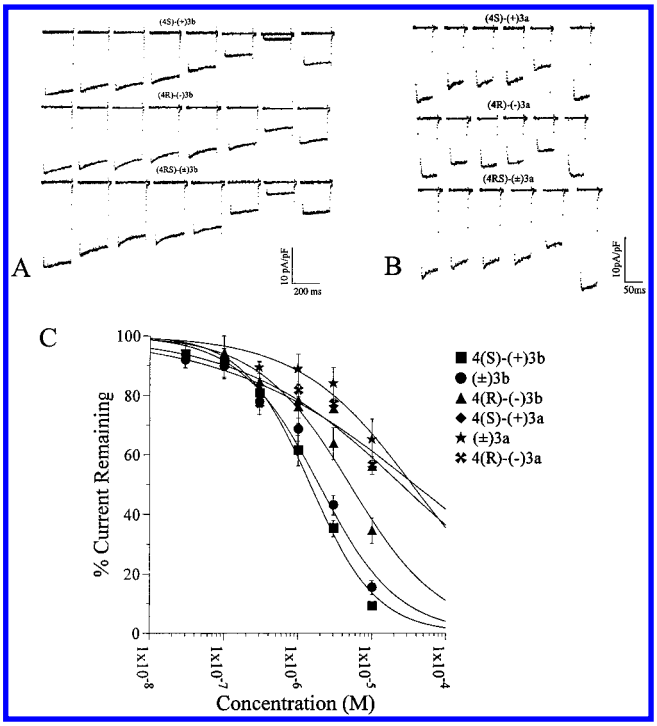

Figure 4. Inhibition of barium currents through L-type calcium channels in GH_4C_1 cells by permanently charged 1,4-dihydropyridine analogues. Currents were elicited by depolarizing pulse to +10 mV from a holding potential of −40 mV. The cell was equilibrated with different concentrations of DHP for 30 s before the test pulse was applied. DHPs (0, 0.03, 0.1, 0.3, 1, 3 and 10 μM) with an eight-carbon linker (A) and DHPs (0, 0.1, 1, 3 and 10 μM) with a two-carbon linker (B) were used in the experiment. The last trace in both (A) and (B) represents the recovery of currents after DHP wash off in external saline at −80 mV. The recordings represent the average data from 6 to 7 cells. (C) The dose response curves for charged DHP inhibition of barium currents. Currents were normalized to the current before the application of drug.

Table 3. Comparative Analysis of Inhibition of Barium Currents in GH_4C_1 Cells by Permanently Charged 1,4-Dihydropyridine Analogues

drug	IC_{50} (μM)	n_H
(4*S*)-(+)3b	1.4 ± 0.3	1.0 ± 0.1
(4*R*)-(−)3b	5.1 ± 1.3	0.8 ± 0.2
(±)3b	1.5 ± 0.3	0.9 ± 0.1
(4*S*)-(+)3a	28.1 ± 4.4	0.4 ± 0.0
(4*R*)-(−)3a	270.9 ± 188.3	0.3 ± 0.0
(±)3a	17.7 ± 3.9	0.7 ± 0.2

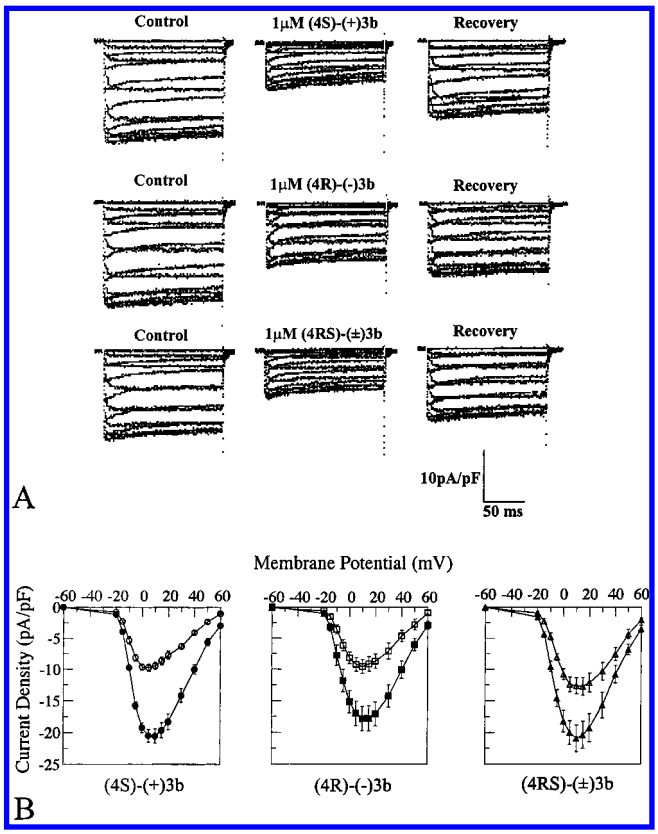

Figure 5. 5. Inhibition of barium currents through L-type calcium channels in GH_4C_1 cells. I−V plots of currents elicited by 200 ms depolarizing steps from a holding potential of −40 mV in the presence and absence of permanently charged 1,4-DHP with an eight-carbon alkyl linker. Control represents currents before drug application. The cells were briefly equilibrated with 1 μM DHP for 30 s, and I−V responses were measured in the presence of DHP. Recovery represents the currents elicited from −40 mV after the drug wash out for 1 min at −80 mV. The current traces as well as the I−V plot represent averages of data from 4 to 9 cells. (A) represents the current traces and (B) represents the I−V plots (filled symbols indicate controls and open symbols indicate drug effect).

was observed. The block of the pituitary L-type calcium channels by these charged DHPs is reversible, and the drug is partially washed out when the cells are held at a hyperpolarizing potential of −80 mV in a stream of drug free external solution. This is unlike the effect of charged DHPs on the cardiac L-type calcium channels, where the block was irreversible.[13] Current−voltage relationships (I−V plots) in the presence of 1 μM of the DHPs with the eight-carbon linker (Figure 5A and B) also showed a decrease in the barium currents similar to the decrease seen in the dose response curve. These DHPs inhibit the barium currents without significantly altering the kinetics of the currents. The stereoselectivity of action is observed as a decrease in the potency of the **(4*R*)-(−)3b** enantiomer as compared with that of the **(4*S*)-(+)3b** enantiomer. I−V experiments were not done for DHPs with the two-carbon alkyl linker because they did not show a significant effect on the currents at 1 μM concentration.

The results from electrophysiology experiments parallel the effects observed in 50 K^+ binding experiments. The compounds with an eight-carbon linker were more potent than the compounds with the two-carbon linker and the *S*-enantiomer is more potent than the *R*-enantiomer.

Discussion

This study brings forth two important conclusions. Our data show that the DHP binding domain is in the membrane and is accessed optimally by charged DHPs that have an eight-carbon alkyl linker between the quaternary ammonium ion and the DHP, but not by DHPs that have a two-carbon alkyl linker. This is in agreement with previous binding[12] and electrophysiological studies[13] and supports the hypothesis that DHPs access the binding site from the extracellular side.[39,40] Our study further strengthens the conclusion that the DHP binding domain is located within the membrane, 11−14 Å from the anchor point of the quaternary trimethylammonium headgroup. The second conclusion from our study is that the stereochemistry at C-4 plays an important role in determining the potency of the charged DHP analogues that were used to characterize the DHP binding site. The *S*-enantiomer is more potent than the *R*-enantiomer. This is in agreement with literature reports on studies done with PN-200-110 and other 1,4-DHP antagonists.[27] However, qualitative differences in terms of antagonist−activator transitions, as seen with BAY K-8644,[23,26] are not seen in these charged DHPs, and both enantiomers are channel antagonists. Our results show also that the charged DHP enantiomers used as probes to characterize the binding site exhibit differences in tissue selectivity. Binding studies on membranes show that the DHPs used in this study had the highest affinity for the cardiac α_{1C}-type channels followed by the α_{1D}-type pituitary channels and the neuronal α_{1D} and α_{1C} channels [Figure 3D]. The charged DHP probes used in the study bind to intact GH_4C_1 cells under depolarized conditions with high affinity, but not to cells under resting conditions, indicating that they exhibit voltage-dependent block, although we did not quantify the difference in affinities in polarized and depolarized preparations. The difference in the potency of DHPs between the GH_4C_1 membranes and intact GH_4C_1 cells under depolarized conditions is probably due to several factors, including the loss of any biochemical control (e.g., phosphorylation) in the isolated membrane preparations. It could also be due to differences in the states of the channel in the membrane preparation and in the intact cells under depolarized conditions. The membrane preparation represents 0 mV membrane potential at which the channels are more inactivated than in the intact GH_4C_1 cells under depolarized condition where the membrane potential is between −15 and −25 mV.

Several studies have identified a total of nine L-type specific and five conserved amino acid residues that define the DHP binding site in the L-type calcium

channel.[18,41] These are located in the transmembrane regions IIIS5 (2 L-type residues), IIIS6 (3 L-type residues), and IVS6 (4 L-type residues). The effects of racemic charged and neutral DHPs were previously characterized on cardiac α_{1Cb} chimeric channels.[14] It was shown that mutations in IVS6 (Tyr 1485Ile, Met1486Phe, Ile1493Leu) lead to a marked decrease in the effects of neutral DHPs, but not of charged DHPs. Similarly, mutations in IIIS6 (Ile1175Phe, Ile1178Phe, Met1183Val) lead to the loss of neutral DHP activity. The (Thr1061Tyr) point mutation in IIIS5 leads to loss of neutral as well as charged DHP interaction with receptor under resting conditions, but only the charged DHP shows a remarkably higher potency under depolarized conditions. Most of these residues are located within 4−5 turns of the α helical structure. Substitution of these nine amino acids into the DHP insensitive P/Q-type α_{1A} subunit, confers DHP sensitivity.[42] Neutralization of the pore region glutamate residues of the channels abolished the spacer-chain length dependence of charged DHPs, suggesting that the quaternary ammonium ion might anchor at negatively charged glutamate residues in the pore.[43]

In concert with previous studies, the present investigation therefore suggests that the DHP binding domain is in the membrane, at a distance of approximately $^1/_3$ to $^2/_3$ the membrane thickness from the extracellular surface. The spacer-chain of eight carbons (11−14 Å) from the point of anchor of charged quaternary ammonium ion provides optimal block. The stereochemistry at C-4 is critical to provide the right conformation for optimal interaction of the charged DHP and the receptor.

Experimental Section

Chemistry. Melting points were all uncorrected and were determined on a Mel-Temp apparatus. ^{1}H NMR spectra were recorded on a Varian Gemini 300 MHz instrument using TMS as internal standard. Elemental analysis was obtained from Atlantic Microlab, Norcross, GA. Optical rotations were measured on a Perkin-Elmer 141 polarimeter using a sodium vapor lamp.

The optical purity of the series of quaternized compounds were determined by ^{1}H NMR by the addition of the chiral shift reagent, tris-[3-(heptafluoropropylhydroxymethylene)-(+)camphorato] europium(III) [(+)-Eu(hfc)$_3$]. In the case of compounds with dimethylaminooctyl side chain, **(4*S*)-(+)-3b** and **(4*R*)-(−)-3b**, the NMR experiments were carried out in $CDCl_3$; and for those with dimethylaminoethyl side chain, **(4*S*)-(+)-3a** and **(4*R*)-(−)-3a**, the optical purity estimation was carried out in CD_3CN. With **(4*S*)-(+)-3b** and **(4*R*)-(−)-3b**, ^{1}H NMR was carried out by addition of 20 μL portions of a solution (100 mg in 1 mL of $CDCl_3$) of the chiral shift reagent to a solution of **(±)-3b** (7 mg in 0.5 mL of $CDCl_3$). The 1,4-dihydropyridine C-2 and C-6 methyl resonances at δ 2.39 and 2.44 were each split into two resonances which appeared at δ 2.56, 2.58 and 2.65, 2.67, respectively. Addition of (+)-Eu(hfc)$_3$ to **(4*S*)-(+)-3b** or **(4*R*)-(−)-3b**, as described above, resulted in single resonances for the C-2 and C-6 methyl groups (ee > 96%). With **(4*S*)-(+)-3a** and **(4*R*)-(−)-3a**, ^{1}H NMR measurements were carried out by addition of 50 μL portions of a solution (100 mg in 1 mL of CD_3CN and filtering the insolubles) of the chiral shift reagent to a solution of **(±)-3a** (5 mg in 0.5 mL of CD_3CN). The 1,4-dihydropyridine C2-methyl resonance at δ 2.4 was split into two resonances which appeared at δ 2.48 and 2.62. Addition of (+)-Eu(hfc)$_3$ to **(4*S*)-(+)-3a** or **(4*R*)-(−)-3a** as described above, resulted in single resonances for the C-2 and C-6 methyl groups (ee > 96%).

The [α] values of 3a enantiomers **(4*S*)-(+)3a** and **(4*R*)-(−)-3a** are in quite good agreement as expected (+72.37 and −71.1, respectively). However, the [α] values of **3b** enantiomers **(4*S*)-(+)3b** and **(4*R*)-(−)-3b** are quite different (+3.8 and −7.77, respectively) though the chiral shift reagent addition NMR experiment indicated the purity to be >96% ee. The difference in optical rotation values for the enantiomers may be due to the accidentally trapped solvents, and this may indeed be the reason for the disagreement of the combustion analysis values.

Preparation of 8-(Dimethylamino)-1-octanol Hydrobromide. 8-Dimethylamino-1-octanol hydrobromide was prepared from 8-bromooctanol and aqueous dimethylamine following the reported procedure.[12]

Preparation of 1,4-Dihydro-5-methoxycarbonyl-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylic Acid (1). Racemic 1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridinecarboxylic acid and the enantiomers were prepared following the previously reported literature procedure.[32]

Esterification of Optically Pure and Racemic DHP Carboxylic Acids: (4*S*)-Dimethylaminoethyl Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (2a). A suspension of (4*R*)-(−)-acid, **1** (400 mg, 1.20 mmol) in CH_2Cl_2 (20 mL), and DMF (2 mL) was cooled in an ice bath to 5−10 °C, and oxalyl chloride (0.14 mL, 1.66 mmol) was added and stirred for 2−3 h. To this acid chloride was added dimethylaminoethanol (0.12 mL, 0.12 mmol) as a solution in CH_2Cl_2. The reaction mixture was stirred overnight, diluted with CH_2Cl_2 (10 mL), washed with saturated $NaHCO_3$ solution (10 mL) and brine, dried, and concentrated to give the crude product. Purification by column chromatography using initially CH_2Cl_2 and grading to CH_2Cl_2 containing 5% MeOH gave the ester, **(4S)-2a** 336 mg, 69%; mp 114−117 °C; ^{1}H NMR ($CDCl_3$) δ 2.21 (s, 6 H), 2.35 (s, 6 H), 2.50 (t, 2 H), 3.62 (s, 3 H), 4.12 (t, 2 H), 5.09 (s, 1 H), 5.8 (br. s, 1 H), 7.31−7.39 (m, 1 H), 7.63 (dd, 1 H), 7.98 (dd, 1 H), 8.12 (s, 1 H).

(4*S*)-(ω-Dimethylaminooctyl) Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (2b). Ester **(4S)-2b** was prepared by the esterification of **(4*R*)-(−)** acid **1**, via the acid chloride using 8-(dimethylamino)-1-octanol hydrobromide instead of dimethylaminoethanol in the above procedure. Yield 39%; ^{1}H-NMR ($CDCl_3$) δ 1.15−1.30 (m, 8 H), 1.3−1.5 (m, 2 H), 1.5−1.65 (m, 2 H), 2.15−2.25 (m, 9 H), 2.35−2.45 (m, 5 H), 3.6 (s, 3 H), 3.9−4.1 (m, 2 H), 5.05 (s, 1 H), 6.15 (s, 1 H), 7.3−7.45 (m, 1 H), 7.65 (dd, 1 H), 8.0 (dd, 1 H), 8.1 (s, 1 H).

(4*R*)-Dimethylaminoethyl Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (2a). Yield 68%; mp 124−126 °C; ^{1}H-NMR ($CDCl_3$) δ 2.22 (s, 6 H), 2.34 (s, 6 H), 2.53 (t, 2 H), 3.63 (s, 3 H), 4.12 (t, 2 H), 5.09 (s, 1 H), 6.15 (s, 1 H), 7.33−7.39 (m, 1 H), 7.64 (dd, 1 H), 7.96 (dd, 1 H), 8.09 (s, 1 H).

(4*R*)-(ω-Dimethylaminooctyl) Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (2b). Yield 37%; ^{1}H-NMR ($CDCl_3$) δ 1.23−1.45 (m, 8 H), 1.5−1.85 (m, 4 H), 2.28 (2s, 6 H), 2.45−2.75 (m, 8 H), 3.63 (s, 3 H), 3.95−4.2 (m, 2 H), 5.07 (s, 1 H), 6.48 (s, 1 H), 7.35−7.55 (m, 1 H), 7.62 (dd, 1 H), 7.97 (dd, 1 H), 8.09 (s, 1 H).

(±)-Dimethylaminoethyl Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (2a). Yield 52%; ^{1}H-NMR ($CDCl_3$) δ 2.22 (s, 6 H), 2.43 (s, 6 H), 2.53 (t, 2 H), 3.63 (s, 3 H), 4.15 (t, 2 H), 5.12 (s, 1 H), 5.9 (s, 1 H), 7.32−7.42 (m, 1 H), 7.65 (dd, 1 H), 8.0 (dd, 1 H), 8.1 (s, 1 H).

(±)−(ω-Dimethylaminooctyl) methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate (2b). Yield 65%; ^{1}H-NMR ($CDCl_3$) δ 1.05−1.4 (m, 8 H), 1.4−1.6 (m, 2 H), 1.65−1.8 (m, 2 H), 2.15−2.28 (m, 8 H), 2.35 (2s, 6 H), 3.65 (s, 3 H), 4.0 (m, 2 H), 5.1 (s, 1 H), 6.2 (br. s, 1 H), 7.3−7.42 (m, 1 H), 7.65 (dd, 1 H), 7.95 (dd, 1 H), 8.1 (s, 1 H).

Preparation of Quaternized Salts: (4*S*)-(+)-Dimethylaminoethyl Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate Methiodide (3a). To a solution of the ester **(4S)-2a** (336 mg, 0.833 mmol) in acetone (5 mL) was added methyl iodide (1 mL) while stirring for 2 h.

The product separated out from the initially clear reaction mixture solution. Filtration of the solid and washing with cold acetone (5 mL) gave the product, **(4*S*)-(+)-3a,** 331 mg; Yield 73%; mp 235−238 °C (dec); ^{1}H NMR (CH_3OH-d_4) δ 2.27 (s, 3 H), 2.37 (s, 3 H), 3.08 (s, 9 H), 3.66 (s, 3 H), 3.68 (t, 2 H), 4.50 (t, 2 H), 5.06 (s, 1 H), 7.42−7.48 (m, 1 H), 7.66 (dd, 1 H), 7.98 (dd, 1 H), 8.05 (s, 1 H) $[\alpha]_D^{22} = +72.37$ (c = 0.7, MeOH); Anal. ($C_{21}H_{28}IN_3O_6$) C, H, N.

(4*S*)-(+)-(*ω*-Dimethylaminooctyl) methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate methiodide (3b). Yield 70%; mp 72−76 °C; ^{1}H NMR ($CDCl_3$) δ 1.15−1.4 (m, 8 H), 1.45−1.65 (m, 2 H), 1.7−1.85 (m, 2 H), 2.40 (s, 3 H), 2.43 (s, 3 H), 3.52 (s, 9 H), 3.55−3.75 (s & m, 5 H), 3.98 (m, 1 H), 4.08 (m, 1 H), 5.09 (s, 1 H), 6.43 (s, 1 H), 7.35−7.45 (m, 1 H), 7.65 (dd, 1 H), 7.98 (dd, 1 H), 8.14 (s, 1 H); $[\alpha]_D^{22} = +3.8$ (c = 0.2, MeOH); Anal. ($C_{27}H_{40}IN_3O_6$ 3.75 H_2O) C, N; H: calcd. 6.82; found 5.98.

(4*R*)-(−)-Dimethylaminoethyl Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate Methiodide (3a). Yield 96%; mp 234−236 °C (dec); ^{1}H NMR (CH_3OH-d_4) δ 2.31 (s, 3 H), 2.41 (s, 3 H), 3.13 (s, 9 H), 3.65 (s, 3 H), 3.73 (t, 2 H), 4.51 (t, 2 H), 5.09 (s, 1 H), 7.48−7.53 (m, 1 H), 7.69 (dd, 1 H), 8.02 (dd, 1 H), 8.09 (s, 1 H); $[\alpha]_D^{22} = -71.1$ (c = 0.5, MeOH); Anal. $C_{21}H_{28}IN_3O_6$ C, H, N.

(4*R*)-(−)-(*ω*-Dimethylaminooctyl) Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate Methiodide (3b). Yield 50%; mp 74−77 °C; ^{1}H NMR ($CDCl_3$) δ 1.17−1.38 (m, 8 H), 1.53−1.63 (m, 2 H), 1.75−1.85 (m, 2 H), 2.39 (s, 3 H), 2.42 (s, 3 H), 3.45 (s, 9 H), 3.55−3.7 (m, 5 H), 3.98 (m, 1 H), 4.08 (m, 1 H), 5.08 (s, 1 H), 6.49 (s, 1 H), 7.35−7.45 (m, 1 H), 7.65 (dd, 1 H), 7.98 (dd, 1 H), 8.15 (s, 1 H); $[\alpha]_D^{22} = -7.77$ (c = 0.26, MeOH); Anal. ($C_{27}H_{40}IN_3O_6$ $2H_2O$) C, N; H: calcd. 6.66; found 6.14.

(±)-Dimethylaminoethyl Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate Methiodide (3a). Yield 63%; mp 212−216 °C; ^{1}H NMR (DMSO-d_6) δ 2.26 (s, 3 H), 2.32 (s, 3 H), 2.99 (s, 9 H), 3.56 (s & t, 5 H), 4.40 (t, 2 H), 4.98 (s, 1 H), 7.54−7.60 (m, 1 H), 7.62 (dd, 1 H), 7.95 (s, 1 H), 7.99 (dd, 1 H), 9.21 (s, 1 H); Anal. ($C_{21}H_{28}IN_3O_6$) C, H, N.

(±)−(*ω*-Dimethylaminooctyl) Methyl 1,4-Dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridinedicarboxylate Methiodide (3b). Yield 77%; mp 73−77 °C; ^{1}H NMR ($CDCl_3$) δ 1.23−1.40 (m, 8 H), 1.5−1.62 (m, 2 H), 1.70−1.95 (m, 2 H), 2.40−2.44 (2s, 6 H), 3.45 (s, 9 H), 3.60−3.75 (m, 5 H), 3.95 (m, 1 H), 4.08 (m, 1 H), 5.08 (s, 1 H), 6.44 (s, 1 H), 7.35−7.48 (m, 1 H), 7.64 (dd, 1 H), 7.98 (dd, 1 H), 8.12 (s, 1 H); Anal. ($C_{27}H_{40}IN_3O_6$ 3.75 H_2O) C, N; H: calcd. 6.82; found 6.12.

Biochemical Experiments: Materials. Radioactive isotope [^{3}H]-(+)-PN200-110 [isopropyl-4-(2,1,3-benzoxdiazole-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate] was purchased from Dupont-New England Nuclear, Boston, MA.

F-10 medium and fetal bovine serum were purchased from Sigma Chemical Co. (St. Louis, MO). Horse serum was purchased from either Sigma Chemical Co. or from GIBCO (Grand Island, NY).

Drugs. Nifedipine [2,6-dimethyl-3,5-dicarbomethoxy-4-(2-nitrophenyl)-1,4-dihydropyridine] analogues, with the ester side chain linked to a permanently charged quaternary ammonium ion through an alkyl linker chain of either eight or two carbons (**(4*S*)-(+)3b**, **(4*R*)-(−)3b**, **(±)3b**, **(4*S*)-(+)3a**, **(4*R*)-(−)3a** and **(±)3a**) were synthesized in our laboratory as described. All drugs were made up as 1 mM stock solutions in EtOH, and appropriate dilutions were made from these. Drugs were stored protected from light.

Methods: Cell Culture. The rat anterior pituitary cell line, GH_4C_1, was obtained from Bayer Inc., West Haven, CT (Dr. Jane Chisholm). Cells were maintained in a monolayer culture in Ham's F-10 media supplemented with 15% horse serum and 2.5% fetal bovine serum at 37 °C in a humidified incubator under an atmosphere containing 5% CO_2. Cells were removed from flasks once a week with 0.05% trypsin and were plated either into flasks or sterile (Corning) 35 mm Petri dishes for electrophysiology experiments. The medium was changed every 2 days. Cells 2−6 days in culture were used for experiments.

Solutions. Normal Tyrode's solution contained the following (in mM): NaCl (132.0), KCl (5.8), $MgCl_2 \cdot 6H_2O$ (1.2), $CaCl_2 \cdot 2H_2O$ (2.0), HEPES (10.0), and dextrose (5.0) (pH adjusted to 7.4 with NaOH). The external recording solution contained the following (in mM): *N*-methyl-D-glucamine (125.0), CsCl (5.0), HEPES (10.0), $MgCl_2 \cdot 6H_2O$ (1.0), dextrose (5.0), and $BaCl_2$ (20.0). A total of 105 mL of 1 N HCl was added per liter of solution (pH adjusted to 7.4 with CsOH). The internal recording solution contained the following (in mM): CsCl (60.0), $CaCl_2 \cdot 2H_2O$ (1.0), $MgCl_2 \cdot 6H_2O$ (1.0), HEPES (10.0), EGTA (11.0), aspartic acid (50.0), and Na_2ATP (5.0) (pH was adjusted to 7.4 with CsOH). Hank's buffer (5.8 K^+) contained the following (in mM): NaCl (127.0), KCl (5.36), $CaCl_2 \cdot 2H_2O$ (1.26), $MgCl_2 \cdot 6H_2O$ (0.98), KH_2PO_4 (0.44), $NaHCO_3$ (4.16), $NaH_2PO_4 \cdot 7H_2O$ (0.63), dextrose (5.56), and HEPES (20.0) (pH adjusted to 7.4 with NaOH). Hank's buffer (50 K^+) contained the following (in mM): NaCl (82.0), KCl (50.0), $CaCl_2 \cdot 2H_2O$ (1.26), $MgCl_2 \cdot 6H_2O$ (0.98), KH_2PO_4 (0.44), $NaHCO_3$ (4.16), $NaH_2PO_4 \cdot 7H_2O$ (0.63), dextrose (5.56), and HEPES (20.0 (pH adjusted to 7.4 with NaOH).

Electrophysiology: Electrodes were pulled from thin-walled borosilicate glass capillaries with an OD of 1.2 mm (TW-120, World Precision Instruments, Sarasota, FL). The pipets were pulled in two stages on a vertical electrode puller (David Kopf Instruments, Model 750, Tujunga, CA) and had a resistance of 8−10 MΩ when filled with internal recording solution.

Whole cell voltage-clamp experiments were carried out using an Axopatch 200 amplifier (Axon Instruments, Inc. Foster City, CA). Voltage commands were generated on a Macintosh IIci computer through a 12-bit digital-to-analog converter using a MacADIOS II/16 board (GW Instruments, Somerville, MA). Data were acquired after 16-bit analog-to-digital conversion. Further analysis was performed with a program written in the laboratory in Think-C (Symantec Corporation, Cupertino, CA). Test currents were digitally sampled every 500 μs, except during the examination of capacitive currents, when they were sampled every 100 μs. Currents were digitally corrected for linear leakage with respect to currents obtained at −60 mV from a holding potential of −40 mV. Current densities, expressed as pA/pF, were calculated by dividing the measured current with the cell capacitance to avoid differences in current amplitude arising from the differences in cell size. Currents were filtered at 5 kHz, with a Lowpass Bessel filter. All voltage-clamp traces represent average data from several different cells. To generate the current voltage profiles (I−V plots), current amplitudes at the peak current value were measured for each voltage command. All data are presented as mean values ± SEM.

Cells were rinsed with 1 mL of normal Tyrode's solution and bathed in 1.5−2.0 mL of the same solution until a cell was patched and the whole cell configuration established. The cell was then perfused with external recording solution locally by a gravity driven perfusion system. Drug solutions were made to a final concentration in recording solution from a 1 mM stock solution in ethanol. Whole cell barium currents were elicited by step depolarizations to different potentials from a holding potential of −40 mV. T-type channels present in these cells inactivate by holding at −40 mV, and the L-type channels can be studied selectively. The cell was allowed to equilibrate for 30 s with each concentration of drug (ranging from 0.01−10 μM in an increasing order), and currents were elicited at the end of each equilibration by either a 200 or a 50 ms test pulse to +10 mV from the holding potential of −40 mV. Capacitative currents settled rapidly within 2 ms in these cells, hence the first 3 ms of recording have been removed from the current traces for clarity.

Competition Binding of 1,4-Dihydropyridine Antagonists. Membrane Preparations. The affinities of the series of charged 1,4-dihydropyridine antagonists were determined in competition binding experiments using techniques in our

laboratory described previously.[15,16] Experiments were done on rat cerebral cortex membranes, rat heart membranes, and GH_4C_1 membranes, and protein was determined by Bradford's method.[44]

Binding in Membrane Preparations. Competition binding assays of calcium channel antagonists with [^{3}H]-(+)-PN200-110 were carried out by methods previously established in our laboratory.[45,46]

Binding in Intact Cells. Competition binding experiments were performed on intact GH_4C_1 cells attached to the culture dish under resting (5.8 mM K^+) as well as depolarizing (50 mM K^+) conditions in Hank's buffer (pH 7.4). These conditions were similar to those previously established by us for binding in intact cardiac cells.[28]

Data Analysis. Data analysis was done using an IBM personal computer with the program EBDA.[47] The concentration for half-maximal inhibition (IC_{50}) and the dissociation constant (K_I) for the 1,4-DHPs tested were calculated using the Hill equation

$$B/B_{max} = 1/(1+ [x/IC_{50}]^n)$$

where B is DPM specific bound in the presence of drug, B_{max} is DPM specific bound in the absence of any drug, x is the concentration of the drug, IC_{50} is the concentration of the drug required to inhibit the specific binding of the radioactive tracer by 50%, and n is the slope factor (Hill coefficient). $n = 1$ represents 1:1 interaction between the ligand and receptor under equilibrium conditions.

$$K_I = IC_{50}/(1+[L]/K_D)$$

[L] is the concentration of the radioligand used, and K_D is the dissociation constant of the radioligand.

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Supporting Information Available: A table describing the elemental analyses for the DHPs synthesized is included. This material is available free of charge via the Internet at http://pubs.acs.org.

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