A Region in IVS5 of the Human Cardiac L-type Calcium Channel Is Required for the Use-dependent Block by Phenyalkylamines and Benzothiazepines*

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Mutations in motif IVS5 and IVS6 of the human cardiac calcium channel were made using homologous residues from the rat brain sodium channel 2a. [3H]PN200-110 and allosteric binding assays revealed that the dihydropyridine and benzothiazepine receptor sites maintained normal coupling in the chimeric mutant channels. Whole cell voltage clamp recording from Xenopus oocytes showed a dramatically slowed inactivation and a complete loss of use-dependent block for mutations in the cytoplasmic connecting link to IVS5 (HHT-5371) and in IVS5 transmembrane segment (HHT-5411) with both diltiazem and verapamil. However, the use-dependent block by isradipine was retained by these two mutants. For mutants HHT-5411 and HHT-5371, the residual current appeared associated with a loss of voltage dependence in the rate of inactivation indicating a destabilization of the inactivated state. Furthermore, both HHT-5371 and -5411 recovered from inactivation significantly faster after drug block than that of the wild type channel. Our data demonstrate that accelerated recovery of HHT-5371 and HHT-5411 decreased accumulation of these channels in inactivation during pulse trains and suggest a close link between inactivation gating of the channel and use-dependent block by phenylalkylamines and benzothiazepines and provide evidence of a role for the transmembrane and cytoplasmic regions of IVS5 in the use-dependent block by diltiazem and verapamil.

Voltage-dependent calcium channels mediate calcium influx in response to membrane depolarization and play a critical role in cellular activities such as excitation-contraction coupling, neurotransmitter release, and hormone secretion (1, 2). L-type voltage-dependent calcium channels are characterized pharmacologically by high sensitivity to the calcium blocking drugs that include dihydropyridines (DHP),1 phenylalkylamines (PAA), and benzothiazepines (BZT) (3, 4). Photolabeling studies of the purified α1 subunit and identification of the proteolyzed fragments by site-directed antibodies demonstrate that the α1 subunit harbors all three classes of binding sites (3). The results from these studies provide substantial evidence that motifs III6 and IVS6 contain the major binding regions for these drugs.

In more recent studies, the molecular identification of the binding sites on the α1 subunit for PAA (5–8), BZT (9, 10), and DHPs (7, 11–14) were revealed. The region YMAI in IVS6 is a common binding site for both BZT (9) and PAA (7, 15). A part of this region in addition to amino acid residues within III6 (16, 17) and IVS6 (8) contribute to the DHP pocket (18, 19). When the YMAI region was mutated to the corresponding amino acids in the DHP-insensitive channel α1A, the resultant channel was still sensitive to PAAs and BZTs (20). Thus, the high affinity binding site YMAI, however, is not the minimal requirement for drug binding.

Experimental Procedures

Construction of α1c cDNA Clones in pBluescript—The wild type and mutant human α1c calcium channel cDNAs were first constructed in pBluescript for expression in Xenopus oocytes and then subsequently
placed into pAGS-3 (24) either as the full-length channel or as a channel deleted at the carboxyl-terminal glycine 1633 (25–27). This cellular strategy was designed to utilize the higher expression level of the carboxyl-terminal truncated form of the channel. Site-directed muta-
tations were introduced by incorporating a two-step polymerase chain reaction (PCR) protocol. Mutant oligonucleotides of IVS5 (except for HHT-5658) were synthesized in the sense direction as follows: HHT-5371, CTGTGACCTCACATGTCCTTGCCGGCCCTGTTCGATATGGGCTCTGAG; HHT-5411, GTGGCCCTGCTCTTCCTGGTGATGTTCATCTACGCG; and HHT-5432, TGTTCTTCACTACCGTCTTCGGGATGTCGAACTTTGCGTACATTGCCCTGAATG (the mutations are underlined). In the first reaction, mutant primers were amplified using an antisense oligonucleotide that harbors the BclI restriction site (GGTT-
GATGATCAGGAAGGCAC) and the wild type a1C as the template. The resultant 300-base pair PCR product was isolated and purified for use in the second round of PCR. In this second step, the antisense oligonucleotide carrying the mutant sequence (mega-primer) was extended using a forward primer harboring the AatII restriction site (GCATA-
ATTGACCTCCAT). Each PCR reaction proceeded for 20–30 cy-
cycles in which the primers were denatured at 94 °C for 1 min followed by annealing at 55 °C for 30 s and a final extension at 72 °C for 30 s. The last cycle consisted of a final 10-min extension at 72 °C. After the second round of PCR, the newly synthesized 500-base pair oligonucleotide containing the mutant sequence and harboring both the AatII and BclII restriction sites was gel-purified and subcloned into the EcoRV site of pBluescript. In the construction of HHT-5658, the antisense oligonucleotide GATGATCAGGAAGAACTATGATGACTGACAAGAAGATACCAACGCTGCTACCACGGTG that contains the BclI site was used with a sense oligonucleotide primer that harbors the BstbI restriction site (CCACCTTCGAAGGGTGGC) for the first step in the PCR reaction. The 1000 base pair product was then isolated and subcloned into pBluescript as described above. The restriction sites and mutations were sequence-verified by the dideoxynucleotide chain termination method.

The wild type human a1C channel was prepared by linearization with BclI followed by partial digestion with AarII in which the 8.5-kilobase fragment was isolated. The sequence-verified IVS5 mutants were digested first with BclII followed by AarII to completion. This mutant oligonucleotide was ligated into the partially digested wild type a1C channel. The full-length channel containing the mutant sequence was again sequence-verified.

![Figure 1](image-url)

**Fig. 1. Strategy of mutagenesis between the a1C human cardiac calcium channel and rat sodium channel 2a.** Conserved residues are boxed and amino acids important for calcium channel blocker binding are boldface. A, comparison of amino acid sequence between the a1C human cardiac calcium channel and rat sodium channel 2a of regions (IIIS5, IIIS6, and IVS6) known to harbor residues critical for high affinity calcium antagonist binding. B, sequence alignment of the mutant calcium channels (HHT-5371, HHT-5411, HHT-5432, and HHT-5658) with the wild type (WT) human heart a1C sequence. Mutations were based on non-conserved residues from the rat brain sodium channel 2a. Intervening amino acid residues among the three IVS5 mutations are conserved between the two channels. C, relative orientation of the mutant and wild type amino acids within transmembrane regions IVS5 and IVS6.
Ligation of IVS5 Mutants into pAGS-3—Both the mutant αCa channel and the full-length channel in pAGS-3 (24) were digested to completion with SalI and HindIII. A 4.8-kilobase band of pAGS-3 was ligated to the 6-kilobase band of the mutant αCa channel.

Ligation of IVS5 Mutants into pAGS-3 as a Δ1633 Carboxyl-terminal Deletant—Deletion I was constructed as a carboxyl-terminal deletion after glycine 1633 (27). In order to ligate the mutations into the truncated channel, the Δ1633 pAGS-3 was digested to completion using HindIII and Sse8387I, and the 4.8-kilobase fragment was isolated. The mutant channels in pBluescript were also digested to completion using HindIII and XbaI. The product from this digestion was partially digested using Sse8387I, and the resultant 4.8-kilobase fragment was isolated. The final step was the ligation of the two 4.8-kilobase bands to complete the Δ1633 pAGS-3 incorporating the IVS5 mutations.

Expression of Calcium Channels in Xenopus Oocytes—Expression of the wild type and mutant calcium channels was done as described previously (26). In vitro synthesized cRNA was made by the Message mMachine synthesis kit (Ambion). Xenopus oocyte isolation and cRNA injection was performed as published elsewhere (11). Briefly, female Xenopus laevis (purchased from Xenopus, Ann Arbor, MI) frogs were anesthetized by exposing them for 15–20 min to 0.15% methane-sulfonate salt of 3-aminobenzoic acid ethyl ester (MS-222; Sigma) solution before pieces of the ovary were removed. The follicular layers of the oocytes were digested with 2.0 mg/ml collagenase (Type IA; Sigma) dissolved in OR-2 medium (in mM): 82.5 NaCl, 1 KCl, 1 MgCl₂, 5 -methyl-D-glucamine, 2 CO₂, 50 N-methyl-D-glucamine, 2 KOH, 5 HEPES, pH 7.5. Stage V–VI oocytes were incubated at 19 °C in P/S medium (in mM): 96 NaCl, 2.0 KCl, 1.0 MgCl₂, 1.8 CaCl₂, 5 HEPES, 2.5 sodium pyruvate, and 0.5 theophylline at pH 7.5. The P/S medium was supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

The wild type and mutant αCa messages were coinjected in a 50-nl solution composed of αCa,b (26) and human β₃ (26, 30) subunits in a 1:1:1 molar ratio. The injected oocytes were incubated in P/S solution at 19 °C. Ca²⁺ current channels were recorded 2–4 days post-injection of the cRNAs at room temperature (20–21 °C). In order to minimize contamination with chloride, oocytes were microinjected with 50 nl of a 40 mM potassium 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetate solution, 10 mM HEPES, pH 7.05, 60 min prior to current recording (29). Whole cell currents were recorded using the standard two microelectrode voltage clamp technique.

The recording medium was a Ca²⁺- and Cl⁻-free solution composed of the following (in mM): 40 Ba(OH)₂, 50 N-methyl-D-glucamine, 2 KOH, 5 HEPES, pH adjusted to 7.4 with methanesulfonic acid. Voltage and current electrodes were filled with 3 M KCl and had a resistance of 0.5–1.5 meq/cm. Currents were recorded using an Axoclamp-2A (Axon Instruments Inc., Foster City, CA) amplifier. Whole cell leakage and capacitative currents were subtracted on-line using the P/4 procedure. Currents were digitized at 1 kHz after being filtered at 1 kHz. The pClamp software (version 5.5 Axon Instruments) was used for data acquisition, and version 6.0.3 was used for analysis.

Ba²⁺ currents were elicited by a 350-ms-long depolarizing pulse from a holding potential of −80 mV to test potentials between −30 mV and +40 mV in 10-mV increments in order to determine the peak potentials of the current voltage relationship of the Ca²⁺ channel construct. Use-dependent block was determined as the inhibition of peak Iᵥ during trains of 15 test pulses of 80-ms duration applied at 0.5 Hz from a holding potential of −60 mV to test potentials +10, +20, and/or +30 mV positive to the peak potential of the I-V curves. Identical pulse protocols were used in the presence of drugs. Drugs were perfused in the bath (for a 2.5-min period) at concentrations of 300 and 100 μM diltiazem (racemic) and verapamil, respectively. These concentrations were selected since they provided sufficient block for the use-dependent measurements in Xenopus oocytes. Tonic block is defined as the peak Iᵥ inhibition during the first pulse after a 2.5-min equilibration in the drug containing solution at −60 mV. The drug block under these conditions (tonic block) reflects steady-state binding to the mixture of closed and closed/inactivated states present at −60 mV. A double-pulse protocol was used in those experiments measuring the voltage dependence of steady-state inactivation. The protocol consisted of a 5-s prepulse that ranged from −80 to +50 mV at a holding potential of −80 mV followed by a 30-ms return pulse to −80 mV. Finally a 400-ms-long test pulse was applied to +10, +20, and/or +30 mV. The curves were fit to the Boltzmann equation y = Aₒ − Aᵣ exp(−x/ kᵣ), Aₒ = Aᵣ exp(−x/ kᵣ), Aₒ = Aᵣ exp(−x/ kᵣ). Recovery of Iᵥ from inactivation was studied after depolarizing the calcium channels during a 3-s prepulse to +10, +20, and/or +30 mV. The time course of Iᵥ recovery from inactivation was estimated at a holding potential of −80 mV by applying a 400-ms test pulse to +10, +20, and/or +30 mV at various times after the conditioning prepulse. Peak Iᵥ values were normalized to the peak current amplitude measured during the prepulse. After the double-pulse protocol, the membrane was hyperpolarized to −100 mV for 3 min to permit complete recovery from inactivation and block.

**Fig. 2.** DHP binding characteristics of mutants in motif IVS5. A, effect of IVS5 mutations on [3H]PN200-110 binding affinity. The vertical bars represent the Kᵦ values from the Scatchard plots. The names of the cDNA constructs are labeled below each vertical bar. The data are means ± S.E. An asterisk represents a p value smaller than 0.05. Two asterisks represent a p value smaller than 0.001. Binding assays were done as described under “Experimental Procedures.” B, effect of diltiazem on [3H]PN200-110 binding for wild type and Δ1633 carboxyl-terminal deletion constructs. Saturation binding of WT (open circles) and Δ1633 (solid circles) were done with saturating (0.8 mM) [3H]PN200-110 with varying concentrations of diltiazem as indicated. The positive allosteric effect was quantitated by the dpm in the presence of diltiazem divided by the DPM bound in the absence of diltiazem (Bₒ) multiplied by 100. The data are means ± S.E. C, maximal allosteric effect of diltiazem on [3H]PN200-110 binding for the calcium channel constructs. Measurements were made from the peak allosteric bound in the presence of diltiazem as in B. Vertical bars represent the positive allosteric effect on each cDNA construct and are labeled at the bottom of the figure. The data are means ± S.E. An asterisk represents a p value smaller than 0.05. Two asterisks represent a p value smaller than 0.001. Binding assays were done as described under “Experimental Procedures.”
7.2, 48–72 h post-transfection. The cells were washed twice in phosphate-buffered saline and disrupted by a Polytron homogenizer in buffer (50 mM Tris, pH 7.4, 1 mM EDTA, pH 7.4, supplemented with 2 mg/ml aprotinin and 0.5 mM phenylmethylsulfonyl fluoride). The membranes were pelleted in a Beckman Ti45 rotor at 40,000 rpm for 35 min and then resuspended in membrane storage buffer (50 mM HEPES, pH 7.4, 1.37 mM MgCl₂ and 1 mM CaCl₂ plus protease inhibitors). Protein was determined using the BCA assay kit (Pierce).

Radioligand Binding—Saturation binding assays of [³H]PN200-110 (Amersham Pharmacia Biotech) were done according to the protocols of Varadi et al. (32) using 50–100 µg of protein for each sample. Binding assays were performed at 25 or 37 °C in binding buffer (100 mM Tris, pH 7.4, 2.4 mM CaCl₂ and 1.4 mM MgCl₂). The bound receptors were collected on glass fiber filters (FP-200 GF/C) and washed three times with ice-cold 50 mM Tris buffer, pH 7.4. Nonspecific and bound radioactivity was estimated in the presence of 10 µM non-labeled isradipine. Radioactivity was measured by liquid scintillation counting.

Positive allosteric binding assays between labeled DHP and BZTs were done at 37 °C for 1 h. In these experiments, varying concentrations of diltiazem hydrochloride and 0.8 nM [³H]PN200-110 were used. The allosteric effect was determined by dividing the specifically bound radioactivity in the presence of diltiazem by that specifically bound in the absence of diltiazem multiplied by 100 for each construct.

Chemicals—[³H]PN200-110 was purchased from Amersham Pharmacia Biotech. Diltiazem was a gift from Marion Merrell Dow (Hoechst-Marion-Roussell). All other chemicals were from Sigma.

Statistical Analysis—The results were analyzed by the KELL program version 4.0 (Biosoft, Cambridge) and Origin version 4.0 (Microcal).
and presented as the means ± S.E. The statistical analysis was done using Student’s t test (p < 0.05).

**RESULTS**

The regions of the a1C calcium channel responsible for high affinity binding of PAA, DHP, and BZT include specific amino acids in IIIS5, IIIS6, and IVS6. However, segments and amino acids responsible for the use-dependent nature of these drugs are found dispersed around the presumed area of calcium blocker-binding sites (20–22, 33). This provided the impetus to investigate other regions of the calcium channel that may play an important role in use-dependent block. We chose amino acid stretches of the rat brain sodium channel 2a (34) since these channels possess a low affinity pattern of inhibition by some calcium antagonists (35, 36). Furthermore, very few amino acids found dispersed around the presumed area of calcium blocker-binding sites (20–22, 33). This provided the impetus to investigate other regions of the calcium channel that may play an important role in use-dependent block. We chose amino acid stretches of the rat brain sodium channel 2a (34) since these channels possess a low affinity pattern of inhibition by some calcium antagonists (35, 36). Furthermore, very few amino acids responsible for the use-dependent nature of these drugs.

**TABLE I**

Activation and inactivation rate constants of wild type and mutant calcium channels

|             | Activation | Inactivation |
|-------------|------------|--------------|
|             | V_{0.5} mV | V_{0.5} mV | k_{inact} | Inact. rate ms |
| Wild type   | 13.6 ± 1.0  | -7.6 ± 1.8  | 11.9 ± 0.5 | 1094.2 ± 40.9 |
| After diltiazem | NDa       | -22.4 ± 1.3 | 9.0 ± 1.0  | ND            |
| HHT-5432    | 14.4 ± 1.4  | 1.7 ± 1.5b  | 10.4 ± 0.4 | 1583.1 ± 83.8b|
| After diltiazem | ND       | -11.6 ± 2.3 | 12.5 ± 0.5 | ND            |
| HHT-5411    | 19.5 ± 0.6b | -1.7 ± 2.6a | 17.2 ± 1.3b| >3000b        |
| After diltiazem | ND       | 8.4 ± 1.3   | 7.7 ± 0.3  | ND            |
| HHT-5371    | 12.7 ± 1.3  | 38.4 ± 18.7 | 15.5 ± 1.7 | >3000b        |
| After diltiazem | ND       | -1.3 ± 1.8  | 11.3 ± 0.9 | ND            |
| HHT-5658    | 3.3 ± 1.7b  | -12.3 ± 0.6 | 11.5 ± 0.6 | 1030.4 ± 44.3 |
| After diltiazem | ND       | -17.9 ± 1.6 | 10.4 ± 0.4 | ND            |

a ND, not determined.
b p value smaller than 0.05 represents a statistically significant difference from wild type. Values are expressed as mean ± S.E. (n = 6–35).

**FIG. 4. Effect of diltiazem inhibition on steady-state inactivation curves.**

The voltage dependence of inactivation was investigated for the wild type, HHT-5411, HHT-5432, HHT-5371, and HHT-5658 channels at the end of a 5-s prepulse. Open symbols, control; filled symbols in the presence of 300 μM diltiazem. A, wild type channel; B, HHT-5432; C, HHT-5411; D, HHT-5371; and E, HHT-5658. The relative peak current at +10 mV or +20 mV was plotted against the different prepulse potentials. Curve fitting was performed by the Boltzmann equation as described under “Experimental Procedures” (n = 5–9).
acids are conserved in the membrane spanning regions between the two channels (Fig. 1A), and the majority of the published residues that have been shown to be critical for the action of calcium antagonists are absent in the rat brain sodium channel (Fig. 1A).

We investigated motif IVS5 since this segment “opposes” IVS6 much like the faces of IIIS5 and IIIS6 are believed to be oriented for DHP binding in the calcium channel. Three separate mutant constructs, termed HHT-5371, HHT-5411, and HHT 5432, were engineered from the intracellular portion (amino acids 1326) of motif IVS5 to the extracellular loop (amino acids 1355) that show a loss of amino acid conservation. In the three mutations there are a total of nine, five and four amino acids that were changed in HHT-5371, -5411, and -5432, respectively (Fig. 1B). The intervening residues between the three mutations in IVS5 are conserved in the two channels. In addition, a motif IVS6 mutant (HHT-5658) 11 amino acids were changed to the corresponding sequence of the rat brain 2a sodium channel. In this construct, the first three of the four amino acids of the YMAI sequence that have been demonstrated to form the primary binding site for calcium antagonists have been changed. The relative location of these mutations within the transmembrane regions are shown in Fig. 1C. These mutations were then assayed for their influence on channel function by the different classes of calcium antagonists.

In a previous report from this laboratory, a carboxyl-terminal deletion of the channel tail after glycine 1633 (Δ1633) was found to increase the $R_{\text{max}}$ of the $\alpha_{1C}$ in transient expression systems without affecting the $K_D$ (27). Thus, we used this carboxyl-terminal deletion construct in all binding assays of HEK 293 cell membranes to enhance expression. Scatchard analysis showed no difference in affinity (0.1–0.2 nM) but an approximately 2-fold increase (140 versus 250 fmol/mg protein) in $R_{\text{max}}$ between the full-length wild type and the Δ1633 construct (data not shown, see Ref. 27). Some of the mutant constructs expressed poorly in HEK 293 cells (HHT-Δ5432) with minor effects on $K_D$ values. Interestingly, some of the mutations within the regions of motif IVS5 also demonstrated a lowered affinity (HHT-55371, >1.2 nM and HHT-Δ4532 0.4 nM) to $[^3H]P_{200-110}$ (Fig. 2A). The mutations in motif IVS5 encompass regions that do not overlap with the published binding sites for calcium channels. The construct HHT-Δ5658 completely lost high affinity binding to $[^3H]P_{200-110}$. Thus, an estimate for DHP affinity could not be determined. These results are consistent with the published literature that motif IVS6 contains the high affinity binding site for DHP binding (7).

We then posed a question to test whether these sites may have any involvement in establishing the allosteric interaction among calcium channel blockers. The concentration-dependent enhancement of subsaturating (0.8 nM) concentrations of $[^3H]P_{200-110}$ by increasing concentrations of diltiazem between the wild type and Δ1633 channel is depicted in Fig. 2B. The maximal allosteric effect of diltiazem between the full-length wild type channel, the Δ1633 wild type channel, and the Δ1633 mutants is shown in Fig. 2C. The full-length wild type construct showed significantly lower (18%) allosteric stimulation by diltiazem compared with the Δ1633 (38%) construct. Maximal binding for the mutant constructs were not significantly different compared with the wild type channel producing a 300% increase in $[^3H]P_{200-110}$ binding than in the absence of diltiazem. The exception was with HHT-Δ5432 in which there was a significant decrease (240%) in binding compared with the Δ1633 control. In all of the constructs the concentration-dependent peak occurred between 30 and 100 μM diltiazem and elicited similar curves as the wild type constructs (data not shown). There was no observable specific DHP binding for the HHT-Δ5658 construct even in the presence of diltiazem. These results are consistent with the loss of the high affinity binding site for DHP.

In order to address whether the motif IVS5 mutant constructs maintained normal biophysical properties and pharmacology to calcium channel antagonists, we expressed cRNAs for the full-length channels in Xenopus oocytes. All of the mutants produced voltage-dependent currents with Ba$^{2+}$ serving as the charge carrier. HHT-5411 produced a substantial positive shift (29.3 ± 0.7 mV), whereas HHT-5658 produced a negative (11.2 ± 1.4 mV) shift in the voltage dependence of the I-V relationship compared with the wild type channel (23.3 ± 1.9 mV). In the case for the constructs HHT-5371 and HHT-5432, there was no significant shift (Fig. 3). As was expected, HHT-5658 produced voltage-dependent Ba$^{2+}$ currents despite demonstrating no binding of $[^3H]P_{200-110}$ to transfected HEK 293 membrane preparations.

Analyses of voltage-dependent activation and inactivation for the mutants used in this study are summarized in Table I. Activation rates revealed small but significant differences between the mutants. The half-maximal voltage for activation significantly shifted toward negative potentials ($V_{1/2}$ = 3.3 mV) for HHT-5658, whereas HHT-5411 shifted toward positive potentials ($V_{1/2}$ = 19.5 mV) compared with the wild type channels ($V_{1/2}$ = 13.5 mV). There was no significant difference among the other mutations (Fig. 4 and Table I). The inactivation time course of Ba$^{2+}$ traces could be fitted well by a single exponential function (Table I). HHT-5371, HHT-5411, and HHT-5432 mutant channels displayed a significantly slower inactivation rate (>3 s, >3 s, and 1.6 ± 0.08 s) than the wild type (1.1 ± 0.04 s) channel. The steady-state inactivation properties of wild type and mutant channels exhibited drastic changes in inactivation of HHT-5411 and HHT-5371 (Fig. 4). HHT-5371 and HHT-5411 revealed incomplete inactivation only to approximately 50% of the normalized current. Upon addition of diltiazem the steady-state inactivation was restored to normal (Fig. 4). The $V_{1/2}$ values of steady-state inactivation at HHT-5371 and HHT-5411 with diltiazem were significantly different from the one obtained for the wild type; however, the slope factors appeared not significantly different from the wild type (Table I). The wild type and HHT-5658 ($V_{1/2}$ = -7.6 mV, $k_o$; 11.9 mV and $V_{1/2}$ = -12.3 mV, $k_i$; 11.5 mV, respectively) inactivated completely, and the midpoint voltages of the curves were
characteristically leftward-shifted in the hyperpolarized direction ($V_{0.5}, -22.4 \text{ mV}; V_{0.5}, -17.9 \text{ mV}$) after diltiazem addition without a significant change in the steepness of the voltage dependence ($k$, 9.0 and 10.4 mV). The absence of change either in the $V_{0.5}$ or in the slope factor of the steady-state inactivation curve for HHT-5658 is in good agreement with the partial loss of the BZT-binding site. Mutant HHT-5432 behaved very similarly to that of the wild type. This mutant inactivated completely either without or with diltiazem showing only modest changes in the steady-state inactivation parameters (Table I).

We further analyzed motif IVS5 and IVS6 mutant calcium channels with regard to tonic and use-dependent block by diltiazem and verapamil. Resting state-dependent (tonic) $I_{\text{Ba}}$ inhibition by diltiazem was 0.69 ± 0.02 ($n = 30$) in the wild type channel. Only the change in HHT-5432 and HHT-5371 produced a significant increase in tonic block (0.56 ± 0.03, $n = 13$ and 0.61 ± 0.03, $n = 16$, respectively). The other mutant calcium channel constructs were not significantly different (HHT-5411, 0.71 ± 0.03, $n = 26$; HHT-5658, 0.62 ± 0.04, $n = 17$) from the wild type channel (Fig. 5). Tonic block was less pronounced with verapamil for the wild type channel (0.92 ± 0.04) suggesting that the drug binds poorly to the resting state of the channel. Similarly to the wild type channel, HHT-5411 (0.86 ± 0.03) and HHT-5371 (0.75 ± 0.07) exhibited very little tonic block with verapamil.

We then studied the impact of the amino acid substitutions on use- or frequency-dependent block by calcium antagonists. Representative current traces are shown for diltiazem and verapamil (Fig. 6 and 7, respectively). Use-dependent block in the wild type channel appeared more efficient with verapamil.

**Fig. 6.** Use-dependent block of wild type and mutant calcium channel currents. Mutations in IVS5 affect $I_{\text{Ba}}$ decay during 0.5-Hz pulse trains. Representative current traces of wild type and mutant channels in the absence (A) and presence (B) of 300 μM diltiazem for wild type, HHT-5432, HHT-5411, HHT-5371, and HHT-5658, respectively. C, use-dependent $I_{\text{Ba}}$ inhibition during 15 consecutive test pulses by 300 μM diltiazem compared with the current decay in the control. 0.5-Hz trains of 15 pulses were applied from a holding potential at −60 mV to a test potential of +10 or +20 mV. Peak currents during each pulse were normalized to the peak $I_{\text{Ba}}$ during the first pulse and are plotted against pulse number.
(30.4 ± 2.1%) than with diltiazem (20.0 ± 2.1%). The IVS6 mutant HHT-5658 displayed less use-dependent block (12.6 ± 1.5%) by diltiazem compared with wild type. The IVS5 mutant HHT-5432 exhibited many similar characteristics to the wild type channel.

A remarkable and unexpected observation was the complete disappearance of use-dependent block of both mutant HHT-5371 and HHT-5411, for either diltiazem (Fig. 6, 0.9 ± 2.4 and 3.1 ± 3.1%, respectively) or verapamil (Fig. 7, E and G) (3.0 ± 0.7 and 5.8 ± 3.3%, respectively). Previously, it has been demonstrated that DHPs also exert use-dependent block at pulse frequencies of 1 Hz or greater (37). By using the same protocol as described above for BZT, we observed use-dependent block for 10 μM isradipine at a frequency of 1 Hz for both the wild type channel (Fig. 7D) and the mutants HHT-5411 and HHT-5371 (Fig. 7, F and H, respectively). Since the mutants tested throughout these studies retained the DHP- and BZT-binding sites (with the exception of the HHT-5658 where the YMA part of the binding site was eliminated), it is logical to assume that the alteration of inactivation properties of the mutant channels is responsible for the loss of use-dependent block by diltiazem and verapamil.

A more complete analysis of the role of channel inactivation in development of block was performed by studying the recovery from depolarization-dependent block (Fig. 8). We measured the INa recovery from inactivation by a two-pulse protocol after maintained 3-s depolarizations. The recovery of the channel from inactivation was estimated at different intervals (recovery interval) from 20 ms to 28 s. Data points in the presence of diltiazem (Fig. 8A) were fitted by a two exponential curve with exception of HHT-5371 in which, due to the very fast recovery, a single exponential fit was employed. The mutant HHT-5371
Time constants of recovery from inactivation for wild type, HHT-5432, HHT-5411, HHT-5371, and HHT-5658 channels in the absence and presence of diltiazem, isradipine, and verapamil

Data generated for this table are derived from experiments summarized in Figs. 8 and 9 and experimental details are given therein. The relative current values were plotted against time, and data points were fitted by a double exponential function when it was possible or for most cases by a single exponential only. The parameters given in the table are as follows: $\tau_1$ and $\tau_2$, time constants of a double exponential function; $\tau_1$ one-exp, time constant of a single exponential function.

|            | Diltiazem | Isradipine | Verapamil |
|------------|-----------|------------|-----------|
| $\tau_1$   | $\tau_2$  | $\tau_1$ one-exp | $\tau_1$   | $\tau_2$  | $\tau_1$ one-exp | $\tau_1$   | $\tau_2$  | $\tau_1$ one-exp |
| Wild type  | 70.4 ± 14.2 | 2066.1 ± 103.2 | 1655.3 ± 172.9 | 70.4 ± 14.2 | 2066.1 ± 103.2 | 1655.3 ± 172.9 | 92.5 ± 26.7 | 1662.9 ± 283.9 | 1531.1 ± 248.2 |
| After drug | 1531.2 ± 179.8 | 5294.4 ± 673.4 | 2516.8 ± 294.1 | 176.2 ± 36.9 | 2396.7 ± 123.8 | 1877.5 ± 133.4 | 572.4 ± 156.3 | 3115.9 ± 427.2 | 2594.8 ± 284.3 |
| HHT-5432   | 31.2 ± 11.4 | 1455.9 ± 208.8 | 917.9 ± 105.0  | 31.2 ± 11.4 | 1455.9 ± 208.8 | 917.9 ± 105.0  | NT          | NT          | NT          |
| After drug | 153.3 ± 18.5 | 3019.3 ± 244.9 | 2517.8 ± 243.5 | 176.2 ± 36.9 | 2396.7 ± 123.8 | 1877.5 ± 133.4 | NT          | NT          | NT          |
| HHT-5411   | 128.6 ± 18.5 | 1369.6 ± 124.9 | 573.4 ± 72.2  | 128.6 ± 18.5 | 1369.6 ± 124.9 | 573.4 ± 72.2  | NT          | ND          | NT          |
| After drug | 112.0 ± 7.7  | 3621.9 ± 191.5 | 2901.8 ± 206.9 | 112.0 ± 7.7  | 3621.9 ± 191.5 | 2901.8 ± 206.9 | NT          | ND          | NT          |
| HHT-5371   | ND        | ND          | ND          | ND          | ND          | ND          | NT          | ND          | NT          |
| After drug | ND        | ND          | ND          | ND          | ND          | ND          | NT          | ND          | NT          |
| HHT-5658   | 71.9 ± 24.4 | 1867.0 ± 60.9 | 1473.4 ± 142.7 | 71.9 ± 24.4 | 1867.0 ± 60.9 | 1473.4 ± 142.7 | NT          | NT          | NT          |
| After drug | 82.8 ± 14.1 | 2407.9 ± 89.5 | 2252.1 ± 61.2 | 82.8 ± 14.1 | 2407.9 ± 89.5 | 2252.1 ± 61.2 | NT          | NT          | NT          |

*NT, not tested.

b Values are statistically significant from wild type, $p < 0.05$. Values are expressed as mean ± S.E. ($n = 6–35$).

c Data are from two experiments.

Data are from two experiments.

a ND, not determined.
The recovery from inactivation for HHT-5371 after isradipine treatment could only be fit using a one exponential function. These results are similar to those observed from recovery in the presence of diltiazem and verapamil (Fig. 8) for both HHT-5411 and HHT-5371. Mutant HHT-5371 IBa recovery appeared to be faster than wild type; however, it should be noted that even after a 5-s conditioning prepulse only 10% of the channels are inactivated (Fig. 9). For HHT-5411 about 30% of the channels were inactivated at the end of the conditioning prepulse. Therefore, determining the time constants for this latter process seemed not to be realistic (Table II). Representative current traces are illustrated in the absence (B, D, and F) and presence of isradipine (Fig. 9, C, E, and G) for wild type, HHT-5411, and HHT-5371, respectively. Displayed currents were recorded after interpulse intervals of 20, 100, 300, 500, 1000, and 3000 ms (wild type, HHT-5411) and 20, 100, 50, 1000, and 3000 ms (HHT-5371). The traces are superimposed. After the 3-s prepulse (to +10 or +20 mV) the IBa recovered within 20 ms to 80–90% of its original value in control (absence of drug) for HHT-5411 and HHT-5371. The data points are fitted by a single exponential function for HHT-5371 and a double exponential function for the wild type and HH-5411. The time constants were calculated from 6 to 11 independent experiments and are summarized in Table II.

**DISCUSSION**

There is little information on the mechanism of use-dependent block, a feature that is critical to the therapeutically useful calcium antagonists. These drugs preferentially inhibit channels during high electric activity as found in certain cardiac arrhythmias and diseases such as ischemic heart disease. According to the guarded receptor hypothesis (23), the molecular elements may be distinct from the structures that specify simple or tonic binding.

In this study we identified a region proximal to the cytoplasmic connecting link of motif IVS5 that contributes to the use-dependent block of BZTs and PAAs. These results are consistent with those reported by Cai et al. (20), in which they demonstrated that a motif I–II replacement with α1A still maintained use-dependent inhibition by verapamil and diltiazem as well as in a mutant in which the high affinity YAI residues had been also mutated to that of the α1A channel. In more recent studies (3, 21, 22), other regions in motifs III and IV that are outside of the high affinity YAI-binding region have been shown to be critical for the use-dependent block observed for these drugs.

We have found that the loss of use-dependent block is closely associated with a decrease in voltage-dependent inactivation. In both HHT-5411 and HHT-5371 diminished inactivation re-
inactivation for the cardiac calcium channels in response to drugs. Then the channel inactivates upon depolarization. The channel exists initially in a resting state that does not bind to BZT and PAA. In this state the drug-binding site is inaccessible and/or has a low affinity to the drugs. Upon depolarization the calcium channel opens allowing the passage of calcium through the pore. In the open state the channel still has a low affinity to the drugs. Then the channel inactivates. Our results have demonstrated that inactivation can be divided into two kinetic states (I\text{fast} and I\text{slow}). Both I\text{fast} and I\text{slow} bind drug. After the drug dissociates, the channel is left in an intermediate inactivated state (I*) that is no longer bound by drug before returning back to the resting state. We have shown that a mutation in motif IVS5 does not completely inactivate, demonstrating an incomplete interconversion from the open to the inactivated state.

This observation can be seen by the incomplete voltage-dependent inactivation shown in the electrophysiological traces of Figs. 5–7. However, upon the addition of BZT or PAA the mutant channels (HHT-5371) completely inactivate. These results demonstrate the coupling between voltage-dependent inactivation and drug binding in the cardiac calcium channel without altering the residues shown to be required for high affinity drug binding.

sulted in the absence of use-dependent block by diltiazem and verapamil. These results are rather specific to these classes of calcium antagonists since use-dependent block by the dihydropyridine antagonist isradipine was not affected. The loss of use-dependent block from the mutations in IVS5 appear to be coupled to a decrease of voltage-dependent inactivation as has been shown for mutants in IISS6 (22).

It appears that motif IVS5 plays an important role in the stability of channel inactivation. In steady-state inactivation studies, both HHT-5411 and HHT-5371 did not completely inactivate except in the presence of calcium antagonists (4). These results provide evidence that the loss of this aspect of calcium channel block is specific rather than due to a general loss in function attributed to gross changes in tertiary structure resulting from the introduction of foreign amino acid residues. There are several reasons why we support this contention. First, only the use-dependent block of these drugs is affected, and the tonic block by BZTs is spared. These results may be due to the fact that the high affinity binding region in motif IV is still intact. However, one mutation, HHT-5658 which we have described in this report, has three of the four residues in the YMAI sequence altered. Despite these changes the channel when expressed in Xenopus oocytes behaves much like the wild type Ba\textsuperscript{2+} currents and exhibits tonic and use-dependent block by calcium antagonists. As anticipated, the high affinity binding to \textsuperscript{3}HJPN200-110 in saturation binding assays was lost. These new data further implicate other regions outside those areas that have been recently published (IISS5, IISS6, and IVS6) to be important for high affinity binding of these classes of drugs (3–22). Second, the loss of use-dependent block is unique to verapamil and diltiazem. Use-dependent block by DHPs is not affected. These results suggest that the use-dependent block by PAA and BZT operates through a mechanism different from DHPs. Furthermore, the conclusion that there is a functional disparity between PAA/BZT and DHP is reasonable since there is more overlap in the high affinity binding region for PAAs and BZTs than for DHPs. Third, HHT-5411 which demonstrates a loss of use-dependent block to BZT and PAA maintains normal DHP binding affinity and allosteric modulation by diltiazem. HHT-5371 which did not retain use-dependent block to BZTs and PAAs shows an approximate 6-fold lowered affinity to DHP binding. A fourth issue in support of our contention that motif IVS5 has a specific structural role for BZT and PAA action is that another mutant, HHT-5432 which is also from the same region, displayed use-dependent block by these drugs and otherwise normal channel currents. When this mutant was expressed in HEK 293 cells, it showed a slightly lowered (2-fold) affinity to DHPs and suggests a role in DHP binding in this region of motif IVS5.

These alterations in function are not likely to be due to the transfer of specific sodium channel sequences into the cardiac calcium channel for several reasons. Motif IVS6 in sodium channels has evolved to harbor specific amino acid residues for local anesthetic action (36). Mutation of these residues reduces not only block but also eliminates the use-dependent characteristics for local anesthetics (36). Thus, unlike the calcium channel, the sodium channel has both its binding site and use-dependent functions contained in motif IVS6.

These results also suggest that the nature of use-dependent block may be different for the calcium antagonists and local anesthetics. Second, it is believed that the intracellular linker between motifs III and IV is responsible for this voltage-dependent inactivation (38).

According to the modulated receptor hypothesis (23), variable affinity channel binding and modification of inactivation kinetics are required for the use-dependent effect of calcium channel drugs. In the case of HHT-5371 and HHT-5411, the very slow inactivation kinetics reflects a slow transition between the open and inactivated state of the channel. Thus, the open state of the channel exerts a lesser affinity to BZT and PAA. The transitions for this mutant to the inactivated state is a less preferred conformation so that repeated depolarizations do not lead to an increased fraction of inactivated channels. However, diltiazem binds to the HHT-5411 and HHT-5371 mutant channels as shown by the change in voltage dependence of inactivation and use dependence. Similar studies performed on these two constructs testing the use-dependence for verapamil confirmed the importance of inactivation in the development of use-dependent block (21, 22). It is a reasonable assumption that a slowly inactivating mutant will be less efficiently blocked by PAA. In the case of calcium channels the only published reports of areas responsible for voltage-dependent inactivation implicates motif IISS6 (39) and the carboxyl terminus in the case for \textalpha\textsubscript{1C} alternative splice variants (40). Therefore, the results suggest a possible third region of the calcium channel for use-dependent inactivation.

This report is the first to demonstrate a functional role of motif IVS5 for calcium channel function and the action of calcium channel antagonists, and the first to dissect the molecular regions involved in use-dependent block by BZT and PAA. We suggest a model (Fig. 10) in which inactivation of the channel occurs in two kinetic states. Both states bind to BZT and PAA. However, in the cardiac calcium channel IVS5 mu-
tants HHT-5411 and HHT-5371, the interconversion between the open and the inactivation state is incomplete. Treatment of either BZT or PAA forces the calcium channel toward complete inactivation. The slowed rate of inactivation in these channels can explain the loss of use-dependent block by these drugs in parallel with the observed decrease in voltage-dependent inactivation without affecting any of the residues for high affinity antagonist binding. We believe that motif IVS5 serves as either an accessory binding domain or a conduit for a conformational movement within the membrane to provide the use-dependent nature characterized by these two classes of calcium-blocking drugs.

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REFERENCES

1. Hofmann, F., Biel, M., and Flockerzi, V. (1994) *Annu. Rev. Neurosci.* 17, 399–418
2. Mori, Y., Mikala, G., Varadi, G., Kobayashi, T., Koch, S., Wakamori, M., and Schwartz, A. (1996) *Jpn. J. Pharmacol.* 72, 83–109
3. Streissnig, J., Grabner, M., Mitterdorfer, J., Hering, S., Sinnegger, M. J., and Glossman, H. (1998) *Trends Pharmacol. Sci.* 19, 108–115
4. Mitterdorfer, J., Grabner, M., Kraus, R. L., Hering, S., Prinz, H., Glossman, H., and Streissnig, J. (1998) *J. Bioenerg. Biomembr.* 30, 319–334
5. Doring, F., Degt’ev, V. E., Grabner, M., Streissnig, J., Hering, S., and Glossman, H. (1996) *J. Biol. Chem.* 271, 11745–11749
6. Johnson, B. D., Hockerman, G. H., Scheuer, T., and Catterall, W. A. (1996) *Mol. Pharmacol.* 50, 1388–1400
7. Schuster, A., Lacinova, L., Klugbauer, N., Ito, H., Birnhaeuser, L., and Hofmann, F. (1996) *EMBO J.* 15, 2365–2370
8. Hockerman, G. H., Johnson, B. D., Abbott, M. R., Scheuer, T., and Catterall, W. A. (1997) *J. Biol. Chem.* 272, 18759–18765
9. Hering, S., Azcel, S., Grabner, M., Doring, F., Berjukow, S., Mitterdorfer, J., Sinnegger, M. J., Streissnig, J., Dektar, V. E., Wang, Z., and Glossman, H. (1996) *J. Biol. Chem.* 271, 24471–24475
10. Kraus, R., Reichl, B., Kimball, S. D., Grabner, N., Murphy, B. J., Catterall, W. A., and Streissnig, J. (1996) *J. Biol. Chem.* 271, 20113–20118
11. Tang, S., Yatani, A., Bahnke, A., Mori, Y., and Schwartz, A. (1993) *Neuron* 11, 1013–1021
12. Grabner, M., Wang, Z., Hering, S., Streissnig, J., and Glossman, H. (1996) *Neuron* 16, 207–218
13. Peterson, B. Z., Tanada, T. N., and Catterall, W. A. (1996) *J. Biol. Chem.* 271, 5293–5296
14. Peterson, B. Z., Johnson, B. D., Hockerman, G. H., Avheson, M., Scheuer, T., and Catterall, W. A. (1997) *J. Biol. Chem.* 272, 18752–18758
15. Hockerman, G. H., Johnson, B. D., Scheuer, T., and Catterall, W. A. (1995) *J. Biol. Chem.* 270, 22119–22122
16. Mitterdorfer, J., Wang, Z., Sinnegger, M. J., Hering, S., Streissnig, J., Grabner, M., and Glossman, H. (1996) *J. Biol. Chem.* 271, 30330–30335
17. He, M., Bodi, I., Mikala, G., and Schwartz, A. (1997) *J. Biol. Chem.* 272, 2629–2633
18. Sinnegger, M. J., Wang, Z., Grabner, M., Hering, S., Streissnig, J., Glossman, H., and Mitterdorfer, J. (1997) *J. Biol. Chem.* 272, 27686–27693
19. Hering, S., Azcel, S., Kraus, R. L., Berjukow, S., Streissnig, J., and Timin, E. N. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 14906–14911
20. Cai, D., Mulle, J. G., and Yue, D. T. (1997) *Protein Sci.* 6, 1875–1876
21. Hwang, S., Mulle, J. G., and Yue, D. T. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 13325–13328
22. Kraus, R. L., Hering, S., Grabner, M., Ostler, D., and Streissnig, J. (1998) *J. Biol. Chem.* 273, 72205–72212
23. Sturman, C. F., Grant, A. O., and Strauss, H. C. (1984) *Biophys. J.* 46, 15–27
24. Miyazaki, J.-I., Takaki, S., Araki, K., Tashiro, F., Tominoya, A., Takate, K., and Yamamura, K. (1989) *Gene (Amst.)* 79, 269–277
25. Wei, X., Neely, A., Lacerda, A. E., Olcese, R., Stefani, E., Perez-Reyes, E., and Birnhaeuser, L. (1994) *J. Biol. Chem.* 269, 1635–1640
26. Klockner, U., Mikala, G., Varadi, M., Varadi, G., and Schwartz, A. (1995) *J. Biol. Chem.* 270, 17306–17313
27. Mikala, G., Klockner, U., Varadi, M., Eisfeld, J., Schwartz, A., and Varadi, G. (1996) *Mol. Cell. Biochem.* 155, 95–109
28. Ellis, S. B., Williams, M. E., Ways, N. R., Brenner, R., Sharp, A. H., Leung, A. T., Campbell, K. P., McKenna, E., Koch, W. J., Hui, A., Schwartz, A., and Harpold, M. P. (1988) *Science* 241, 1661–1664
29. Massey, N., Grob, D., Oh, Y., Birnhaeuser, L., and Stefani, E. (1994) *Biophys. J.* 66, 1895–1903
30. Collin, T., Lory, P., Tavaix, S., Courtieu, C., Guilbaud, P., Berta, P., and Nappo, J. (1994) *Eur. J. Biochem.* 230, 257–262
31. Chen, C., and Okawara, H. (1987) *Mol. Cell. Biol.* 7, 2745–2752
32. Varadi, G., Lory, P., Glaize, D., Varadi, M., and Schwartz, A. (1991) *Nature* 352, 159–162
33. Hering, S., Berjukow, S., Azcel, S., and Timin, E. N. (1998) *Trends Pharmacol. Sci.* 19, 439–443
34. Noda, M., Ikeda, T., Suzuki, H., Yakeshima, H., Takahashi, T., Kunou, M., and Numa, S. (1986) *Nature* 322, 826–828
35. Yatani, A., Kunou, D. L., and Brown, A. (1988) *Am. J. Physiol.* 254, H140–H147
36. Ragsdale, D. S., Scheuer, T., and Catterall, W. A. (1991) *Mol. Pharmacol.* 40, 756–765
37. Sanguinetti, M. C., and Kass, R. S. (1984) *Circ. Res.* 55, 336–48
38. Stuhmer, W., Comi, F., Suzuki, H., Wang, X., Noda, M., Yahagi, N., Kudo, H., and Numa, S. (1989) *Nature* 339, 597–603
39. Zhang, J.-F., Ellinor, P. T., Aldrich, R. W., and Tsien, R. W. (1994) *J. Biol. Chem.* 269, 22119–22122
40. Soldatov, N. M., Oz, M., O’Brien, K. A., Abernethy, D. R., and Morad, M. (1998) *J. Biol. Chem.* 273, 957–963