Subunit-Dependent Modulation of Recombinant L-Type Calcium Channels
Molecular Basis for Dihydropyridine Tissue Selectivity

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At least four calcium channel subtypes (P, T, N, and L) have now been classified on the basis of their biophysical and/or pharmacological properties. L-type channels, a channel family particularly important to physiological function of the cardiovascular system, are identified by their slow voltage- and calcium-dependent inactivation as well as their sensitivity to dihydropyridine (DHP) calcium channel antagonists. In this study, we report the results of experiments in which we have measured the DHP modulation of recombinant calcium channel activity in cells transfected with α1 subunits of cardiac and smooth muscle L-type calcium channels. We find subunit-dependent differences in the voltage and concentration dependence of channel modulation. Our results provide evidence for a molecular basis for DHP sensitivity of heart and smooth muscle calcium channels and, additionally, indicate that, even within one family of calcium channels, slight differences in channel structure can cause marked differences in channel pharmacology. (Circ Res. 1993;73:974-980.)

Key Words • L-type Ca\textsuperscript{2+} channels • dihydropyridine

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Contribute to the targeting of these cells by DHP drugs.\textsuperscript{6,8}

The present study was designed to take advantage of the cloning and discovery of tissue-specific expression of the cDNAs for L-type calcium channel α1 subunits from heart and SM cells\textsuperscript{9,12} to test directly for a molecular basis for DHP tissue selectivity. We investigated the modulation of recombinant channel activity of cells transfected with cDNA encoding the α1 subunit of either the cardiac or SM L-type calcium channel by the DHP nisoldipine. Our results not only indicate that the α1 subunit of the L-type channel is sufficient to reconstitute most voltage-dependent properties of DHP channel modulation, but, importantly, that in the same cells and under identical experimental conditions, channel activity expressed by transfection of SM α1 cDNA is more sensitive to DHP modulation than channel activity expressed by transfection with cardiac α1 cDNA. These experiments provide the first direct evidence for a molecular basis for the tissue selectivity of this important class of drugs.

Materials and Methods

Experiments on recombinant channel activity were carried out in Chinese hamster ovary (CHO) cells that had been stably transfected with the α1 subunit of the cardiac L-type calcium channel as previously described.\textsuperscript{13} Transfected cells were grown in tissue culture conditions using a modified essential medium supplemented with fetal calf serum, penicillin, and glutamine. Electrophysiological experiments could be carried out 2 to 3 days after splitting cells and plating coverslips to appropriate densities. Cardiac cells were obtained from guinea pig ventricles using previously described conventional enzymatic dissociation procedures.\textsuperscript{14}
The generation of the stable cell line CHOCa1 expressing the SM \( \alpha_i \) subunit (type 2b calcium channel \( \alpha_i \) subunit) has been described. To obtain the stable cell lines CHOCa1 and CHOCa4, CHO cells were stably transfected with the recombinant plasmids pKN\( \alpha_i \)12a and p91\( \alpha_i \)12a, respectively. Both plasmids contain the entire protein coding region of the cardiac \( \alpha_i \) subunit (type 2a calcium channel \( \alpha_i \) subunit) GenBank accession number X15539 under the control of the simian virus 40 enhancer/promoter and the adenovirus promoter, respectively. In p91\( \alpha_i \)12a the 5' untranslated sequence of the cardiac cDNA was removed and replaced with the consensus sequence for initiation of translation in vertebrates. Transfection of CHO cells and propagation of cell lines were as described by Bosse et al. In the case of transfection with pKN\( \alpha_i \)12a pCHO cells were grown in the presence of G418 (200 \( \mu g/mL \)). Membrane currents were measured at room temperature with the whole-cell configuration of the patch-clamp method using a Yale Mark IV amplifier. Recording conditions and solutions were as previously described. \( Ba^{2+} \) (30 mmol/L) was used as the divalent ion charge carrier, except where otherwise specifically noted. Data were sampled at 6.7 kHz and filtered at 3 to 5 kHz. DHP channel inhibition was measured during trains of 40-millisecond test pulses applied once every 5 seconds after changing the holding potential from -80 to -40 mV. Recovery from inhibition was studied by returning the holding potential to -80 mV and applying 20-millisecond test pulses every 5 seconds to minimize depolarization during recovery. The test pulse voltage was +30 mV, near the peak of the current-voltage relation under these experimental conditions. Tonic block was measured by applying 40-millisecond test pulses once every 5 seconds from -80 mV. Data were plotted, and statistical analysis was carried out using ORIGIN 2.8 software (MicroCal, Inc, Northampton, Mass). Pooled data are shown as mean±SEM.

**Results**

Because previous studies have suggested that the voltage-dependent modulation of native L-type channels by DHPs is due to a preferential interaction between the drug and the inactivated state of the channel, we first investigated the calcium and voltage dependence of the inactivation of \( \alpha_i \) channels. Fig 1 compares families of current tracings obtained from...
native cardiac channels (left) with recordings from recombinant SM α1 channels (right) when calcium or barium carries the inward charge and shows that, like the native channels, recombinant channel inactivation is faster when calcium (upper tracings) rather than barium (lower tracings) is the charge carrier. Over the duration of the brief (40-millisecond) voltage pulses shown in this figure, there is virtually no inactivation of the barium currents but more than 50% inactivation of calcium currents at some voltages. Recombinant Ba2+ currents, measured after long (5-second) conditioning pulses (data not shown), do inactivate in a manner consistent with voltage-dependent inactivation with similar properties for both SM α1 and cardiac α1 channels.19 Thus, like native channels, channels comprised solely of α1 subunits inactivate via voltage- as well as calcium-dependent pathways.

Nisoldipine, a DHP that has been shown to inhibit native cardiac L-type channels,4 inhibits SM α1 channels in a potent and voltage-dependent manner. Fig 2A shows that 1 nmol/L nisoldipine inhibits SM α1 channel activity for pulses applied from −40 mV (▲) but not from −80 mV (■). Thus, as is the case for native heart and SM L-type channels, DHP modulation of SM α1 channel activity is voltage dependent. Apparently, allosteric interactions with other subunits are not necessary to account for the voltage dependence of nisoldipine channel modulation. However, in this experiment, the threshold nisoldipine concentration that causes channel inhibition at −40 mV is lower for SM α1 channels than for native heart channels (data not shown). The effects of 10 nmol/L nisoldipine on SM α1 channels is illustrated in Fig 2B; here, channel inhibition that occurs in the presence (●) but not in the absence (○) of 10 nmol/L of the drug is much more rapid than in the presence of 1 nmol/L nisoldipine. Additionally, after channels have been inhibited, a return to −80 mV fails to relieve block (□), used to mark the beginning of the recovery protocol and not

![Figure 2](http://circres.ahajournals.org/)

**Fig 2.** Nisoldipine inhibition of smooth muscle α1 channels: concentration dependence of onset. A. Recording and graph show that exposure to nisoldipine (1 nmol/L) inhibits smooth muscle Chinese hamster ovary α1 channel activity recorded during pulse train protocol with −40 mV (▲), but not −80 mV (■), conditioning potential. Control recordings indicate no change in current amplitude at −40 mV in the absence of drug (○). The test potential was +30 mV. Bars=175 pA and 20 milliseconds. B. Recording and graph show that nisoldipine (10 nmol/L) block of smooth muscle α1 channels is not relieved by hyperpolarization. Pulse train protocol with −40-mV conditioning potential promotes block of smooth muscle α1 channel activity in the presence (●, graph and left inset), but not absence (○ and ▽, graph only), of 10 nmol/L nisoldipine. Subsequent return to −80-mV conditioning voltage (□, graph and right inset) does not promote relief of block. The test potential was +30 mV. Bars=200 pA and 10 milliseconds.
the removal of drug). The inability to relieve block of SM α1 channels at −80 mV was a consistent finding: the mean percent of recovered current after block by 10 nmol/L nisoldipine was 9.1±6% (SM CHO, n=6). Surprisingly, the inhibition of SM α1 channels at −80 mV was due in part to tonic block, block that developed in the presence of 10 nmol/L nisoldipine even if the cell membrane was held at −80 mV and not depolarized to −40 mV (see Fig 4A).

Fig 3 illustrates the effects of nisoldipine on cardiac α1 channel activity. Important differences emerge compared with nisoldipine modulation of SM α1 channels. First, nisoldipine (10 nmol/L), applied while the cell was held at −80 mV and pulsed every 5 seconds to +30 mV to assay channel activity, did not inhibit cardiac α1 channel activity (■). Second, after the holding potential was changed to −40 mV, inhibition of cardiac α1 channel activity was clearly apparent (○), indicating voltage-dependent effects of the drug on the cardiac α1 subunit, but in this case, the time course of the development of block appeared slower than under similar conditions for SM α1 channels. Third, after channel activity was completely inhibited at −40 mV, the membrane potential was returned to −80 mV; a pulse protocol was applied to assay removal of drug block; and partial recovery was observed (△), again in contrast with the results obtained with SM α1 channels. In a total of five cardiac α1 CHO cells, 53±10% of the current was recovered by returning the membrane potential to −80 mV. This compares favorably with the recovery of native heart L-type channels at −80 mV after block by 10 nmol/L nisoldipine: 57±6% (n=10) (data not shown). Finally, at a nisoldipine concentration of 1 nmol/L, little change in cardiac α1 channel activity was observed.

Fig 4 summarizes two aspects of nisoldipine modulation of channel activity for cardiac and SM α1 channels and, for comparison, native heart L-type channels: tonic block at −80 mV and the time course of the development of block at −40 mV. Fig 4A shows that tonic block (at −80 mV) of SM CHO channels is significantly different from both cardiac CHO (P<.02) and native heart (P<.01) channels. Interestingly, tonic block of native heart channels is not significantly different from tonic block of cardiac CHO channels (P=.128). Using the train protocols, we confirmed that the time course of the development of nisoldipine block of SM α1 channels at −40 mV was consistently faster than nisoldipine block of cardiac α1 channels under identical experimental conditions. Fig 4B summarizes the differences in the time course of the onset of nisoldipine block at −40 mV for 3 and 10 nmol/L nisoldipine. For both SM α1 and cardiac α1 channels, the time course of the onset of nisoldipine block is concentration dependent, but in each nisoldipine concentration, the block of SM α1 channels is approximately twice as fast as block of the cardiac α1 channels. To emphasize the consistency of these differences, we compared currents remaining at 90 seconds (3 nmol/L) and 70 seconds (10 nmol/L) after the start of the depolarizing train protocols for each nisoldipine concentration. In both concentrations, the SM α1 currents were significantly smaller than the cardiac α1 currents for 3 nmol/L (P<.015) and 10 nmol/L (P<.002) nisoldipine. Thus, in the same cells and under identical experimental conditions, channel activity expressed by transfection of SM α1 cDNA is more sensitive to DHP modulation than channel activity expressed by transfection with cardiac α1 cDNA.

Discussion

The results of the present study indicate that calcium channel activity expressed by cells transfected with cDNA encoding either the cardiac or SM α1 subunit alone is characterized by most of the voltage-dependent modulatory actions of DHP calcium channel blockers. Nisoldipine modulation of cardiac CHO α1 channels is remarkably similar to nisoldipine modulation of intact native heart L-type channels and markedly distinct from the voltage-dependent modulation of SM CHO α1 channels. Taken together, these results provide strong
Distinct Interactions With Smooth Muscle and Cardiac Subunits

Previous studies of recombinant cardiac, SM, and skeletal muscle a1 calcium channels have provided evidence that high concentrations of agonists or antagonists can modulate measured currents11,13,20-23 but did not address the modulation of channel activity by drug concentrations that approach the affinities predicted by binding data. Bosse et al13 reported an affinity constant of 0.1 nmol/L for [3H]nisoldipine binding to crude membranes of CHO cells stably transfected with SM a1 subunits, and Hofmann et al24 have reported comparable binding site numbers and high-affinity binding in CHO cells expressing cardiac and SM a1 channels. The evidence in favor of a molecular basis for the clinically relevant tissue selectivity of DHP calcium channel blockers.

Figure 4. Comparison of nisoldipine modulation of calcium channel types: smooth muscle a1 (sm), native heart (native), and cardiac a1 (hrt). A, Bar graph shows tonic block measured at -80 mV vs channel type. Tonic block was measured as the percent decrease in current amplitude measured from a -80-mV holding potential ("Materials and Methods") after exposing cells to 10 nmol/L nisoldipine. Time of drug application was approximately 3 minutes in all cases. Plotted is the average percent block under these conditions. The numbers of cells were as follows: sm, n=6; native, n=10; and hrt, n=7. ** significantly different (P<.0001); *** significantly different (P<.0002); and **** not significantly different (P=.128). B, Graphs show onset of nisoldipine block at -40 mV: concentration and subunit dependence. Conditioning train protocols using identical parameters ("Materials and Methods") were used to measure the onset of nisoldipine inhibition for each channel type and nisoldipine concentration. Currents were normalized, averaged, and plotted against time after changing from a -80-mV holding potential to the -40-mV conditioning potential of the train protocol for each case. The left graph summarizes the pooled data for Chinese hamster ovary cardiac (CHO HRT) cells and, for comparison, data obtained from native heart channels. The time constants describing the onset of block of CHO HRT channels were 70 seconds for 3 nmol/L (●, n=6) and 54 seconds for 10 nmol/L (▲, n=13) nisoldipine. The open circles (○) show pooled responses of native heart channels (n=5) under identical experimental conditions. The right graph summarizes pooled data for Chinese hamster ovary smooth muscle (CHO SM) cells. The time constants of the development of nisoldipine block were 34 seconds for 3 nmol/L (●, n=4) and 20 seconds for 10 nmol/L (▲, n=8) nisoldipine.
results they report are as follows: for cardiac CHO, 73.5±5.4 (mean±SEM) fmol/mg (DHP binding sites, n=3) and 0.2 nmol/L (affinity); and for SM CHO, 141±3 (DHP binding sites, n=3) and 0.1±0.04 (DHP binding sites, n=3). Thus, in the present experiments, we focused on the effects of low DHP concentrations on and voltage-dependent modulation of the expressed channel activity.

The DHP response of cells transfected with SM α1 cDNA differed in two important ways from cells transfected with cardiac α1 cDNA. First, the SM α1 channels were sensitive to lower (1 nmol/L) DHP concentrations than either native or recombinant α1 cardiac channels. Second, still at very low DHP concentrations (10 nmol/L), we found that the SM α1, but not the native or cardiac α1, channels were markedly inhibited at negative membrane potentials. Block of SM α1 channel activity was still voltage dependent at this and higher (100 nmol/L) nisoldipine concentrations, because the rate of block was significantly faster at depolarized potentials than it was at −80 mV (data not shown); however, once blocked, SM α1 channels did not recover their availability to conduct when membrane potential was returned to −80 mV or more negative potentials. At roughly 10-fold higher DHP concentrations, several investigators have reported tonic block of SM L-type channel activity at negative membrane potentials,6,25,26 and Yatani et al8 found greater resting block by the DHP nitrrendipine for SM, compared with cardiac muscle, calcium channels. This property clearly distinguishes SM from cardiac L-type channel activity, and our data show that the distinct SM sensitivity to DHPs is most likely due to structural differences of the α1 subunits in the two tissues. It will be important to test in future experiments whether cotransfection with other subunits, such as α2 or β2,27 further modifies the voltage-dependent effects of DHPs on recombinant channel activity and contributes to the marked differences we report here.

A Molecular Basis for Tissue Selectivity

The amino acid sequences of cardiac and SM α1 subunits have been deduced from cloned cDNAs, and more than 95% of the two proteins are identical.8,12 Although both clones do not differ in the putative DHP binding domain,28-31 differences do exist within the amino terminal portions of the two proteins and the hydrophobic segments IS6 and IVS3, which are supposed to cross the cell membrane. In addition, the SM protein contains an insertion within the linker between I and II.12,13 The functions of these different amino acid sequences within the cardiac and SM α1 subunits have not been determined. In current topographical models, the amino terminus and the linker between repeat I and II are localized intracellularly,32 and cytoplasmic elements have been shown to be involved in both activation and inactivation gating in voltage-activated potassium33,34 and sodium35 channels. However, both recombinant calcium channels share similar activation and inactivation kinetics, and the distinct sensitivity to DHPs of the SM protein might therefore depend on the transmembrane segments IS6 and IVS3. In fact, DHPs are strongly bound by lipid bilayers and appear to bind to hydrophobic regions of the calcium channel.16 Additionally, it has been demonstrated that a protein that

imitates the sequence of segment IVS3 reconstitutes a channel in planar lipid bilayers containing pharmacological properties of authentic calcium channels.37 Thus, the different IVS3 sequences might contribute to the distinct sensitivity toward DHPs of SM and cardiac muscle α1, and in this case, exchanging the cardiac versus the SM IVS3 sequence should confer SM DHP sensitivity to the recombinant cardiac channel. Identification of the structural elements underlying the unique tissue-selective properties of this family of organic compounds will allow the design of more selective drugs and should provide key insights into the mechanism that underlies the well-known regulation of indigenous channel gating by DHPs.3,4,8

The results of the present study imply that slight differences in the molecular structure of an ion channel protein can have marked effects on the interactions between the channel protein and modulatory agents. In addition to the implications for the design of tissue-selective compounds, our results suggest that it might be possible that expression of ion channel protein variants that may occur during a chronic disease such as hypertension could account for differences in drug activity in the diseased state compared with normal tissue.

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