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Synthesis and Evaluation of a New Class of Nifedipine Analogs with T-Type Calcium Channel Blocking Activity

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ABSTRACT

We have synthesized a novel series of 18 dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates from anacardic acid, a natural compound found in cashew nut shells, and investigated their blocking action on L- and T-type calcium channels transiently expressed in tSA-201 cells. The IC_{50} values for L-type calcium channel block obtained with the series ranged from 1 to ~40 μM, with higher affinities being favored by increasing the size of the alkoxy group on the 4-phenyl ring and ester substituent in the 3,5 positions. A detailed analysis of the strongest L-type channel blocker of the series (PPK-12) revealed that block was poorly reversible and mediated an apparent speeding of the time course of inactivation. Moreover, in the presence of PPK-12, the midpoint of the steady state inactivation curve was shifted by 20 mV toward more hyperpolarized potentials, resulting in an increase in blocking efficacy at more depolarized holding potentials. Surprisingly, PPK-12 blocked T- and L-type calcium channels with similar affinities. One of the weakest L-type channel inhibitors (PPK-5) exhibited a T-type channel affinity that was similar to that seen with PPK-12, resulting in a 40-fold selectivity of PPK-5 for T- over L-type channels. Thus, dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates may serve as excellent candidates for the development of T-type calcium-channel specific blockers.

Voltage-gated calcium channels are important regulators of calcium influx in a number of cell types. Calcium entry through these channels activates a plethora of intracellular events, from the broad stimulation of gene expression, calcium-dependent second messenger cascades, and cell proliferation to the specific release of neurotransmitter within the nervous system and contraction in smooth and cardiac muscle (Tsien et al., 1991; Wheeler et al., 1994; Dunlap et al., 1995). A number of different types of calcium channels have been identified in native tissues and divided based on their biophysical profiles into low-voltage–activated (LVA) and high-voltage–activated (HVA) channels (Nowycky et al., 1985; Tsien et al., 1991). LVA channels first activate at relatively hyperpolarized potentials and rapidly inactivate (Akaike et al., 1989; Takahashi et al., 1991). By contrast, HVA channels require stronger membrane depolarizations to activate and can be divided further into N-, P/Q-, R- and L-types based on their pharmacological properties (for review, see Stea et al., 1995; Zamponi, 1997). Molecular cloning has revealed that HVA channels are heteromultimers composed of a pore-forming α_1 subunit plus ancillary α_2-δ, β, and possibly γ subunits (Pragnell et al., 1994; Klugbauer et al., 1999, 2000; for review, see Catterall, 2000), whereas LVA channels seem to contain only the α_1 subunit (Lacinova et al., 2000). So far, 10 different types of calcium channel α_1 subunits have been identified and shown to encode the previously identified native calcium channel isoforms. Expression studies show that alternative splicing of α_{1A} generates both P- and Q-type Ca^{2+} channels (Bourinet et al., 1999); α_{1B} encodes *N*-type channels (Dubel et al., 1992); α_{1C}, α_{1D}, and α_{1F} are L-type channels (Williams et al., 1992; Bech-Hansen et al., 1998); α_{1G}, α_{1H}, and α_{1I} form T-type channels (i.e., McRory et al., 2001); α_{1E} may encode *R*-type channels (Soong et al., 1993; Tottene et al., 1996); and α_{1S} encodes the skeletal muscle L-type channel isoform (Tanabe et al., 1987).

Dihydropyridine (DHP) antagonists of L-type calcium channels are widely used therapeutics in the treatment of hypertension, angina, arrhythmias, congestive heart failure, cardiomyopathy, atherosclerosis, and cerebral and peripheral vascular disorders (Janis and Triggle, 1990). The ability of DHPs to both block and enhance native L-type calcium

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ABBREVIATIONS: LVA, low-voltage–activated; HVA, high-voltage–activated; DHP, dihydropyridine.

currents has been well documented (Bean, 1984; Bechem and Hoffmann, 1993; Bangalore et al., 1994; Peterson and Catterall, 1995). Although they are considered to be selective inhibitors of the L-type, a number of reports have suggested that native T-type channels may also show sensitivity to certain commonly used DHPs (Akaike et al., 1989; Romanin et al., 1992; Formenti et al., 1993; Santi et al., 1996). In contrast, for cloned calcium channels, only α_{1S}, α_{1C}, and α_{1D} have been shown to be sensitive to the most commonly used DHPs (Williams et al., 1992; Tomlinson et al., 1993; Grabner et al., 1996; Berjukow et al., 2000; Koschak et al., 2001). None of the remaining calcium channel subtypes cloned from brain exhibit significant DHP sensitivity; however, information on DHP block of cloned T-type calcium channels is still sparse.

Here, we report the calcium channel blocking action of a novel series of DHP derivatives [dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates] derived from anacardic acid, a phenolic constituent present in cashew nut shell liquid (Paul and Yeddanapalli, 1956). Our data show that these novel DHPs have the propensity to mediate high-affinity inhibition of cloned L-type calcium channels. However, more interestingly, these compounds also effectively block T-type calcium channels, in some cases with a substantial unparalleled selectivity over the L-type channel.

Materials and Methods

Chemistry. Anacardic acid was isolated from cashew nut shell liquid by a novel method reported by Paramashivappa et al. (2001). As illustrated in Fig. 1, the ene mixture of anacardic acid obtained by this method was hydrogenated using Pd/C to obtain saturated anacardic acid (Fig. 1, scheme 1.1), which after alkylation with dimethyl or diethyl sulfate using potassium carbonate gave the dialkylated derivative (Fig. 1, scheme 1.2). Di isopropyl anacardic acid was obtained by using isopropyl bromide (Fig. 1 , scheme 2). Dialkylated anacardic acid (Fig. 1, scheme 1.2) was reduced to alcohol (Fig. 1, scheme 1.3) using lithium aluminum hydride and oxidized to corresponding aldehyde (Fig. 1, scheme 1.4) using pyridinium chloro chromate. The aldehyde is then converted to 1,4-dihydropyridine using the modified Hantzsch procedure. All the 1,4-dihydro pyridines were converted to pyridine derivatives (Yadav et al., 2000; Fig. 1, scheme 3) for further characterization of the compounds.

All of the compounds were characterized by IR, ^{1}H-NMR, ^{13}C-NMR, and electrospray mass spectrometry. The melting points for all of the compounds were recorded on an electrically heated melting point apparatus and are uncorrected. IR spectra were recorded on a Galaxy 4020 FT-IR instrument (Mattson, Madison, WI) as KBr discs. NMR (^{1}H and ^{13}C) spectra were recorded on a DPX200 (Bruker, Fallenden, Switzerland; 40 MHz for ^{13}C and 200 MHz for ^{1}H), Fourier transform-NMR in CDCl3 using tetramethylsilane as an internal standard. In the case of ^{1}H NMR, typically 500 scans were accumulated. All signals were referenced to tetramethylsilane to within ±0.01 ppm. Typically 1000–2000 scans were accumulated for the proton noise decoupled ^{13}C NMR spectra. TLC was done using

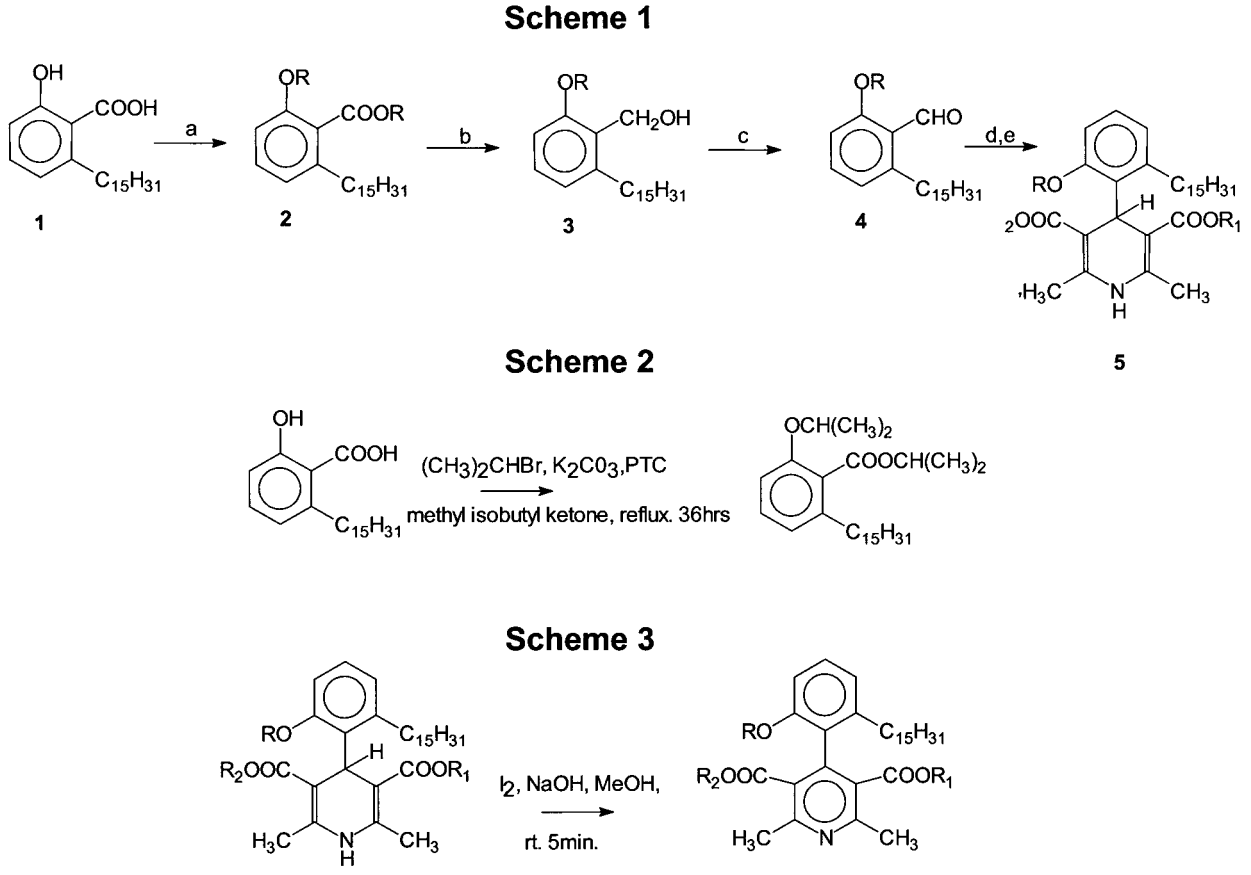


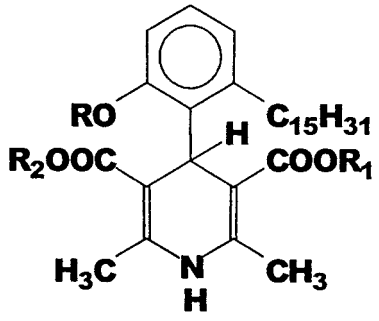
Fig. 1. Synthesis of the series of dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates from anacardic acid as described in detail under *Materials and Methods* section. In scheme 1, the reagents used in each step were as follows: $(CH_3)_2SO_4/K_2CO_3$, acetone, reflux 3 h (a); $LiAlH_4$, Tetrahydrofuran, reflux 3 h (b); PCC, dichloromethane, rt. 3 h (c); $CH_3COCH_2COOR_1$/piperidine/acetic acid, *n*-butanol, rt. 3 h (d); and $(CH_3)(NH_2)C = CH(COOR_2)$, *n*-butanol, reflux 30 h (e).

precoated silicagel GF_{254} plates (Merck, Darmstadt, Germany) with hexane/EtOAc/acetic acid (7:3:0.1) as the developing solvent and visualized by UV at 254 and 360 nm. Electrospray mass spectra were recorded on VG QUATTRO II from Micromass, UK.

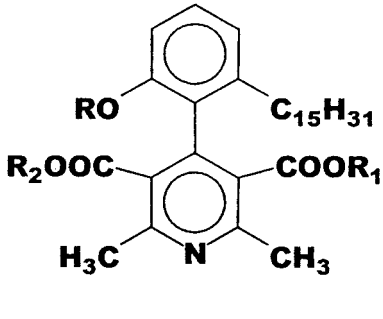
Preparation of Dialkyl 1,4-Dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 Pyridine Dicarboxylates. 2-Ethoxy-6-pentadecyl-2-benzaldehyde (3 g, 8.3 mmol) and ethylacetoacetate (1.08 g, 8.3 mmol) were dissolved in *n*-butanol (20 ml). Acetic acid (0.5 g, 8.3 mmol) and piperidine (0.7 g, 8.3 mmol) were added and stirred at room temperature for 3–4 h. Ethyl-3-amino crotonate (1.1 g, 8.3 mmol) was then added and refluxed for 30 h. 2-*n*-Butanol was distilled off under vacuum and product was purified by column chromatography using silicagel (100–200 mesh) with hexane/EtoAc (94:6) solvent system to give dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates (PPK4) as white powder (0.69 g, 14.2%).

Cell Culture and Transfections. The human embryonic kidney tSA-201 cell line was used to transiently express cloned rat α_{1C} and human α_{1G}) calcium channels. The cells were grown in standard Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, and 0.5 mg/ml penicillin streptomycin. Once the cells were at 90% confluence, they were split with trypsin-EGTA, and plated on glass coverslips at 10% confluence 12 h before transfection. Immediately before transfection, fresh media was given to the cells. A standard calcium phosphate protocol was used to transiently transfect the cells with cDNA constructs encoding for the calcium channel α_1 subunit (α_{1C} or α_{1G}) and the green fluorescent protein EGFP (7 and 4 μg of DNA, respectively). DNA constructs encoding ancillary β_{1b} and α_2-δ subunits were added to the α_{1C} mix (7 μg of each DNA). After a 12-h incubation at 36°C, the cells were washed with fresh DMEM and allowed to recover for an additional 12 h. Subsequently, the cells were incubated at 28°C in 5% CO_2 for 1 to 3 days before recording. The cDNA constructs encoding calcium channel α_{1C}, α_2-δ, and β_{1b} subunits were kindly donated by Dr. Terry Snutch, and the "b" splice variant of the human α_{1G} construct was isolated as we have described elsewhere (A. M. Beedle, J. Hamid, and G. W. Zamponi, submitted). The electrophysiological and biophysical properties of our human α_{1G} clone (i.e., half-activation potential of −47 mV, half-inactivation potential of −79 mV in 2 mM barium) closely parallel those described for the "b" splice variant by Monteil et al. (2000).

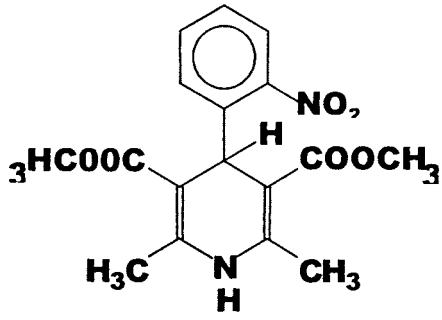
Electrophysiological Recordings. Expressed calcium channels were screened at room temperature for macroscopic currents using an Axopatch 200B amplifier (Axon Instruments, Union City, CA) linked to a personal computer equipped with pCLAMP version 6.0. Patch pipettes (Sutter borosilicate glass, BF150–86-15), pulled using a microelectrode puller (P87; Sutter Instruments, Novato, CA) to a typical resistance of ~4 MΩ and fire-polished with a Narashige microforge, were used for whole-cell patch clamp recordings. Typically, series resistance and capacitance were compensated by at least 85% to minimize contamination of records by voltage errors caused by insufficient voltage clamp. The internal pipette solution consisted of 108 mM cesium methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, and 9 mM HEPES, pH 7.2 with cesium hydroxide. The external bath solution consisted of 5 mM $BaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 40 mM tetraethylammonium chloride, 10 mM glucose, and 90 mM CsCl, pH 7.2 with tetraethylammonium hydroxide. The DHP derivatives were solubilized in dimethyl sulfoxide to a concentration of 100 mM. From the stock, the compounds were diluted to the appropriate concentration in external solution. The final concentration of dimethyl sulfoxide in the recording solution was kept below 1:1000, which did not affect calcium channel activity. External solutions were applied to cells expressing calcium channels through a sole-



PPK 1-16



PPK 17-18



NIFEDIPINE

TABLE 1

Nomenclature, chemical structures, and chemical properties of the series of compounds examined in this study

Note that PPK-17 and -18 do not belong to the dihydropyridine class.

Sample Name	R	R_1	R_2	Melting Point
PPK-1	CH_3	CH_2CH_3	CH_3	52–54°C
PPK-2	CH_3	CH_2CH_3	CH_2CH_3	42–44°C
PPK-3	CH_2CH_3	CH_2CH_3	CH_3	36–38°C
PPK-4	CH_2CH_3	CH_2CH_3	CH_2CH_3	65–68°C
PPK-5	$CH(CH_3)_2$	CH_3	CH_3	Viscous liquid
PPK-6	$CH(CH_3)_2$	CH_2CH_3	CH_3	Viscous liquid
PPK-7	$CH(CH_3)_2$	CH_2CH_3	CH_2CH_3	Viscous liquid
PPK-8	CH_3	$CH(CH_3)_2$	CH_3	Viscous liquid
PPK-9	CH_3	$CH(CH_3)_2$	CH_2CH_3	Viscous liquid
PPK-10	CH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	Viscous liquid
PPK-11	CH_2CH_3	$CH(CH_3)_2$	CH_3	Viscous liquid
PPK-12	CH_2CH_3	$CH(CH_3)_2$	CH_2CH_3	Viscous liquid
PPK-13	CH_2CH_3	$CH(CH_3)_2$	$CH(CH_3)_2$	48–50°C
PPK-14	$CH(CH_3)_2$	$CH(CH_3)_2$	CH_3	Viscous liquid
PPK-15	$CH(CH_3)_2$	$CH(CH_3)_2$	CH_2CH_3	Viscous liquid
PPK-16	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$	Viscous liquid
PPK-17	CH_3	CH_2CH_3	CH_2CH_3	48–50°C
PPK-18	CH_2CH_3	CH_2CH_3	CH_2CH_3	Low melting solid

noid-driven perfusion system. In our experiments, rundown of α_{1C} and α_{1G} currents was typically minimal (<10% over the course of a typical experiment), and the few cells that exhibited significant rundown were discarded. To minimize the possibility of contamination from rundown during prolonged drug applications, cells were repeatedly pulsed in the presence of 5 mM barium control solution until a stable baseline was obtained. Only after currents had reached equilibrium were the compounds bath-applied at the concentrations detailed in the figures. The time course of the effects of the various PPK-compounds was determined at a test potential of 0 mV (α_{1C}) or −20 mV (α_{1G}) applied every 10 s for 200 ms from a holding potential of −80 mV. After the drug effect had stabilized, the cells were washed with control external solution for up to 10 min to determine whether any effects were reversible. To determine the effect of the holding potential on drug affinity, the holding potential was changed to either −60 mV or −40 mV and the duration of the test potential was reduced to 30 ms and 15 ms, respectively. Steady-state inactivation curves were obtained in the presence and absence of compounds by providing conditioning pulses between −100 mV and + 20 mV in 10 mV increments for 5 s and measuring the corresponding currents at a standard potential of 10 mV (α_{1C}) or −20 mV (α_{1G}).

Data Analysis. All data were analyzed using Clampfit (Axon Instruments) and Sigmaplot 4.0 (Jandel Scientific, Costa Madre, CA). Steady-state inactivation curves were fit with the Boltzmann equation to obtain half inactivation potentials. The dose-response curves were fit with the Hill equation to determine the IC_{50} values of the compounds. The data are given as mean ± S.E. and the numbers in parentheses reflect the number of experiments per group. Student's *t* tests were carried out to ascertain the statistical significance of the results ($P < 0.05$).

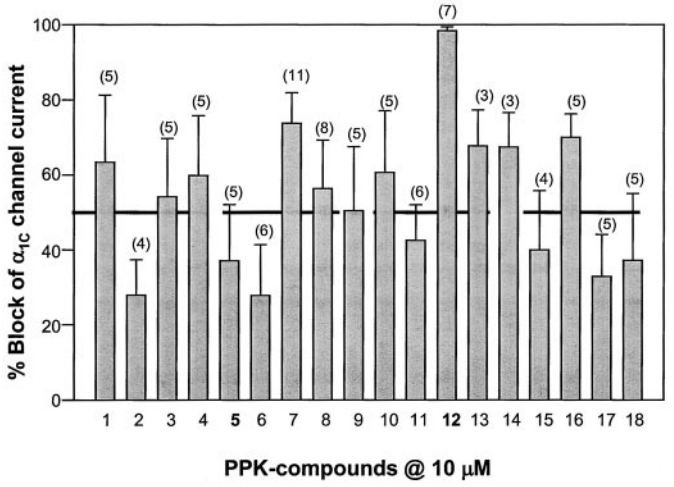


Fig. 2. Inhibitory effects of the PPK series on L-type (α_{1C} +α_2- δ + β_{1b}) calcium channels transiently expressed in tSA-201 cells. Whole-cell currents were recorded in 5 mM external barium by stepping from a holding potential of −80 mV to a test depolarization of 0 mV. The degree of block was determined from the percentage of peak current inhibition obtained in the presence of each of the compounds. The bars reflect means, error bars are S.D., and numbers in parentheses indicate the number of experiments.

Results

Structure Activity Relationship of the Dialkyl 1,4-Dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 Pyridine Dicarboxylates. Anacardic acid is a phenolic constituent present in cashew nut shell liquid (Paul and Yeddanapalli, 1956) and is reported to exhibit antibacterial (Kubo et al., 1993a), antitumor (Kubo et al., 1993b) and antiacne (Kubo et al., 1994a) properties. It is also known to inhibit such medicinally important enzymes as prostaglandin synthase (Grazzini et al., 1991), lipoxygenase (Shobha et al., 1994), and tyrosinase (Kubo et al., 1994b). We have used saturated anacardic acid (2-hydroxy-6-pentadecyl benzoic acid) to prepare a series of 18 novel dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates (termed PPK-1 through 18; Table 1). Because these derivatives are structurally related to DHP compounds of the nifedipine class, we investigated their abilities to block transiently expressed L-type calcium channels via whole-cell patch-clamp recordings. In these experiments, cells were held at −80 mV to approximate the membrane potential of native excitable cells and to isolate tonic block from any

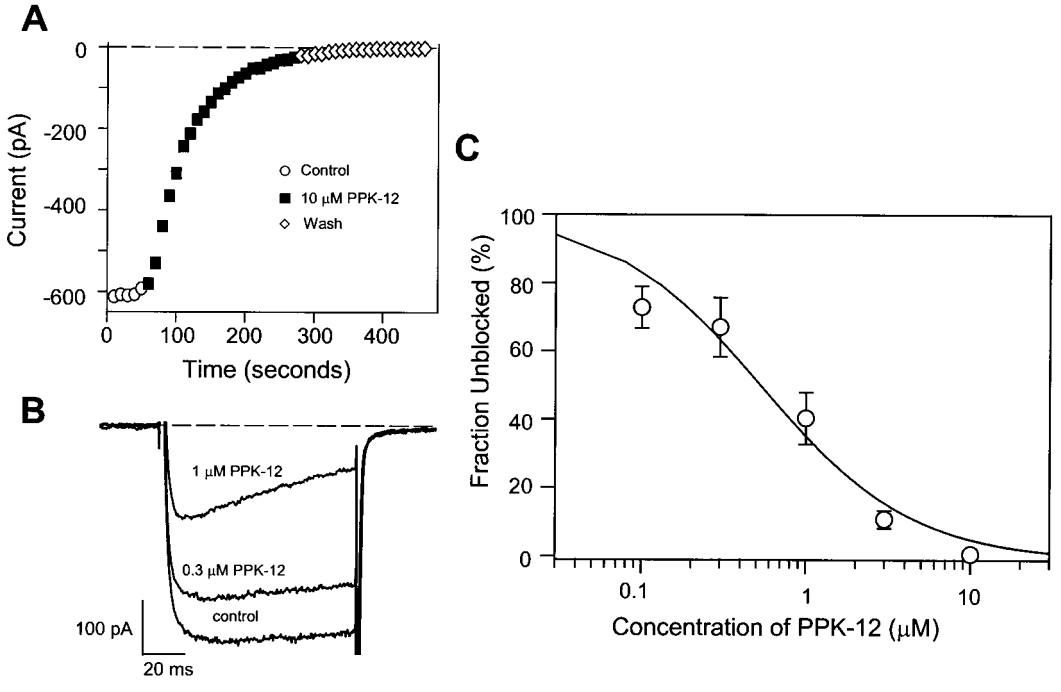


Fig. 3. Effects of PPK-12 on transiently expressed L-type channels at a holding potential of −80 mV. A, representative time course of PPK-12 block of transiently expressed L-type calcium channels. Symbols reflect peak current amplitudes in the presence and absence of 10 μM PPK-12. B, representative current records obtained before and after application of PPK-12. Note the inhibition of peak current amplitude and the speeding of the rate of current decay. C, ensemble dose-response curve obtained from 10 experiments. The data were fitted with the Hill equation (solid line). The parameters obtained from the fit were as follows; IC_{50} = 0.54 μM, n_H=0.95). Error bars reflect S.E. and the symbols are means of 7 to 10 independent determinations of drug effect.

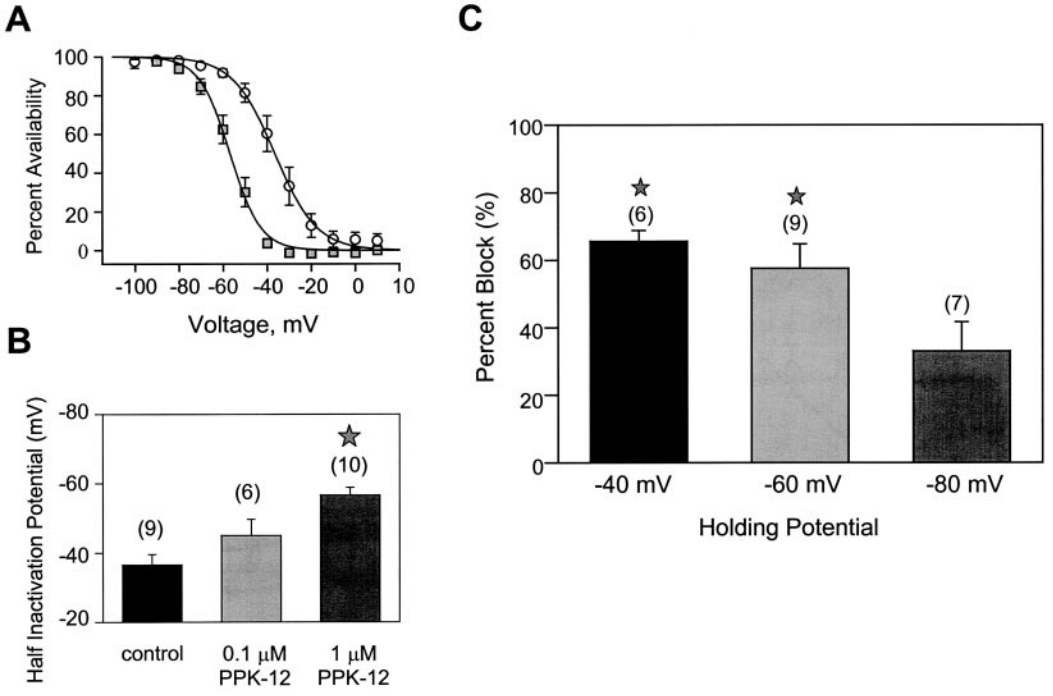


Fig. 4. Dependence of PPK-12 block of L-type calcium channel on holding potential. A, ensemble steady-state inactivation curves obtained in the absence (○) and presence (□) of 1 μM PPK-12. The data were fitted with the Boltzmann equation (solid lines). The half inactivation potentials obtained from the fits were −36.5 mV and −56.8 mV in the absence and the presence of 1 μM PPK-12, respectively. In each case, steady-state inactivation curves in the absence and presence of PPK-12 were recorded from the same cell for a total of 10 cells. Error bars are S.E. B, dependence of half-inactivation potential on the concentration of PPK-12. The asterisks indicate statistical significance relative to control ($P < 0.05$; Student's t test). C, dependence of the blocking effects of 300 nM PPK-12 on the holding potential. Note the significant increase in the degree of block ($P < 0.05$) at more depolarized membrane potentials. The asterisks indicate significance relative to the data obtained at −80 mV ($p < 0.05$).

putative inactivation state dependent effects. As seen in Fig. 2, under these conditions, the application of a standard concentration for each compound (10 μM) results in varying degrees of inhibition of $\alpha_{1C} + \alpha_2\text{-}\delta + \beta_{1b}$ calcium channels transiently expressed in tSA-201 cells ranging from about 25% (i.e., PPK-2) to virtually complete block (PPK-12). Examination of the R group structures of these compounds reveals a moderate structure activity relationship: increasing the size of alkoxy group on 4-phenyl ring and ester substituent in the 3,5 positions increases the efficacy of these compounds, where the role of the C_{15} alkyl chain is to ensure perpendicularity between 4-aryl and dihydropyridine rings. Moreover, modification of the dihydropyridine ring of derivatives with inherent symmetry to nonsymmetric analogs seems to result in more potent block, as suggested by earlier work (Rovnyak et al., 1992) on nifedipine analogs. Hence, dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates can yield effective blockers of L-type calcium channels with SAR in both the phenyl and DHP rings.

Analysis of PPK-12 Block of L-Type Calcium Channels. Figs. 3 and 4 more precisely characterize the effects of the most efficient blocker identified in the initial screen, PPK-12. Figure 3A illustrates a typical time course of the effects of PPK-12 on transiently expressed L-type calcium channels. Block developed rapidly, but was not reversible after washout, indicating either a tight interaction with the blocking site, or accumulation of the compounds in the plasma membrane. Figure 3B displays current records obtained with transiently expressed L-type calcium channels in the presence and the absence of PPK-12. As seen from the records, significant block already occurs at 300 nM concentrations, whereas block is greater than half-maximal at 1 μM PPK-12. Upon inspection of the current records, a significant speeding of the macroscopic time constant for current inactivation becomes apparent. This could reflect additional open channel block developing during the time course of the membrane depolarization or, alternatively, a drug-induced inactivation of the currents similar to what has been proposed to occur for certain DHPs (Berjukow et al., 2000). Irrespective of the mechanism, this speeding would serve to further depress calcium influx during prolonged membrane depolarization. Figure 3C displays a complete dose-response curve for PPK-12 block obtained at a holding potential of −80 mV. The data are nicely described by the Michaelis-Menten equation, yielding an apparent IC_{50} of 540 nM. For comparison, the IC_{50} obtained for nifedipine block under identical conditions was 170 nM (see Fig. 6), indicating that PPK-12 is only about 3-fold less effective in inhibiting L-type currents compared with established DHP compounds.

The blocking affinities of a number of DHPs are strongly increased at more depolarized membrane potentials (i.e., Sun and Triggle, 1995). This strong inactivated state dependence of blocking action is typically reflected at the whole-cell levels as a hyperpolarizing shift in the midpoint of the steady state inactivation curve. Figure 4 illustrates just such a property for PPK-12. In the presence of 1 μM PPK-12, the steady-state inactivation curve was shifted about 20 mV into the hyperpolarizing direction (Fig. 4A). This effect was observed at concentrations as low as 300 nM PPK-12 and would predict significant additional current inhibition as the membrane became more depolarized. This is shown quantitatively in

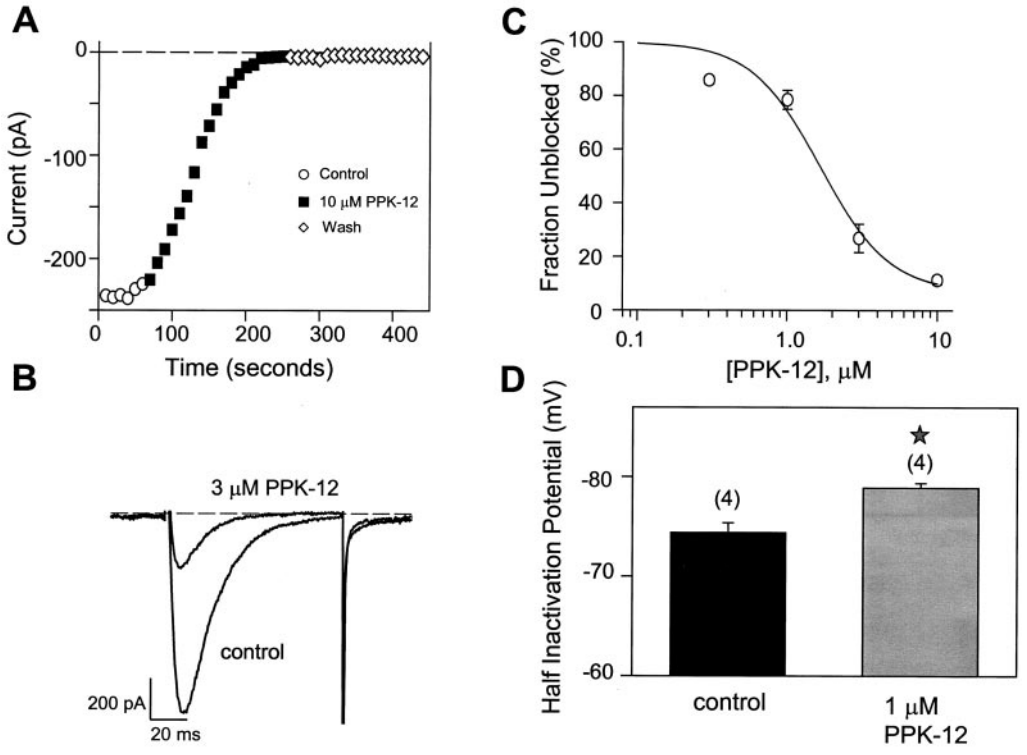


Fig. 5. Effects of PPK-12 on transiently expressed T-type calcium channels. A, representative time course of peak current block induced by 10 μM PPK-12. Note that the inhibition is virtually complete and not reversible after washout. B, effect of 3 μM PPK-12 on whole-cell current records elicited by stepping from -80 mV to a test potential of -20 mV. C, dose dependence of the effects of PPK-12 on transiently expressed T-type calcium channels. Data from 12 experiments are included in the figure. Symbols reflect means from 8 to 12 individual determinations, the solid line is a fit according to the Hill equation (IC_{50} = 1.65 μM, n_H=1.88), error bars reflect S.E. D, comparison of the half-inactivation potentials obtained in the absence and presence of 1 μM PPK-12. The asterisk indicates statistical significance ($P < 0.005$, paired Student's t test).

Fig. 4C, where the degree of block inducing a fixed concentration of PPK-12 is examined at different holding potentials. As seen from the figure, the degree of PPK-12 block increases significantly such that an inhibition of greater than 50% is obtained in the presence of 300 nM PPK-12. Hence, PPK-12 block of L-type calcium channels can occur with submicromolar affinity.

PPK-12 and PPK-5 Block T-Type Calcium Channels. In native cells, a number of DHPs have been shown to exert a low-affinity block of T-type calcium channels. To determine whether this occurred for dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates, we examined the effects of PPK-12 on α_{1G} (Cav 3.1) T-type calcium channels transiently expressed in tSA-201 cells. Application of 3 μM PPK-12 mediated a dramatic reduction in α_{1G} peak current amplitude without any additional speeding of the macroscopic time course of inactivation that was typically irreversible after washout (Figs. 5, A and B). Interestingly, the dose dependence of PPK-12 block of α_{1G} was surprisingly steep (n_H=1.88; Fig. 5C). Given the negative inactivation range of T-type calcium channels and our standard holding potential of -80 mV, a drug induced hyperpolarizing shift in the midpoint of the steady-state inactivation curve could result in additional inhibition, thus increasing the slope of the dose-response curve. To examine this possibility, we assessed the effects of PPK-12 on half-inactivation potential. As seen in Fig. 5D, 1 μM PPK-12 resulted in a small ($\sim$5 mV) but statistically significant ($p < 0.05$, paired t test) hyperpolarizing shift in the midpoint of the steady state inactivation curve (Fig. 5D). Although the magnitude of this effect did not approach that seen with the L-type calcium channel isoform, it may contribute to the shape of the dose-response curve shown in Fig. 5C. Overall, these data indicate that at hyperpolarized membrane potentials, PPK-12 blocks L- and T-type calcium channels with almost equal efficacy.

The experiments shown in Fig. 5 raise the intriguing possibility that if the drug structural requirements underlying block of T- and L-type calcium channels were to be distinct, then members of the dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylate series may offer the potential to yield a selective T-type calcium channel inhibitor. To examine this possibility, we tested the actions of one of the least effective L-type calcium channel blockers of the series (PPK-5) on transiently expressed T-type calcium channels and compared the selectivity of this compound relative to that seen with nifedipine and PPK-12. Figure 6, A and B, illustrates block of T-type calcium channels by PPK-5. As seen from the figure, the application of a 3 μM concentration of this compound mediated a robust inhibition of T-type channels that, similar to the action of PPK-12, could not be reversed with washes. The dose dependence of the PPK-5 effects indicates an IC_{50} of 1.14 μM with a Hill coefficient of 2.06 (Fig. 6C), the latter of which may be reflective of additional effects on steady-state inactivation, like the ones seen with PPK-12. Hence, despite the dramatic reduction in L-type calcium channel blocking efficacy seen with PPK-5 compared with PPK-12, the abilities of PPK-5 and PPK-12 to inhibit T-type calcium channels are remarkably similar. The relative selectivities of PPK-5 and PPK-12 for L- and T-type calcium channels are illustrated in Fig. 6D in comparison with nifedipine. Whereas nifedipine blocked L-type channels more effectively than T-type channels by almost 3 orders of magnitude, PPK-12 blocked both channel types with comparable affinities, and PPK-5 exhibited a 40-fold selectivity for T-type over L-type channels. Thus, L-type and T-type calcium channels require distinct drug structural requirements for effective DHP block.

Nifedipine Antagonizes PPK-5 Action. In view of the overall structural similarity between our series of compounds and nifedipine, it is possible that nifedipine may bind to

T-type calcium channels without significantly affecting current activity. To examine this possibility, we compared the time course of development of PPK-5 block with and without prior application of 30 μM nifedipine. Figure 7 shows that although channels that were exposed to nifedipine could still be completely blocked by 3 μM PPK-5, the time constant for development of PPK-5 block was slowed 2-fold [from 70.6 $\pm$ 11.6 s (n = 5) to 138.9 $\pm$ 12.6 s (n = 4), $p < 0.05$]. The simplest explanation for this effect is that nifedipine may indeed occupy part of the PPK-5 binding site without actually blocking current activity and that for PPK-5 to mediate its blocking effects, nifedipine may have to first dissociate from the channel. If so, these would suggest that T-type calcium channels contain a binding pocket for the DHP pharmacophore, but that channel block requires the presence of specific R groups on the DHP molecule.

Discussion

Comparison with Previous Work. Of the DHP blockers, dialkyl 1,4-dihydro-4-aryl-2,6-dimethyl-3,5-pyridine dicarboxylates of the nifedipine class have been found to offer longer bioavailability and greater tissue selectivity (Goldmann and Stoltefuss, 1991). The superior blocking efficacy of this class of compounds containing bulky ortho substituents on the 4-aryl ring has been attributed to a forced perpendicular orientation between 4-aryl ring and 1,4-dihydropyridine ring (Loev et al., 1974). Although 4-aryl(2′,6′-di substituted)1,4 DHPs were predicted to possess high affinity for the L-type calcium channel (Loev et al., 1974; Coburn et al., 1988), these derivatives received less attention because of low product yield obtained even with modified Hantzsch synthesis (Loev et al., 1974). For this study, we prepared a series of dialkyl 1,4-dihydro-4-(2′alkoxy-6′-pentadecylphenyl)-2,6-dimethyl-3,5 pyridine dicarboxylates starting from saturated anacardic acid (2-hydroxy-6-pentadecyl benzoic acid). These compounds are structurally related to nifedipine (see Table 1), and it is thus not surprising that they are able to inhibit L-type calcium channel activity, albeit to a somewhat lesser extent compared with nifedipine per se. The most efficacious L-type channel blocker of the series, PPK-12, showed several similarities to nifedipine action: first, like nifedipine, PPK-12 accelerated the time course of current decay (Lee and Tsien, 1983), which may reflect either a true effect on inactivation of the channel (Berjukow et al., 2000) or additional open channel block that develops during the test depolarization. Second, like nifedipine and several other DHPs (Lee and Tsien, 1983; Shen et al., 2000), PPK-12 mediated a robust shift in the midpoint of the steady-state inactivation curve toward more hyperpolarized potentials, indicative of strong inactivated channel block. Unlike nifedipine, however, the action of PPK-12 or any of the other derivatives examined, could not be reversed during the time course of a typical experiment. In view of evidence that DHPs must at least partially partition into the plasma membrane to mediate L-type channel block (Kass et al., 1991; Strubing et al., 1993; Bangalore et al., 1994), it is possible that the added hydrophobicity arising from the pentadecyl side chain results in accumulation of these compounds in the plasma membrane, thus precluding rapid reversal of the blocking action.

In our experiments, nifedipine only weakly inhibited T-type calcium channel activity. This is consistent with a recent report by Lacinova et al. (2000), who reported a 14% inhibition of α_{1G} current activity by 10 μM nifedipine. These authors also found a weak dependence of nifedipine blocking affinity on holding potential. Although we did not examine this property for nifedipine, PPK-12 mediated a small but

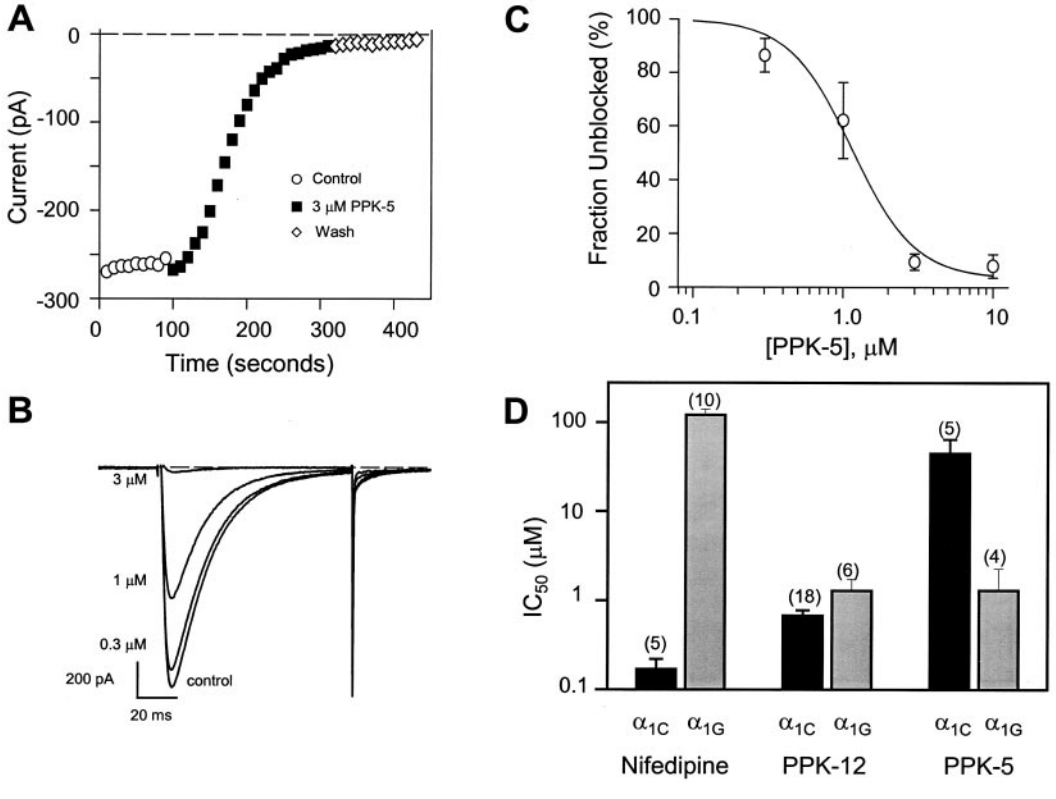


Fig. 6. Block and selectivity of PPK-5 for T-type calcium channels. A, representative time course of block of T-type calcium channel by 3 μM PPK-5. Note the lack of reversibility. B, current records obtained in the absence and presence of PPK-5 under the same conditions as those in Fig. 5B. C, dose dependence of PPK-5 action on transiently expressed T-type channels obtained from 11 different cells. The experimental conditions were the same as in Fig. 5C, the IC_{50} and Hill coefficient obtained from the fit were 1.14 μM and 2.06, respectively. D, relative selectivities of nifedipine, PPK-12, and PPK-5 among L- and T-type calcium channels. Note that the IC_{50} values shown on the ordinate are on a logarithmic scale. Error bars are S.E., numbers in parentheses reflect the number of cells used to calculate the means.

statistically significant leftward shift in the position of the steady-state inactivation curve toward more hyperpolarizing potentials, which is qualitatively consistent with the results of Lacinova et al. (2000).

Mechanism of T-Type Calcium Channel Block. Perhaps the most striking result from our study is the strong inhibition of T-type calcium channel activity by PPK-5 and PPK-12. Moreover, the 40-fold selectivity of PPK-5 for T-type over L-type calcium channels is particularly interesting, although one must be aware that this value was obtained at a holding potential of −80 mV, where little inactivated channel block of the L-type isoform is expected. A large (~20 mV) hyperpolarizing shift in the midpoint of the voltage dependence of L-type channel inactivation (such as that observed with PPK-12, Fig. 4A) would be expected to reduce the degree of selectivity for T-type channels at very depolarized holding potentials; at membrane potentials more negative than −40 mV, however, significant T-type channel selectivity would remain (for example, the apparent blocking affinity for PPK-12 increased by less than 5-fold when the holding potential was switched from −80 mV to −40 mV).

The DHP receptor site in the L-type calcium channel comprises as few as nine single amino acid residues in the domain IIIS5, IIIS6, and IVS6 regions of the α_{1C} subunit (Ito et al., 1997; Yamaguchi et al., 2000; Wappl et al., 2001). These residues are not conserved in T-type calcium channels, suggesting that the DHP blocking site on the latter channels is of a fundamentally different nature. This is also consistent with our observation that PPK-12 mediated much more pronounced effects on the steady-state inactivation behavior of L-type channels compared with the T type. Without molecular biological or biochemical studies, however, it is difficult even to speculate on a possible location of the DHP blocking site on the α_{1G} subunit. Moreover, it will be interesting to examine the abilities of these compounds to inhibit other T-type calcium channel isoforms such as α_{1H} and α_{1I} (i.e., McRory et al., 2001) to determine whether this site of action is conserved across all members of the T-type channel family.

Yet qualitative features of this site can be inferred from the observation that nifedipine, although on its own being able to mediate only a small reduction in the peak current amplitude at 30 μM concentrations, antagonized PPK-5 block of α_{1G} channels. If this concentration of nifedipine were to act on only a fraction of T-type channels in a given cell, then the remaining (nifedipine-free) channels would be expected to respond normally to the application of PPK-5. Instead, the time course of development of PPK-5 block was slowed dramatically in channels pretreated with nifedipine. It is unlikely that this effect is caused by a perfusion artifact, because our microperfusion system achieves complete solution exchanges in less than 1 s and is thus much faster than the time course of development of block observed in our experiments. We also do not expect the formation of inactive nifedipine-PPK-5 complexes, because PPK-5 was not applied as part of a nifedipine-PPK-5 mixture. Thus, we conclude that nifedipine is able to bind to a DHP interaction site on the T-type calcium channel molecule without significantly inhibiting current flux. By doing so, nifedipine might perhaps allosterically reduce the affinity of the channel for PPK-5 acting at a distinct site. However, given the structural homology between nifedipine and PPK-5, we favor a model in which the two compounds compete for the same site, but because of its bulkier substituents, PPK-5 is able to effectively block channel activity whereas nifedipine is not. For PPK-5 to be able to occupy the blocking site, nifedipine would then have to first dissociate from the channel, thereby ac-

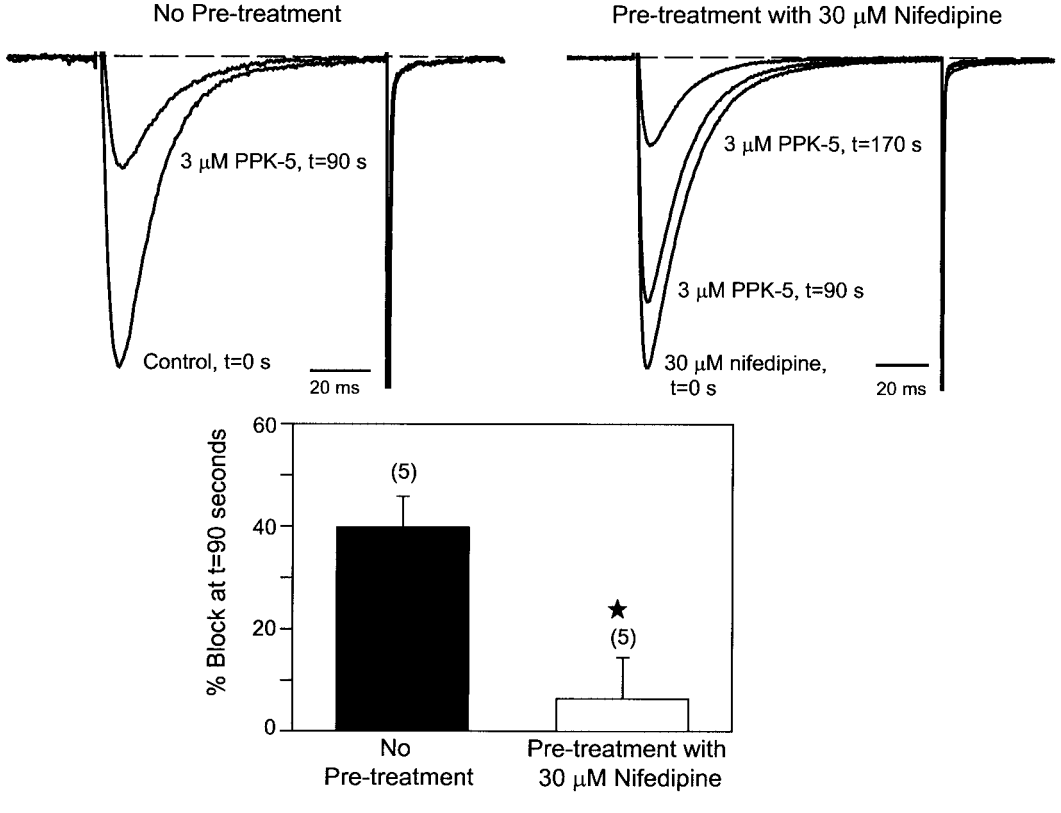


Fig. 7. Slowing of the development of PPK-5 block of T-type calcium channels by prior application of nifedipine. A, whole-cell current records obtained from α_{1G} calcium channels with (right) and without (left) prior application of 30 μM nifedipine (for 3 min), measured 90 s after application of 3 μM PPK-5. The currents obtained in the absence of PPK-5 were normalized relative to each other to facilitate comparison of the PPK-5 effect. Note that in the absence of pretreatment with nifedipine, a 90-s application of PPK-5 mediates 65% block, whereas the same exposure in cells pretreated with nifedipine results in a much smaller degree of block (~20%), and longer exposure times are required achieve a comparable level of inhibition (in this example, 170 s is required for 3 μM PPK-5 to block 70% of the current). B, quantitative comparison of the effects illustrated in A. Note that nifedipine dramatically increases the time constant for development of PPK-5 block. A total of five experiments is included in the figure.

counting for the slowed kinetics of development of block. The pentadecyl chain on the aryl ring is conserved across the entire PPK series, but absent in nifedipine, and is thus most likely to be responsible for the inhibitory effects of PPK-5 and PPK-12 on T-type calcium channel activity. This is supported by preliminary observations that PPK-17, which also carried this structure, but is technically not a DHP compound, also effectively inhibited T-type channels (S. C. Stotz and G. W. Zamponi, unpublished observations). Within the confines of our model, the aromatic moieties may serve to anchor these compounds to the channel protein, whereas the long alkyl chain may be responsible for the physical inhibition of current activity. In future experiments, it will be interesting to examine whether (and at what point) shortening the pentadecyl chain results in a loss of T-type channel blocking activity.

Overall, regardless of the detailed molecular mechanisms involved, we have identified a novel series of DHP derivatives that exhibit a unique ability to inhibit T-type calcium channels. The observed structure-activity relationship for L-type calcium channel block could potentially be used to design additional derivatives that completely lack the ability to block L-type calcium channels but in which the inhibitory effects on T-type channels are maintained or possibly even enhanced. In lieu of any presently known specific blockers of T-type calcium channels, this could pave the road toward the identification of novel, clinically active therapeutics for disorders such as epilepsy.

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