Syntheses, Calcium Channel Agonist−Antagonist Modulation Activities, Nitric Oxide Release, and Voltage-Clamp Studies of 2-Nitrooxyethyl 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate Enantiomers

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Received August 20, 2001

The novel (−)-(*S*)-**2** and (+)-(*R*)-**3** enantiomers of 2-nitrooxyethyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate were synthesized for evaluation as calcium channel modulators. Determination of their in vitro calcium-channel-modulating activities using guinea pig left atria (GPLA) and ileum longitudinal smooth muscle (GPILSM) showed that the (−)-(*S*)-**2** enantiomer acted as a *dual cardioselective calcium channel agonist (GPLA)/smooth muscle selective calcium channel antagonist (GPILSM).* In contrast, the (+)-(*R*)-**3** enantiomer exhibited calcium channel antagonist activity on both GPLA and GPILSM. The 2-nitrooxyethyl racemate is a nitric oxide (•NO) donor that released 2.7% •NO, relative to the reference drug glyceryl trinitrate (5.3% •NO release/ONO_2 moiety), in the presence of *N*-acetylcysteamine. Whole-cell voltage-clamp studies using isolated guinea pig ventricular myocytes indicated that both enantiomers inhibit calcium current but that the (−)-(*S*)-**2** enantiomer is a weaker antagonist than the (+)-(*R*)-**3** enantiomer. These results indicate that replacement of the methyl ester substituent of (−)-(*S*)-methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate [(−)-(*S*)-**1**] by the 2-nitrooxyethyl ester •NO donor substituent present in (−)-(*S*)-**2** provides a useful drug design concept to abolish the contraindicated calcium channel agonist effect of (−)-(*S*)-**1** on vascular smooth muscle. The novel (−)-(*S*)-**2** enantiomer is a useful lead compound for drug discovery targeted toward the treatment of congestive heart failure, and it provides a useful probe to study the structure−function relationships of calcium channels.

Introduction

The 1,4-dihydropyridine (1,4-DHP) L-type voltage-sensitive calcium ion channel represents an important drug target that possesses specific binding sites for both antagonist and agonist ligands that modulate the closed or open conformational state of the channel. Different states of the channel have different affinities and/or access for drugs, and drugs may exhibit both a quantitative and a qualitative structure−activity difference, including stereoselectivity between channel states.[1] In this regard, ion channels can be considered to be multiple-drug binding receptors typically having four to eight discrete binding sites that may be individually linked to each other and to the gating and permeation action of the ion channel by complex allosteric interactions.[2]

The development of tissue-selective 1,4-DHP calcium channel (CC) modulators suitable for the treatment of congestive heart failure (CHF) requires that their adverse smooth muscle vasoconstrictor effect be eliminated and/or separated from the target cardiac positive inotropic stimulant action.[3] For example, racemic methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (Bay K 8644) acts as a CC agonist on both smooth and cardiac muscle because the agonist (−)-(*S*)-enantiomer (**1**) is approximately 10-fold more potent as an activator compared to the (+)-(*R*)-antipode that acts as an antagonist.[4] The discovery that nitric oxide (•NO) serves as an endogenous activator of guanylate cyclase, an enzyme responsible for vascular muscular relaxation, and that organic nitrovasodilators exert their effect in vivo by bypassing the •NO-production system in the endothelium to deliver •NO directly to muscle cells in the artery prompted a number of studies[5−8] to design hybrid Hantzsch-type 1,4-DHP CC antagonists that also have the potential to simultaneously release •NO. In a preliminary communication,[9] we reported that (−)-(*S*)-2-nitrooxyethyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (**2**), similar to (−)-(*S*-)-**1**, acts as a cardiac CC agonist, but in contrast to (−)-(*S*)-**1**, (−)-(*S*)-**2** is a smooth muscle CC antagonist that also acts as a •NO donor in vitro (see Figure 1). This dual combination of tissue-selective CC modulating activities satisfies the clinical requirements for the treatment of CHF. In our ongoing program to acquire structure−activity relationships with respect to CC modulation and to design compounds to study CC structure−function relationships, we now report the chiral syntheses of (−)-(*S*)-**2** and its (+)-(*R*)-enantiomer (**3**), their in vitro CC modulating effects on smooth and cardiac muscle and modu-

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10.1021/jm010394k CCC: $22.00
Published on Web 01/22/2002

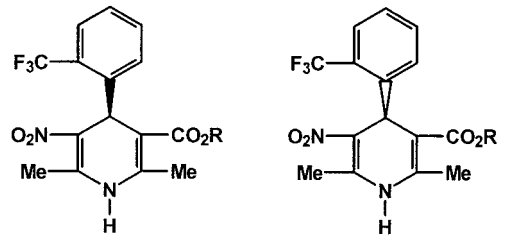


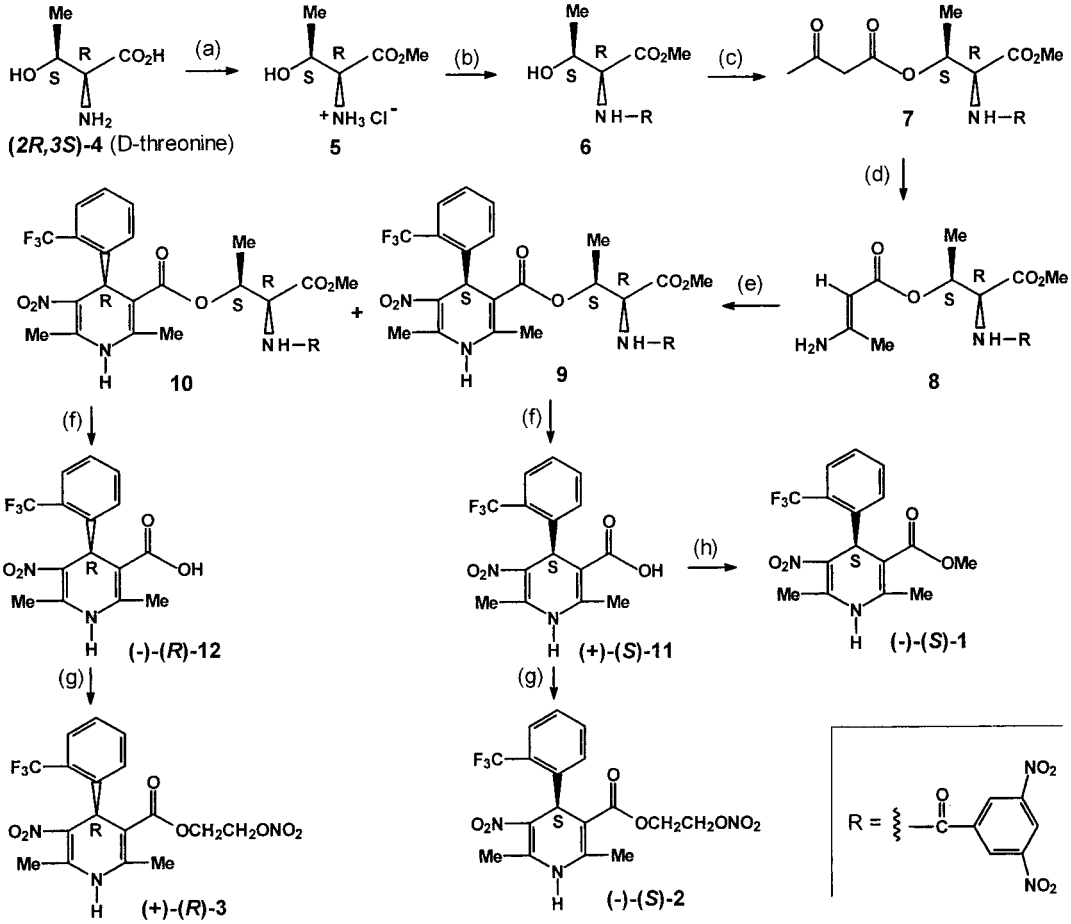
Figure 1. Structures of (−)-(*S*)-methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate [(−)-(*S*)-**1**], (−)-(*S*)-2-nitrooxyethyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (**2**), and the (+)-(*R*)-enantiomer (**3**).

lation of L-type voltage-sensitive CCs in isolated guinea pig ventricular myocytes, and •NO release data for the racemic mixture of 2-nitrooxyethyl compounds (−)-(*S*)-**2** and (+)-(*R*)-**3**.

Chemistry

The first synthetic strategy investigated to prepare the individual enantiomers (−)-(*S*)-**2** and (+)-(*R*)-**3** by a variant of the Hantzsch dihydropyridine synthesis involving the asymmetric Michael addition of a metalated chiral aminocrotonate, derived from D-valine or L-valine, to a Knoevenagel acceptor as reported earlier[10] for the efficient synthesis of chiral dialkyl 1,4-dihydropyridine-3,5-dicarboxylates afforded (−)-(*S*)-**2** or (+)-(*R*)-**3** in unacceptably low enantiomeric excess (70% ee). Alternatively, it was anticipated that use of a chiral esterifying group such as D-threonine, which could be readily removed by a β-elimination reaction using a nonnucleophilic base such as 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU), would be a suitable derivative for separation of diastereomeric esters (**9**, **10**) that could be converted to the chiral acids (**11**, **12**).[11] Accordingly, the reaction of methyl (2*R*,3*S*)-2-amino-3-hydroxybutanoate hydrochloride (**5**), prepared by esterification of D-threonine with $SOCl_2$ in MeOH, with 3,5-dinitrobenzoyl chloride gave the *N*-(3,5-dinitrobenzoyl) derivative **6** (see Scheme 1). Reaction of the alcohol **6** with 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (a diketene precursor) yielded the acetoacetate ester **7**. Elaboration of **7** to the β-aminocrotonate derivative **8** and its subsequent modified Hantzsch condensation with 2-trifluoromethylbenzaldehyde and nitroacetone afforded a mixture of the 1,4-dihydropyridine diastereomers **9** and **10**. These two diastereomers, which differ in configuration (*S* or *R*) at the C-4 position of the 1,4-dihydropyridine ring, were separated by silica gel column chromatography. Cleavage of the individual esters **9** and **10** using DBU at 25 °C yielded the individual (+)-(*S*)-**11** and (−)-(*R*)-

Scheme 1[a]



[a] Reagents and conditions: (a) $SOCl_2$, MeOH, 25 °C, 48 h; (b) K_2CO_3, 3,5-dinitrobenzoyl chloride, EtOAc/H_2O, 25 °C, 17 h; (c) 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one, xylene, 150 °C, 30 min; (d) 4-Me−C_6H_4−SO_3H, NH_3, toluene, 130 °C, 4 h; (e) 2-CF_3−C_6H_4−CHO, $MeCOCH_2NO_2$, EtOH, reflux, 80 °C, 17 h; (f) 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 25 °C, 4 h; (g) $BrCH_2CH_2ONO_2$, DMF, K_2CO_3, 25 °C, 24 h; (h) CH_2N_2, MeOH, 25 °C, 15 min.

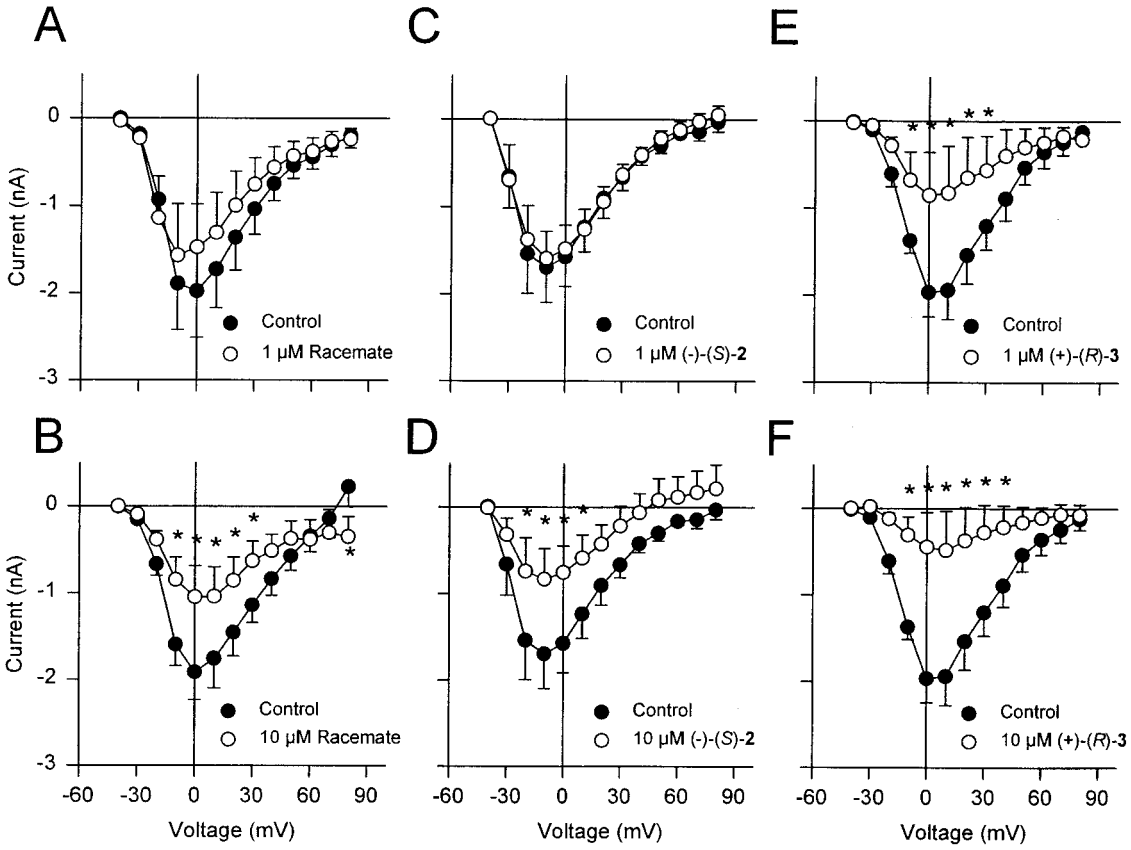


Figure 2. I_{Ca} is inhibited by the 5-(2-nitrooxyethyl) racemate [(−)-(*S*)-**2**/(+)-(*R*)-**3**], as well as by the (−)-(*S*)-**2** and (+)-(*R*)-**3** enantiomers. Shown are the mean ± SEM current−voltage relationships for I_{Ca} under control conditions and in the presence of 1 μM (A) and 10 μM (B) racemate (−)-(*S*)-**2**/(+)-(*R*)-**3**. At a concentration of 1 μM, the (−)-(*S*)-**2** enantiomer had no effect on I_{Ca} (C), but 10 μM (−)-(*S*)-**2** inhibited I_{Ca} (D). In contrast, the (+)-(*R*)-**3** enantiomer markedly inhibited I_{Ca} at both 1 μM (E) and 10 μM (F). Asterisks indicate points that are significantly different from the control ($n = 4-7$ cells per group; $p < 0.05$).

12 carboxylate enantiomers, respectively. Reaction of (+)-(*S*)-**11** or (−)-(*R*)-**12** with $BrCH_2CH_2ONO_2$ in the presence of K_2CO_3 in DMF afforded the respective (−)-(*S*)-**2** or (+)-(*R*)-**3** enantiomers of 2-nitrooxyethyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate.

The absolute configuration of the (−)-(4*S*)-**2** enantiomer {$[\alpha]^{23}_D$ −48.75°} was assigned based on the fact that (+)-(*S*)-**11** was elaborated to the known (−)-(*S*)-**1**, which shows an optical rotation {$[\alpha]^{23}_D$ −56.75°} identical to the reported value {$[\alpha]^{23}_D$ −56.7°}.[4] Therefore, the dextro enantiomer (+)-(4*R*)-**3** {$[\alpha]^{23}_D$ +48.75°} must have the (4*R*)-configuration. The two enantiomers (−)-(*S*)-**2** and (+)-(*R*)-**3** each exhibited a single resonance for the dihydropyridine C-6 methyl resonance upon addition of the ^{1}H NMR chiral shift reagent (+)-Eu(hfc)$_3$, indicating a very high optical purity (≥96% ee).

Results and Discussion

Two hybrid Hantzsch-type 1,4-DHP enantiomers [(−)-(*S*)-**2** and (+)-(*R*)-**3**] having a 2-nitrooxyethyl ester nitric oxide (•NO) donor moiety in place of the methyl ester substituent in methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate were synthesized. It was anticipated that the smooth muscle vasorelaxant effect of released •NO offered a drug design concept[5−8] to counteract the contraindicated smooth muscle vasconstrictor effect exhibited by methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate while retaining its desired cardiac positive inotropic (calcium agonist) effect. Credence for this concept is based on reports that the CC antagonist nitrendipine enhances the release of •NO from vascular endothelium, which may increase it vascular relaxation effect,[12] that •NO modulates the activity of the Ca^{2+} release channel by preventing oxidation of regulatory sulfhydryls,[13] and that organic nitrates and •NO donors evoke a small but constant positive inotropic effect in vivo that is not induced by coronary vasodilation.[14]

In vitro calcium channel (CC) modulating activities of the (−)-(*S*)-**2** and (+)-(*R*)-**3** enantiomers were determined using guinea pig ileum longitudinal smooth muscle (GPILSM) and guinea pig left atrium (GPLA). (−)-(*S*)-**2** [GPLA $ED_{50} = (9.2 \pm 6.8) \times 10^{-7}$ M, $n = 3$], like (−)-(*S*)-**1**,[4] acts as a cardiac CC agonist (positive inotrope) but, unlike (−)-(*S*)-**1**, is a smooth muscle CC antagonist [GPILSM $IC_{50} = (1.6 \pm 0.1) \times 10^{-5}$ M, $n = 3$]. The EC_{50} value for agonist activity (GPLA) is the molar concentration eliciting 50% of the maximum contractile response produced by the test drug on GPLA, as determined graphically from the dose−response curve. In contrast, the (+)-(*R*)-**3** enantiomer exhibited CC antagonist effects on both cardiac [GPLA $IC_{50} = (1.6 \pm 8.2) \times 10^{-5}$ M, $n = 3$] and smooth muscle [GPILSM $IC_{50} = (7.9 \pm 0.4) \times 10^{-8}$ M, $n = 3$].

In vitro •NO release was determined by quantitation of nitrite using the Griess reaction.[15] The percentage of •NO release for the 2-nitrooxyethyl racemate [(−)-(*S*)-**2**/(+)-(*R*)-**3**] was 1.09 ± 0.02% and 2.70 ± 0.02% in the

absence and presence of *N*-acetycysteamine, respectively ($n = 3$). In comparison, the percentage of •NO release for the reference drug glycerol trinitrate was 0.18 ± 0.00% per ONO_2 moiety and 5.30 ± 0.00% per ONO_2 moiety in the absence and presence of *N*-acetylcysteamine, respectively ($n = 3$). These •NO release data are consistent with the previous observation that (−)-(*S*)-**2** acts as a CC antagonist on GPILSM, in contrast to (−)-(*S*)-Bay K8644, which acts as a CC agonist on smooth muscle[4] and which is most likely the result of smooth muscle relaxation induced by release of •NO.

The modulation of L-type sensitive calcium channels by the 2-nitrooxyethyl racemate [(−)-(*S*)-**2**/(+)-(*R*)-**3**] and the (−)-(*S*)-**2** and (+)-(*R*)-**3** enantiomers in guinea pig ventricular myocytes using the whole-cell voltage-clamp technique was investigated. In Figure 2 are the mean (±SEM) current−voltage relations for I_{Ca} under control conditions and in the presence of 1 and 10 μM concentrations of the racemate (panels A and B), (−)-(*S*)-**2** (panels C and D), and (+)-(*R*)-**3** (panels E and F). In all experimental groups under control conditions, the threshold for activation of I_{Ca} was between −40 and −30 mV, and the current reached a maximum between potentials of −10 and 0 mV (Figure 2, panels A−F). At a concentration of 1 μM, the 2-nitrooxyethyl racemate had little effect on the magnitude of I_{Ca} (panel A). However, 10 μM racemate significantly inhibited I_{Ca}, in particular near the peak of the current−voltage curve (panel B). (−)-(*S*)-**2** had no effect on the magnitude of I_{Ca} at 1 μM but exerted a weak antagonist effect at the higher concentration (10 μM, panel D). In contrast, (+)-(*R*)-**3** exerted significant antagonist effects at both 1 and 10 μM concentrations (panels E and F). All three compounds inhibited peak inward current but did not appear to shift the voltage dependence of I_{Ca}. These results suggest that (−)-(*S*)-**2** is a weaker antagonist of calcium current than (+)-(*R*)-**3** in guinea pig ventricular myocytes. Furthermore, the effects of the 2-nitrooxyethyl racemate appear to be between the effects of the two enantiomers. Although 1 μM (−)-(*S*)-**2** had little effect on the magnitude of I_{Ca} as measured in isolated guinea pig ventricular myocytes, similar concentrations of (−)-(*S*)-**2** actually enhanced contractility in guinea pig atria. This difference in results may be due to differences in atrial and ventricular myocytes or due to differences in the voltage dependence of calcium channel blockade in the two preparations. Ventricular myocytes were voltage-clamped from a membrane potential of −40 mV, whereas atrial cells in the isolated tissue experiments were at their resting membrane potential, presumably more negative than −40 mV. Certain 1,4-DHP's act as calcium channel agonists at more negative activating potentials and have much less effect at more positive potentials.[16] It is therefore possible that the calcium channel agonist activity of (−)-(*S*)-**2** also is voltage-dependent. Additional experiments will be required to test this possibility.

Conclusions

The (−)-(*S*)-**2** and (+)-(*R*)-**3** enantiomers of 2-nitrooxyethyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate represent a novel type of compound with different calcium channel modulation activities. In particular, the (−)-(*S*)-**2** enantiomer exhibits a distinctive profile in vitro that encompasses a *dual cardioselective agonist/smooth muscle selective antagonist activity* that could provide a potentially new approach to drug design targeted toward the treatment of congestive heart failure. These (−)-(*S*)-**2** and (+)-(*R*)-**3** enantiomers may also be valuable probes to study the structure−function relationship of calcium channels.

Experimental Section

Melting points were recorded with a Thomas-Hoover capillary apparatus and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker AM-300 spectrometer (300 MHz). The assignment of exchangeable protons (N*H*, NH_2, O*H*) was confirmed by the addition of D_2O. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. Infrared spectra were acquired using a Nicolet IR-500 series II spectrometer. Silica gel column chromatography was carried out using Merck 7734 (60−200 mesh) silica gel. Microanalyses were within ±0.4% of theoretical values for all elements listed, unless otherwise stated. D-Threonine, 2-bromoethanol, α,α,α-trifluoro-*o*-tolualdehyde, 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one, *N*-acetylcysteamine, and tris[3-(heptafluoropropylhydroxymethylene)-(+)-camphorato]europium(III) [Eu(hfc)$_3$] were purchased from Aldrich Chemical Co. Nitroacetone,[17] nitroglycerin,[18] and racemic 2-nitrooxyethyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate[19] were prepared using literature procedures.

2-Nitrooxyethyl Bromide. 2-Bromoethanol (12.5 g, 100 mmol) was added dropwise to a mixture of HNO_3 (11 mL of 70% w/v) and H_2SO_4 (24 mL of 98% w/v) at 0 °C with stirring, the reaction was allowed to proceed at 0 °C for 1 h, and the resulting mixture was poured into cold water (100 mL). Extraction with CH_2Cl_2 (4 × 100 mL), drying of the CH_2Cl_2 extract ($MgSO_4$), and removal of the solvent in vacuo gave the title compound (16 g, 94%) as an oil. ^{1}H NMR ($CDCl_3$): δ 3.56 (t, $J = 6.4$ Hz, 2H, H-1), 4.76 (t, $J = 6.4$, 2H, H-2). The title compound was used immediately for the subsequent syntheses of (−)-(*S*)-**2** and (+)-(*R*)-**3**.

Methyl (2*R*,3*S*)-2-Amino-3-hydroxybutanoate Hydrochloride (5). A solution of thionyl chloride (74.9 g, 0.63 mol) in MeOH (100 mL) was cooled to 0 °C, D-threonine (25.0 g, 0.21 mol) was added in aliquots with stirring, and the reaction mixture was allowed to proceed at 0 °C for 1 h and then at 25 °C for 48 h. Removal of the solvent in vacuo afforded **5** as a viscous oil (35.6 g, 100%), which was used immediately for the subsequent preparation of **6**.

Methyl (2*R*,3*S*)-2-(3,5-Dinitrophenylcarbonylamino)-3-hydroxybutanoate (6). A mixture of **5** (35.6 g, 0.21 mol) in EtOAc (300 mL) and K_2CO_3 (43.0 g, 0.31 mol) in water (150 mL) was cooled to 0 °C, and 3,5-dinitrobenzoyl chloride (48.4 g, 0.21 mol) was added in small aliquots with stirring. The reaction was allowed to proceed at 5−10 °C for 3 h and then at 25 °C for 17 h, the organic layer was separated and washed with brine (5 × 40 mL), the organic fraction was dried (Na_2SO_4), and the solvent was removed in vacuo to give a solid that was recrystallized from EtOAc/Et_2O to yield **6** as a white solid (57.8 g, 84.1%): mp 69−70 °C; $[\alpha]^{23}_D$ − 19.50° (*c* 0.4, MeOH). IR ($CHCl_3$): 1743 (CO_2), 3320 (OH), 3419 (NH) cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.32 (d, $J_{Me,CH} = 7.0$ Hz, 3H, CH−*Me*), 2.83 (br s, 1H, OH), 3.82 (s, 3H, CO_2*Me*), 4.55 (qd, $J_{CH,Me} = 7.0$ Hz, $J_{CH,CH} = 2.0$ Hz, 1H, C*H*−Me), 4.87 (dd, $J_{CH,NH} = 9.0$ Hz, $J_{CH,CH} = 2.0$ Hz, 1H, −CHC*H*NH), 7.62 (d, $J_{NH,CH} = 9.0$ Hz, 1H, CHN*H*), 9.06 (d, $J = 2.0$ Hz, 2H, phenyl H-2 and H-6), 9.17 (dd, $J = 2.0$, $J = 2.0$ Hz, 1H, phenyl H-4). Anal. ($C_{12}H_{13}N_3O_8$) C, H, N.

(1*S*,2*R*)-2-(3,5-Dinitrophenylcarbonylamino)-2-methoxycarbonyl-1-methylethyl Acetoacetate (7). A solution of **6** (45.57 g, 139.37 mmol) and 2,2,6-trimethyl-4*H*-1,3-dioxin-4-one (19.81 g, 139.37 mmol) in xylene (80 mL) was placed in a 250 mL Erlenmeyer flask. The flask was immersed in an oil bath preheated to 150 °C, and the reaction was allowed to proceed with vigorous stirring for 30 min at which time no further evolution of acetone occurred. The reaction mixture

was cooled to 25 °C, and the product was filtered and recrystallized from EtOAc/Et_2O to yield **7** as white crystals (53.96 g, 94.2%): mp 79−80 °C; $[\alpha]^{23}_D$ − 105.75° (*c* 0.4, $CHCl_3$). IR ($CHCl_3$): 1718 (CO), 1748 (CO_2), 3350 (NH) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.42 (d, $J_{Me,CH}$ = 7.0 Hz, 3H, CH*Me*), 2.34 (s, 3H, CO*Me*), 3.60 (d, J_{gem} = 16.0 Hz, 2H, COCH_2CO), 3.81 (s, 3H, CO_2*Me*), 4.98 (dd, $J_{CH,NH}$ = 9.0 Hz, $J_{CH,CH}$ = 2.0 Hz, 1H, CHC*H*NH), 5.60 (qd, $J_{CH,Me}$ = 7.0 Hz, $J_{CH,CH}$ = 2.0 Hz, 1H, O−C*H*−Me), 8.07 (d, $J_{NH,CH}$ = 9.0 Hz, 1H, CHN*H*), 9.21 (dd, *J* = 2.0, *J* = 2.0 Hz, 1H, phenyl H-4), 9.24 (d, *J* = 2.0 Hz, 2H, phenyl H-2, and H-6). Anal. ($C_{16}H_{17}N_3O_{10}$) C, H, N.

(1*S*,2*R*)-2-(3,5-Dinitrophenylcarbonylamino)-2-methoxycarbonyl-1-methylethyl 3-Aminocrotonate (8). Ammonia gas was bubbled into a solution of **7** (23.2 g, 56.4 mmol) and *p*-toluenesulfonic acid (150 mg, 0.87 mmol) in dry toluene (300 mL) that was preheated to 130 °C for 20 min. The reaction was allowed to proceed with azeotropic removal of water using a Dean−Stark apparatus for 4 h. Removal of the solvent in vacuo, purification of the residue by silica gel column chromatography (EtOAc/hexane; 1:1, v/v), and recrystallization of the product from EtOAc/hexane afforded **8** as yellowish crystals (12.6 g, 54.7%): mp 170 °C; $[\alpha]^{23}_D$ −83.25° (*c* 0.4, $CHCl_3$). IR ($CHCl_3$): 1680 and 1760 (CO_2), 3320 and 3408 (NH_2), 3500 (NH) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.38 (d, $J_{Me,CH}$ = 6.4 Hz, 3H, CH*Me*), 1.94 (s, 3H, =C−*Me*), 3.81 (s, 3H, CO_2*Me*), 4.55 (s, 1H, C*H*=C−NH_2), 4.73 (dd, $J_{CH,NH}$ = 7.3 Hz, $J_{CH,CH}$ = 6.1 Hz, 1H, CHC*H*NH), 4.90 (br s, 1H, =C−N*H*H′), 5.41 (qd, $J_{CH,Me}$ = 6.4 Hz, $J_{CH,CH}$ = 6.1 Hz, 1H, OC*H*Me), 7.93 (d, $J_{NH,CH}$ = 7.3 Hz, 1H, CHN*H*), 8.09 (br s, 1H, =C−NH*H*′), 9.02 (d, *J* = 2.1 Hz, 2H, phenyl H-2, and H-6), 9.18 (dd, *J* = 2.1, *J* = 2.1 Hz, 1H, phenyl H-4). Anal. ($C_{16}H_{18}N_4O_9 \cdot {}^1/_2H_2O$) C, H, N.

(1*S*,2*R*)-2-(3,5-Dinitrophenylcarbonylamino)-2-methoxycarbonyl-1-methylethyl 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate Diastereomers (9 and 10). A solution of **8** (10.0 g, 24.4 mmol), α,α,α-trifluoro-*o*-tolualdehyde (4.25 g, 24.4 mmol), and nitroacetone (3.51 g, 34.0 mmol) in EtOH (150 mL) was stirred at 25 °C for 1 h prior to heating at 80 °C for 17 h. Removal of the solvent in vacuo gave a foamlike solid that was separated by silica gel column chromatography using EtOAc/hexane (1:1, v/v) as eluent. Upon elution, **9** and **6** eluted first (ratio of 1:2 according to the ^1H NMR spectrum), which could not be separated using the EtOAc/hexane system but which were successfully separated using toluene/EtOAc (2:1, v/v) as eluent. Further elution gave a mixture of the two diastereomers **9** and **10** followed by fractions containing pure **10**. The fractions containing the mixture of **9** and **10** were rechromatographed using the same procedure. In this way, after five column purifications, similar fractions were combined and the solvent was removed in vacuo to afford **9** and **10** as yellow crystals after recrystallization from EtOAc/hexane.

Diastereomer 9: 1.85 g; 11.65%; mp 231 °C; $[\alpha]^{23}_D$ +87.50° (*c* 0.4, MeOH). IR ($CHCl_3$): 1309 and 1474 (NO_2), 1670 and 1738 (CO_2) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.39 (d, $J_{Me,CH}$ = 6.4 Hz, 3H, CH*Me*), 2.48 (s, 3H, C-6 *Me*), 2.49 (s, 3H, C-2 *Me*), 3.73 (s, 3H, CO_2*Me*), 4.56 (dd, $J_{CH,NH}$ = 7.9 Hz, $J_{CH,CH}$ = 7.0 Hz, 1H, CHC*H*NH), 5.45 (qd, $J_{CH,CH}$ = 7.0 Hz, $J_{CH,Me}$ = 6.4 Hz, 1H, OC*H*Me), 5.93 (s, 1H, H-4), 5.96 (s, 1H, dihydropyridyl N*H*), 7.02 (d, $J_{NH,CH}$ = 7.9 Hz, 1H, CH−N*H*), 7.07 (dd, *J* = 7.9, *J* = 7.3 Hz, 1H, trifluoromethylphenyl H-4), 7.15 (dd, *J* = 7.9, *J* = 7.3 Hz, 1H, trifluoromethylphenyl H-5), 7.39 (d, *J* = 7.9 Hz, 2H, trifluoromethylphenyl H-3 and H-6), 8.70 (d, *J* = 2.0 Hz, 2H, dinitrophenyl H-2 and H-6), 9.18 (dd, *J* = 2.0, *J* = 2.0 Hz, 1H, dinitrophenyl H-4). Anal. ($C_{27}H_{24}N_5O_{11}F_3$) C, H, N.

Diastereomer 10: 1.59 g; 10.0%; mp 214 °C; $[\alpha]^{23}_D$ +136.75° (*c* 0.4, MeOH). IR ($CHCl_3$): 1309 and 1474 (NO_2), 1670 and 1738 (CO_2) cm^{-1}. ^1H NMR ($CDCl_3$): δ 1.11 (d, $J_{Me,CH}$ = 6.2 Hz, 3H, CH*Me*), 2.34 (s, 3H, C-6 *Me*), 2.41 (s, 3H, C-2 *Me*), 3.64 (s, 3H, CO_2*Me*), 4.82 (dd, $J_{CH,NH}$ = 8.2 Hz, $J_{CH,CH}$ = 3.8 Hz, 1H, CHC*H*NH), 5.46 (m, 1H, OC*H*Me), 5.81 (s, 1H, H-4), 5.84 (s, 1H, dihydropyridyl N*H*), 7.15 (d, $J_{NH,CH}$ = 8.2 Hz, 1H, CHN*H*), 7.22 (m, 1H, trifluoromethylphenyl H-4), 7.36 (m, 2H, trifluoromethylphenyl H-5 and H-6), 7.43 (d, *J* = 7.6 Hz, 1H, trifluoromethylphenyl H-3), 8.93 (d, *J* = 2.0 Hz, 2H, dinitrophenyl H-2 and H-6), 9.15 (dd, *J* = 2.0, *J* = 2.0 Hz, 1H, dinitrophenyl H-4). Anal. ($C_{27}H_{24}N_5O_{11}F_3$) C, H, N.

1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylic Acid Enantiomers [(+)-(*S*)-11 and (−)-(*R*)-12]. A solution of either **9** or **10** (0.8 g, 1.23 mmol) and DBU (0.56 g, 3.70 mmol) in MeOH (40 mL) was stirred at 25 °C for 4 h, the solvent was removed in vacuo, and water (40 mL) was added. The water fraction was washed with ether (3 × 100 mL), acidified to pH 2 with 0.5 N HCl, and extracted with EtOAc (3 × 100 mL). The combined organic extracts were dried (Na_2SO_4), and the solvent was removed in vacuo to give the crude yellow product as a tacky residue that was recrystallized from MeOH/ether to afford (+)-(*S*)-**11** or (−)-(*R*)-**12** as yellow crystals, respectively.

Enantiomer (+)-(*S*)-11: 236.7 mg, 56.3%; mp 205−206 °C (dec); $[\alpha]^{23}_D$ + 48.75° (*c* 0.4, MeOH). IR (KBr): 1300 and 1480 (NO_2), 1671 (CO_2), 2500−3500 (CO_2H) cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.24 (s, 3H, C-6 *Me*), 2.47 (s, 3H, C-2 *Me*), 5.76 (s, 1H, H-4), 7.36 (dd, *J* = 8.0, *J* = 7.3 Hz, 1H, phenyl H-4), 7.43 (d, *J* = 8.0 Hz, 1H, phenyl H-6), 7.51 (d, *J* = 8.0 Hz, 1H, phenyl H-3), 7.56 (dd, *J* = 8.0, *J* = 7.3 Hz, 1H, phenyl H-5), 9.49 (s, 1H, N*H*), 12.19 (br s, 1H, COO*H*). Anal. ($C_{15}H_{13}N_2O_4F_3 \cdot {}^1/_9H_2O$) C, H, N.

Enantiomer (−)-(*R*)-12: 232.5 mg, 55.3%; mp 204−205 °C (dec); $[\alpha]^{23}_D$ −49.75° (*c* 0.4, MeOH). IR (KBr) and ^1H NMR (DMSO-d_6) spectral data for (−)-(*R*)-**12** were the same as those for (+)-(*S*)-**11**. Anal. ($C_{15}H_{13}N_2O_4F_3 \cdot {}^1/_9H_2O$) C, H, N.

2-Nitrooxyethyl 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate Enantiomers [(−)-(*S*)-2 and (+)-(*R*)-3]. A solution of either (+)-(*S*)-**11** or (−)-(*R*)-**12** (136.8 mg, 0.40 mmol), 2-nitrooxyethyl bromide (74.8 mg, 0.44 mmol), and K_2CO_3 (66.3 mg, 0.48 mmol) in dry DMF (12 mL) was stirred at 25 °C for 24 h. Water (40 mL) was added, the mixture was extracted with EtOAc (3 × 100 mL), and the organic extracts were washed with water (2 × 40 mL) and then brine (40 mL). The organic fraction was dried (Na_2SO_4), the solvent was removed in vacuo, and the residue was purified by silica gel column chromatography using EtOAc/hexane (1:1, v/v) as eluent to yield (−)-(*S*)-**2** or (+)-(*R*)-**3** as a yellow oil, respectively.

Enantiomer (−)-(*S*)-2: 139.2 mg; 80.7%; $[\alpha]^{23}_D$ −48.75° (*c* 0.4, $CHCl_3$). IR ($CHCl_3$): 1271, 1308, 1492, and 1638 (ONO_2 and NO_2), 1704 (CO_2), 3315 (NH) cm^{-1}. ^1H NMR ($CDCl_3$): δ 2.36 (s, 3H, C-6 *Me*), 2.51 (s, 3H, C-2 *Me*), 4.21−4.28 (m, 1H, C*H*H′ONO_2), 4.36−4.44 (m, 1H, CH*H*′ONO_2), 4.56 (t, *J* = 4.58 Hz, 2H, CO_2CH_2), 5.95 (s, 2H, H-4 and N*H*), 7.30−7.34 (m, 1H, phenyl H-4), 7.44 (m, 2H, phenyl H-5 and H-6), 7.55 (d, *J* = 7.63 Hz, 1H, phenyl H-3). Anal. ($C_{17}H_{16}N_3O_7F_3$) C, H, N.

Enantiomer (+)-(*R*)-3: 139.6 mg; 81.0%; $[\alpha]^{23}_D$ +48.75° (*c* 0.4, $CHCl_3$). IR ($CHCl_3$) and ^1H NMR ($CDCl_3$) spectra for (+)-(*R*)-**3** were the same as those for (−)-(*S*)-**2**. Anal. ($C_{17}H_{16}N_3O_7F_3$) C, H, N.

(−)-(*S*)-Methyl 1,4-Dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate [(−)-(*S*)-1]. To a solution of (+)-(*S*)-**11** (16 mg, 0.0468 mmol) in MeOH (2 mL) was added a saturated solution of diazomethane in ether with stirring for 15 min until no further gas evolution occurred. Evaporation of solvents in vacuo gave a residue that was purified by silica gel chromatography (EtOAc/hexane 1:1, v/v) to yield (−)-(*S*)-**1** as a yellow oil (12.4 mg, 74.5%), $[\alpha]^{23}_D$ −56.75° (*c* 0.4, dioxane) [lit.[4] $[\alpha]_D$ −56.7° (dioxane)]. ^1H NMR ($CDCl_3$): δ 2.34 (s, 3H, C-6 *Me*), 2.51 (s, 3H, C-2 *Me*), 3.60 (s, 3H, CO_2*Me*), 5.88 (br s, 1H, N*H*), 5.95 (s, 1H, H-4), 7.27−7.31 (m, 1H, phenyl H-4), 7.42 (m, 2H, phenyl H-5 and H-6), 7.54 (d, *J* = 7.9 Hz, 1H, phenyl H-3).

Optical Purity of (−)-(*S*)-2, (+)-(*R*)-3, and (−)-(*S*)-1. The optical purity of (−)-(*S*)-**2**, (+)-(*R*)-**3**, and (−)-(*S*)-**1** were determined by ^1H NMR spectrometry. When 50 μL of a solution (100 mg in 1 mL $CDCl_3$) of the chiral shift reagent (+)-Eu(hfc)$_3$ was added to a solution of racemic 2-nitrooxyethyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)pyridine-5-carboxylate (5 mg in 0.5 mL of $CDCl_3$), the original 1,4-dihydropyridyl C-6 methyl resonance at δ 2.36 was separated into two resonances that appeared at δ 2.52 and 2.53. Addition

of (+)-Eu(hfc)$_3$ (80 μL) to (−)-(*S*)-**2** or (+)-(*R*)-**3**, as described above, resulted in retention of a single resonance for the C-6 methyl resonance (≥96% ee). Similarly, addition of (+)-Eu(hfc)$_3$ (30 μL) to racemic Bay K 8644 (5 mg in 0.5 mL of $CDCl_3$) resulted in the separation of 1,4-dihydropyridyl C-6 methyl and the ester methyl resonances at δ 2.34 and 3.60 into two resonances that appeared at δ 2.59 and 2.61, and 3.90 and 3.91, respectively. Addition of (+)-Eu(hfc)$_3$ (80 μL) to (−)-(*S*)-**1**, as described above for racemic Bay K 8644, resulted in retention of single resonances for the C-6 methyl and the ester methyl resonances (≥96% ee).

In Vitro Calcium Channel Antagonist and Agonist Assays. Calcium channel antagonist activities were determined as the molar concentration of the test compound required to produce 50% inhibition of the muscarinic receptor-mediated (carbachol, 1.6×10^{-7} M) Ca^{2+}-dependent contraction (tonic response) of guinea pig ileum longitudinal smooth muscle (GPILSM) using the procedure reported previously.[11] The IC_{50} value (±SEM, $n = 3$) was determined graphically from the dose−response curve.

Calcium channel modulation activity (positive or negative inotropic effect) was calculated as the molar concentration of the test compound required to produce a 50% increase (positive inotropic, or CC agonist, effect) or a 50% decrease (negative inotropic, or CC antagonist, effect) in contractile force of isolated guinea pig left atrium (GPLA) relative to its basal contractile force in the absence of test compound.[11]

Nitric Oxide Release. In vitro nitric oxide release was assayed using a modification of the previously reported procedure.[15] The test compound (0.0075 mmol) was added to a thoroughly mixed solution of 0.1 M phosphate buffer (pH 7.4) and acetonitrile (1:1, v/v) (1.5 mL) containing *N*-acetylcysteamine (1 equiv per $-ONO_2$ moiety), or without *N*-acetylcysteamine, with stirring under argon at 37 °C for 1 h. After exposure to air for 10 min, the reaction mixture (0.2 mL) was diluted with water (0.6 mL), and this solution was treated with 0.2 mL of Griess reagent [sulfanilamide (4 g), *N*-naphthylenediamine dihydrochloride (0.2 g), and 85% phosphoric acid (10 mL) in distilled water (final volume, 100 mL)] for 10 min at 25 °C. After dilution of this solution with water to a volume of 5 mL, ultraviolet absorbance was measured at 540 nm. Solutions of 3−24 μM sodium nitrite were used to prepare a nitrite absorbance versus concentration curve. A control experiment (absence of test compound) was performed for each measurement ($n = 3$). The percent nitric oxide release (quantitated as nitrite ion) was calculated from the nitrite absorbance versus concentration curve.

Whole-Cell Voltage-Clamp Studies. 1. Myocyte Preparation. Male or female guinea pigs (350−400 g, Charles River) were injected with heparin (3.3 IU/g) and sodium pentobarbital (160 mg/kg). The heart was cannulated in situ and perfused retrogradely through the aorta (10−12 mL/min, 7−8 min) with calcium-free solution (mM): 120 NaCl, 4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 HEPES, 12 glucose (100% O_2, 36 °C, pH 7.4) followed by 4−5 min of perfusion with 50 mL of calcium-free solution plus 25 mg of collagenase A (Boehringer Mannheim) and 4.8 mg of protease XIV (Sigma). Ventricles were minced and myocytes were released by gentle agitation of the tissue in high potassium solution (mM): 90 KOH, 50 glutamic acid, 30 KCl, 30 KH_2PO_4, 20 taurine, 10 HEPES, 10 glucose, 3 $MgSO_4$, 0.5 EGTA (pH 7.4 with KOH). Myocytes were superfused with buffer solution (mM): 145 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose, 2 $CaCl_2$ (36 °C, 100% O_2, pH 7.4 plus 200 μM lidocaine to block sodium currents).

2. Electrophysiological Recording. Cells were impaled with intracellular microelectrodes (18−25 MΩ, 2.7 M KCl), and currents were recorded with a discontinuous single-electrode voltage clamp (7−10 kHz). Current and voltage were recorded with an Axoclamp-2A amplifier (Axon Instruments, Inc.), digitized with a Labmaster A/D interface at 125 kHz (TL1-125, Axon Instruments, Inc.), and stored on computer. pClamp software (Axon Instruments, Inc.) was used to generate voltage clamp protocols and to acquire and analyze data. Test steps were preceded by a 200 ms conditioning step to 0 mV from the holding potential of −80 mV, followed by a return to −40 mV. The calcium current (I_{Ca}) was activated by 200 ms test steps to potentials between −40 and +80 mV in 10 mV increments.

3. Experimental Protocol. Stock solutions of drugs were prepared by dissolving the test compound in distilled DMSO. Because DMSO exerts a weak inhibitory effect[11] on I_{Ca}, we controlled for solvent effects by including the same amount of DMSO in all control and drug solutions. Cells were first exposed to control solution for 10 min, then to 1 or 10 μM concentrations of the test compound for 10 min. Drug was washed out for up to 15 min, although the effects of test compound on I_{Ca} was not reversed within that time.

4. Analysis. The peak amplitude of I_{Ca} was measured as the difference between the peak inward current and the current at 200 ms. In a separate series of experiments, the validity of this measure of I_{Ca} was tested in cells exposed to known calcium channel antagonists. Current−voltage relationships were constructed by plotting the peak amplitude of I_{Ca} as a function of membrane potential. Data are presented as mean ± SEM where "*n*" is the number of myocytes sampled. Differences between drug treatment and control recordings were assessed with two-way repeated measures analysis of variance. Differences were significant when $p < 0.05$.

Acknowledgment. We thank the Canadian Institutes of Health Research (Grant No. MT-8892) for financial support of this research and for a fellowship to R.S. The authors also acknowledge the excellent technical assistance of C.-A. McEwen, P. Nicholl, and C. Mapplebeck.

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JM010394K