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Identification of *R*(−)-isomer of efonidipine as a selective blocker of T-type Ca^{2+} channels

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1 Efonidipine, a derivative of dihydropyridine Ca^{2+} antagonist, is known to block both L- and T-type Ca^{2+} channels. It remains to be clarified, however, whether efonidipine affects other voltage-dependent Ca^{2+} channel subtypes such as N-, P/Q- and R-types, and whether the optical isomers of efonidipine have different selectivities in blocking these Ca^{2+} channels, including L- and T-types.

2 To address these issues, the effects of efonidipine and its *R*(−)- and *S*(+)-isomers on these Ca^{2+} channel subtypes were examined electrophysiologically in the expression systems using *Xenopus* oocytes and baby hamster kidney cells (BHK tk-ts13).

3 Efonidipine, a mixture of *R*(−)- and *S*(+)-isomers, exerted blocking actions on L- and T-types, but no effects on N-, P/Q- and R-type Ca^{2+} channels.

4 The selective blocking actions on L- and T-type channels were reproduced by the *S*(+)-efonidipine isomer.

5 By contrast, the *R*(−)-efonidipine isomer preferentially blocked T-type channels.

6 The blocking actions of efonidipine and its enantiomers were dependent on holding potentials.

7 These findings indicate that the *R*(−)-isomer of efonidipine is a specific blocker of the T-type Ca^{2+} channel.

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Abbreviations: DHP, dihydropyridine; DMSO, dimethyl sulfoxide

Introduction

Efonidipine, a derivative of dihydropyridine (DHP) Ca^{2+} antagonist, has a unique pharmacological profile, characterized by blockade of both L- and T-type Ca^{2+} channels (Tanaka & Shigenobu, 2002). The effects of the blockade of T-type Ca^{2+} channels on the cardiovascular system were studied in the late 1990s using mibefradil, a selective non-DHP blocker of T-type channels (Mishra & Hermsmeyer, 1994). Thereafter, a substantial number of both basic and clinical studies have shown the hemodynamic advantages of blocking T-type channels in the control of hypertension and ischemic heart disease (Hermsmeyer *et al*., 1997; Van der Vring *et al*., 1999). Efonidipine is also considered to have a similar favorable hemodynamic effect, based on the blockade of T-type channels. However, the effects of efonidipine on voltage-dependent Ca^{2+} channels other than L- and T-types have not been studied. Since some DHP Ca^{2+} antagonists, such as amlodipine, can block N- and P/Q-type Ca^{2+} channels as well as the L-type (Furukawa *et al*., 1999), it is essential to determine the selectivity for channel subtypes in understanding the basic and clinical features of the drug.

Furthermore, DHP usually has its optical isomers (enantiomers), and only one of them is capable of blocking L-type channels (Triggle & Rampe, 1989). Therefore, the drug action of any DHP should be specified by its isomers. This is true for efonidipine, which is known to have *R*(−)- and *S*(+)-enantiomers (Sakoda *et al*., 1992). However, the effects of each enantiomer of efonidipine on L- and T-type Ca^{2+} channels have not yet been characterized.

To address these issues, five different types of voltage-dependent Ca^{2+} channels (L, N, P/Q, R and T) were functionally expressed in *Xenopus* oocytes, and the effects of efonidipine on these channels were investigated to clarify the selectivity in channel blockade by efonidipine. For the channels sensitive to efonidipine, the effects of efonidipine were further characterized by *R*(−)- and *S*(+)-enantiomers, using both *Xenopus* oocyte and mammalian cell line expression systems.

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Methods

cDNA cloning

Based on the cDNA sequence for the rat α_{1G} subtype (Genbank accession number, AF027984), 14 oligodeoxyribonucleotide primers were synthesized. The sense primers corresponded to the amino-acid residues 1–6 (primer SAG1), 312–319 (SAG2), 638–645 (SAG3), 936–944 (SAG4), 1275–1283 (SAG5), 1643–1651 (SAG6) and 1927–1934 (SAG7): SAG1, CCACATGCTCCC(A/G/C/T)CA(C/T)CG(A/G/C/T)GT; SAG2, GATCCTGCAGGAG(C/T)GT(A/G/C/T)CC(A/G/C/T)AC; SAG3, GCACTAGTGGAGGT(A/G/C/T)GC(A/G/C/T)CC(A/G/C/T)A; SAG4, GAAGAATTTCGACTC(A/G/C/T)CT(A/G/C/T)CT(A/G/C/T)TG; SAG5, GTTTCGTCTCCTGTGTCA(C/T)CG(A/G/C/T)AT; SAG6, CTGAAGATCTG(C/T)AA(C/T)TA(C/T)AT(A/T/C)TT(C/T)AC; and SAG7, GATGGTACCCCA(C/T)CC(A/G/C/T)GA(A/G)GA. The antisense primers corresponded to the amino-acid residues 309–316 (primer AAG1), 633–641 (AAG2), 932–940 (AAG3), 1273–1281 (AAG4), 1640–1647 (AAG5), 1924–1931 (AAG6) and 2281–2286 (AAG7): AAG1, ACTCCTGCAGGA(C/T)CTCAT(A/G/C/T)CC(A/G)TT; AAG2, TCCACTAGTGCTTT(A/G)TC(C/T)TT(A/G/C/T)AG(A/G/T)AT; AAG3, CGAAATTCTTCCG(A/G)TC(A/G/C/T)GG(C/T)AA(A/G/C/T)GT; AAG4, GACACAGGAGACG(A/G)AA(C/T)CT(A/G/C/T)GA(C/T)TG; AAG5, TGCAGATCTT(A/G/C/T)AG(A/G/C/T)GC(C/T)TC(A/G)TC; AAG6, GGGGTACCAT(A/G/C/T)GT(A/G/C/T)GG(A/G)TG(C/T)TC; and AAG7, ACTCAGGG(A/G)TCCAT(A/G)TC(A/G/C/T)GT(A/G/C/T)GG. Then, seven parts of the rat α_{1G} cDNA were cloned from rat brain poly-$(A)^+$RNA, using a reverse transcription-based polymerase chain reaction (RT–PCR) (Wada *et al*., 2000) with seven sets of the sense and antisense primers, SAG1/AAG1, SAG2/AAG2, SAG3/AAG3, SAG4/AAG4, SAG5/AAG5, SAG6/AAG6 and SAG7/AAG7. To construct a plasmid containing the entire protein-coding sequences of rat α_{1G} (pBlA1G), each α_{1G} cDNA was excised with *Eco*RV/*Sse*8387I, *Sse*8387I/*Spe*I, *Spe*I/*Apo*I, *Apo*I/*Bsm*BI, *Bsm*BI/*Bgl*II, *Bgl*II/*Kpn*I or *Kpn*I/*Not*I, and recombined into the *Eco*RV/*Not*I site of pBluescript SK(+) (Stratagene, CA, U.S.A.). Nucleotide sequence analysis was performed using a DNA sequencer (ABI Prism 377, PE Applied Biosystems, CA, U.S.A.).

The determination of the nucleotide and the predicted amino-acid sequences of the inserts of clone pBlA1G revealed 139 nucleotide changes from the sequence of rat α_{1G} (Perez-Reyes *et al*., 1998), which resulted in 17 amino-acid substitutions and an insertion of 23 amino acids: His-1060, Cys-1096, Ala-1666, Leu-1699, His-1734, Pro-1811, Ser-1812, Thr-1901, Phe-2148 and Phe-2169 were determined as Leu, Ser, Gly, Ala, Asp, Thr, Leu, Pro, Ser and Ser, encoded by CTA, TCT, GGC, GCT, GAC, ACC, CTC, CCT, TCC and TCC, respectively. The amino-acid residues, EIGKREDASGQLS CIQLPVNSQG (encoded by GAA, ATC, GGC, AAA, CGG, GAA, GAT, GCG, AGT, GGA, CAG, TTA, AGC, TGT, ATT, CAG, CTG, CCT, GTC, AAC, TCT, CAG and GGG), were inserted at the position of 1004. Seven amino-acid residues, SKEKQMA, corresponding to 1580–1586, were substituted by 18 amino-acid residues, NLMLDDVIASGS SASAAS (encoded by AAT, CTA, ATG, TTG, GAC, GAT, GTA, ATT, GCT, TCC, GGC, AGC, TCA, GCC, AGC, GCT, GCG and TCA). In all, 33 nucleotide changes in the sequences were shown not to alter the coding amino-acid residues: C (9), G (15), G (816), T (927), G (951), C (954), C (1059), C (1542), G (1902), T (1908), G (1926), C (1929), A (2802), C (2805), C (2823), C (2829), C (3370), G (3819), G (3825), T (3828), C(3843), G (3846), T (4926), G (4929), C (4938), C (4947), G (5772), C (5778), G (5781), C (5793), C (5796), T (6054) and A (6846) were T, T, A, C, T, A, T, T, C, C, A, G, G, T, G, T, T, A, A, C, T, T, C, C, T, T, A, G, A, T, T, C and G in our clones, respectively. Thus, the insert of this clone contained a cDNA sequence encoding a splice variant of α_{1G}, α_{1G-bce} (Cribbs *et al*., 2000; Monteil *et al*., 2000).

In vitro *transcription*

The 50-bp *Kpn*I/*Eco*RI fragment was excised from pBluescript SK(+), blunted and inserted into the *Hin*dIII (blunted) site of pSPA2 (Nakamura *et al*., 1994) to produce pSPA3. For *in vitro* transcription, the 7.0-kb *Eco*RV/*Not*I (blunted) fragment containing the entire coding sequence for α_{1G} from pBlA1G was inserted into the *Eco*RV site of pSPA3 to yield pSPA1G. cRNA specific for the α_{1G} subtype of the T-type Ca^{2+} channel was synthesized *in vitro* using a MEGAscript SP6 kit (Ambion, TX, U.S.A.).

Transfection

The 4.0-kb *Age*I/*Bsr*GI fragment from pECFP-N1 (Clontech) was blunted with T4 DNA polymerase and circularized with T4 DNA ligase to yield pEnon-N1. In the same orientation with respect to cytomegalovirus gene transcription, the 7.0-kb *Eco*RV/*Not*I fragment containing the entire coding sequence for α_{1G} from pBlA1G was ligated with the 4.0-kb *Sma*I/*Not*I fragment from pEnon-N1 to yield pEA1G1. Similarly, the 7.0-kb *Hin*dIII fragment containing the entire coding sequence for α_{1C} from pSPCDR (Furukawa *et al*., 1998) was blunted and ligated with the 5.5-kb *Sma*I fragment from pCI-neo (Promega, WI, U.S.A.) to yield pCIA1C.

Functional expression and electrophysiology

The methods for the *in vitro* transcription of cRNAs specific for the Ca^{2+} channel α_1, α_2/δ and β subunits, and the procedures for the functional expression of Ca^{2+} channels in *Xenopus* oocytes have been described previously (Furukawa *et al*., 1999). Briefly, defolliculated *Xenopus* oocytes were injected with cRNA specific for Ca^{2+} channel subunits. cRNAs were used either with 0.3 μg μl^{-1} of α_{1G} cRNA alone or 0.3 μg μl^{-1} α_1 (α_{1C} (Mikami *et al*., 1989), α_{1B} (Fujita *et al*., 1993), α_{1A} (Mori *et al*., 1991) or α_{1E} (Niidome *et al*., 1992)) cRNA in combination with 0.2 μg μl^{-1} α_2/δ_1 (Mikami *et al*., 1989) cRNA and 0.1 μg μl^{-1} β_{2a} (Hullin *et al*., 1992) cRNA. The oocytes were cultured for 2 to 4 days and then subjected to electrophysiological measurement. The oocytes were positioned in a recording chamber (0.4 ml in volume) perfused with an extracellular solution containing 40 mM $Ba(OH)_2$, 50 mM NaOH, 2 mM KOH and 5 mM HEPES (pH 7.5 with methanesulfonic acid), and Ba^{2+} currents through expressed channels were measured by the two-microelectrode voltage-clamp method using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA, U.S.A.). The chamber was perfused continuously (2.0–3.0 ml min^{-1}) with the extracellular

solution. Commercial software (pClamp version 6.04, Axon Instruments) was used for generating voltage pulses, acquiring data and analyzing the currents. Oocytes were clamped at a holding potential of −80 mV and depolarized to +10 mV for 200 ms every 15 s, unless otherwise noted. The microelectrodes were filled with 3 M KCl, and those showing a resistance of 0.5–1.2 MΩ were used.

Membrane currents recorded from *Xenopus* oocytes injected with α_{1G} cRNA were completely blocked by 15 μM mibefradil ($n=4$), indicating that T-type Ca^{2+} channels were functionally expressed in *Xenopus* oocytes, as in the case of L-type ($\alpha_{1C}\alpha_2/\delta_1\beta_{2a}$), N-type ($\alpha_{1B}\alpha_2/\delta_1\beta_{2a}$), P/Q-type ($\alpha_{1A}\alpha_2/\delta_1\beta_{2a}$) and R-type ($\alpha_{1E}\alpha_2/\delta_1\beta_{2a}$) channels (Furukawa *et al*., 1999).

The baby hamster kidney (BHK) tk-ts13 cell line (Talavera & Basilico, 1977) and its transformant, BHK6, which stably expressed Ca^{2+} channel α_2/δ_1 and β_{1a} subunits (Wakamori *et al*., 1998), were cultured in DMEM medium (Invitrogen, Carlsbad, CA, U.S.A.) containing 5% fetal bovine serum (Invitrogen), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. For BHK6 cells, the culture medium was further supplemented with 600 μg ml^{-1} G418 (Sigma, NY, U.S.A.). Cells were cotransfected with the plasmid pEGFP-N1 (Clontech) together either with pEA1G1 or pCIA1C (see above) at a ratio of 1:20 (1 μg in total), using a Lipofectamine reagent (Invitrogen). To express transiently T- and L-type Ca^{2+} channels, pEA1G1 and pCIA1C were introduced into tk-ts13 and BHK6 cells, respectively. Transfected cells were seeded onto glass coverslips and subjected to electrophysiological measurements after 24–48 h.

The experimental chamber was 0.5 ml in size, and was perfused at a rate of 1 ml/min^{-1} with a solution containing (in mM) $MgCl_2$ (0.5), $BaCl_2$ (5), CsCl (5.4), tetraethyl-ammonium chloride (TEA-Cl) (132), HEPES (10), 4-aminopyridine (3.0) and glucose (5.5). The solution was titrated to a pH of 7.4 with tetraethyl-ammonium hydroxide. Micropipettes were pulled from 1.5-mm o.d.-walled thin glass tubes (G-1.5, Narishige, Tokyo, Japan) using a horizontal puller (Model P-87, Sutter Instrument Co., Novato, CA, U.S.A.), and were filled with an internal solution having a composition of (in mM): CsCl (150), $MgCl_2$ (3.0), Na_2-ATP (5.0), EGTA (5.0) and HEPES (5.0) (pH 7.2 with CsOH). The microelectrodes filled with the internal solution had a tip resistance of 1.5–3 MΩ. An Axopatch 200A amplifier (Axon Instruments, Foster City, CA, U.S.A.) was used for a single-electrode whole-cell voltage clamp (Hamill *et al*., 1981). Current traces were acquired at 10 kHz and filtered at 2 kHz with an eight-pole low-pass Bessel filter (CyberAmp 320, Axon Instruments). Compensation for series resistance was applied to reduce approximately 75 to 80% of the total resistance. Capacitative and leak currents were eliminated digitally using scaled hyperpolarizing steps of one-fourth amplitude (P/N4). Commercial software (pClamp version 6.04, Axon Instruments) was used for generating voltage pulses, acquiring data and analyzing the currents. All experiments were performed at room temperature (22±1°C). The membrane potential was held at −80 mV, and step depolarization of a 100-ms duration was executed to +10 mV every 10 s.

The drug effects were evaluated after a 5-min perfusion with bath solution containing an agent. In experiments to obtain concentration–blockade relationships, the concentration of DHPs was changed successively. Each experiment was finished within 20 min to avoid a possible run-down of Ba^{2+} currents. As no detectable current changes were observed during exposure to the vehicle for DHP (0.2% dimethyl sulfoxide (DMSO)), as reported previously (Furukawa *et al*., 1997), the concentration of DMSO in the bath solution was maintained at 0.2% throughout the experiments. DHPs were dissolved into DMSO just before each experiment and added to the bath solution to make the final concentration. Efonidipine, a racemic mixture of $S(+)$- and $R(-)$-enantiomers, and both enantiomers were generous gifts from Nissan Chemical Industries Ltd (Tokyo, Japan). Mibefradil was the generous gift of F. Hoffmann-La Roche, Ltd (Basel, Switzerland). Other drugs were purchased from Sigma (U.S.A.), unless otherwise noted. Concentration-response curves were fitted to the Hill equation: Block (%) = 100 $(1+([IC_{50}/[D])nH)^{-1}$, where IC_{50} is the concentration at half-blockade, [D] is the drug concentration, and nH is the Hill coefficient.

Data from multiple experiments are expressed by means±s.e.m. Statistical analyses were performed with analysis of variance followed by a Dunett *post hoc* test. A *P*-value less than 0.05 was considered significant.

Results

A combination of cRNAs for L-type ($\alpha_{1C}\alpha_2/\delta_1\beta_{2a}$), N-type ($\alpha_{1B}\alpha_2/\delta_1\beta_{2a}$), P/Q-type ($\alpha_{1A}\alpha_2/\delta_1\beta_{2a}$), R-type ($\alpha_{1E}\alpha_2/\delta_1\beta_{2a}$) or T-type ($\alpha_{1G}$) channels was injected into *Xenopus* oocytes as described previously (Furukawa *et al*., 1999). L-, N-, P/Q-, R- and T-type Ca^{2+} channels were functionally expressed in these oocytes (Figure 1, top, open circles). After a 5-min exposure to 10 μM efonidipine, membrane currents through L- and T-type channels were inhibited to a half-level, as compared with those before application of the drug (Figure 1, top, filled circles; bottom). Efonidipine inhibited N- and P/Q-type channels by less than 10% and failed to block R-type channels.

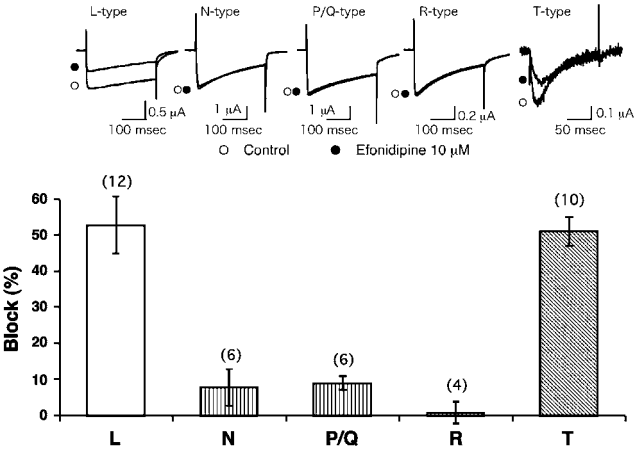

Figure 1 Effects of efonidipine on five subtypes of Ca^{2+} channels (L-, N-, P/Q-, R- and T-types) expressed in *Xenopus* oocytes. Membrane currents in response to step depolarizations of a 300- or 150-ms duration from a holding potential of −80 to +10 mV (L, N-, P/Q- and R-types), or −20 mV (T-type) before (Control) and after a 5-min perfusion of 10 μM efonidipine are presented with current traces (top). The decrease in the amplitude of the peak inward currents after a 5-min perfusion of 10 μM efonidipine is expressed as a channel blockade in percent (bottom).

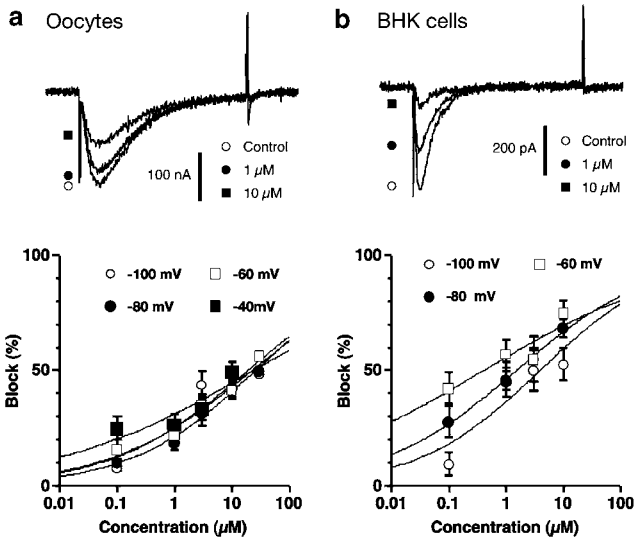

Figure 2 Effects of efonidipine on T-type α_{1G} channels expressed in *Xenopus* oocytes (a) and BHK cells (b). Membrane currents in response to step depolarizations of a 100-ms duration from a holding potential of −80 to −20 mV before (Control) and after a 5-min perfusion of 1 and 10 μM efonidipine are presented with current traces (top). Concentration–blockade relationships (bottom) for efonidipine at various holding potentials were obtained in oocytes and BHK cells. The block amounts were obtained after a 5-min perfusion of efonidipine. Each data point is an average of more than eight observations.

To further characterize the blockade of the T-type channel by efonidipine, the channel was expressed in the cell line BHK tk-ts13 (Talavera & Basilico, 1977), as well as in *Xenopus* oocytes. As shown in Figure 2, the α_{1G} channel blockade by efonidipine was significantly enhanced when the channel was expressed in a BHK cell. The IC_{50} values for efonidipine block on the T-type channel in both expression systems are summarized in Figure 6 (top). Moreover, the concentration-blockade relationships (Figure 2, bottom) show that the voltage dependences of the channel blockade were also different. Enhancement of the channel blockade by depolarizing the holding potential was obvious at each holding potential (−100, −80 and −60 mV) for BHK cells, while a detectable increase was observed only at −40 mV for oocytes. In order to elucidate the drug effect on channel inactivation, steady-state current availabilities at various membrane potentials were examined in two expression systems. Figure 3 shows the results. Conditioning pulses of 5 s were delivered before the test pulse, and current availability was measured by step depolarization to the membrane potential at which the maximal inward current was observed (usually −20 mV). A similar current inhibition at a very hyperpolarized membrane potential (−120 mV) was observed by 10 μM efonidipine for oocytes (31.5 ± 5.2%, $n = 8$), and by 1 μM efonidipine for BHK cells (30.4 ± 6.8%, $n = 8$). The conditioning pulse voltage at which a half of the channel was inactivated (V_h) was about −60 mV for oocytes, and it was not significantly affected by 10 μM efonidipine. However, the voltage dependence of the channel inactivation was apparently shifted to a hyperpolarizing direction in BHK cells. The V_h was 10 mV negative to that in oocytes, and further shifted −7 mV by exposure to 1 μM efonidipine. Slope factors were not modulated by efonidipine in either system.

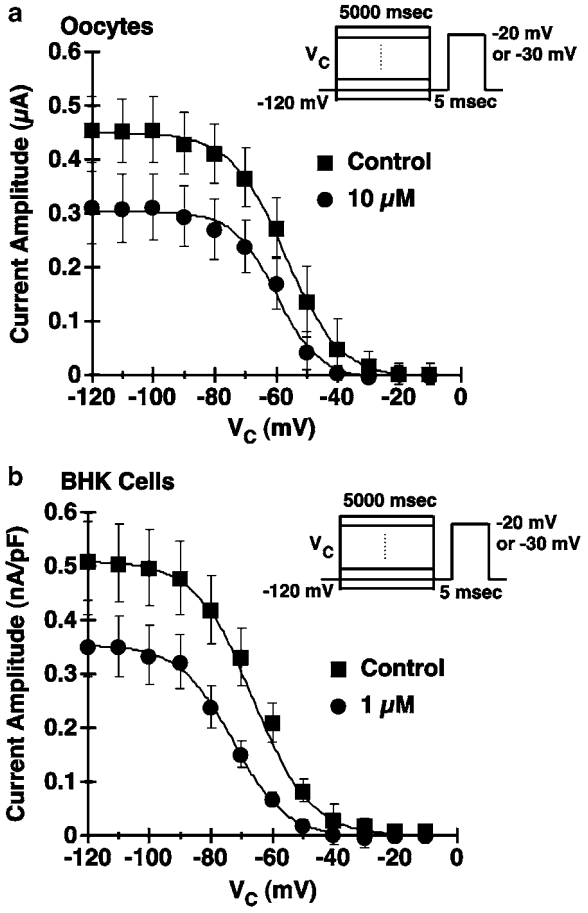

Figure 3 Effects of efonidipine on the voltage dependence of α_{1G} channel availability in oocytes and BHK cells. The membrane current amplitudes after a 5-s conditioning pulse to various potentials were measured by test pulse in the control and 5 min after exposure to efonidipine. The concentration of efonidipine was 10 μM for oocytes (a) and 1 μM for BHK cells (b). The membrane voltage of the test pulse was chosen to elicit the maximal inward current (−30 or −20 mV). The raw value of the current amplitude was averaged in *Xenopus* oocytes ($n = 8$). The current amplitude was normalized to membrane capacitance in BHK cells, and the values (current density) were averaged ($n = 8$). The data was summarized by the following equation:

$$I_m = I_{max}/(1 - e^{(V_c - V_h)/s})$$

where I_m is membrane current amplitude, I_{max} is maximal amplitude of current availability, V_c is the voltage of conditioning pulse, V_h is the conditioning pulse voltage at which a half of the channel is inactivated and s is the slope factor of channel inactivation. The values were the following: oocytes in the control, $I_m = 0.46 \pm 0.06$ μA, $V_h = -56.8 \pm 2.8$ mV, $s = 4.8 \pm 1.8$ mV; oocytes in 10 μM efonidipine, $I_m = 0.31 \pm 0.07$ μA, $V_h = -59.6 \pm 5.6$ mV, $s = 5.4 \pm 1.6$ mV; BHK cells in the control, $I_m = 0.51 \pm 0.07$ nA/pF, $V_h = -67.2 \pm 4.7$ mV, $s = 5.8 \pm 2.4$ mV; BHK cells in 1 μM efonidpine, $I_m = 0.35 \pm 0.06$ nA/pF, $V_h = -74.9 \pm 5.9$ mV, $s = 6.3 \pm 1.9$ mV.

The effects of the optical isomers of efonidipine on L- and T-type channels were examined in the two expression systems. Figure 4 illustrates the representative current traces showing the effects of the optical isomers, and Figure 5 shows the concentration–blockade relationships for the block of each isomer on two channel subtypes. $S(+)$-efonidipine inhibited L-type $\alpha_{1C}\alpha_2/\delta_1\beta_{2a}$ and T-type α_{1G} channels expressed in *Xenopus* oocytes (Figure 4a) in a similar concentration-dependent manner (Figure 5a) as that of enantiomer-unspeci-

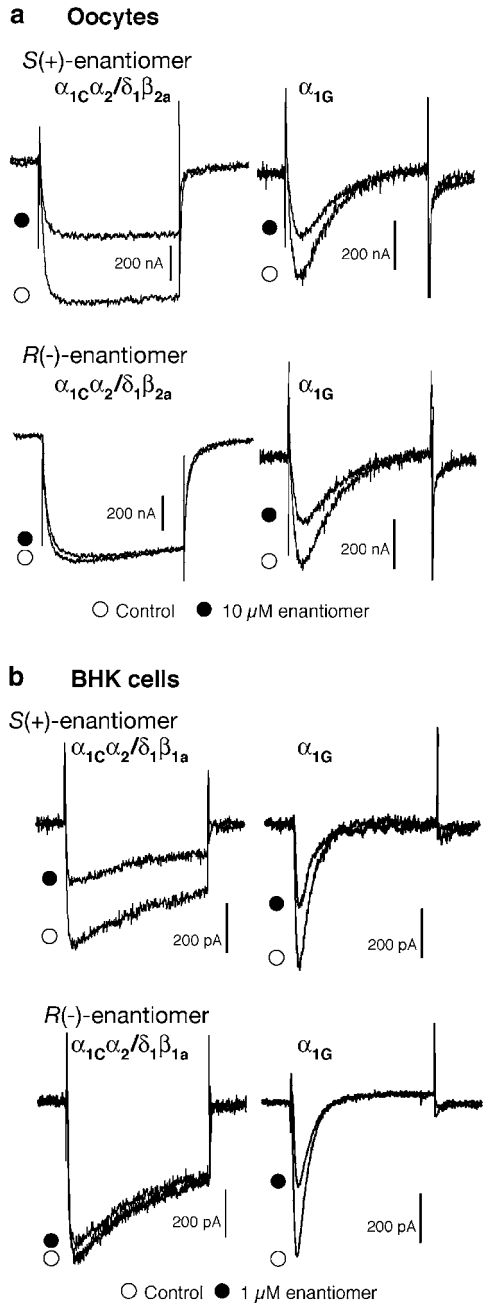

Figure 4 Effects of the optical isomers of efonidipine, $S(+)$- and $R(-)$-efonidipine on L- and T-type Ca^{2+} channels expressed in *Xenopus* oocytes and BHK cells. (a and b) Membrane currents in response to the step depolarization of a 200- (L-type)- or 100-ms duration (T-type) from a holding potential of −80 to +10 mV before (Control) and after a 5-min perfusion of $S(+)$- (top) or $R(-)$-efonidipine (bottom) are presented with current traces. L-type $\alpha_{1C}\alpha_2/\delta\beta_{2a}$ and T-type α_{1G} Ca^{2+} channels were expressed in *Xenopus* oocytes (a) or BHK cells (b). In BHK cells, β_{2a} subunit was substituted with β_{1a} subunit. Note that the $S(+)$-isomer kept the blocking ability of efonidipine for both L- and T-type channels (see Figure 1), but the $R(-)$-isomer scarcely affected L-type channels.

fied efonidipine. The blocking actions of $S(+)$-efonidipine on the L- and T-type channels were comparable, and the actions were not voltage-dependent in either type of channel, as in the case of enantiomer-unspecified efonidipine. $S(+)$-efonidipine also inhibited the L- and T-type channels expressed in BHK cells (Figure 4b). The concentration–blockade relationships for both channels were similar, and the blocks were dependent on the holding potential (Figure 5b). The channel sensitivity to $S(+)$-efonidipine and the voltage dependence were larger in BHK cells compared to those in oocytes. These findings indicate that the $S(+)$-enantiomer of efonidipine blocked L- and T-type α_{1G} channels as in the case of enantiomer-unspecified efonidipine (Figures 1 and 2), although the channel blockade was more pronounced in BHK cells compared with that in *Xenopus* oocytes.

On the other hand, $R(-)$-efonidipine inhibited T-type α_{1G} channels expressed in both *Xenopus* oocytes (Figure 4a, bottom) and BHK cells (Figure 4b, bottom). In contrast to the effect by $S(+)$-efonidipine, $R(-)$-efonidipine failed to inhibit L-type α_{1C} channels, regardless of whether they were expressed in *Xenopus* oocytes or BHK cells (Figure 4, bottom). The $R(-)$-efonidipine block on the T-type channel was steeply voltage-dependent in BHK cells. The blockade of membrane current by 0.1 μM $R(-)$-efonidipine was increased four-fold by depolarizing the holding potential from −100 to −60 mV. The increase was 2.8-fold in the case of $S(+)$-efonidipine.

Figure 6 summarizes the IC_{50}'s for the blockade of the L- and T-type Ca^{2+} channels by efonidipine, $S(+)$-efonidipine and $R(-)$-efonidipine. In each combination of drug and channel, the IC_{50} for channel in BHK cells was smaller than that in *Xenopus* oocytes. This was true at each holding potential. $R(-)$-efonidipine blocked T-type channels in *Xenopus* oocytes slightly more potently than $S(+)$-enantiomer, and the IC_{50} was not changed by holding potentials in the range of −100 to −60 mV. Both $S(+)$- and $R(-)$-efonidipine enantiomers blocked T-type α_{1G} channels in BHK cells with comparable IC_{50} values. However, the voltage dependence of channel blockade was steeper in $R(-)$-efonidipine than in $S(+)$-efonidipine. The Hill coefficients were not markedly influenced by the enantiomers or the expression systems. These findings taken together indicate that $R(-)$-efonidipine, but not $S(+)$-efonidipine, is a selective blocker of the T-type Ca^{2+} channel.

Discussion

In the present study, the high-voltage-activated L- (α_{1C}), N- (α_{1B}), P/Q- (α_{1A}) and R-type (α_{1E}) Ca^{2+} channels and the low-voltage-activated T-type (α_{1G}) Ca^{2+} channel were functionally expressed in the expression systems using *Xenopus* oocytes and cultured BHK cells. The high-voltage-activated channels were expressed with auxiliary α_2/δ and β subunits and the α_{1G} channel was expressed as a single molecule, as the forms that are known to work as the channels found in native tissues (Perez-Reyes, 2003b). We found that efonidipine blocked L- and T-type Ca^{2+} channel activities and failed to influence N-, P/Q- and R-type Ca^{2+} channels. Of the two optical isomers of efonidipine, only $S(+)$-isomer blocked both T- and L-type channels, indicating that the selectivity of efonidipine for channel blockade is derived from the $S(+)$-isomer of efonidipine. By contrast, the other isomer of efonidipine, $R(-)$-isomer, blocked T-type channels without affecting the L-type. These results provide evidence that the $R(-)$-isomer of efonidipine is a T-type-specific blocker.

The efonidipine employed in this study is commercially available as a DHP L-type Ca^{2+} channel antagonist with less marked reflex sympathomimetic actions (Masuda & Tanaka,

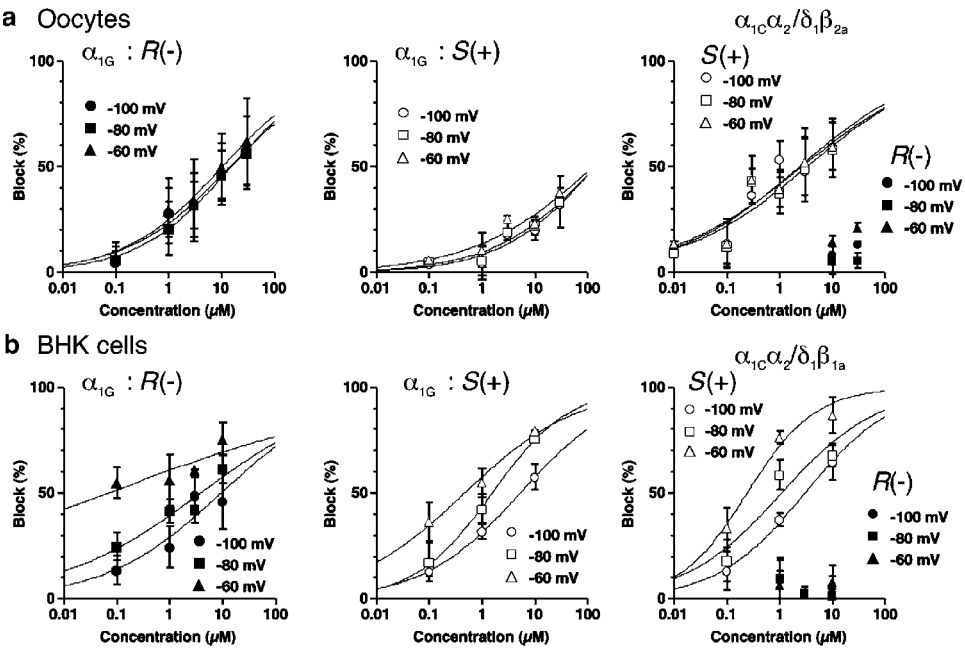

Figure 5 Concentration-blockade curves for $S(+)$- (top) and $R(-)$-efonidipine (bottom) at three holding potentials (−60, −80 and −100 mV). Blockades of the peak inward currents through T- (left and middle) and L-type channels (right) expressed in *Xenopus* oocytes (a) and BHK cells (b) were measured after a 5-min perfusion of various concentrations of the isomers.

1994). In addition, efonidipine has been shown to inhibit myocardial T-type Ca^{2+} channels (Masumiya *et al*., 1997; 1998) and the molecularly identified α1H subtype of T-type Ca^{2+} channels (Ono *et al*., 2000). Therefore, efonidipine is classified into the category of dual blocker of T- and L-type Ca^{2+} channels (Tanaka & Shigenobu, 2002). It is also known that some DHP derivatives block N- and P/Q-type channels as well as L-type channels (Uneyama *et al*., 1997; Furukawa *et al*., 1999). However, in the present study, efonidipine and its $S(+)$-isomer blocked only L-type channels among all the high-voltage-activated Ca^{2+} channels examined.

Generally, DHP acts on L-type Ca^{2+} channels as a blocker or a stimulator. For example, one of the two optical isomers of Bay K-8644 stimulates the L-type channel, whereas the other blocks it (Triggle & Rampe, 1989). With respect to the L-type Ca^{2+} channel, the $S(+)$-enantiomer, but not the $R(-)$-enantiomer, of efonidipine blocked L-type α_{1C} channels, which is consistent with a previous report (Sakoda *et al*., 1992). With respect to the T-type Ca^{2+} channel, efonidipine blocked T-type α_{1G} channels as reported regarding T-type α1H Ca^{2+} channels (Ono *et al*., 2000), and unlike the L-type, the channel blockades were not optical isomer-specific.

The transmembrane segments S5 and S6 of repeat III and the transmembrane segment S6 of repeat IV have been assigned to the interaction sites of L-type Ca^{2+} channels with DHP (Hockerman *et al*., 1997; Hering *et al*., 1998; Striessnig *et al*., 1998). In these regions of Ca^{2+} channels, amino-acid sequences of T-type α_{1G}, α1H and α_{1I} channels are the most diverse compared to those of L-type α_{1C}, α_{1S} and α_{1D} channels among the voltage-dependent Ca^{2+} channels known (Perez-Reyes, 2003a). These findings suggest that efonidipine interacts with L- and T-type Ca^{2+} channels in a different mode or through different sites.

In this study, two different heterologous expression systems were used for L-type $\alpha_{1C}\alpha_2/\delta\beta$ channels and T-type α_{1G} channels. The steady-state current availability curve (Figure 3) showed that the voltage dependence of inactivation in BHK cells was shifted about 10 mV for the hyperpolarizing direction compared to that in oocytes. As shown in Figure 2, the time course of membrane current inactivation at −20 mV seemed faster in BHK cells. Although the cause of the shift was not clarified, the differences in recording methods, that is, double microelectrodes vs whole cell patch, and 5 vs 40 mM external Ba^{2+}, partially contributed to the different voltage dependence of channel inactivation. Such altered voltage dependences of the α_{1G} channel in different expression and recording systems have been seen in previous reports (Perez-Reyes *et al*., 1998; Lee *et al*., 1999; Cribbs *et al*., 2000; Marksteiner *et al*., 2001).

Changing the expression system from that of oocytes to that of BHK cells apparently increased the channel sensitivity to efonidipine in both L- and T-type channels. The estimated IC_{50} for block on T-type channels by enantiomer-unspecified efonidipine at −100 mV was 10.8-fold larger in oocytes than that in BHK cells. A similar difference (10.3-fold) was observed for L-type channels as well. Moreover, the voltage dependence of blockades was also different between the two systems. As in Figure 6, the IC_{50} for the $R(-)$-enantiomer in BHK cells was decreased 60-fold by depolarizing the holding potential from −100 to −60 mV, while the value was not significantly changed in oocytes. A similar distinct voltage dependence of block was observed for the effect of enantiomer-unspecified efonidipine and the $S(+)$-enantiomer. Explanations for the different effects have not been fully provided at this point. Altered voltage dependence may partially contribute to the different effects of efonidipine and its isomers. However, the results in Figure 3 could not be explained by the different voltage dependence of inactivation, in which 1 μM efonidipine in BHK cells and 10 μM efonidipine in oocytes showed a similar block at a very hyperpolarized membrane potential (−120 mM). These discrepancies may arise due to different lipid compositions of the cell membrane

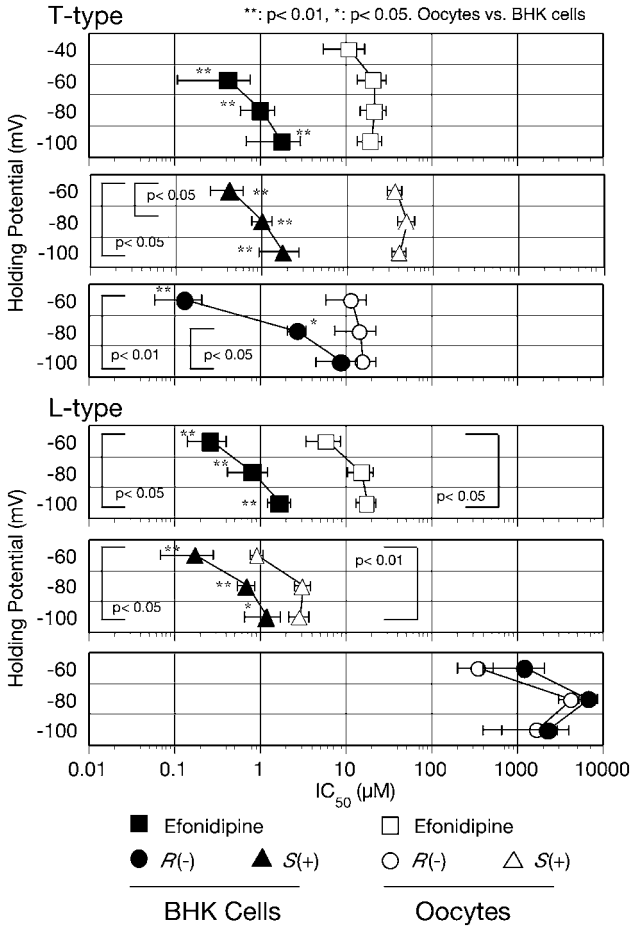

Figure 6 The IC_{50} values for efonidipine and its enantiomers on L- and T-type Ca^{2+} channels in oocytes (open symbols) and BHK cells (filled symbols) are summarized. The IC_{50} at each holding potential was estimated from the data in Figures 2 and 5. The values for oocytes are denoted with open symbols, and those for BHK cells with filled symbols. Asterisks denote that the IC_{50} for BHK cells was significantly smaller than that for oocytes. The Hill's coefficients were the following: enantiomer-unspecified efonidipine in oocytes, 0.8 ± 0.1 at -100 mV, 0.8 ± 0.2 at -80 mV, 0.6 ± 0.2 at -60 mV; $S(+)$-efonidipine in oocytes, 0.9 ± 0.1 at -100 mV, 0.8 ± 0.2 at -80 mV, 0.7 ± 0.3 at -60 mV; $R(-)$-efonidipine in oocytes, 0.8 ± 0.1 at -100 mV, 0.8 ± 0.2 at -80 mV, 0.8 ± 0.2 at -60 mV; enantiomer-unspecified efonidipine in BHK cells, 0.7 ± 0.2 at -100 mV, 0.7 ± 0.3 at -80 mV, 0.6 ± 0.2 at -60 mV; $S(+)$-efonidipine in BHK cells, 0.9 ± 0.2 at -100 mV, 0.7 ± 0.1 at -80 mV, 0.7 ± 0.2 at -60 mV; $R(-)$-efonidipine in BHK cells, 1.1 ± 0.2 at -100 mV, 0.9 ± 0.3 at -80 mV, 0.9 ± 0.3 at -60 mV. Each data point is an average of more than eight observations. Significant differences in IC_{50} values at different holding potentials are denoted in the figure.

between *Xenopus* oocytes and BHK cells, which influence activities of Ca^{2+} channels (Cannon *et al*., 2003) and Ca^{2+} channel antagonists (Mason, 1993). Further evaluations are necessary to fully elucidate the different efonidipine action in two systems. Nevertheless, the relative selectivity of efonidipine and its enantiomers for L- and T-type channel subtypes were similar in the two expression systems. In both expression systems, the IC_{50} for $R(-)$-efonidipine block of L-type channels was more than 100-fold larger than that of T-type channels at a holding potential of -100 mV. Therefore, the L- and T-type selective effect of efonidipine on the series of Ca^{2+} channel subtypes shown in Figure 1 is considered to be independent of the expression system used.

Studies with mibefradil, a non-DHP blocker specific for T-type Ca^{2+} channels, have implied that selective inhibition of T-type Ca^{2+} channels is advantageous in the treatment of hypertension or angina pectoris (Hermsmeyer *et al*., 1997; Van der Vring *et al*., 1999). However, mibefradil was withdrawn from the market because of serious pharmacokinetic and pharmacodynamic interactions with other drugs frequently administered to patients with cardiovascular diseases (Krayenbuhl *et al*., 1999). Therefore, the development of a new T-type Ca^{2+} channel blocker without adverse effects has been considered necessary for medication. Efonidipine has been clinically evaluated as an agent which shows cardiac (Harada *et al*., 2003) and renal protective functions (Hayashi *et al*., 2003). These clinical effects of efonidipine are consistent with the high expression of α_{1G} mRNA in the cardiac muscle and kidneys after hypertrophy or myocardial infarction (Sen & Smith, 1994; Martinez *et al*., 1999; Andreasen *et al*., 2000) and our finding that efonidipine blocked the α_{1G} channel. Therefore, the clinical use of the $R(-)$-isomer of efonidipine, which specifically blocks T-type Ca^{2+} channels, is perhaps the most intriguing.

In conclusion, the characterization of agents for their specificity to each channel subtype, as we did in this study, should be essential for clarifying the relationship between the three-dimensional structure of Ca^{2+} channel antagonists and their specific actions on Ca^{2+} channel subtypes, to develop more effective and safer drugs.

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