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Molecular mechanisms of vasoselectivity of the 1,4-dihydropyridine lercanidipine

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1 The effects of (S)- and (R)-lercanidipine on CHO cells stably expressing the cardiac ($Ca_v1.2a$) or vascular ($Ca_v1.2b$) splice variant of the L-type calcium channel pore subunit were studied, using whole-cell and single-channel patch-clamp measurements.

2 Lercanidipine block of $Ca_v1.2b$ current was enantioselective. (S)-lercanidipine was 4.1-fold more potent.

3 Experiments using acidic solutions (pH 6.8) revealed a 6.4-fold enhanced inhibitory effect of (S)-lercanidipine compared with physiological conditions (pH 7.4) indicating that the charged form mediates inhibition.

4 At depolarised holding potential (−40 mV), (S)-lercanidipine exhibited a 35-fold greater potency, compared with standard conditions (−80 mV).

5 A comparison of the concentration-dependent inhibition of $Ca_v1.2a$ with $Ca_v1.2b$ subunit currents by (S)-lercanidipine revealed only a 1.8-fold difference in IC_{50}, but the slope of the dose–response curve was much steeper ($n_H = 2.3$) with $Ca_v1.2a$, compared with $Ca_v1.2b$ ($n_H = 0.8$). This indicates overlap between agonistic and antagonistic effects, predominant with the cardiac $Ca_v1.2a$ subunit. This idea is supported by transient stimulatory effects, and a slight leftward shift of the IV curves. These effects were more prominent for $Ca_v1.2a$ than for $Ca_v1.2b$.

6 Single-channel experiments confirmed typical features of calcium channel agonists such as prolonged channel openings, a component of lengthened openings, and an enhanced open probability in the presence of (S)-lercanidipine. Again, these findings were concentration-dependent and more pronounced for $Ca_v1.2a$ than for $Ca_v1.2b$.

7 Our data indicate a splice-variant predominant agonism as a new mechanism contributing to the vasoselectivity of lercanidipine, along with marked voltage-dependence of action.
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Abbreviations: $Ca_v1.2a$, cardiac splice variant of the L-type calcium channel pore subunit; $Ca_v1.2b$, vascular splice variant of the L-type calcium channel pore subunit; DHP, dihydropyridine; HP, holding potential; n_H, Hill coefficient; IC_{50}, half-maximal inhibitory concentration; NS, not significant; sqrt, square root; TTX, tetrodotoxin

Introduction

L-type calcium channel antagonists prevent calcium entry through L-type calcium channels in vascular and cardiac muscle cells. Tissue selectivity has been one of the main properties for the development of new calcium channel blockers.

Lercanidipine is a more recently introduced 1,4-dihydropyridine (DHP) L-type calcium channel antagonist, which has been shown in both *in vivo* and *in vitro* studies to block L-type calcium channels in a markedly vasoselective manner without exerting negative inotropic effects (Guarneri *et al*., 1996; 1997; Angelico *et al*., 1999).

Structurally, lercanidipine shows two different ester groups in position 3 and 5 of the DHP ring, leading to the existence of two enantiomers. Binding experiments and functional studies revealed that the (S)-enantiomer is more potent than (R)-lercanidipine (Guarneri *et al*., 1996; Leonardi *et al*., 1997; Sironi *et al*., 1997), as with other DHPs (Romanin *et al*., 1992; Handrock & Herzig, 1996). However, while most DHPs are neutral at physiological pH, lercanidipine carries an amino group with a pK_a of 6.8. At pH 7.4, 20% of the lercanidipine molecules are ionised. It is unclear whether the inhibitory effect is mediated by the charged or the uncharged form of lercanidipine. Permanently ionised DHPs like SDZ 207-180 or UK-118,434-05 exhibited the same potency on both cardiac and vascular isoforms of the L-type calcium channel pore, or even a greater potency on the cardiac isoform, respectively (Heath *et al*., 1997; Morel *et al*., 1998). In contrast, neutral DHPs are known to inhibit the vascular pore subunit to a greater extent (Welling *et al*., 1993; 1997; Morel *et al*., 1998), This leads to the question how lercanidipine, as a potentially ionised DHP, would behave in terms of potency regarding these splice variants. Several functional studies proved lercanidipine to be more active in vascular than in cardiac tissues. Moreover, it has been claimed to be the most vasoselective substance compared to other known vasoselective DHPs, like lacidipine and amlodipine (Angelico *et al*., 1999). We were interested in the vasoselectivity of lercanidipine

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and its underlying mechanisms. We addressed several possibilities:

1. A voltage-dependence of the effect. This is well-known for DHP calcium channel blockers (Bean, 1984; Sanguinetti & Kass, 1984) and has already been confirmed for lercanidipine in guinea-pig myocytes (Cerbai *et al*., 1997).
2. A selectivity towards the vascular $Ca_v1.2b$ splice variant of the pore subunit. This would be a typical feature of neutral DHPs (Welling *et al*., 1993; Morel *et al*., 1998).
3. A $Ca_v1.2a$ splice-variant selective agonistic effect overlapping with antagonism. Previous studies have already observed stimulatory effects by DHPs. Examples include nitrendipine at the single-channel level (Hess *et al*., 1984), and nifedipine, nitrendipine and nisoldipine in isolated hearts (Strauer, 1974; Thomas *et al*., 1984). Specifically, calcium current increase has been described for (S)-lercanidipine at a holding potential of −80 mV in guinea-pig ventricular myocytes (Cerbai *et al*., 1997).

The first specific aim of the present study was to examine if stereoselectivity of lercanidipine could be confirmed electrophysiologically in the vascular L-type calcium channel. Secondly, to elucidate the structure responsible for the effects we investigated the pH dependence of inhibition by lercanidipine. Finally, the third and main aim was to analyse the mechanisms underlying the vasoselectivity of lercanidipine, focussing on voltage-dependent and agonistic effects. Lercanidipine effects were studied in chinese hamster ovary cells that stably express the cardiac and vascular $Ca_v1.2$ pore subunit of the L-type calcium channel. The whole-cell and single-channel configuration of the patch-clamp technique was used. Some of the results have been previously reported in abstract form (Wirtz & Herzig, 2003).

Methods

Cells

Chinese hamster ovary cells stably transfected with the $Ca_v1.2b$ subunit cloned from rabbit lung (CHOCa9) (Bosse *et al*., 1992), or with the $Ca_v1.2a$ subunit cloned from rabbit heart (CHOαH10) (Welling *et al*., 1993) were used. Cells were subcultured using Dulbecco's modified Eagle's medium (DMEM; Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (PAA, Cölbe, Germany), penicillin (30 U ml^{-1}; Sigma, Taufkirchen, Germany), streptomycin (30 μg ml^{-1}; Sigma, Taufkirchen, Germany), 1% nonessential amino acids (100 ×; Biochrom AG, Berlin, Germany), 0.01% sodium pyruvate (Biochrom AG, Berlin, Germany), 0.3% sodium bicarbonate (Biochrom AG, Berlin, Germany), 0.3% L-glutamine (Biochrom AG, Berlin, Germany) and in the case of CHOαH10 2% HT-supplement (50 ×; Biochrom AG, Berlin, Germany) and 0.02% geneticin (Gibco, Karlsruhe, Germany). For electrophysiological experiments the cells were used within 48–96 h after plating.

Electrophysiological recordings

L-type calcium channel currents were measured at room temperature using the whole-cell and single-channel configuration of the patch-clamp technique as described previously (Schröder *et al*., 1998; Handrock *et al*., 1999).

Whole-cell experiments were performed in an external solution containing (mM): NaCl 120, $BaCl_2$ 10.8, $MgCl_2$ 1, CsCl 5.4, dextrose 10, HEPES 10 (pH 7.4 or 6.8 with NaOH). Pipettes (borosilicate glass, 5–7 MΩ) were filled with (mM): CsCl 120, MgCl 3, MgATP 5, EGTA 10, HEPES 5 (pH 7.4 with CsOH). Since Na^+ currents are observed occasionally in these cells, Na^+ channels were blocked by application of 1 μM tetrodotoxin (TTX) to the bath solution. Using a rapid solution changer (RSC 200, Bio-Logic), cells were continuously superfused with drug-free bath solution and then switched to a solution containing lercanidipine at one indicated concentration per experiment. Whole-cell Ba^{2+} currents were elicited by 100 ms depolarising voltage steps from a holding potential of −80 and −40 mV at 0.2 Hz. For each cell a current–voltage relationship was established. The test potential at which maximum current occurred was used for the rest of the experiment (−10 to +20 mV). Currents were sampled at 10 kHz and filtered (−3 dB) at 2 kHz (EPC-9, HEKA, Lambrecht, Germany). Leak and capacitive currents were substracted by using a P/N pulse protocol. The software PULSE (version 9.12; HEKA) was used for data acquisition.

Single calcium channels were recorded in the cell-attached configuration using depolarising test pulses of 100 or 150 ms duration to +10 mV from a holding potential of −100 mV at 1.67 Hz, as reported previously (Lauven *et al*., 1999). Cells were bathed in a solution containing (mM): K-glutamate 120, KCl 25, $MgCl_2$ 2, HEPES 10, EGTA 2, $CaCl_2$ 1, Na_2-ATP 1, dextrose 10 (pH 7.4 with NaOH). Pipettes (5–8 MΩ) were filled with (mM): $BaCl_2$ 110, HEPES 10 (pH 7.4 with TEA-OH). An Axopatch 1D amplifier and PClamp 5.5 or 6.0 software (both Axon Instruments, Union City, CA, U.S.A.) were used for pulse generation, data acquisition (10 kHz), and filtering (2 kHz, −3 dB, 4-pole Bessel). After recording control traces (at least 180 sweeps), (S)-lercanidipine was applied directly into the bath solutions. Only one experiment using one concentration was performed per dish. Linear leak and capacitive currents were digitally subtracted. Openings and closures were identified by the half-height criterion. The availability (fraction of sweeps containing at least 1 channel opening), the open probability, and the peak ensemble average current (obtained visually) were analysed from single-channel and multichannel patches. In the latter case, they were corrected for n, the numbers of channels in the patch. n was defined as the maximum current amplitude observed divided by the unitary current. Peak current was corrected by division through n. The availability was corrected by the square root method: $(1-\text{availability}_{\text{corrected}})$ is the nth root of $(1-\text{availability}_{\text{uncorrected}})$. The corrected open probability was calculated on the basis of the corrected number of active sweep, that is, total open time divided by $[n \times \text{availability}_{\text{corrected}} \times \text{number of test pulses} \times \text{pulse length}]$. Time constants of open-time histograms were obtained by maximum-likelihood estimation (PStat software version 6.0; Union City, CA, Axon Instruments, U.S.A.).

Drugs

The enantiomers of lercanidipine (UCB, Kerpen, Germany) were prepared as stock solutions in dimethylsulphoxide (DMSO) (10 mM) and diluted in DMSO and bath solution

immediately before use. The highest final concentration of DMSO in the superfusate was 0.1%, a concentration that has no direct effect on $Ca_v1.2$ subunit currents. All the solutions were protected from light.

Statistics and data analysis

Whole-cell peak currents were determined using the average of a 5 ms time window within the first 30 ms of the test pulse. Lercanidipine effects on whole-cell currents were measured 600 s after application of the drug. Concentration–response curves were fitted by nonlinear regression analysis using the Hill equation with maximum effect fixed to 100%. The Hill coefficient n_H was fixed to 1 in the cases of curves consisting of three points. Whole-cell and single-channel data are given as means ± s.e.m. Statistical comparison of data was made using paired or unpaired Student's *t*-test, ANOVA followed by Bonferroni-corrected post-tests, or Fisher's exact test as appropriate. $P<0.05$ was considered statistically significant.

Results

Enantioselectivity of lercanidipine action on the vascular $Ca_v1.2b$ subunit current

The action of (S)- and (R)-lercanidipine on whole-cell peak currents was studied on CHO cells expressing the vascular $Ca_v1.2b$ pore subunit of the L-type calcium channel. A typical time course and original traces of a single experiment using (S)-lercanidipine 10^{-7} M is exemplified in Figure 1. The development of block occurred very slowly (~500 s), confirming the slow onset of functional lercanidipine effects (Leonardi *et al*., 1997; Angelico *et al*., 1999). Lercanidipine effects were analysed 600 s after application in all experiments, eliminating major confounding by spontaneous run-down (not shown). At that time (S)-lercanidipine 10^{-7} M inhibited the current through the pore subunit almost completely (87.5 ± 6.0%, $n=6$), whereas equimolar (R)-lercanidipine (Figure 2) blocked the current to 56.6 ± 5.7% ($n=6$). The corresponding concentration–response curves for (S)- and (R)- lercanidipine are shown in Figure 2. Both enantiomers blocked the current in a concentration-dependent manner. The data were fitted by the Hill equation, yielding IC_{50} values of 1.8×10^{-8} M (Hill coefficient $n_H=0.8$) in the case of (S)-lercanidipine, and $IC_{50}=7.4\times10^{-8}$ M (Hill coefficient fixed at $n_H=1$) in the case of (R)-lercanidipine. Thus, (S)-lercanidipine was 4.1-fold more potent than the (R)-enantiomer, indicating enantioselective calcium channel block by lercanidipine. The following experiments were carried out using the (S)-enantiomer of lercanidipine.

pH dependency of (S)-lercanidipine effects on the $Ca_v1.2b$ subunit

To study whether the charged or the uncharged form of lercanidipine is responsible for the effects on the L-type calcium channel current, (S)-lercanidipine was examined at pH = 6.8. Under this condition 50% of lercanidipine is charged, while under physiological conditions only 20% are ionised. Figure 3 presents the concentration–response curve for (S)-lercanidipine obtained with the $Ca_v1.2b$ subunit in solutions buffered to pH 6.8, compared with physiological pH 7.4. The data are

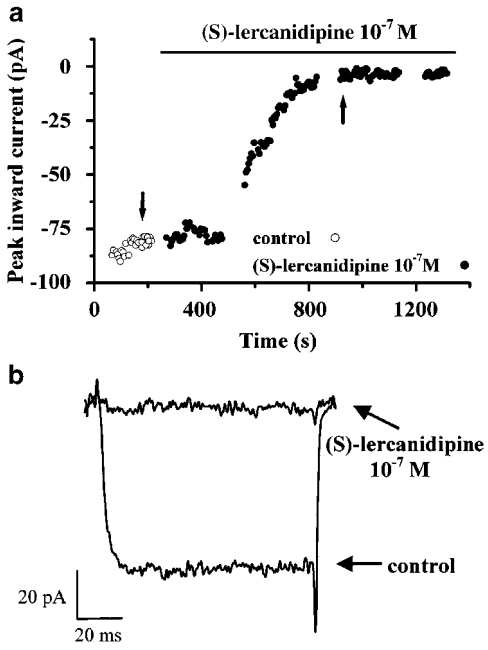

Figure 1 (a) Time course of whole-cell peak current during a single experiment with CHO cells that stably express the vascular $Ca_v1.2b$ pore subunit before and after addition of 10^{-7} M (S)-lercanidipine. Open symbols indicate control, filled symbols (S)-lercanidipine. The current was elicited from a holding potential of −80 mV, the test potential was +10 mV. The arrows indicate the time points of the traces presented in (b). (b) Original traces in the absence and presence of (S)-lercanidipine 10^{-7} M at the time points indicated by arrows in (a).

adequately described by the Hill equation with $IC_{50}=2.8\times10^{-9}$ M (Hill coefficient fixed at $n_H=1$) for pH 6.8. The values indicate that the inhibitory effect of (S)-lercanidipine was 6.4-fold enhanced at pH 6.8 compared with pH = 7.4, suggesting that the charged form mediates inhibition.

Voltage-dependent modulation of $Ca_v1.2b$ subunit currents by (S)-lercanidipine

To study the quantitative impact of voltage-dependence we compared the modulation of currents through the vascular $Ca_v1.2b$ subunit at two different holding potentials, −40 and −80 mV. Figure 4a presents a typical time course of (S)-lercanidipine (10^{-8} M) block at the holding potential of −40 mV. This concentration inhibited the current completely (96.3 ± 1.1%, $n=3$), while the same concentration reduced the current to only 33.5 ± 13.8% ($n=6$, $P<0.05$) at the holding potential of −80 mV. A comparison of both concentration–response curves (Figure 4b) revealed a markedly enhanced inhibition at −40 mV, leading to a leftward shift of the curve. IC_{50} values indicates a 35-fold stronger inhibition at the depolarised holding potential ($IC_{50}=5.2\times10^{-10}$ vs 1.8×10^{-8} M).

Concentration-dependent inhibition of $Ca_v1.2a$ current by (S)-lercanidipine

To study the splice variant selectivity of lercanidipine we investigated the effects of (S)-lercanidipine on CHO cells that stably express the cardiac $Ca_v1.2a$ splice variant of the L-type calcium channel pore subunit, and compared it with $Ca_v1.2b$

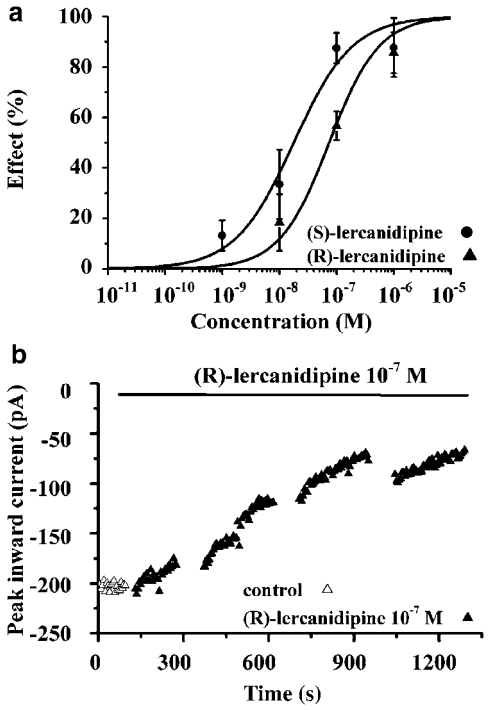

Figure 2 (a) Concentration–response curves for (S)- and (R)-lercanidipine obtained with CHO cells expressing the vascular $Ca_v1.2b$ pore subunit of the L-type calcium channel. Each point represents data of six to eight experiments in the case of (S)-lercanidipine and of four to six experiments in the case of (R)-lercanidipine. The data were fitted by the Hill equation yielding an IC_{50} value of 1.8×10^{-8} M, Hill coefficient determined as $n_H = 0.8$ for (S)-lercanidipine and $IC_{50} = 7.4 \times 10^{-8}$ M, Hill coefficient fixed at $n_H = 1$ for (R)-lercanidipine. (b) Time course of whole-cell peak current during a single experiment with CHO cells that stably express the vascular $Ca_v1.2b$ pore subunit before and after addition of 10^{-7} M (R)-lercanidipine. Open symbols indicate control, filled symbols (R)-lercanidipine. The current was elicited from a holding potential of −80 mV, the test potential was +10 mV.

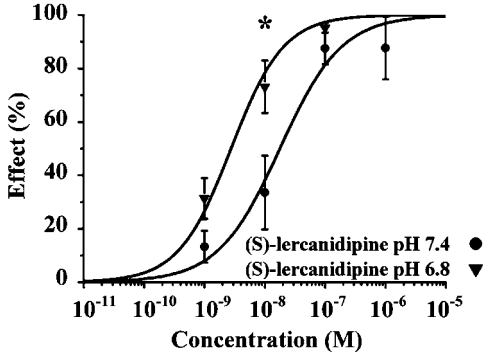

Figure 3 Concentration–response curves for (S)-lercanidipine obtained in solutions buffered to pH 6.8 or 7.4 with CHO cells expressing the vascular $Ca_v1.2b$ pore subunit of the L-type calcium channel. Each point represents means±s.e.m. of four to five experiments (pH 6.8) or six to eight experiments (pH 7.4). * Indicates $P<0.05$ (post-tests among identical concentrations). The data were fitted by the Hill equation yielding an IC_{50} value of 2.8×10^{-9} M, Hill coefficient fixed at $n_H = 1$ for pH 6.8 and $IC_{50} = 1.8 \times 10^{-8}$ M, Hill coefficient $n_H = 0.8$ for pH 7.4.

(Figure 5). The concentration–response curves and corresponding IC_{50} values (cardiac $Ca_v1.2a$ pore subunit: $IC_{50} = 3.3 \times 10^{-8}$ M) revealed only a slight, 1.8-fold selectivity

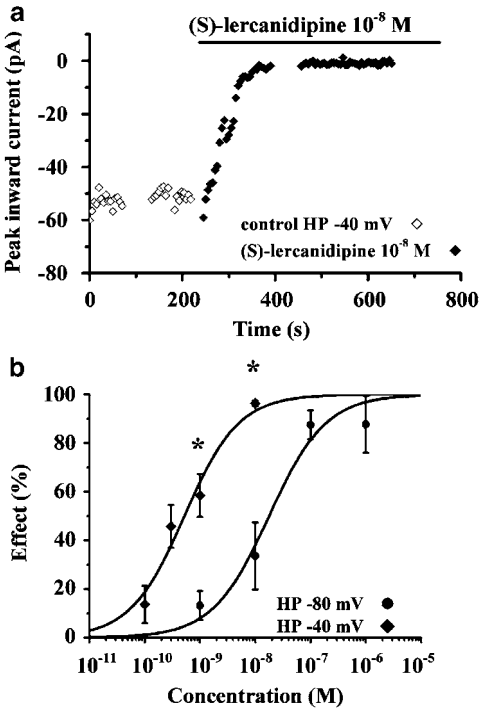

Figure 4 (a) Time course of whole-cell peak current during a single experiment with CHO cells that stably express the vascular $Ca_v1.2b$ pore subunit before and after addition of 10^{-8} M (S)-lercanidipine at a holding potential (HP) of −40 mV. The test potential was +10 mV. Open symbols indicate control, filled symbols (S)-lercanidipine 10^{-8} M. (b) Concentration–response curves for (S)-lercanidipine obtained with CHO cells expressing the vascular $Ca_v1.2b$ subunit of the L-type calcium channel at two different holding potentials of −40 and −80 mV. Values indicate means±s.e.m. of three to five experiments (HP −40 mV) and of six to eight experiments (HP −80 mV). * Indicates $P<0.05$ (post-tests among identical concentrations). IC_{50} determined by Hill analysis of the data is 5.2×10^{-10} M, Hill coefficient $n_H = 0.9$ for the depolarised holding potential −40 mV and $IC_{50} = 1.8 \times 10^{-8}$ M, Hill coefficient $n_H = 0.8$ for the holding potential −80 mV.

towards $Ca_v1.2b$. However, the analysis of the slope of the dose–response curves revealed marked differences. The Hill coefficient obtained for the vascular pore subunit was close to unity, $n_H = 0.8$, whereas the value for the cardiac subunit was 2.3. Accordingly, effects of 10^{-7} M (S)-lercanidipine were similar on both subunits ($Ca_v1.2a$: 93.5±5.0% ($n=5$); $Ca_v1.2b$: 87.5±6.0% ($n=6$)), but 10^{-8} M (S)-lercanidipine seemed to inhibit the current through both subunits to a different extent ($Ca_v1.2a$: 7.7±5.8% ($n=6$); $Ca_v1.2b$: 33.5±13.8% ($n=6$, NS). The apparent deviation of the $Ca_v1.2a$ data from simple law-of-mass action is rather unusual for DHPs (e.g. Bean, 1984; Morel *et al*., 1998; Handrock *et al*., 1999). It could be due to an overlap between agonistic (Cerbai *et al*., 1997) and antagonistic effects, which become evident as less-than expected inhibition at lower drug concentrations. Evidence for this idea is presented in the next two sections.

Stimulatory effects on the $Ca_v1.2$ subunit current caused by (S)-lercanidipine

In some experiments, inhibitory steady-state effects of lercanidipine were preceded by a transient stimulatory effect.

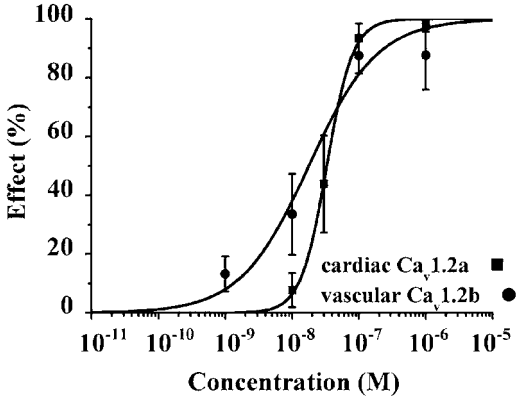

Figure 5 Concentration–response curves for (S)-lercanidipine obtained with CHO cells expressing the cardiac $Ca_v1.2a$ subunit or the vascular $Ca_v1.2b$ subunit of the L-type calcium channel. Values indicate means ± s.e.m. of three to six experiments ($Ca_v1.2a$) and of six to eight experiments ($Ca_v1.2b$). IC_{50} determined by Hill equation is 3.3×10^{-8} M, Hill coefficient $n_H = 2.3$ for the cardiac $Ca_v1.2a$ subunit and $IC_{50} = 1.8 \times 10^{-8}$ M, Hill coefficient $n_H = 0.8$ for the vascular $Ca_v1.2b$ pore subunit.

If we define such transient stimulation as

- an increase of peak current by at least 10% of pre-drug values,
- starting to develop after drug application, and
- lasting above baseline for at least 300 s,

transient stimulation was observed in six out of 96 experiments. It was observed at threshold concentrations (i.e. the lowest respective concentration used at a given condition, 6/30), but not ant any higher concentrations (0/66, $P < 0.05$). Most frequently, it was seen in $Ca_v1.2a$ (3/22). Sporadically, transient stimulation occurred in $Ca_v1.2b$ under similar (1/27, NS) or all experimental conditions (3/74, NS), including those at pH 6.8 and −40 mV. This finding directly indicates the presence of agonism, and gives a hint towards predominance for the $Ca_v1.2a$ pore isoform. Figure 6 illustrates an example for transient stimulation.

Another typical feature of calcium channel agonism is a leftward shift of the current–voltage relationship. Therefore, IV-curves were inspected before and after drug, as illustrated in Figure 6c. Indeed, we observed a slight leftward shift of the ascending limb and of the potential of maximum current in all those cases were transient stimulation was observed. However, the aggregated data for $Ca_v1.2a$ (10^{-8} M (S)-lercanidipine, $n = 8$) showed only a minor shift of the maximum (from 5.6 ± 1.6 to 2.5 ± 1.8 mV, NS). Corresponding data for $Ca_v1.2b$ were rather similar (10^{-9} M (S)-lercanidipine, from 8.0 ± 2.0 mV to 7.0 ± 3.7 mV, $n = 5$, NS).

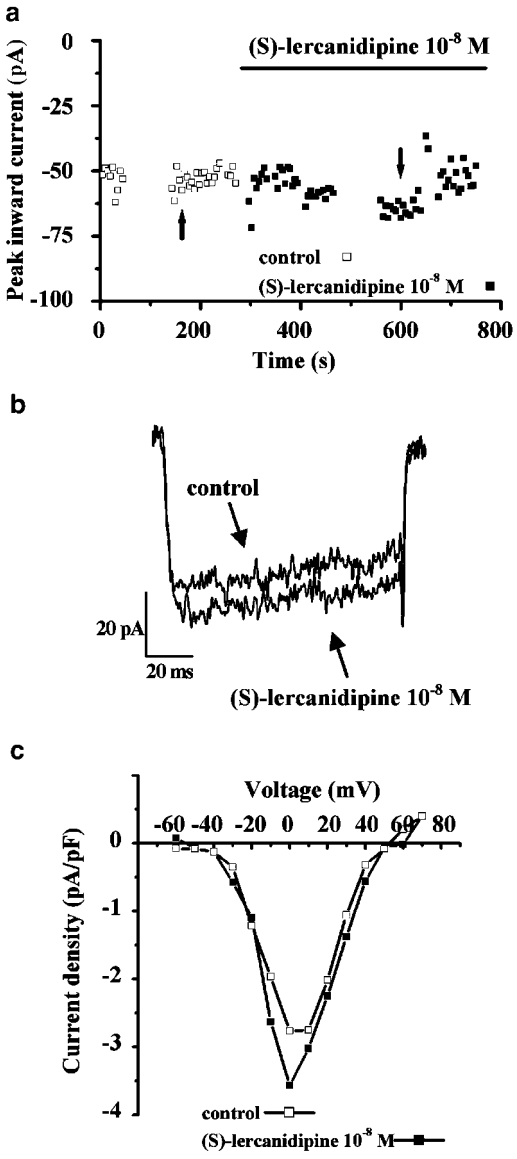

Figure 6 A slight stimulatory effect of 10^{-8} M (S)-lercanidipine on the cardiac $Ca_v1.2a$ subunit current was observed in three of nine experiments. (a) Time course of whole-cell peak current. Open symbols indicate control, filled symbols (S)-lercanidipine. The arrows indicate the time points of the traces presented in (b). (b) Original traces before and after addition of 10^{-8} M (S)-lercanidipine. (c) Current density–voltage relationship in the absence and presence of 10^{-8} M (S)-lercanidipine of the same experiment as in (a) and (b). Open symbols indicate control data, filled symbols data at time ~300 s after (S)-lercanidipine application.

Effects at the single-channel level

As a more sensitive, qualitative means to detect calcium channel agonism, single-channel experiments were performed. Figure 7a illustrates the response of a single cardiac $Ca_v1.2a$ pore subunit to 10^{-7} M (S)-lercanidipine. Before drug addition, open times were short ($\tau = 0.15$ ms, Figure 7b). In the presence of (S)-lercanidipine 10^{-7} M mean open time increased (Table 1), and distribution of open times revealed two components (Figure 7b). Sweeps with long openings (Figure 7a) formed a second component of lengthened openings ($\tau = 1.14$ ms) in the open time histogram. The time course of open probability (Figure 7c) demonstrates a sustained increase of open probability by (S)-lercanidipine 10^{-7} M, leading to an increased ensemble average current (Figure 7a). These results are typical for calcium channel agonism (Hess *et al*., 1984; Lauven *et al*., 1999) and are representative for results from three patches (Table 1). Experiments using higher concentrations of (S)-lercanidipine did not show such behaviour. Figure 8 demonstrates that 10^{-5} M (S)-lercanidipine inhibited the current, due to a decreased fraction of active sweeps (availability), see Table 1. (S)-lercanidipine 10^{-6} M ($n = 2$, data not shown) caused an intermediate phenomenology: availability was decreased, but the open times and open probability were

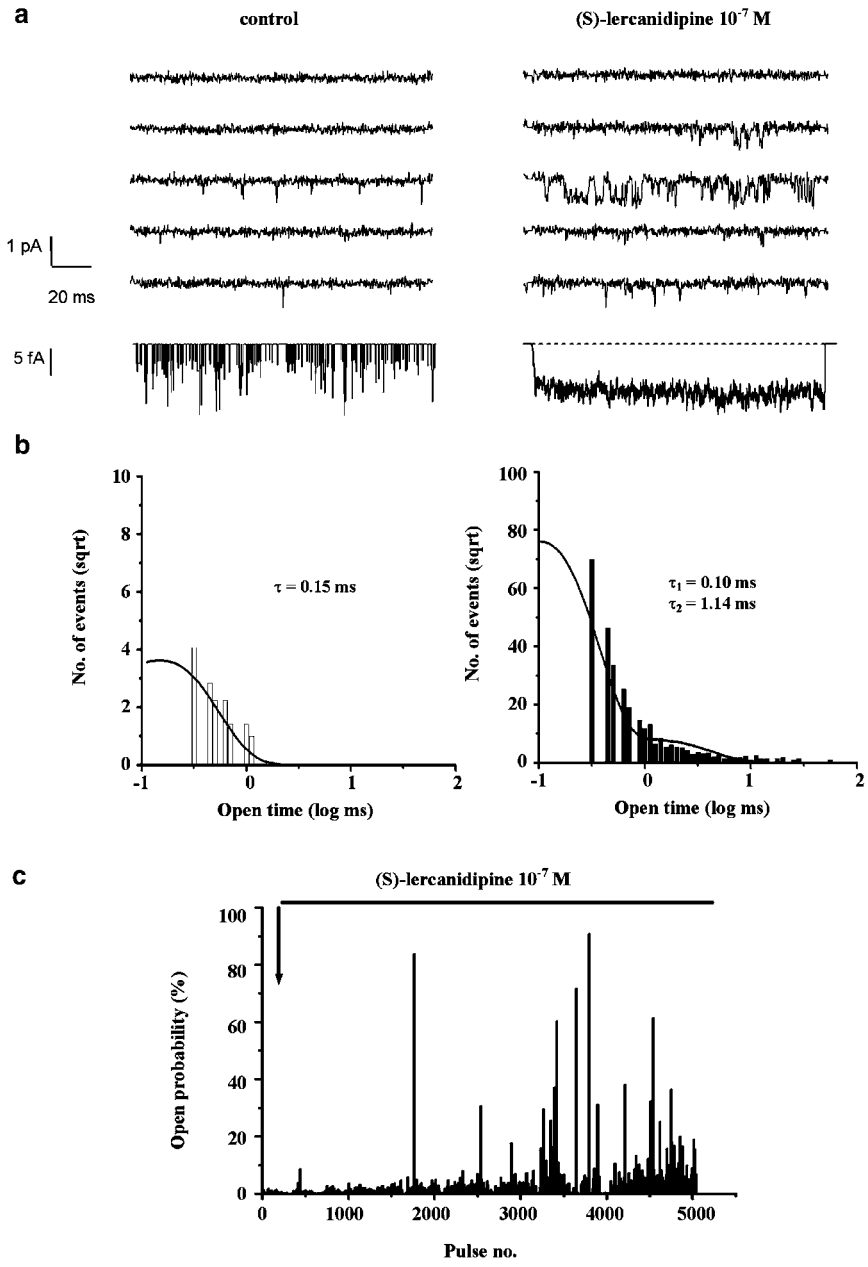

Figure 7 Effect of 10^{-7} M (S)-lercanidipine on the L-type calcium current through a single cardiac $Ca_v1.2a$ pore subunit in a cell-attached patch (150 ms pulse length, holding potential −100 mV, test pulse +10 mV, 1.67 Hz). (a) Five consecutive traces before (left) and after application (right) of 10^{-7} M (S)-lercanidipine. Bottom rows: ensemble average current of all traces of each experiment. Scale bars: 20 ms/1 pA (single traces) and 20 ms/5 fA (ensemble average current). (b) Open time distribution before (left, open columns) and after 10^{-7} M (S)-lercanidipine (right, filled columns). (c) Time course of open probability before and after application of 10^{-7} M (S)-lercanidipine.

increased, reflecting signs of agonism and antagonism simultaneously.

In order to analyse if this agonistic behaviour is splice variant-selective or -predominant we investigated the effects of (S)-lercanidipine 10^{-7} M on the activity of the vascular $Ca_v1.2b$ pore subunit at the single-channel level (Figure 9). Here, a second component of increased openings was found in only two of six experiments. Additionally, the time course (Figure 9b) reveals a transient nature of stimulation. Single-channel analysis (Table 2) demonstrates no net effect on ensemble average current ($n=6$). This is due to slight increases of open probability and open times, compensated by depressed availability. Thus, we qualitatively detected agonistic behaviour also in $Ca_v1.2b$, but this effect was less pronounced than with the cardiac $Ca_v1.2a$ pore subunit.

Discussion

The present study is the first to describe inhibition of vascular L-type calcium channels by the 1,4-DHP lercanidipine. By using recombinant channels, we were able to compare drug

Table 1 Effects of (S)-lercanidipine 10^{-7} M and 10^{-5} M on single-channel properties of cardiac $Ca_v1.2a$ subunits of the L-type calcium channel

	Mean open time (ms)	*Open probability* (%)	*Availability* (%)	*Ensemble peak current* (fA)	*n*
Predrug control	0.21±0.00	0.49±0.07	50.94±11.92	5.03±1.11	3
10^{-7} M (S)-lercanidipine	0.26±0.03	1.49±0.21	69.56±9.02	10.63±0.19	3
Predrug control	0.25±0.03	0.80±0.21	59.74±11.53	10.07±3.08	3
10^{-5} M (S)-lercanidipine	0.21±0.03	0.50±0.05	28.07±6.01	2.93±0.64	3

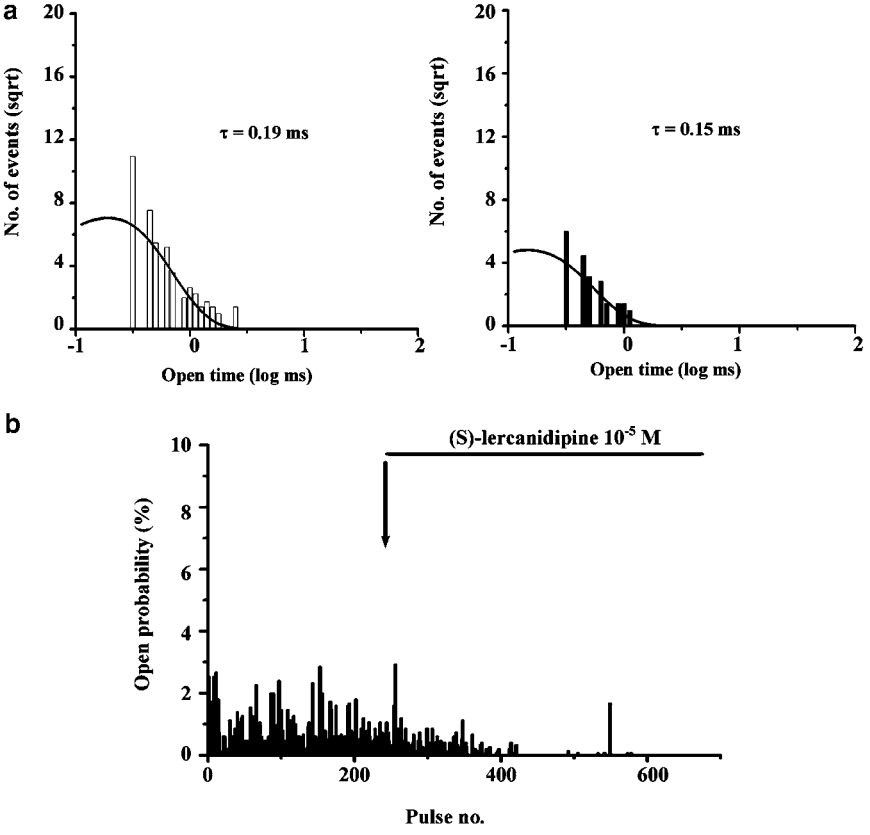

Figure 8 Effect of 10^{-5} M (S)-lercanidipine on the L-type calcium channel current obtained with a single CHO cell stably expressing the cardiac $Ca_v1.2a$ pore subunit using the cell-attached configuration (100 ms pulse length, holding potential −100 mV, test pulse +10 mV, 1.67 Hz). (a) Open time distributions before (left, open columns) and after application of 10^{-5} M (S)-lercanidipine (right, filled columns). (b) Time course of open probability before and after application of 10^{-5} M (S)-lercanidipine.

effects on vascular- ($Ca_v1.2b$) and cardiac-type ($Ca_v1.2a$) channels under otherwise identical conditions. We then dissected molecular mechanisms contributing to the pronounced functional vasoselectivity of this compound. Our analysis focussed on the active (S)-enantiomer (see Figure 1, and Guarneri *et al*., 1996; Leonardi *et al*., 1997; Sironi *et al*., 1997) of lercanidipine.

By varying the pH, we observed enhanced block of the current at more acidic bath solution, indicating that the block is mediated by the charged form. Similar studies on amlodipine had revealed opposing results (Kass *et al*., 1988). Consistently, UK-118,434-05, a permanently charged amlodipine analog, is less potent than the parent compound (Heath *et al*., 1997). Lercanidipine and amlodipine differ in the position of their amino group. Amlodipine contains an aminomethoxymethyl side chain, a primary amine, at position 2 of the DHP ring while lercanidipine possesses a diphenylpropylaminoalkyl side chain, a tertiary amine, at position 3 of the DHP ring. Given the structure–activity relationship of amine-substituted DHPs (Baindur *et al*., 1993; Bangalore *et al*., 1994; Peri *et al*., 2000), the distance between the charged group and the DHP ring could be more close to optimum in the case of lercanidipine. Importantly, pH variations in the physiological range could modulate the potency in the case of lercanidipine. Charged and neutral DHPs possess different structural requirements regarding their binding sites (Lacinova *et al*., 1999). In particular, only neutral DHPs have been shown to block the vascular pore isoform $Ca_v1.2b$ more potently than $Ca_v1.2a$ (Welling *et al*., 1993; 1997; Morel *et al*., 1998). Charged DHPs, in contrast, have similar (Morel *et al*., 1998), or even higher (Heath *et al*., 1997), potency at the cardiac isoform, resembling phenylalkylamines in this regard (Morel *et al*., 1998). Taken together, these observations raise the question of the mechanism of vasoselectivity of lercanidipine.

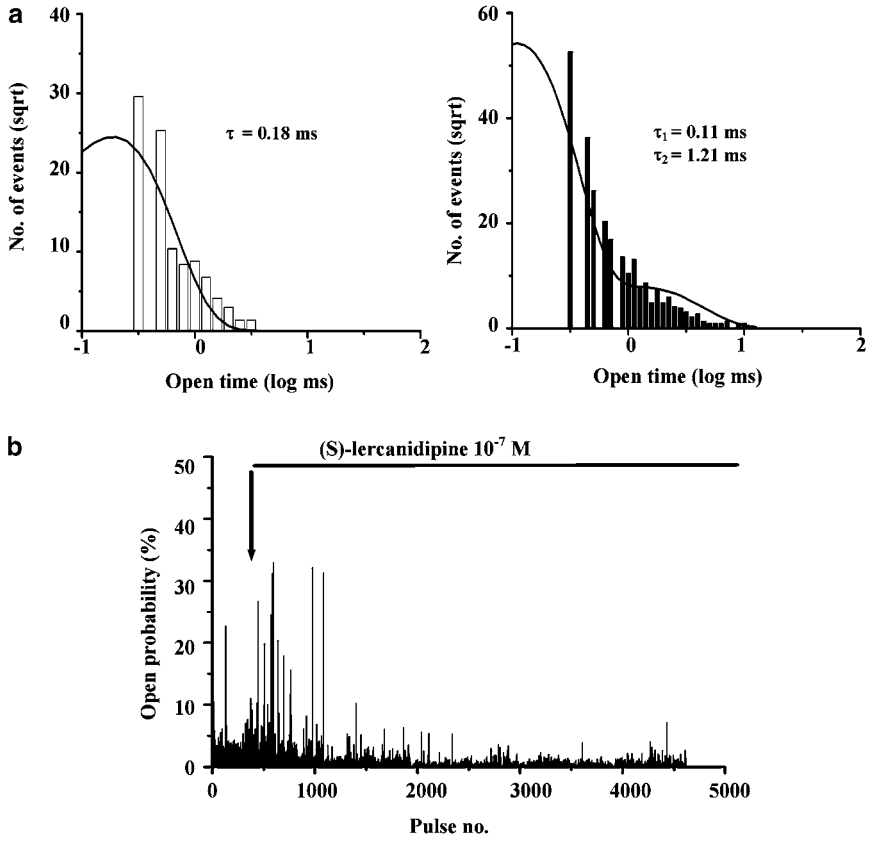

Figure 9 Effects of (S)-lercanidipine 10^{-7} M on the L-type calcium channel activity in a cell-attached patch (150 ms pulse length, holding potential −100 mV, test pulse +10 mV, 1.67 Hz) obtained with a CHO cell stably expressing the vascular $Ca_v1.2b$ pore subunit. (a) Open time distributions before (left, open columns) and after application of 10^{-7} M (S)-lercanidipine (right, filled columns). (b) Time course of open probability before and after application of 10^{-7} M (S)-lercanidipine.

Table 2 Effects of (S)-lercanidipine 10^{-7} M on single-channel properties of vascular $Ca_v1.2b$ subunits of the L-type calcium channel

	Mean open time (ms)	*Open probability* (%)	*Availability* (%)	*Ensemble peak current* (fA)	*n*
Predrug control	0.22±0.01	0.74±0.15	77.31±6.49	11.00±1.99	6
10^{-7} M (S)-lercanidipine	0.33±0.08	1.09±0.36	56.98±6.88	9.60±2.18	6

Voltage-dependence of action is well known for DHPs (Bean, 1984), and likely is a major factor contributing to vasoselectivity. For (S)-lercanidipine, we found a 35-fold higher potency at a holding potential of −40 mV compared to −80 mV. This figure is higher than values obtained for (S)-isradipine (factor 4.5, Handrock *et al*., 1999; factor 4.8, Morel *et al*., 1998), (R)-isradipine (factor 3.4, Handrock *et al*., 1999), amlodipine (factor 10, Hughes & Wijetunge, 1993), nisoldipine (factor 3.7, Morel *et al*., 1998) or nifedipine (factor 2.7, Morel *et al*., 1998) under identical or very similar conditions, respectively. Qualitatively, Cerbai *et al*. (1997) have already observed voltage-dependence of (S)-lercanidipine in guinea pig ventricular myocytes, but their findings were confounded by stimulatory effects observed at a holding potential of −80 mV. Therefore, voltage-dependence seems to be particularly pronounced in case of (S)-lercanidipine. What about the structural features discriminating the vascular and cardiac pore subunit?

Comparison of the IC_{50} values of (S)-lercanidipine on the vascular pore subunit (1.8×10^{-8} M) and on the cardiac isoform (3.3×10^{-8} M) revealed only slight, insignificant differences. The 1.8-fold greater potency on the vascular subunit is lower than values known for neutral DHPs (Morel *et al*., 1998). However, inspection and analysis of the shape of concentration–response curves revealed marked differences in the Hill coefficients with $n_H = 0.8$ for the smooth muscle isoform and $n_H = 2.3$ for the cardiac pore subunit. A possible explanation for the high coefficient of the cardiac pore might be an overlap of stimulatory (Cerbai *et al*., 1997) and antagonistic effects on the cardiac pore subunit. Direct hints on an agonistic behaviour of (S)-lercanidipine were rather subtle in our study at the whole-cell level: in some experiments a transient stimulatory effect on the L-type calcium channel current and a slight leftward shift of the maximum of the current–voltage relationship was observed in the presence of (S)-lercanidipine. These phenomena were more obvious in $Ca_v1.2a$ than in $Ca_v1.2b$, but their incidence and extent was too small to reach statistical significance.

Experiments through single-channels make it possible to detect the typical qualitative features of calcium channel agonism and antagonism, even simultaneously. With (S)-

lercanidipine, we observed an increase in mean open probability and lengthened openings as typical features of calcium channel agonism (Hess *et al*., 1984, Lacerda & Brown, 1989; Bechem & Hoffmann, 1993), and a decrease in availability typical for antagonism (Kawashima & Ochi, 1988). Again, agonistic effects were only detectable using lower (S)-lercanidipine concentrations, with 10^{-5} M (S)-lercanidipine leading to pure inhibition. The vascular isoform revealed qualitative features of channel agonism, but in contrast to the cardiac isoform, this effect was less pronounced. It never gave rise to a net stimulation of single-channel average currents (Table 2). The discrepancy between absolute concentrations required for whole-cell and single-channel effects likely result from differences in the experimental procedures. The cell-attached configuration may impede access of drug when applied to the bath solution. Furthermore, the difference in divalent cation concentrations (whole-cell: 10.8 mM, single-channel: 110 mM Ba^{2+}) should reduce DHP affinity in the latter case (Peterson & Catterall, 1995). The use of Ba^{2+} (instead of physiological Ca^{2+}) as charge carrier should not hamper our interpretation qualitatively: agonistic and antagonistic behaviour have been detected for various calcium channel modulators using Ca^{2+} (Bechem & Hoffmann, 1993; Kass & Arena, 1989) as well as Ba^{2+} (Hess *et al*., 1984; Kokubun *et al*., 1986). Furthermore, both the voltage-dependence of action as well as the transient agonism were observed for (S)-lercanidipine with currents carried by Ca^{2+} in ventricular myocytes (Cerbai *et al*., 1997).

In conclusion, the marked vasoselectivity of lercanidipine known from functional studies is explained by two of the three proposed molecular mechanisms. (1) (S)-Lercanidipine exerts pronounced voltage-dependent inhibition of vascular $Ca_v1.2b$ channels. (2) It does not discriminate between $Ca_v1.2b$ and cardiac $Ca_v1.2a$ isoforms regarding apparent affinity, unlike neutral DHPs. (3) (S)-Lercanidipine exerts agonistic and antagonistic effects like some other DHPs, but agonistic behaviour is more pronounced – and functionally relevant – with the cardiac $Ca_v1.2a$ isoform. This adds a quantitatively minor but qualitatively novel mechanism of DHP vasoselectivity.

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