

# Lercanidipine and T-type calcium current

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**Abstract. – OBJECTIVE:** Lercanidipine is a calcium antagonist with no cardiodepressant activity, long lasting antihypertensive action and reno-protective effect. Our previous data demonstrated that lercanidipine blocks L-type calcium channels (CaL). However, no data are available concerning its effects on T-type calcium channels (CaT). The aim of this study was to evaluate the effect on both CaL and CaT and the selectivity ratio of R-lercanidipine, S-lercanidipine and RS-lercanidipine. A comparison with other dihydropyridines (amlodipine and lacidipine) and the CaT blocker mibefradil was also performed.

**MATERIALS AND METHODS:** In patch-clamped guinea-pig ventricular myocytes, a voltage protocol was applied mimicking a normal action potential: HP of -90 mV, 200 ms depolarizing steps to -50/+50 mV. Lercanidipine was tested at concentrations (1-10  $\mu$ M) able to block  $\approx$  50% CaL evoked from a HP in the range of -50 to -30 mV. Cells were superfused with a Na<sup>+</sup> and K<sup>+</sup> free solution pre-warmed to 35°C to abolish overlapping currents.

**RESULTS:** Using the described voltage protocol, all dihydropyridines at 1  $\mu$ M blocked less than 20% CaL, with the exception of lacidipine, that reduced CaL >60% of control. All calcium channel blockers (CCBs) blocked a significant amount of CaT, varying from 28% (mibefradil) to 4.3% (amlodipine). Based on the ratio between CaT and CaL blockade in each cell (T/L), mibefradil, as expected, showed the highest T affinity (T/L=1.3). Lercanidipine, either racemate or enantiomers, showed a noticeable T selectivity, T/L varying from 1.05 (S-lercanidipine) to 1.15 (R-lercanidipine).

**CONCLUSIONS:** All CCBs examined in this study showed both T- and L-channel blocking activities and can be differentiated based on their relative affinity. Among tested dihydropyridines, lercanidipine showed the highest T/L selectivity.

*Key Words:*

Lercanidipine, Dihydropyridines, Calcium channel blockers, T-type calcium antagonists, Renal protection, Electrophysiology.

## Introduction

The discovery, development and uses of Calcium Channels Blockers (CCB) have been recently reviewed<sup>1</sup>. Voltage gated Ca<sup>2+</sup> channels are the

primary source of Ca<sup>2+</sup> in resistance artery and arteriolar smooth muscle cells thus controlling the vascular tone and consequently blood pressure<sup>2</sup>. Tykocky et al<sup>2</sup> have discussed the physiology and pharmacology of these channels in resistance arteries and arterioles, their modulation by vasoconstrictors and vasodilators, their role in the functional regulation of tissue blood flow and their dysfunction in diseases such as hypertension, obesity, and diabetes.

L-type and T-type Ca<sup>2+</sup> channels play a pivotal role in this setting. In the cardiovascular system, the two channels play a different role depending on cell type (smooth or cardiac cells) and subtype (e.g., pacemaker cells vs. atrioventricular tissue). Indeed, cardiac myocytes usually express both types of Ca<sup>2+</sup> channels, L- (CaL) and T-type Ca<sup>2+</sup> channels (CaT)<sup>3-5</sup>. In the last twenty years, molecular studies have identified several different genes coding for the alpha subunits of the two type of channels, which are classified into the subfamily of Ca<sub>v</sub>1 and Ca<sub>v</sub>3 channels for L-type and T-type, respectively<sup>6</sup>. In turn, splice isoforms of channel subtypes produce a large variety of conductances whose function differs according to cell type and system. CaT are involved in sinoatrial node automaticity<sup>7</sup>, together with CaL subtypes Ca<sub>v</sub>1.2 and/or Ca<sub>v</sub>1.3<sup>8</sup>, while CaL are essential for excitation-contraction coupling mechanisms in the working myocardium<sup>6</sup>. Abnormal expression of CaT has been linked to a role myocardial hypertrophy and ventricular arrhythmias<sup>9,10</sup>. The function of CaT and CaL subtypes in non-cardiac tissue, and especially in renal smooth muscle cells physiology, has been deeply investigated. In the renal microvasculature, the vasodilator response of L-type CCBs is mainly observed in preglomerular microvessels (i.e., afferent arterioles), whereas efferent arterioles are refractory to the dilator action of these agents, suggesting a predominance of CaL in the afferent arteriole<sup>11</sup>. In contrast, a growing body of evidence has accumulated indicating that CaT have a different distribution in the kidney, being expressed primarily on the

postglomerular arterioles and thus having a critical role in mediating the efferent arteriolar tone. It has been demonstrated that CCBs that act on both CaL and CaT dilate afferent and efferent arterioles, favour reduced glomerular capillary pressure and exert a beneficial action on kidney function. These effects may alleviate glomerular hypertension, reduce proteinuria and improve renal survival in patients with chronic kidney disease. The ability to vasodilate both afferent and efferent renal arterioles, which is not induced by the majority of CCBs, has been demonstrated for lercanidipine in a murine model of hypertension<sup>12</sup> where the drug countered the luminal narrowing of efferent arterioles and improved glomerular morphology.

Based on recent preclinical and clinical observations<sup>13-16</sup> we wished to re-evaluate the effect of lercanidipine in terms of CaT and CaL selectivity. We were prompted also by previously published results from our lab showing that, at variance with the common belief of CCB as “L-type calcium channel blockers”, they may exhibit different degrees of CaT blockade<sup>17</sup>. As previously described, we tested the selectivity of lercanidipine vs.  $I_{Ca,T}$  and  $I_{Ca,L}$  evoked by a voltage-clamp protocol allowing to elicit and compare, in the same cell, T- and L-type calcium current. For sake of comparison, we also report data obtained with two other dihydropyridines (lacidipine, amlodipine) and with mibefradil, the selective CaT blocker.

## Materials and Methods

### Isolation of Guinea-Pig Ventricular Myocytes

The methods for cell isolation from male guinea pigs have been previously described<sup>17</sup>. All experiments, including present original data, were carried out after approval by the Animal Care and Research Ethics Committee of the University of Florence in compliance with current national and European directive at time of animal use.

### Solutions

The cells were placed in the experimental chamber (0.2 mL) and superfused at a rate of 2 mL/min using a six-flow line system controlled by electronic valves to allow rapid change from control to experimental solutions. The extracellular solution was a modified Tyrode's solution prewarmed at 35°C containing (mM): NaCl 137, KCl 5.4, MgCl<sub>2</sub> 1.2, CaCl<sub>2</sub> 1.8, glucose 10, pH adjusted to 7.4 with

HEPES/NaOH. T- and L-type calcium current were recorded in Na<sup>+</sup> and K<sup>+</sup>-free solution containing (mM): Tris 137, CsCl 20, MgCl<sub>2</sub> 1, CaCl<sub>2</sub> 5.4, Glucose 5, pH 7.4 with HCl. Pipettes, having a resistance of about 2 Mohm, contained (mM): CsCl 125, TEA-Cl 20, Mg-ATP 5, EGTA 15, pH 7.2 with HEPES/CsOH. Stock solutions (10 mM) of S-, R-, and RS-lercanidipine, lacidipine and amlodipine in dimethylsulfoxide (DMSO) were used to prepare the final solutions of drugs at the concentration of 0.1-10 μM in Tyrode's solution. DMSO was adjusted in order to have the same percentage in all solutions. Mibefradil was dissolved in water and then in Tyrode's solution to achieve the final concentration of 3-10 μM.

### Electrophysiological Set-Up

The electrical activity was recorded by using the patch-clamp technique in the whole-cell configuration. Details of the experimental protocols and equipment are given elsewhere<sup>17</sup>. Patch pipettes (Corning Capillaries 7052, Garner Glass, Claremont, CA, USA) had a resistance of about 2 Mohm. The electrical signal was recorded by a patch amplifier (Axopatch 1D, Axon Instrument Inc., Union City, CA, USA) and digitized (Labmaster TL-1 DMA, Scientific Solutions). The cut-off frequency was 10 kHz. Current and voltage protocol generation, data acquisition, and analysis were performed using the pClamp software (version 10.2, Molecular Devices Inc.). MicroCal Origin (version 2018 MicroCal Software Inc.) and Prism (version 6, GraphPad Software Inc., La Jolla, CA, USA) were used for further analysis.

### Electrophysiological Protocols

Recording was started after 5 min dialysis of the cell and external perfusion with the Na<sup>+</sup>-K<sup>+</sup> free solution in order to eliminate Na<sup>+</sup> and K<sup>+</sup> currents completely. In guinea-pig ventricular cells, a distinct fraction of  $I_{Ca,T}$  can be activated at a membrane potential of -30 mV, whereas, at this potential, the channels conducting  $I_{Ca,L}$  remain inactive. On the other hand, at threshold potential for activation of  $I_{Ca,L}$  (0/10 mV), the contribution of  $I_{Ca,T}$  is negligible. Thus, the different potential ranges of activation can be used to separate the two components of the calcium current.  $I_{Ca}$  was elicited by 200-ms depolarizing steps to increasing positive voltages (-40 to +50 mV) from a holding potential (HP) of -90 mV. Steps were applied at low frequency (maximum rate: 0.2 Hz) and sampled at 5 kHz.  $I_{Ca}$  amplitude

**Table 1.** Percentage blockade of  $I_{Ca,T}$  and  $I_{Ca,L}$  by calcium channel blockers.

	CaL		CaT	
	[CCB <sub>low</sub> ]	[CCB <sub>high</sub> ]	[CCB <sub>low</sub> ]	[CCB <sub>high</sub> ]
RS-LER <sup>a</sup>	5.1±3.5	6.7±3.0	10.5±5.6	15.9±5.5
S-LER <sup>a</sup>	5.8±4.2	19.9±9.3	9.9±3.8	21.6±9.2
R-LER <sup>a</sup>	11.1±4.4	7.9±2.8	20.3±8.3	16.5±7.4
Amlodipine <sup>a</sup>	13.1±7.2 <sup>d</sup>	39.7±2.8	4.3± 3.8 <sup>d</sup>	39.0±2.3
Lacidipine <sup>b</sup>	26.6±6.8 <sup>d</sup>	61.3±11.2	5.7± 4.7 <sup>d</sup>	22.9±4.9
Mibefradil <sup>c</sup>	14.3±2.1 <sup>d</sup>	16.3±1.3	27.7± 4.9 <sup>d</sup>	42.1±2.3

Each value represents the mean±S.E.M. of 5-6 cells challenged with CCBs at different concentrations, as follows: <sup>a</sup>low=1  $\mu$ M; high=10  $\mu$ M; <sup>b</sup>low=0.1  $\mu$ M; high=1  $\mu$ M; <sup>c</sup>low=3  $\mu$ M, high=10  $\mu$ M. <sup>d</sup>Data are from Figure 5 in 17.

was measured as difference between steady state current, measured at the end of the depolarizing step, and peak inward current.

The ratio between T- and L-type blockade allowed to rank the drugs according to their T/L selectivity. The effect of S-, R- and RS-lercanidipine was compared to that of the most selective T-type channel blocker, mibefradil, and with two dihydropyridines that, according to previous work from our lab<sup>17-19</sup>, resulted to be preferentially L-type channel blocker (lacidipine) or not (amlodipine).

### Statistical Analysis

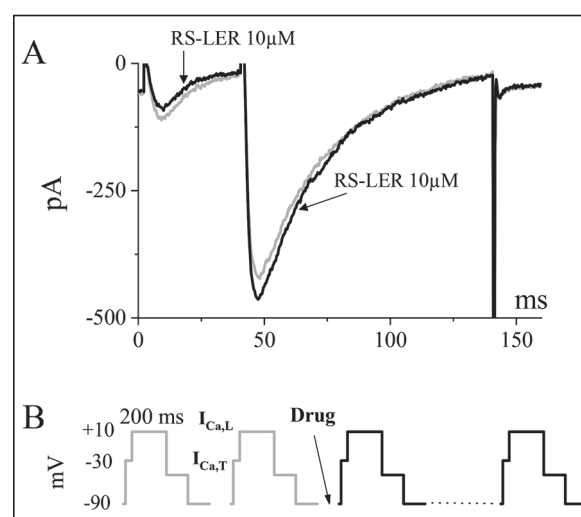
Data are expressed as mean±SEM. Results were compared by using ANOVA followed by the Dunnett's test. A  $p < 0.05$  was considered statistically significant.

## Results

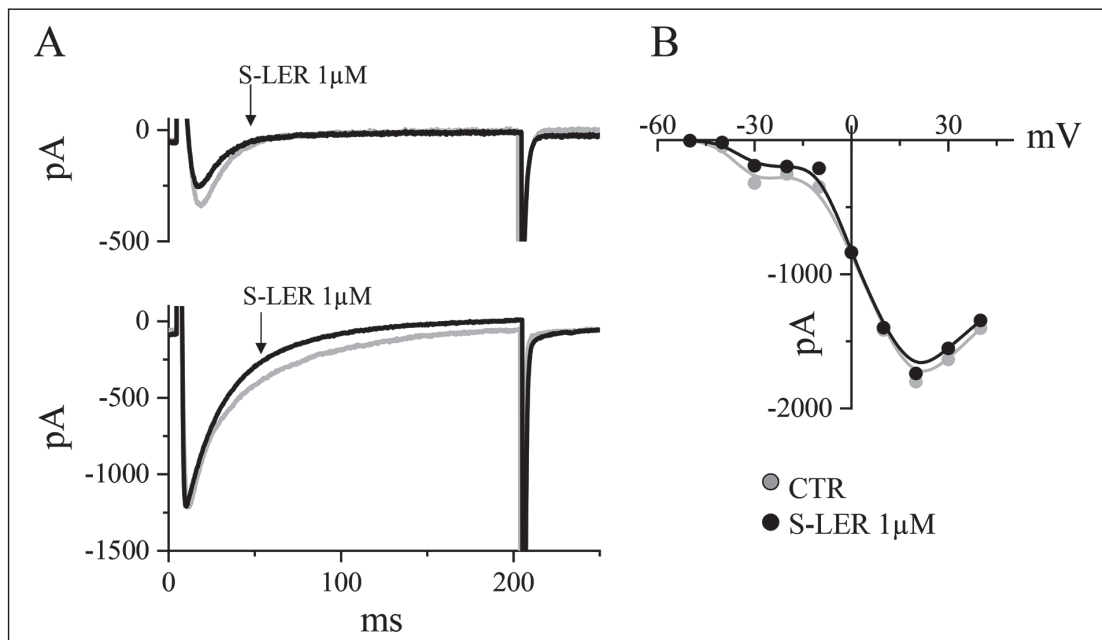
Lercanidipine, either as enantiomers or racemate, was used at a concentration of 1 and 10  $\mu$ M. As shown in Figure 1B, the voltage-clamp protocol allowed to elicit T-type and L-type Ca-currents in the same cell and to evaluate the percent blockade by the different drugs of the two components under conditions (HP= -90mV) likely resembling those present by the development of a normal cardiac action potential. Figure 1A shows an example of T- and L-type calcium current recorded in the absence and presence of 10  $\mu$ M RS-lercanidipine (RS-LER).

A similar protocol was used for all tested drugs. Figures 2A and 3A show examples of recordings obtained with the two enantiomers, S- and R-LER, respectively, at 1  $\mu$ M. In both figures, panels show peak  $I_{Ca,T}$  (upper traces) and  $I_{Ca,L}$  (bot-

tom traces), recorded in the absence or presence of drugs. It is worth noting that the effect of S-LER and R-LER on peak  $I_{Ca,L}$  is quite small under these experimental conditions, although the current decay was accelerated in both cases. However, we have demonstrated that, when  $I_{Ca,L}$  is elicited from a more positive holding potential, LER reduced it by about 50%<sup>18</sup>. Peak inward currents evoked by a family of voltage-clamp steps were reported in the corresponding I-V relationship in Figure 2B and 3B panel. The two peaks measured at voltages around -30 mV ( $I_{Ca,T}$ ) and +20 mV ( $I_{Ca,L}$ ) are clearly distinguishable; in these experimental conditions, S-LER and R-LER reduced  $I_{Ca,T}$  amplitude while peak  $I_{Ca,L}$  was almost unchanged. Table I summarizes the percentage blockade of the two



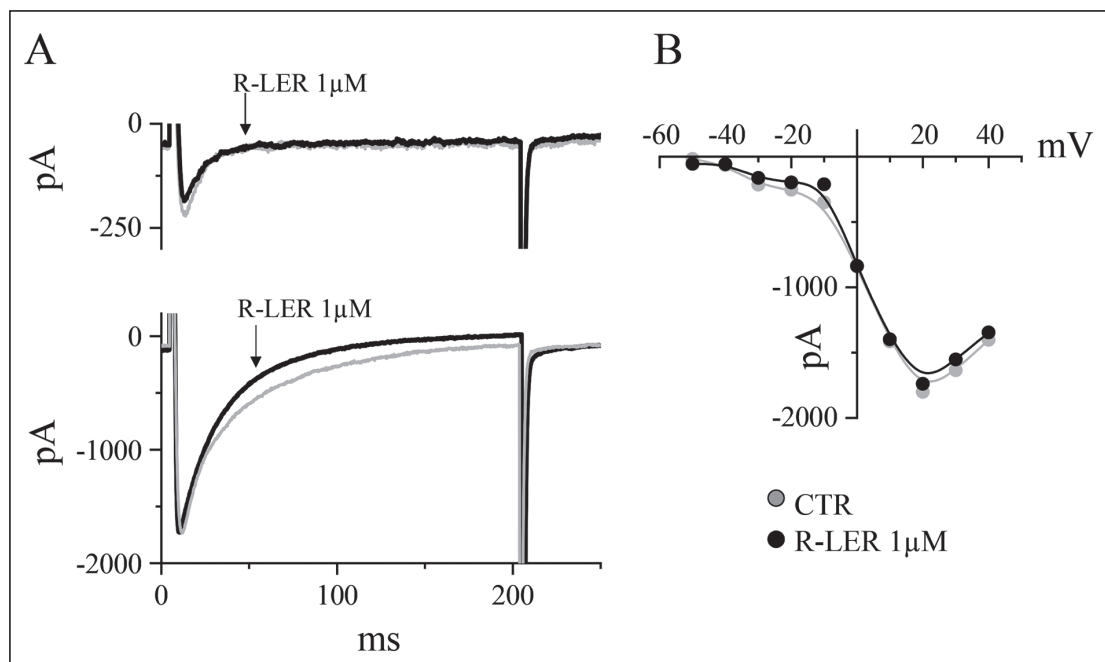
**Figure 1.** Effect of RS-lercanidipine on calcium currents. Panel A shows inward currents evoked by a typical two-step protocol as in panel B, in control conditions (grey line) and after superfusion with 10  $\mu$ M RS-LER (black line).



**Figure 2.** Effect of S-lercanidipine on L- and T-type calcium currents. Panel A shows inward currents evoked at -30 mV ( $I_{Ca,T}$ , upper traces) and +20 mV ( $I_{Ca,L}$ , lower traces), in control conditions (grey lines) and after superfusion with 1  $\mu$ M S-LER (black lines). The corresponding I-V plot is reported in panel B.

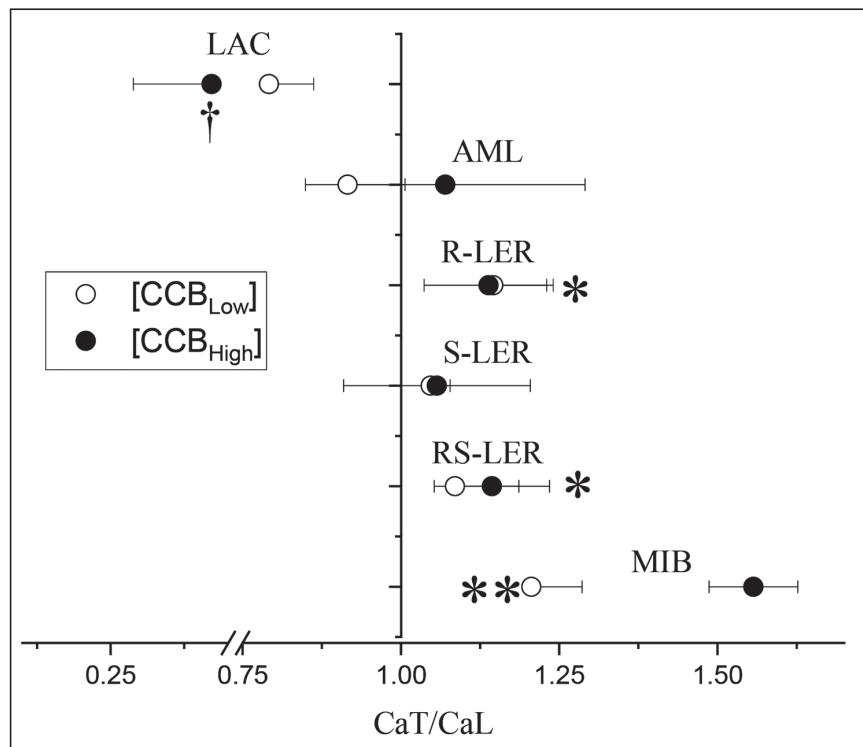
types of channels, by the lowest (1  $\mu$ M, [CCB<sub>low</sub>]) or the highest (10  $\mu$ M, [CCB<sub>high</sub>]) concentration of lercanidipine tests, either enantiomers or racema-

te. For sake of comparison, the values obtained with equipotent concentrations of reference CCBs are also reported.



**Figure 3.** Effect of R-lercanidipine on L- and T-type calcium currents. Panel A shows inward currents evoked at -30 mV ( $I_{Ca,T}$ , upper traces) and +20 mV ( $I_{Ca,L}$ , lower traces), in control conditions (grey lines) and after superfusion with 1  $\mu$ M R-LER (black lines). The corresponding I-V plot is reported in panel B.

**Figure 4.** CaT vs. CaL selectivity ratio obtained as described in the RESULTS section. LAC, lacidipine, AML, amlodipine; MIB, mibefradil; \* $p < 0.05$ , \*\* $p < 0.01$  vs. LAC (low concentrations); † $p < 0.01$  LAC vs. all other CCBs (high concentrations).



Altogether, data suggest that RS-LER, S-LER and R-LER reduce both  $I_{CaL}$  and  $I_{CaT}$  at micromolar concentrations. This effect was not completely surprising since, as previously demonstrated<sup>17</sup>, other so-called “L-type calcium channel blockers” exhibit a similar behavior. Figure 4 summarizes data obtained with amlodipine (AML), lacidipine (LAC) and the selective CaT blocker mibefradil (MIB), in comparison with data obtained with lercanidipine. The figure applies the same formalism as adopted for previously published data<sup>17</sup>. CaT/CaL represents the ratio between the percentage blockade of the two types of current, in individual cells at any drug concentration: 1 or 10  $\mu$ M for AML, S-LER, R-LER and RS-LER, 0.1 or 1  $\mu$ M LAC, 3 or 10  $\mu$ M MIB. A value  $>1$  (right side of the plot) means that the drug is more selective for CaT than CaL; a ratio  $<1$  (left side) indicates selectivity for CaL. LAC and MIB show, as expected, preferential blockade of CaL and CaT respectively at any concentration, while AML did not show any selectivity. Contrary to lacidipine and amlodipine, lercanidipine showed a detectable selectivity toward T-type calcium channels: the ratio was  $>1$  for both enantiomers and the racemate.

Statistical comparison was then performed using one-way ANOVA by comparing all drugs tested, respectively, at the lowest ( $[CCB]_{low}$ ) or

at the highest ( $[CCB]_{high}$ ) concentration used. At  $[CCB]_{low}$ , CaT/CaL values resulted to be statistically significant different for R-LER and RS-LER ( $p < 0.05$ ), as well as for MIB ( $p < 0.01$ ), compared to LAC. At  $[CCB]_{high}$ , all CCB were statistically significantly different from lacidipine for which the CaT/CaL ratio was smaller than 0.5.

## Discussion

These results demonstrated that, using an appropriated voltage protocol, all the calcium antagonists examined in this study showed both T- and L-channel blocking activities. The substances differed however in their selectivity and therefore the T/L channel inhibition ratio was different. As previously demonstrated<sup>17</sup>, when compared to widely used CCBs, lacidipine had the highest L- and mibefradil the highest T-selectivity. Here we report data for another widely used dihydropyridine, lercanidipine, tested as racemate or R and S enantiomers. Using the same formalism and voltage-clamp protocol as previously described, we observed that lercanidipine displayed a CaT/CaL ratio  $>1$ , that is, the percentage blockade of T-type current was higher than that of L-type current measured in the same cell. In particular, values

calculated for 1  $\mu\text{M}$  R-LER and RS-LER (1.15 and 1.09, respectively) were statistically significant different from that of 0.1  $\mu\text{M}$  LAC (0.79). However, present data complete and integrate previous ones also from another point of view. Table I and Figure 4 report values for percentage blockade of the two types of current and, hence, corresponding CaT/CaL calculated in cells challenged with higher CCB concentrations (10  $\mu\text{M}$  for all but lacidipine, tested at 1  $\mu\text{M}$ ). It is interesting to note that, while the percentage blockade of either CaL or CaT generally increases dose-dependently as expected, T vs. L selectivity was maintained. Indeed, lacidipine was even more L-selective (0.42) and mibefradil T-selective (1.56); CaT/CaL for R-, S- and RS-LER was consistently  $>1$  (1.14, 1.06 and 1.14 respectively).

CaT blockade has been proposed as a new therapeutic strategy for preventing calcium-mediated remodeling and/or arrhythmogenic mechanisms in different cardiac diseases (atrial fibrillation, myocardial hypertrophy and failure). Our study demonstrates that widely used calcium channel blockers, which (at variance with mibefradil) possess a safe well-documented pharmacokinetic profile, may exert beneficial actions beyond those classically attributed to their CaL-blocking activity. Knowledge of the CaT affinity of the calcium antagonists likely may help to compensate for the loss of mibefradil as a hopeful drug in the treatment of cardiovascular diseases; the different influences of calcium antagonists in the diseased heart on the other hand likely may help in the future to further understand the pathophysiological role of the T-type calcium channel. In this regard a very relevant area is renal protection. In fact, a growing body of evidence indicates that CCBs inhibiting both L- and T-type channels are able to alleviate glomerular hypertension through the dilation of both afferent and efferent arterioles, thus offering more beneficial action on proteinuria and renal survival rate in patients with chronic kidney disease<sup>11,15</sup>.

As previously stated CaT have a different distribution in the kidney, being expressed primarily on the postglomerular arterioles. T-type calcium channels are coded by three different genes belonging to the  $\text{Ca}_v3$  family. Besides their relevant role in neuronal cells, CaT are considered potential candidate target for antihypertensive drugs due to their involvement in angiotensin-mediated aldosterone secretion<sup>20</sup>. A different distribution of CaT and CaL in intrarenal arteries have been clearly detected; in particular, among T-type channels both  $\text{Ca}_v3.1$  and  $\text{Ca}_v3.2$  isoforms

are expressed in renal glomerular vessels<sup>21</sup>. The complex interaction between different types and subtypes of calcium channels, joint to their site specific distribution in renal arteries<sup>21</sup>, may explain the preferential renoprotective effect of CCBs combining T- and L-type blockade over selective blockade<sup>22</sup>. In this line, the combined T- and L-type blocking activity of lercanidipine may explain its vasodilatory action on efferent arterioles resulting in beneficial nephroprotective effects. A reduction of filtration fraction and proteinuria, with a nephroprotective effect similar to that exerted by blockers of the renin-angiotensin system has been described in clinical practice<sup>16</sup>. Furthermore, in the ZAFRA study<sup>13</sup> lercanidipine showed a high antihypertensive effect in chronic renal failure patients with improvement in renal function, measured through creatine clearance. In addition, the results of the RED LEVEL study<sup>14</sup> lend further support to the anti-albuminuric effect of the lercanidipine and to the long-term renal-protective effect in patients with hypertension.

## Conclusions

We found that all the calcium antagonists examined in this study showed both T- and L-channel blocking activities and can be differentiated on the basis of their relative affinity, with lercanidipine showing the highest T/L selectivity. This peculiar pharmacological property may translate in beneficial clinical effects.

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## Conflict of Interest

The Authors declare that they have no conflict of interest.

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