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Characteristics of L-type calcium channel blockade by lacidipine in guinea-pig ventricular myocytes

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1 The Ca^{2+}-antagonistic properties of lacidipine were investigated in patch-clamp guinea-pig ventricular myocytes.

2 In basal conditions, 0.1 μM lacidipine reduced the action potential duration, associated with a decrease in the L-type calcium current ($I_{Ca,L}$) to 66±4% of the control value, without a change in the current-voltage relationship. Sodium current and background potassium currents were not affected. All the effects reached a steady state within 2 min.

3 The Ca^{2+}-antagonistic effect of lacidipine was voltage-dependent: a marked negative shift (about 20 mV) of the steady-state inactivation curve was observed with long (10 s) conditioning prepulses, but not with short (350 ms) prepulses.

4 The onset of and recovery from the voltage-dependent effect caused by 0.1 μM lacidipine were significantly slower when compared to those of equiactive concentrations of nimodipine (0.5 μM) and nisoldipine (0.1 μM). $I_{Ca,L}$ measured after prepulses at −40 mV lasting 500 ms or less was unchanged (95±5% of maximum current value) while it was reduced to 49±10% by nimodipine and 43±9% by nisoldipine ($P<0.05$ vs lacidipine for both).

5 Similarly, the recovery from block in the presence of lacidipine was slower than with nimodipine and nisoldipine. After a prepulse of 1 s at −80 mV, $I_{Ca,L}$ recovered up to 54±2% of the maximum current value in the presence of lacidipine, and up to 91±3% and 93±5% in the presence of nimodipine and nisoldipine, respectively ($P<0.05$ vs lacidipine).

6 Blockade of $I_{Ca,L}$ by lacidipine was use-dependent. After ten 200 ms long pulses (1 Hz) from −80 mV, $I_{Ca,L}$ was reduced to 55±7% of the current measured at the first pulse. In the presence of nimodipine and nisoldipine, $I_{Ca,L}$ elicited by the tenth pulse amounted to 93±3% and 80±6% of the first pulse value, respectively ($P<0.05$ vs lacidipine). Lacidipine did not cause use-dependent blockade of $I_{Ca,L}$ in cells stimulated with 10 ms long pulses.

7 These results demonstrate that lacidipine selectively inhibits $I_{Ca,L}$ in isolated cardiomyocytes and suggest that this effect occurs mainly through binding to the inactivated Ca^{2+} channels.

Keywords: Lacidipine; L-type calcium current; sodium current; ventricular myocytes; nimodipine; nisoldipine; patch-clamp

Introduction

Dihydropyridine calcium channel blockers have been widely used in the treatment of arterial hypertension (Weber & Graettinger, 1994). Their pharmacological and therapeutic properties are attributable to the block of the influx of calcium ions through L-type calcium channels in the membrane of vascular smooth muscle cells (Catterall & Striessnig, 1992). Recently, many new calcium antagonists have been developed, especially in the class of dihydropyridine compounds, which have considerably higher vascular selectivity and longer duration of action with respect to the prototype compound, nifedipine (Nayler, 1988). Lacidipine is a 'second-generation' dihydropyridine (DHP) derivative showing long-lasting anti-hypertensive action (Micheli *et al*., 1990; 1991). Recent data suggest that this property can be largely explained by the marked liposolubility of the compound which may allow accumulation of the drug in cell membranes: in fact, the membrane partition coefficient of lacidipine is significantly higher than that of other second-generation DHP derivatives, such as amlodipine, isradipine and nimodipine (Herbette *et al*., 1994). It has been suggested that lacidipine may reach its receptor, the L-type calcium channel, via a 'membrane bilayer pathway' (Herbette *et al*., 1993) with a two-step process: first, it binds and accumulates in the membrane lipid bilayer and then diffuses within the membrane to the calcium channel receptor. However, these findings also imply that the kinetics of block and unblock and therefore, the modulation of cardiac and smooth muscle calcium channels by lacidipine, may be substantially different from those of other dihydropyridine compounds.

The electrophysiological properties of lacidipine have been studied only in multicellular cardiac preparations (Cerbai *et al*., 1990). Lacidipine decreased the plateau of the action potential recorded in sheep Purkinje fibres and blocked histamine-induced slow action potentials in potassium-depolarized guinea-pig papillary muscles. These effects, which can be considered a reliable index of calcium antagonistic activity, remained unchanged 2 h after drug wash-out, in agreement with the persistent anti-hypertensive action of lacidipine. However, detailed studies of the inhibitory action of lacidipine on L-type calcium current have not yet been performed.

The purpose of the present study was to examine the electrophysiological effect of lacidipine on cardiac L-type calcium current ($I_{Ca,L}$), in order to get more information about the mechanism of long-lasting effects of the drug. In fact, $I_{Ca,L}$ measurement in patch-clamped cardiomyocytes can be a suitable model for studying the interaction of calcium-channel blockers with their receptors, as demonstrated by previous analogue studies carried out with several DHP (Kamp *et al*., 1989; Kass & Arena, 1989; Kass *et al*., 1991) and non-DHP compounds (Yasui & Palade, 1995). The action of lacidipine has been compared to that of equiactive concentrations of nimodipine and nisoldipine, two 'second-generation' dihydropyridine derivatives whose calcium antagonistic properties have been investigated previously (Sanguinetti & Kass, 1984; Schwartz *et al*., 1984; Kass & Arena, 1988), and like lacidipine, are uncharged at neutral pH.

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Methods

Experimental preparation

The investigation conforms to the rules for the care and use of laboratory animals of the European Community (86/609/CEE).

Isolated ventricular myocytes were obtained by a protocol based on previously described procedures (Cerbai *et al*., 1991). Guinea-pigs (200–300 g) were anaesthetized with ether and killed by a blow on the neck. The heart was rapidly excised, mounted in a Langendorff apparatus and perfused for 5 min with a low-calcium solution (LCS) of the following composition (mM): NaCl 120, KCl 10, KH_2PO_4 1.2, $MgCl_2$ 1.2, glucose 10, taurine 20, pyruvate 5, pH 7.2 with HEPES/NaOH. The LCS was thermostated at 37°C and equilibrated with 100% O_2. The solution was then quickly changed to LCS plus 30 mg l^{-1} pronase E (Serva) and 1 g l^{-1} albumin; when the heart became soft, the ventricles were sliced and slowly stirred in enzymatic solution for about 1 h. Cardiomyocytes that appeared in the supernatant were purified by gravity sedimentation, collected and stored in LCS at room temperature. Cells were kept in LCS supplemented with 1 mM $CaCl_2$, penicillin (50 iu ml^{-1}) and streptomycin (50 μg ml^{-1}) (Gibco), and used within 10 h of their isolation.

The experimental set-up was similar to that described by Cerbai *et al*. (1995). A drop of cells was placed in the experimental chamber (0.2 ml) mounted on the plate of an inverted microscope (TMS, Nikon), connected to a monitor (Hitachi VM 122) via a camera (Hitachi HM30).

The whole-cell configuration of the patch-clamp technique was used to record action potential and membrane currents. Patch pipettes (Corning Capillaries 7052, Garner Glass), having a resistance of 1–2 MΩ, were pulled by a two-step puller (Hans Otchozki, Homburg), fire polished and filled with a solution of the following composition (mM): KCl 140, $MgCl_2$ 1, Na_2-ATP 3, ethyleneglycol-*bis*-[β-aminoethylether]-N,N,N′,N′-tetraacetic acid (EGTA) 5 and HEPES 10; pH 7.2 with KOH. The electrical signal was recorded by a patch amplifier (Axopatch 1A, Axon Instrument Inc.), digitized (Labmaster TL-1 DMA, Scientific Solutions) and displayed on the monitor of a 386 personal computer. The cut-off frequency was 20 kHz. Current and voltage protocol generation, data acquisition and analysis were performed by use of the pClamp software (Vers 5.5.1, Axon Instrument Inc.). MicroCal Origin (MicroCal Software Inc.) was used for further analysis.

Recording was started after a 5 min dialysis of the cell. Action potentials were elicited at a rate of 0.2 Hz and sampled at 1 kHz. L-type calcium current ($I_{Ca,L}$) was elicited by 200 ms depolarizing steps to 0 mV from a holding potential of −80 mV, preceded by a brief (10 ms) step to −40 mV to inactivate sodium current. Steps were applied at low frequency (maximum rate: 0.2 Hz) and sampled at 5 kHz. $I_{Ca,L}$ amplitude was measured as difference between steady-state current, measured at the end of the depolarizing step at 0 mV, and peak inward current. The voltage of half-maximal inactivation (V_H) and the slope factor (k) describing the steepness of the inactivation curve were obtained by using a computer-calculated Boltzmann fit according to the equation:

$$I = 1/\{1 + \exp[(V_H - V_m)/k]\}$$

where I is the fraction of available current and V_m the membrane potential.

Measurements of sodium current (I_{Na}) were performed at room temperature with small ventricular cells and low resistance pipettes (1 MΩ). Zero-current membrane potential was chosen as the holding potential (−80 to −70 mV); I_{Na} was then elicited by 35 ms pulses in the range −60 to +50 mV and sampled at 50 kHz. In these experiments, series resistance was compensated by 80% and membrane capacitance was corrected to minimize the amplitude of the capacitative transient.

Solutions and drugs

Cells were superfused at a rate of 2 ml min^{-1} with a three-flow line system controlled by electronic valves to allow rapid change from control to experimental solutions. Control solution was a modified Tyrode solution containing (mM): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.2, glucose 10 and HEPES 5, pH adjusted to 7.35 with NaOH, at a temperature of 35°C. The experiments designed to determine the effect of lacidipine on I_{Na} were performed at room temperature (20–22°C).

A stock solution of 1 mM lacidipine in ethanol (10%) was diluted in Tyrode solution to obtain the final concentration (0.1 μM) just before each experiment. In our experimental conditions, this concentration was able to reach its maximum effect within 2 min and to reduce 'basal' $I_{Ca,L}$ to 66±4% (n=7) of the control value. Nimodipine and nisoldipine were also dissolved in ethanol and then in Tyrode solution, to achieve final concentrations of 0.5 and 0.1 μM, respectively. In preliminary experiments, 0.5 μM nimodipine and 0.1 μM nisoldipine decreased $I_{Ca,L}$ amplitude to 67±4% (n=6) and 62±6% (n=6), respectively, of the initial value in 2 min, thus producing a quantitatively similar block as that resulting in cells superfused with 0.1 μM lacidipine. Higher concentrations of lacidipine were used in preliminary experiments. At 1 μM lacidipine, $I_{Ca,L}$ blockade was almost complete and the analysis of use- and voltage-dependent effect of the drug was therefore impossible. All the experiments designed to test the voltage-dependence and the use-dependence of the calcium-antagonistic activity of drugs were performed after their effect on $I_{Ca,L}$ had reached a steady-state (that is, after 3 min superfusion). At the concentration used, ethanol had no direct electrophysiological effect on action potential and membrane currents in the absence of calcium antagonists.

Analysis of data

Data are expressed as mean±s.e.mean. Results were compared by using one-way analysis of variance or Student's t test for grouped data, as appropriate; $P<0.05$ was considered significant.

Results

Basal electrophysiological effects of lacidipine

Figure 1 shows the typical effect of 0.1 μM lacidipine on the action potential recorded in a guinea-pig ventricular myocyte. As previously observed in polarized multicellular preparations (Cerbai *et al*., 1990), the only evident effect of the drug was a

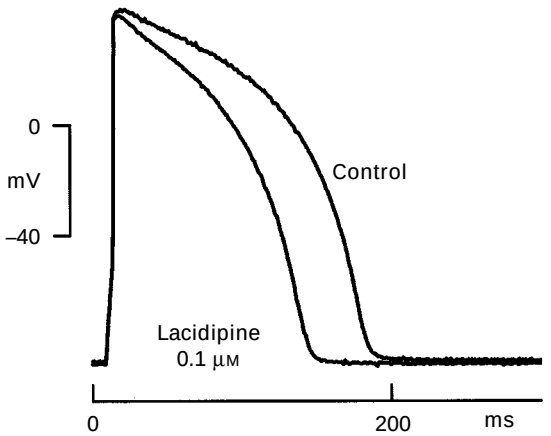

Figure 1 Superimposed action potentials recorded in a guinea-pig ventricular myocyte in control and after 2 min superfusion with 0.1 μM lacidipine.

marked reduction in action potential duration, while amplitude and resting potential remained unchanged. This result can be explained by the selectivity of the drug toward $I_{Ca,L}$, as demonstrated by the following experiments. Figure 2a illustrates current traces recorded in the same myocyte during a depolarizing step to 0 mV, in control and after 2 min of superfusion with lacidipine. In the presence of the drug, the peak $I_{Ca,L}$ was reduced by about 40% while late currents (I_{late}) recorded at the end of the step were practically unchanged. The lack of effect of lacidipine on I_{late} is better demonstrated in Figure 2b, where current traces recorded at -110, -90 and $+40$ mV in the absence and presence of drug are superimposed. Current-voltage (I-V) relationships for both $I_{Ca,L}$ and I_{late} are plotted in Figure 2c: the latter is practically superimposable on the control and in the presence of lacidipine over a large range of test potentials (-100 to $+40$ mV). In the control, $I_{Ca,L}$ had a threshold of around -20 mV and reached its maximum at 0 mV; lacidipine (0.1 μM) caused a decrease in $I_{Ca,L}$ at all test potentials, without modifying the shape of the I-V curve (Figure 2c). Similar results were obtained in 3 cells. On the other hand, I_{Na} recorded in small ventricular myocytes, was not modified by the drug, as shown in Figure 3. Both the peak current recorded at -5 mV (Figure 3a) and I-V relationship were similar in the absence and presence of 0.1 μM lacidipine ($n=3$).

Voltage-dependent effect of lacidipine

To test the voltage-dependence of the effect of lacidipine, steady-state inactivation curves of $I_{Ca,L}$ were obtained before and after application of 0.1 μM lacidipine by use of a two-pulse protocol. Conditioning prepulses of either 350 ms or 10 s to various potentials (from -80 to 0 mV) were applied, and the peak $I_{Ca,L}$ was measured during the subsequent test pulse to 0 mV; the holding potential was kept at -80 mV. Typical normalized curves obtained in the absence and presence of 0.1 μM lacidipine are shown in Figure 4. With the short prepulses (Figure 4a), the relationships between voltage and availability of $I_{Ca,L}$ (inactivation curves) were identical in the absence and presence of the drug. Fitting of data points with a Boltzmann function gave a V_H of -27.8 ± 2.4 mV in control and -29.7 ± 3.1 mV in the presence of lacidipine ($n=4$, not significant).

When the duration of prepulses was increased to 10 s (Figure 4b), the control curve shifted to more negative potentials ($V_H = -31.9\pm0.8$ mV, $n=3$) compared with those seen with 350 ms prepulses, possibly as a consequence of slow inactivation of calcium channels (Datyner & Cohen, 1993; Yasui & Palade, 1995). However, the difference between the control curves (obtained after a conditioning pulse of either 350 ms or 10 s) was not statistically significant. Lacipidine produced a further marked hyperpolarizing shift in the steady-state inactivation curve ($V_H=-57.8\pm3.7$ mV, $n=3$), which was statistically significant ($P<0.05$) compared to the control value and to that obtained, in the presence of lacidipine, with a 350 ms conditioning pulse. In the presence of lacidipine, calcium channel inactivation occurred at more negative potentials, as a consequence of $I_{Ca,L}$ blockade by the drug during depolarizing prepulses; these results also suggest that the binding rate of lacidipine to the inactivated channels requires more than 350 ms to develop.

Onset of and recovery from the voltage-dependent effect

To obtain further insight into the time-course of the voltage-dependent effect of lacidipine, conditioning prepulses of increasing duration were applied to different potentials (-80 mV or -40 mV), at which the voltage-dependent block of lacidipine was absent or almost maximal, respectively (see Figure 4b). The effect of lacidipine was compared to that of nimodipine and nisoldipine used at 0.5 and 0.1 μM, respectively; they gave a degree of $I_{Ca,L}$ reduction similar to that of 0.1 μM lacidipine in basal conditions (see Methods).

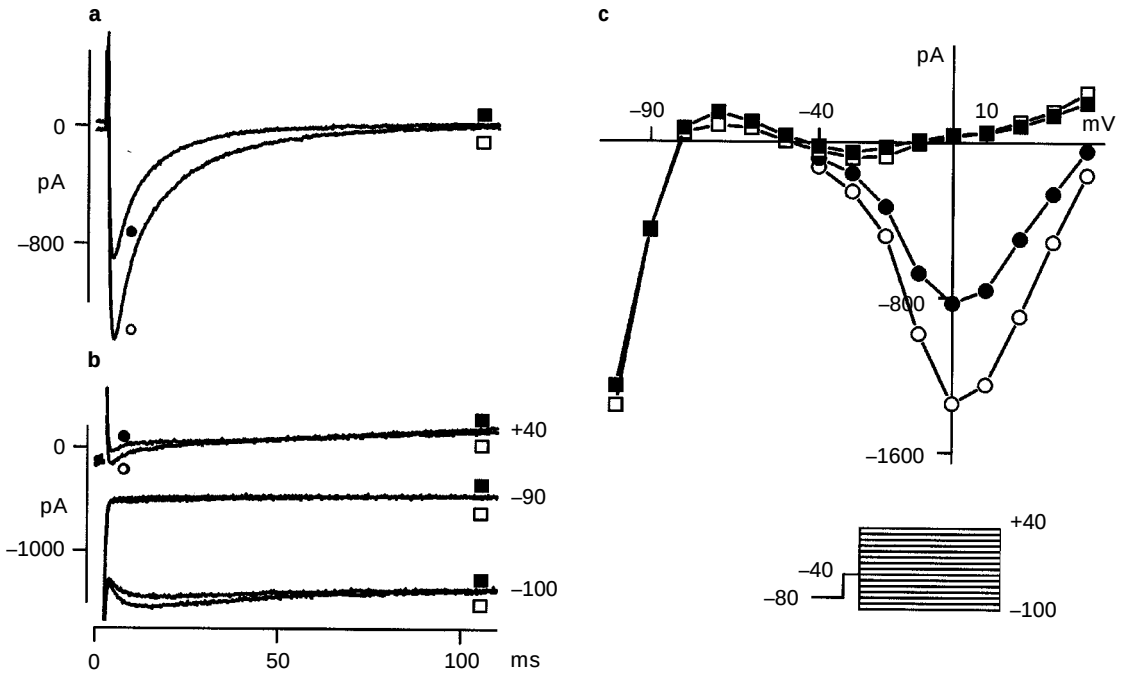

Figure 2 Effect of 0.1 μM lacidipine on calcium current ($I_{Ca,L}$, ○,●) and late current (I_{late}, □,■) recorded in the same myocyte as in Figure 1. (a) Current traces obtained in the absence (open symbols) and presence (solid symbols) of lacidipine during a voltage clamp step to 0 mV from a holding potential of -80 mV; a prepulse to -40 mV was used to inactivate sodium current (not shown). (b) Current traces obtained in the absence (open symbols) and presence (solid symbols) of lacidipine during a voltage clamp step to -100, -90, and $+40$ mV. (c) Current voltage relationships of $I_{Ca,L}$ and I_{late} before and after the application of lacidipine. Pulses of 200 ms were applied from -80 mV to different potentials ranging from -40 to $+40$ mV for $I_{Ca,L}$ and from -100 to $+40$ mV for I_{late} according to the voltage protocol. In (c) (○) $I_{Ca,L}$, control; (●) $I_{Ca,L}$, lacidipine; (□) I_{late}, control (■) I_{late}, lacidipine.

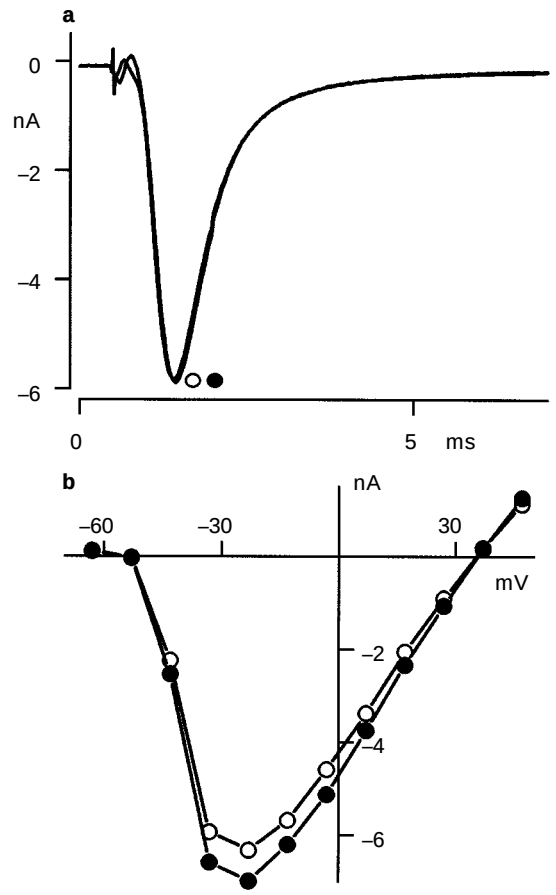

Figure 3 Typical experiment showing the effect of 0.1 μM lacidipine on sodium current (I_{Na}). (a) Current traces were recorded during a step to −5 mV from a holding potential of −70 mV, before (○) and after (●) superfusion with the drug. (b) Current-voltage relationship of I_{Na} before (○) and after (●) superfusion with lacidipine; the peak I_{Na} was measured during pulses applied from a holding potential of −75 mV and ranging from −65 to +45 mV.

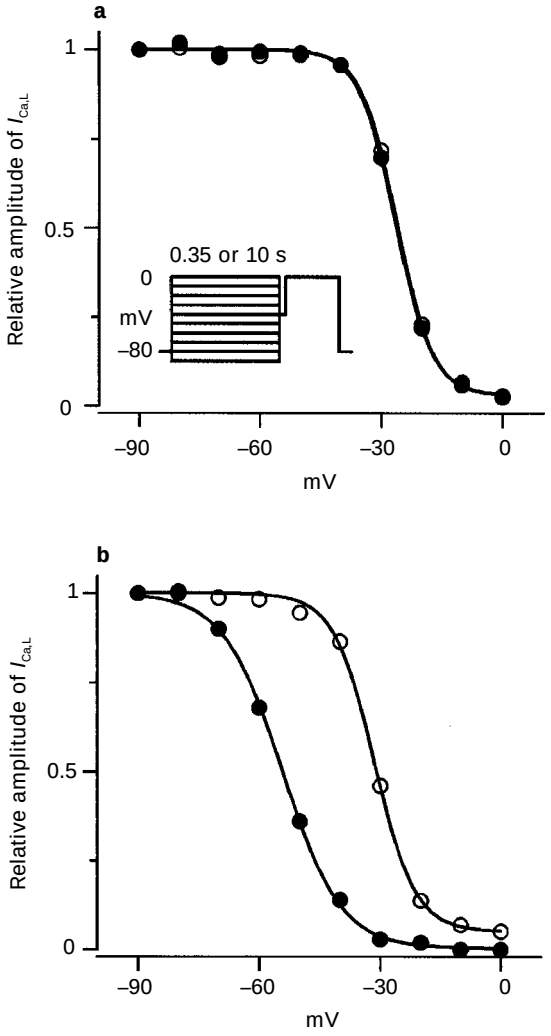

Figure 4 Typical effect of 0.1 μM lacidipine on steady state inactivation curves of $I_{Ca,L}$. Graph shows normalized plot of current measured during the test pulse in the absence (○) and presence (●) of the drug, after short (350 ms, a) or long (10 s, b) conditioning prepulses to various potentials; the normalized current is plotted against prepulse potential. Lines represent best fit with a Boltzmann function (see Methods). Voltage protocol is shown in the inset; a brief (10 ms) pulse to −40 mV preceding the test pulse to 0 mV was used to inactivate I_{Na}.

From a holding potential of −80 mV, prepulses to −40 mV lasting 50 ms to 30 s were applied followed by a test pulse of 0 mV. In Figure 5a, the $I_{Ca,L}$ amplitude, measured during the test pulse and normalized compared to the value (I_{max}) obtained after the shortest (50 ms) prepulse, is plotted as a function of prepulse duration. In controls, the amplitude of $I_{Ca,L}$ was slightly reduced after a 30 s prepulse to −40 mV. In the presence of 0.1 μM lacidipine, $I_{Ca,L}$ measured after prepulses of 500 ms or less was also scarcely reduced (95±5% of I_{max}, $n=4$). However, after 15 s at −40 mV, most of the available current was blocked by the drug (22±9% of I_{max}). With the same experimental procedure, nimodipine (0.5 μM) and nisoldipine (0.1 μM) showed a similar voltage-dependent blockade of $I_{Ca,L}$ (Figure 5a). However, the onset of their effect was significantly faster than that of lacidipine: the amplitude of the current was reduced to 49±10% of control value by nimodipine ($n=3$) and 43±9% by nisoldipine ($n=3$), after a 500-ms prepulse to −40 mV, a figure which was significantly different ($P<0.05$) from that observed with lacidipine.

Recovery from block was evaluated in cells constantly kept at −40 mV, by applying conditioning prepulses to −80 mV of increasing duration (50 ms to 15 s) followed by a test pulse to 0 mV to ascertain the relative amount of current present (Figure 5b). Again, $I_{Ca,L}$ amplitude was normalized to I_{max} and plotted as a function of prepulse duration. In controls, increasing prepulse duration did not modify the $I_{Ca,L}$ amplitude substantially. In the presence of 0.1 μM lacidipine, setting the holding potential at −40 mV provoked, as expected, a marked reduction in $I_{Ca,L}$. The block was progressively removed by increasing the duration of the prepulse, and $I_{Ca,L}$ returned to the maximum current amplitude (99±2% of I_{max}, $n=4$) when the membrane potential was maintained at −80 mV for 15 s (Figure 5b). As for the onset, the recovery from block in the presence of nimodipine and nisoldipine showed a faster time-course. Block was in fact completely absent after 3.5 s (103±2% for nimodipine and 100±2% for nisoldipine, $n=3$). It is worth noting that, with a conditioning prepulse of 1 s, only 54±2% of $I_{Ca,L}$ recovered in the presence of lacidipine, vs. 91±3% and 93±5% in the presence of nimodipine and nisoldipine, respectively ($P<0.05$).

Use-dependent block

The use-dependence of the effect of 0.1 μM lacidipine was studied by evaluating changes in $I_{Ca,L}$ amplitude during repetitive stimulation, i.e., throughout a series of ten steps to 0 mV at 1 Hz, from a holding potential of −80 (Figure 6) or −40 mV (Figure 7). The duration of voltage-clamp pulses was 200 ms or 10 ms, thus allowing (200 ms) or not allowing (10 ms) the current to inactivate completely after reaching the peak. The relative amplitude of $I_{Ca,L}$ was then plotted as a

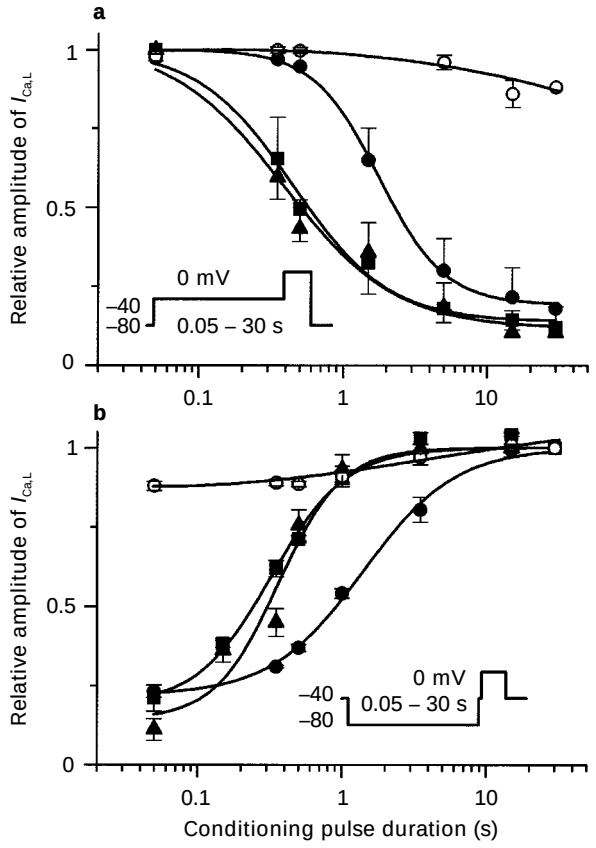

Figure 5 Onset of (a) and recovery from (b) the voltage-dependent block of $I_{Ca,L}$ by 0.1 μM lacidipine (●). The time-course of the effect of nimodipine (0.5 μM, ■) and nisoldipine (0.1 μM, ▲) is shown for comparison. (a) Normalized plot of current measured during the test pulse in the absence (○) and presence of drugs, after a prepulse to −40 mV of increasing duration (0.05 to 30 s); the normalized current is plotted against conditioning pulse duration. Inset: voltage protocol. (b) $I_{Ca,L}$ amplitude measured during the test pulse in the absence and presence of drugs, after a conditioning prepulse to −80 mV of increasing duration (0.05 to 15 s) from a holding potential of −40 mV; the normalized current is plotted against conditioning pulse duration. In (a) and (b), each point represents the mean value obtained from 3–4 different cells; vertical lines show s.e.mean. Voltage protocol is shown in the inset; a brief (10 ms) pulse to −40 mV preceding the test pulse to 0 mV was used to inactivate I_{Na}.

function of number of pulses. In the presence of lacidipine and with 200 ms test pulses from −80 mV, $I_{Ca,L}$ blockade developed throughout the stimulation, the mean peak current amplitude evoked by the tenth pulse being 55±7% ($n=5$) compared to the first pulse (Figure 6a). Traces from a representative experiment are shown in Figure 6c. In the absence of lacidipine, peak $I_{Ca,L}$ underwent little reduction during repetitive pulsing; the amplitude of the current elicited by the last pulse was 90±1% of that recorded during the first one ($n=4$). No blockade developed in cells stimulated with 10 ms pulses, which are long enough to induce calcium current activation but not complete inactivation (Figure 6b): even in the presence of lacidipine (see also Figure 6a), the amplitude of $I_{Ca,L}$ evoked by the tenth pulse did not differ from that measured at the first one (108±6% in 5 cells).

At depolarized holding potential (−40 mV, Figure 7a), a marked reduction in $I_{Ca,L}$ in the presence of lacidipine was also observed during stimulation with 10 ms pulses, as a consequence of the superimposed voltage-dependent block. The relative current amplitude gradually decreased, reaching a steady-state value around the sixth pulse (38±9% of the current evoked by the first pulse, $n=4$). Longer test pulses (200 ms) caused an additional reduction in $I_{Ca,L}$ (Figure 7a), which can be referred to as a real use-dependent block. This extra block was more evident by comparing pulses at the beginning of the train: $I_{Ca,L}$ amplitude at the second pulse was reduced to 74±7% ($n=4$) and 48±9% ($n=3$) of the first one with 10 ms and 200 ms steps, respectively. Representative current traces recorded in the presence of 0.1 μM lacidipine at the first, second and sixth pulse lasting 10 or 200 ms are shown in Figure 7b and c, respectively. In controls, after a series of ten 200 ms long pulses applied at 1 Hz, the current was reduced to 72±6% of that recorded during the first pulse ($n=3$) (Figure 7a) and was almost unchanged at the end of repetitive stimulation with 10 ms pulses (93±3%, $n=3$, data not shown).

Use-dependent effect of nimodipine and nisoldipine

The voltage protocols employed to study the use-dependent block by lacidipine were applied to cells superfused with 0.5 μM nimodipine or 0.1 μM nisoldipine. At the holding potential of −40 mV (Figure 8a, b), repetitive stimulation resulted in a rapid $I_{Ca,L}$ blockade either with 10 ms or 200 ms pulses. In the presence of nimodipine and nisoldipine, respectively, the peak $I_{Ca,L}$ elicited by the second pulse was reduced to 66±2% and 48±4% ($n=3$) of the first pulse value with 10 ms steps and to 41±5% and 42±2% ($n=3$) of the first pulse value with 200 ms steps. The percentage block induced by nimodipine was not significantly different from that observed in the presence of lacidipine, with the same voltage protocol. With nisoldipine, the percentage value of peak $I_{Ca,L}$ elicited by the second 10 ms pulse was significantly smaller than that obtained with lacidipine (74±7%, $P<0.05$).

However, when a train of 200 ms pulses was applied from the holding potential of −80 mV, the degree of $I_{Ca,L}$ block developing throughout the stimulation either in the presence of nimodipine or nisoldipine was much smaller compared to that observed with lacidipine (Figure 8a, b). In the presence of nimodipine and nisoldipine, respectively, $I_{Ca,L}$ elicited by the tenth pulse amounted to 93±3% ($n=3$) and 80±6% ($n=3$) of the first pulse value; both figures were significantly different ($P<0.05$) from the percentage $I_{Ca,L}$ measured at the tenth pulse in the presence of lacidipine (55±7%).

Discussion

In the present work, the calcium antagonistic action of lacidipine has been studied in isolated ventricular cells from the guinea-pig heart. At 0.1 μM, lacidipine reduced $I_{Ca,L}$ to about 60% of its initial value within 2 min. By using appropriate voltage protocols, both voltage- and use-dependent block of $I_{Ca,L}$ could be observed. While its behaviour resembled that described for other dihydropyridine compounds (Lee & Tsien, 1983; Sanguinetti & Kass, 1984; Kass & Arena, 1988), the time course of onset and recovery from block were slower than those of nisoldipine and nimodipine, studied in the same experimental conditions.

The dihydropyridine family includes a series of molecules which are characterized by the capacity to block the long-lasting calcium current ($I_{Ca,L}$) in a very potent and specific manner (Triggle, 1991). Since the dihydropyridine affinity for cardiac calcium channel depends on membrane potential (Bean, 1984; 'modulated receptor hypothesis', Sanguinetti & Kass, 1984), a prominent feature of their action is the ability to change voltage-dependence of calcium current kinetics. In this respect, the blocking action of lacidipine resembled that of other dihydropyridine derivatives. When long-lasting conditioning pulses (10 s) were applied to different voltages, the resulting steady-state inactivation curve was shifted to the hyperpolarizing direction by about 20 mV. A similar effect has been described for nisoldipine and explained by the affinity of calcium antagonists for the inactivated state of the calcium channel (Sanguinetti & Kass, 1984).

In this context, it is not surprising that the blocking effect of lacidipine, as well as that of nisoldipine and nimodipine, could

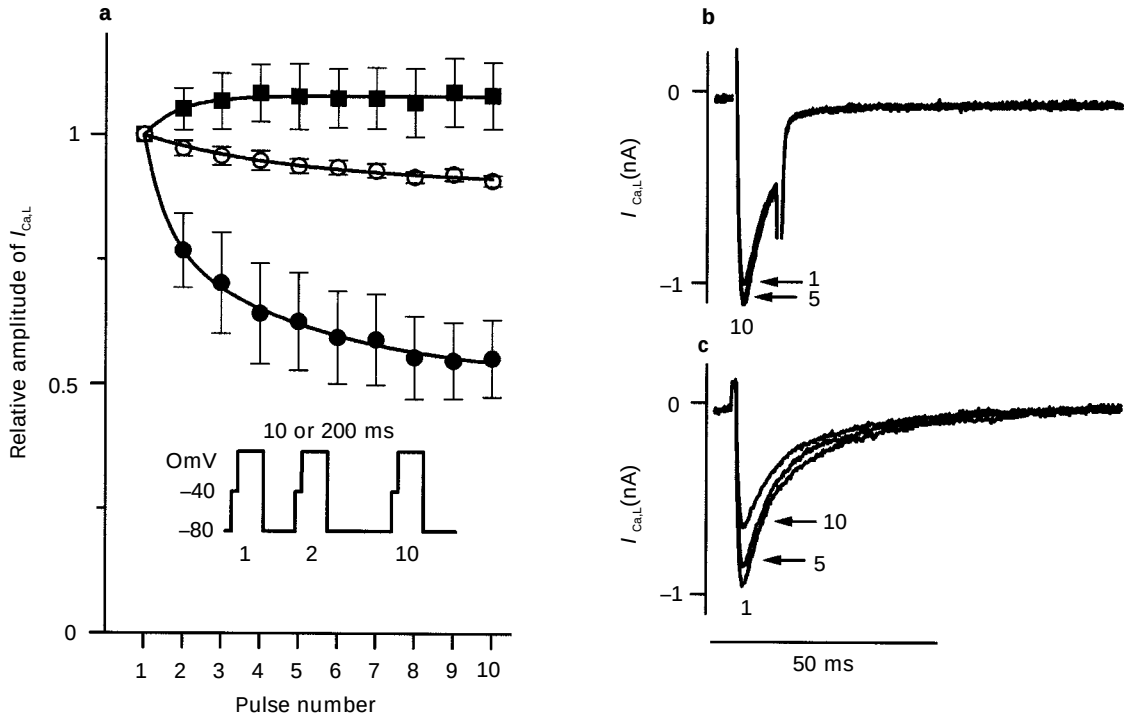

Figure 6 Use-dependent block of $I_{Ca,L}$ by 0.1 μM lacidipine. A train of ten pulses to 0 mV was applied at a frequency of 1 Hz, from a holding potential of -80 mV; (○) 200 ms pulses, control; (●) 200 ms pulses, lacidipine; (■) 10 ms pulses, lacidipine. Voltage protocol is shown in the inset, a brief (10 ms) pulse to -40 mV preceding the test pulse to 0 mV was used to inactivate I_{Na}. The peak $I_{Ca,L}$ was normalized with respect to the value measured during the first step and plotted against the number of steps. Each point represents the mean value obtained from 4–5 different cells; vertical lines shown s.e.mean. Here and in the following figures, curves were obtained by fitting data points with a double exponential decay function. (b) Typical current traces recorded during the first, fifth and tenth 10 ms step in the presence of lacidipine. (c) Typical current traces recorded during the first, fifth and tenth 200 ms step in the presence of lacidipine.

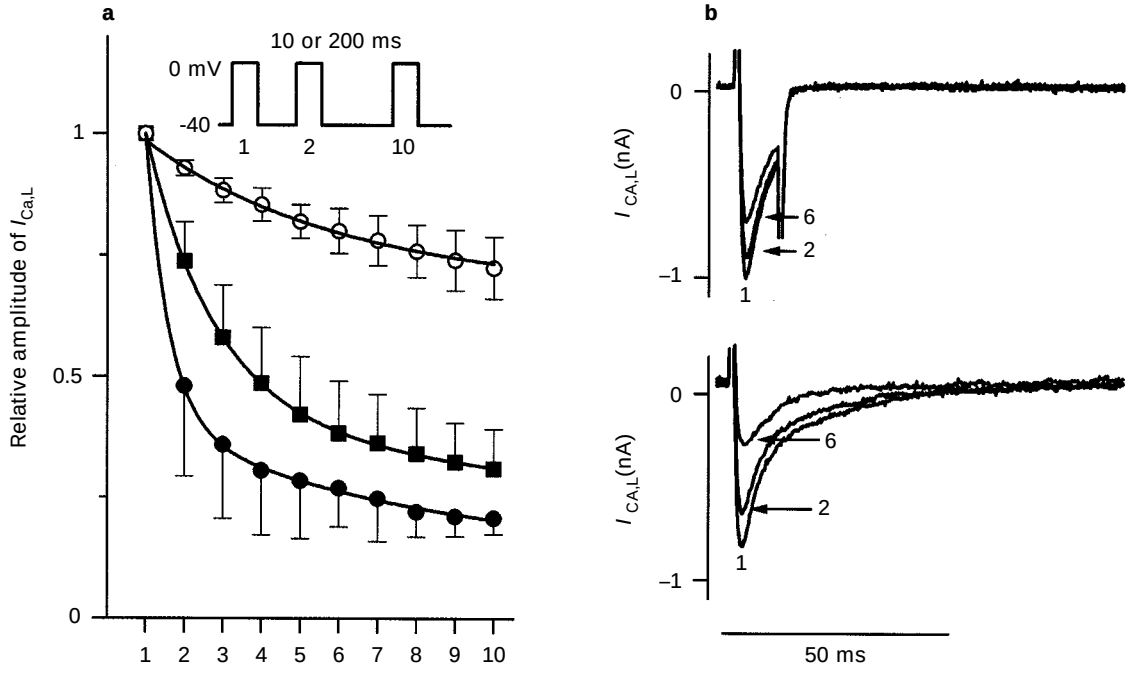

Figure 7 (a) Use-dependent block of $I_{Ca,L}$ by 0.1 μM lacidipine. A train of ten pulses to 0 mV was applied at the frequency of 1 Hz, from a holding potential of -40 mV; (○) 200 ms pulses, control; (●) 200 ms pulses, lacidipine; (■) 10 ms pulses, lacidipine. Voltage protocol is shown in the inset. The peak $I_{Ca,L}$ was normalized with respect to the value measured during the first step and plotted against the number of steps. Each point represents the mean value obtained from 3–4 different cells; vertical lines show s.e.mean. Typical current traces recorded during the first, second and sixth 10 ms step in the presence of lacidipine. (c) Typical current traces recorded during the first, second and sixth 200 ms step in the presence of lacidipine.

be greatly enhanced by changing the holding potential from −80 to −40 mV, and *vice versa*. However, it is of interest to observe that the time needed for the onset of – and the recovery from – drug blockade in the presence of lacidipine was significantly slower than that of nimodipine and nisoldipine. The latter two compounds are highly lipid-soluble molecules, which show tissue-selectivity for cerebral and coronary arteries, respectively (Godfraind *et al*., 1987; Langley & Sorkin, 1989; van Zwieten & Pfaffendorf, 1993). Like lacidipine, they are more or less completely uncharged at physiological pH, and, due to their lipophilicity, are thought to be able to interact with the channel-associated receptor by penetration of the lipid bilayer (Chester *et al*., 1987; Triggle, 1991). Recent molecular (Nakayama *et al*., 1991; Striessnig *et al*., 1991) and electrophysiological (Kwan *et al*., 1995) studies suggest that the dihydropyridine binding site is located within the bilayer or channel pore closer to the extracellular membrane surface. It is thought that dihydropyridines reach their binding domain via a membrane pathway; therefore, an increase in the probability of channel opening (i.e., during repetitive stimulation) does not enhance channel blockade (Nayler, 1988). This appears to be true for nimodipine and nisoldipine, which show a small use-dependent blockade at −80 mV (Figure 8). However, this consideration does not seem to apply to lacidipine, which actually demonstrates a marked use-dependent blockade of $I_{Ca,L}$ at normal resting potential (i.e., −80 mV).

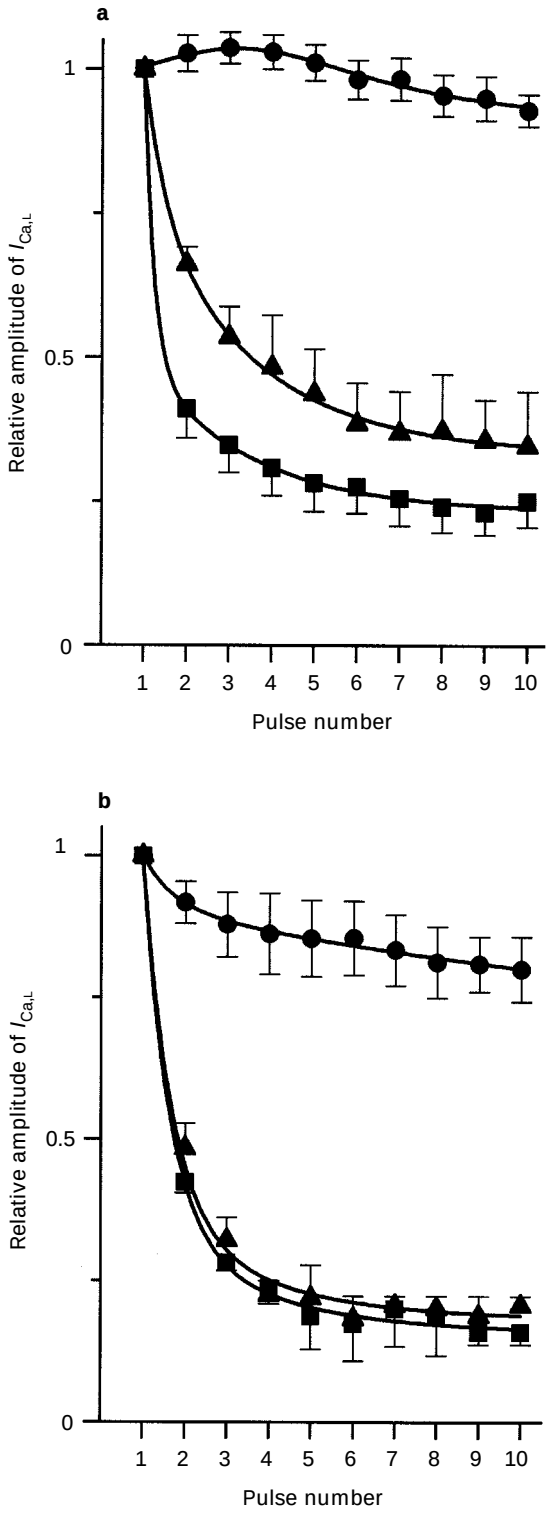

Figure 8 Use-dependent block of $I_{Ca,L}$ by 0.5 μM nimodipine (a) and 0.1 μM nisoldipine (b). A train of ten pulses to 0 mV was applied at the frequency of 1 Hz, from a holding potential of −80 mV or −40 mV; for the holding potential of −80 mV, a brief (10 ms) pulse to −40 mV preceding the test pulse to 0 mV was used to inactivate I_{Na} (see also voltage protocols of Figures 6 and 7). (●) −80 mV, 200 ms pulses; (■) −40 mV, 200 ms pulses; (▲) −40 mV, 10 ms pulses. The peak $I_{Ca,L}$ was normalized with respect to the value measured during the first step and plotted against the number of steps. Each point represents the mean value obtained from 3 different cells; vertical lines show s.e.mean.

Use-dependent reduction is directly related to voltage-dependent interaction with the calcium channel current by 1,4-dihydropyridine derivatives, and particularly to the slowing of current recovery from inactivation, which is a unique effect of dihydropyridines. When subsequent pulses are applied with an interval of time shorter than that needed for current recovery from inactivation, the reduction in current becomes additive (use-dependent effect). Our results demonstrate that, at a stimulation frequency of 1 Hz, the time interval between two pulses is long enough to allow the current to recover from inactivation almost completely in the presence of nimodipine and nisoldipine, but not in the presence of lacidipine. The difference was much less evident when pulses were applied from depolarized membrane level (−40 mV), due to predominance of a voltage-dependent effect of the three drugs. This is not surprising if one observes the time-course of recovery from blockade (see Figure 4b): after 1 s at −80 mV, only 50% of channels are available in the presence of lacidipine, while current recovery is almost complete both with nimodipine and nisoldipine. Thus, these latter two compounds seem to exert a predominantly tonic rather than use-dependent blockade, as previously shown for dihydropyridine derivatives (Lee & Tsien, 1983; Uehara & Hume, 1985).

Our results strongly suggest that lacidipine preferentially blocks the inactivated state of the calcium channel. In the presence of lacidipine, no use-dependent blockade of $I_{Ca,L}$ occurred when calcium current inactivation was precluded by using short pulses to a hyperpolarized potential (−80 mV). By setting the holding potential at −40 mV, block developed rapidly regardless of whether brief (10 ms) or long pulses (200 ms) were applied, but the $I_{Ca,L}$ reduction was faster and greater with a 200 ms pulse train. In the presence of lacidipine, the fraction of L-type calcium channels blocked within 10 s at −40 mV without repetitive stimulation (Figure 5a) was not significantly different from that blocked at the same holding potential but during application of 10 short (10 ms) pulses. Also, at −80 mV lacidipine was able to reduce $I_{Ca,L}$ only during repetitive stimulation with long pulses which facilitate channel inactivation and binding of the drug. Taken together, these results suggest that drug binding to the open state of the calcium channel, in the absence of inactivation, may be negligible. This behaviour of lacidipine resembles that of other dihydropyridine derivatives which are not ionized at physiological pH, such as nisoldipine (present data; Kass & Arena, 1988) and nifedipine (Brown *et al*., 1986), while it may differ from the blocking effect of amlodipine and nicardipine, which are partially charged at physiological pH (Kass & Arena, 1988; 1989).

The prominent pharmacological properties of lacidipine are due to its long-lasting antihypertensive effect and its vascular

selectivity (Carpi *et al*., 1986; Cerbai *et al*., 1990; Micheli *et al*., 1990; 1991). As for tissue selectivity, our data show that, in isolated myocytes, 0.1 μM lacidipine (a concentration at least 100 times higher than that active on vasculature) reduced $I_{Ca,L}$ to 60% of its control value, thus confirming our previous observation on multicellular preparations (Cerbai *et al*., 1990). Our data also demonstrate the specificity of lacidipine for calcium current: the sodium current and background potassium currents were not altered by the drug.

The molecular mechanism which underlies the slow time-course of drug interaction with the L-type calcium channel cannot be easily deduced from the present data. The only dihydropyridine which has been investigated with a similar experimental approach and which also shows a long-lasting persistent inhibition of Ca^{2+}-dependent activity is amlodipine (Burges *et al*., 1987). Amlodipine is a charged molecule at neutral pH and the slow kinetics of its effect are due to the delayed washout of the ionized molecular from its receptor (Kass & Arena, 1989). Lacidipine is uncharged at neutral pH, but its membrane partition coefficient is much higher than that of amlodipine, as well as those of nimodipine and nisoldipine (Herbette *et al*., 1993; 1994). As a consequence, it washes into membranes (and out of them) slowly, a characteristic not pertaining to most other calcium antagonists (Herbette *et al*., 1993; 1994). Therefore, it is tempting to speculate that the kinetics of lacidipine binding to its receptor are in turn modulated by drug interactions with the local environment of the calcium channel. Even if lacidipine affects cardiac and vascular muscle cell contractility with a great difference in potency, its properties at the level of cardiac Ca^{2+} channels are probably also relevant in vascular smooth muscle, thus determining its long-lasting antihypertensive action.

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References

BEAN, B.P. (1984). Nitrendipine block of cardiac calcium channel: high affinity binding to the inactivated state. *Proc. Natl. Acad. Sci. U.S.A.*, **81,** 6388–6392.

BROWN, A.M., KUNZE, D.L. & YATANI, A. (1986). Dual effects of dihydropyridines on whole cell and unitary currents in single ventricular cells of guinea-pig. *J. Physiol.*, **379,** 495–514.

BURGES, R.A., GARDINER, D.G., GWILT, M., HIGGINS, A.J., BLACKBURN, K.J., CAMPBELL, S.F., CROSS, P.E. & STUBBS, J.K. (1987). Calcium channel blocking properties of amlodipine in vascular smooth muscle and cardiac muscle in vitro: evidence for voltage modulation of vascular dihydropyridine receptors. *J. Cardiovasc. Pharmacol.*, **9,** 110–119.

CARPI, C., GAVIRAGHI, G. & SEMERARO, C. (1986). GX 1048 (GR 43659X), a new dihydropyridine calcium antagonist with potent and long-lasting antihypertensive action. *Br. J. Pharmacol.*, **89,** 758P.

CATTERALL, W.A. & SRIESSNIG, J. (1992). Receptor sites for Ca^{2+} channel antagonists. *Trends Pharmacol. Sci.*, **13,** 256–262.

CERBAI, E., AMBROSIO, G., PORCIATTI, F., CHIARIELLO, M., GIOTTI, A & MUGELLI, A. (1991). Cellular electrophysiological basis for oxygen radical-induced arrhythmias: a patch-clamp study in guinea-pig ventricular myocytes. *Circulation*, **84,** 1773–1782.

CERBAI, E., DE BONFIOLI CAVALCABÒ, P., MASINI, I., VISENTIN, S., GIOTTI, A. & MUGELLI, A. (1990). Cardiac electrophysiology of a new calcium antagonist, lacidipine. *J. Cardiovasc. Pharmacol.*, **15,** 604–609.

CERBAI, E., GUERRA, L., VARANI, K., BOREA, P.A., BARBIERI, M. & MUGELLI, A. (1995). β-Adrenoceptor subtypes in young and old rat ventricular myocytes: a combined patch-clamp and binding study. *Br. J. Pharmacol.* **116,** 1835–1842.

CHESTER, D.W., HERBETTE, L.G., MASON, R.P., JISLYN, A.F., TRIGGLE, D.J. & KOPPEL, D.E. (1987). Diffusion of dihydropyridine calcium channel antagonists in cardiac sarcolemmal lipid multibilayers. *Biophys. J.*, **52,** 1021–1030.

DATYNER, N.B. & COHEN, I.S. (1993). Slow inactivation of L-type calcium current distorts the measurement of L-type and T-type calcium current in Purkinje myocytes. *J. Gen. Physiol.*, **102,** 859–869.

GODFRAIND, T., EGLEME, C., FINET, M. & JAUMIN, P. (1987). The actions of nifedipine and nisoldipine on the contractile activity of human coronary arteries and human cardiac tissue in vitro. *Pharmacol. Toxicol.*, **61,** 79–84.

HERBETTE, L.G., GAVIRAGHI, G., TULENKO, T. & MASON, R.P. (1993). Molecular interaction between lacidipine and biological membranes. *J. Hypertens.*, **11** (Suppl 1), S13–S19.

HERBETTE, L.G., PRESTON MASON, T. & TULENKO, T.N. (1994). Calcium antagonists and membrane binding - the unique properties of lacidipine. *Res. Clin. Forums*, **16,** 17–28.

KAMP, T.J., SANGUINETTI, M.C. & MILLER, R.J. (1989). Voltage- and use-dependent modulation of cardiac calcium channels by the dihydropyridine (+)-202-791. *Circ. Res.*, **64,** 338–351.

KASS, R.S. & ARENA, J.P. (1988). Membrane potential and dihydropyridine block of calcium channels in the heart: influence of drug ionization on blocking activity. In *The Calcium Channel: Structure, Function and Implications*. ed. Morad, M., Nayler, W., Kazda, S & Schramm, M. pp. 81–91. Berlin: Springer Verlag.

KASS, R.S. & ARENA, J.P. (1989). Influence of pH_o on calcium channel block by amlodipine, a charged dihydropyridine compound: implications for location of the dihydropyridine receptor. *J. Gen. Physiol.*, **93,** 1109–1127.

KASS, R.S., ARENA, J.P. & CHIN, S. (1991). Block of L-type calcium channels by charged dihydropyridines: sensitivity to side of application and calcium. *J. Gen. Physiol.*, **98,** 63–75.

KWAN, Y.W., BANGALORE, R., LAKITSH, M., HARMUT, G. & KASS, R. (1995). Inhibition of cardiac L-type calcium channels by quaternary amlodipine: implications for pharmacokinetics and access to dihydropyridine binding sites. *J. Mol. Cell. Cardiol.*, **27,** 253–262.

LANGLEY, M.S., & SORKIN, E.M. (1989). Nimodipine: a review of its pharmacodynamic and pharmacokinetic properties, and therapeutic potential in cerebrovascular disease. *Drugs*, **37,** 669–699.

LEE, K.S. & TSIEN, R.W. (1983). Mechanism of calcium channel blockade by verapamil, D-600, diltiazem and nitrendipine in single dialyzed heart cells. *Nature*, **302,** 790–794.

MICHELI, D., COLLODEL, A., SEMERARO, C., GAVIRAGHI, G. & CARPI, C. (1990). Lacidipine: a calcium antagonist with potent and long-lasting antihypertensive effects in animal studies. *J. Cardiovasc. Pharmacol.*, **15,** 666–675.

MICHELI, D., RATTI, E., TOSON, G. & GAVIRAGHI, G. (1991). Pharmacology of lacidipine, a vascular selective calcium antagonist. *J. Cardiovasc. Pharmacol.*, **17,** (Suppl 4), S1–S8.

NAKAYAMA, H., TAKI, M., STRIESSNIG, J., GLOSSMANN, H., CATTERALL, W.A. & KANAOKA, Y. (1991). Identification of 1,4-dihydropyridine binding regions within the α_1 subunit of skeletal muscle Ca^{2+} channel by photoaffinity labelling of diazepine. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 9203–9207.

NAYLER, W.G. (1988). *Calcium Antagonists*. London: Academic Press.

SANGUINETTI, M.C. & KASS, R.S. (1984). Voltage-dependent block of calcium channel current in the calf Purkinje fiber by dihydropyridine calcium channel antagonists. *Circ. Res.*, **55,** 336–348

SCHWARTZ, A., GRUPP, I.L., GRUPP, G., WILLIAMS, J.S. & VAGHY, P.L. (1984). Effects of dihydropyridine calcium channel modulators in the heart: pharmacological and radioligand binding correlations. *Biochem. Biophys. Res. Commun.*, **25,** 387–394.

STIESSNIG, J., MURPHY, B.J. & CATTERALL, W.A. (1991). Dihydropyridine receptor of L-type Ca channels: identification of binding domains of ^{3}H-PN200-110 and ^{3}H-azidopine within the alpha 1 subunit. *Proc. Natl. Acad. Sci. U.S.A.*, **88,** 10769–10773.

TRIGGLE, D.J. (1991). Calcium-channel drugs: structure-function relationships and selectivity of action. *J. Cardiovasc. Pharmacol.*, **18** (Suppl. 10), S1–S6.

UEHARA, A. & HUME, J.R. (1985). Interactions of organic calcium channel antagonists with calcium channels in frog atrial cells. *J. Gen. Physiol.*, **89,** 621–647.

VAN ZWIETEN, P.A. & PFAFFENDORF, M. (1993). Similarities and differences between calcium antagonists: pharmacological aspects. *J. Hypertens.*, **11** (Suppl. 1), S3–S11.

WEBER, M.A. & GRAETTINGER, W.F. (1994). Calcium channel blockers as hypotensive agents. In *Cardiovascular Pharmacology and Therapeutics*. ed. Singh, B.N., Dzau, V.J., Vanhoutte, P.M. & Woosley, R.L. pp. 931–944 New York: Churchill Livingston Inc.

YASUI, K. & PALADE, P. (1995). Inhibitory action of SR33557 on L-type calcium current in single ventricular myocytes of rat. *Br. J. Pharmacol.*, **114,** 468–474.

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