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Mechanism of L- and T-type Ca^{2+} channel blockade by flunarizine in ventricular myocytes of the guinea-pig

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Abstract

Flunarizine is a substance known to block voltage-dependent Ca^{2+} channels in smooth muscle and neuronal cells. Reports on the effect on voltage-dependent cardiac Ca^{2+} channels are however sparse. Therefore, the mechanism of action of flunarizine on two types of voltage-dependent cardiac Ca^{2+} channels, the L- and T-type, in single ventricular myocytes of the guinea-pig was investigated using the whole-cell voltage clamp technique. Both channel types can be blocked by flunarizine in a time-, frequency-, voltage-, Ca^{2+}-, and proton-dependent way. While the overall mechanism of action on cardiac myocytes is similar to the one reported for other cell types, we found that cardiomyocytes are less susceptible to block (K_d 3.3–11 mM). We also describe a complete analysis of the different components of block, together with evidence for open channel state block and drug-induced changes in channel gating. These findings provide new insights into the mechanism of action of flunarizine on voltage-dependent Ca^{2+} channels.

Keywords: Myocyte, ventricular; Ca^{2+} channel; Flunarizine

1. Introduction

For many cell types with voltage-dependent Ca^{2+} conductances, a low-threshold transient T-type Ca^{2+} current ($I_{Ca2+,T}$) has been described together with a high-threshold long-lasting L-type Ca^{2+} current ($I_{Ca2+,L}$). Reports on the specific modulation of $I_{Ca2+,T}$ by drugs or neurotransmitters are, however, sparse. Terada et al. (1987) and Akaike et al. (1989a) have studied the effect of flunarizine on Ca^{2+} currents in smooth muscle cells, demonstrating that the drug dose dependently reduces $I_{Ca2+,L}$ and $I_{Ca2+,T}$ with a K_d of 0.1–1 μM. Akaike et al. (1989b) have shown that, in neuronal cells, flunarizine, among various Ca^{2+} antagonists, is most effective to block $I_{Ca2+,T}$ (K_d 0.7 μM), while the suppression of $I_{Ca2+,L}$ is somewhat less sensitive (K_d 3 μM). In preliminary reports, we have shown that flunarizine reduces both myocardial $I_{Ca2+,L}$ and $I_{Ca2+,T}$ (Tytgat et al., 1988), as well as neuronal N-type ($I_{Ca2+,N}$) Ca^{2+} current (K_d 0.8 μM) (Tytgat et al., 1991). In an early study by Van Nueten and Janssen (1973), flunarizine was found to be much more active at peripheral sites than on cardiac tissue (only minor changes in inotropy and chronotropy without effects on the contractile force were observed at concentrations 0.5–25 μM). The aim of this study was to obtain a more comprehensive insight into the mechanism of action of flunarizine on voltage-dependent Ca^{2+} channels in cardiac cells, and to compare the results with those obtained for smooth muscle and neuronal cells, to try and find why cardiac Ca^{2+} channels are less susceptible to block. We therefore studied the effect of flunarizine on $I_{Ca2+,L}$ and $I_{Ca2+,T}$ in single ventricular myocytes of the guinea-pig by means of the whole-cell voltage clamp technique.

2. Materials and methods

2.1. Cell isolation

Single ventricular myocytes of the guinea-pig were dissociated by enzymatic dispersion as described earlier (Tytgat, 1994). In short, the heart was quickly

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removed after decerebration of the animal and was mounted on a Langendorff perfusion column. The aorta was cannulated and the heart was perfused at 37°C with: (i) Ca^{2+}-free standard solution (see Solutions) for 5 min, (ii) 50 ml Ca^{2+}-free standard solution containing 40 mg collagenase (126 U/mg, Worthington Biochem. Corp., New Jersey, USA) and 6 mg protease (5.8 U/mg, Sigma, St Louis, USA) for 5 min, and (iii) low Ca^{2+} (180 μM) standard solution for 5 min. After the perfusion, the heart was placed in a Petri dish containing low-Ca^{2+} standard solution. The single cells were dispersed in this solution. Finally after 10 min, the low-Ca^{2+} standard solution was replaced by a normal Hepes-containing Tyrode solution.

2.2. Electrical measurements

We applied the two-electrode voltage clamp technique with suction pipettes (Hamill et al., 1981) to guinea-pig ventricular myocytes. The pipettes (i.e. electrodes) had resistances ranging from 2 to 5 MΩ, and were prepared from Pyrex glass (Jencons H15/10, Leighton Buzzard, England). After a two-phase pulling procedure based on heating, the tip of all pipettes was fire-polished. Final diameters of the tip of the pipettes were 1–2 μm. The two electrodes were positioned by means of Leitz micro-manipulators (type M; Leitz, FRG) under visual control via a Zeiss microscope (type IM 35; Zeiss, FRG) and a closed TV-circuit. The voltage clamp amplifier used in our experiments was the Axoclamp 2A-amplifier (Axon Instruments, Foster City, USA). The current signals were filtered with an 8-pole Bessel filter (Kemo, Beckenham Kent, UK; 3 dB at 500 Hz, unless mentioned elsewhere). Thereafter, the currents were sampled at a frequency of 2 kHz (unless mentioned elsewhere) using a 12-bit analog-to-digital converter (LabMaster TM40 interface, Scientific Solutions, Solon, USA), which was connected to an IBM-compatible PC. Data acquisition and analysis were controlled by pCLAMP software (version 5, Axon Instruments, Foster City, USA). All experiments were performed at room temperature (19–23°C). Exponential and linear fits were obtained by regression analysis, using the least square method (Bevington, 1969). Whenever possible, quantitative results were given as means ± standard error of the mean (S.E.M.); $P < 0.05$ was considered as statistically significant (Student's t-test).

In the experiments where we used the Na^+-free, Tris-containing solution (see below), $I_{Ca2+,L}$ was separated from $I_{Ca2+,T}$ by applying voltage steps to different test potentials (V_{test}) from holding potentials (V_{hold}) of −90 mV and −50 mV. $I_{Ca2+,T}$ was defined as the difference between the currents from the two V_{hold}, since the T-type Ca^{2+} channel is inactivated at −50 mV (Bean, 1985; Tytgat et al., 1988). Unless specified otherwise, $I_{Ca2+,L}$ was defined as the current evoked from V_{hold} −50 mV. When we used the normal Hepes-containing Tyrode solution, the current evoked from V_{hold} −40 or −50 mV was designated as $I_{Ca2+,L}$. Absolute values of in- and outward currents were measured from the zero current level (indicated by a horizontal dotted line), or from the 100% blocked or fully inactivated current level. The two approaches gave similar results. Since run-down of $I_{Ca2+,L}$ in our experiments appeared to be more prominent at the early stage of internal cell perfusion, all experiments were only started after a 10- to 15-min period of perfusion, when a further decline of $I_{Ca2+,L}$ had become much smaller ($\approx$ 15% run-down for 60-min internal perfusion). For comparison between different cells, whole-cell currents were expressed as current densities ($\mu A/cm^2$). A small hyperpolarizing step from V_{hold} −50 to V_{test} −55 mV was delivered at the beginning of each experiment. Such a small hyperpolarizing step did not evoke any voltage-activated, time-dependent current. The area under the capacitative current, obtained by integration, is equal to the amount of charge needed for charging of the membrane. Dividing this charge by the applied voltage step gives the total cell capacitance. Since it is assumed that biological membranes have a specific membrane capacitance of 1 $\mu F/cm^2$, we were able to express all Ca^{2+} currents as current densities ($\mu A/cm^2$). The average total cell capacitance of our cells was 201.2 ± 7.7 pF ($n = 156$), corresponding to a membrane area of 2.10^{-4} cm^2.

2.3. Solutions

For cell isolation, the Ca^{2+}-free standard solution contained (in mM): 130 NaCl, 5.4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 glucose, 6 Hepes, and was brought to pH 7.2 with NaOH. For the low-Ca^{2+} standard solution, 180 μM $CaCl_2$ was added to the Ca^{2+}-free standard solution. The enzymes were added to the Ca^{2+}-free standard solution. The pipette solution (= intracellular) contained (in mM): 125 CsCl, 5 MgATP, 15 EGTA, 20 TEA-Cl, 10 Hepes, and was titrated with CsOH to pH 7.2. The following two main bath solutions were used. (i) Normal Hepes-containing Tyrode solution with (in mM): 137.6 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$. 11.6 Hepes, 5 glucose, brought to pH 7.4 with NaOH; 20 mM CsCl was always added to this solution. (ii) Na^+-free, Tris-containing solution containing (in mM): 137 Tris, 1 $MgCl_2$, 1.8 or 5.4 $CaCl_2$ (for $I_{Ca2+,L}$ and $I_{Ca2+,T}$, respectively), 5 glucose, 20 CsCl, and brought to pH 7.4 with HCl.

Flunarizine, (1-[bis(4-fluorophenyl)methyl]-4-(3-phenyl-2-propenyl)piperazine), was dissolved at the concentrations indicated in the bath solution, except for the experiments where it was applied intracellularly and was therefore dissolved in the pipette solution.

3. Results

3.1. Effect on the L-type Ca^{2+} current

Lee and Tsien (1983) have shown that organic inhibitors of Ca^{2+} entry prevent inward as well as outward current through cardiac Ca^{2+} channels, without exerting an effect on the current at the reversal potential through the Ca^{2+} channel ($\approx +60$ mV). Where the inward current through Ca^{2+} channels can be carried by Ca^{2+}, Ba^{2+}, or Na^{+} ions (according to the experimental conditions), the outward current can be carried by K^{+} or Cs^{+} ions. We started our study by investigating if flunarizine inhibits the inward Ca^{2+} current and outward Cs^{+} current through the cardiac L-type Ca^{2+} channel. Therefore, 300-ms clamp steps were applied from V_{hold} −50 mV to V_{test} +5, +60, or +100 mV, in the absence and presence of 5 μM flunarizine in the bath solution after 5-min equilibration (Fig. 1A). The drug caused a reduction of the inward current at V_{test} +5 mV, as well as of the outward current at V_{test} +100 mV. At V_{test} +60 mV, flunarizine did not affect the small residual, time-independent outward current.

Next, we applied a train of six steps from V_{hold} −50 mV to V_{test} +5 mV for 300 ms at 0.2 Hz both in the control and in the presence of 5 μM flunarizine to see if the block displayed tonic and/or frequency-dependent characteristics (Fig. 1B). The presence of tonic and total block was evaluated respectively from the peak current during the 1st and 6th pulse of the train in the presence of the drug, relative to control peak currents. Subtraction of the tonic block from the total block resulted in the frequency-dependent component of the block. The time course of the peak amplitude of $I_{Ca2+,L}$ during this experiment is illustrated in the box of panel B. The tonic block, developed during a 5-min equilibration with 5 μM flunarizine at V_{hold} −50 mV, amounted to 16.3 ± 5.3% ($n = 6$). Repetitive depolarizations caused a more pronounced block. The current during the 6th pulse of the train showed a total block of 42.1 ± 4.7% ($n = 6$), and frequency-dependent block of 25.8 ± 2.6% ($n = 6$). The frequency-dependent block by flunarizine showed a slow rate of onset and could be relieved by hyperpolarizing pulses in a potential-dependent way (data not shown). We also found that the substance acts irreversibly, i.e. washout could never be obtained and was independent of membrane voltage. The presence of a tonic block may reflect the block of channels in the resting (closed) state, while the frequency-dependent block may correspond to block developed during activation and/or consecutive inactivation of the channel.

To find whether flunarizine interacts preferentially with activated (open) Ca^{2+} channels, or with inactivated (closed) channels, we performed the experiment shown in panel C of Fig. 1. The effect of flunarizine is

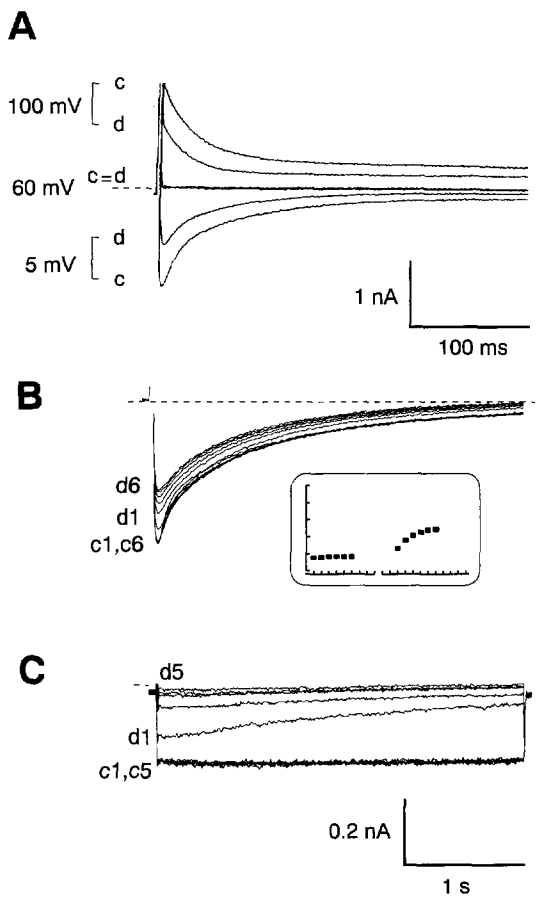

Fig. 1. Effect of flunarizine on $I_{Ca2+,L}$. (A) Inward (V_{test} +5 mV) and outward currents (V_{test} +100 mV) as evoked from V_{hold} −50 mV were reduced in the presence of 5 μM flunarizine (d), as compared to control conditions (c) (filtering 3 dB at 250 Hz, sampling at 1 kHz). Note the lack of effect of the substance on the small residual, time-independent outward current (c = d) at V_{test} +60 mV. (B) Six pulses were given every 5 s in control (c_1 to c_6) and in the presence of 5 μM flunarizine (d_1 to d_6) from V_{hold} −50 mV to V_{test} +5 mV. The current during the 1st and the 6th pulse of the train is taken as tonic (d_1) and total (d_6) block, respectively (filtering 3dB at 250 Hz, sampling at 1 kHz). The time course of the peak amplitude of $I_{Ca2+,L}$ from this experiment is boxed. (C) Five pulses were given every 5 s under control conditions (c_1 to c_5) and in the presence of 5 μM flunarizine (d_1 to d_5) from V_{hold} −50 mV to V_{test} −30 mV. The charge carrier was 1.8 mM Ba^{2+} (filtering 3 dB at 25 Hz, sampling at 100 Hz). Note the drug-induced decay of the current.

shown in an experiment where Ca^{2+} ions were replaced by Ba^{2+} ions as charge carriers. Under these conditions, it is known that the inactivation of $I_{Ca2+,L}$ is much slower (Kohlhardt et al., 1972), since Ca^{2+}-induced inactivation is absent (Brehm and Eckert, 1978). When we clamped the cell from V_{hold} −50 mV to V_{test} −30 mV under control conditions, we could evoke a 3-s-long, non-inactivating Ba^{2+} current. This means that approximately similar numbers of channels are open for the duration of the depolarization, including the possibility of channels 'cycling' between closed (available) and open states without undergoing inacti-

vation. In the presence of 5 μM flunarizine, a clear drug-induced decay of the current was observed. The simplest explanation is that flunarizine binds to open channels, acting as an active state blocker, and brings these channels into a drug-bound, non-conducting state.

The current-voltage relationship (I/V) of $I_{Ca2+,L}$ in the presence of flunarizine (10 min drug equilibration at V_{hold} −40 mV) was obtained in experiments in which clamp steps were applied from V_{hold} −40 mV to V_{test} ranging from −30 to +70 mV at 0.1 Hz (Fig. 2A). The current density of peak $I_{Ca2+,L}$ as a function of membrane potential averaged from 4 to 11 cells is shown. The presence of 5 μM flunarizine suppressed $I_{Ca2+,L}$ at the peak of the I/V relationship (+5 mV) by about 41%, without shifting the peak along the voltage axis. The drug reduced the maximal conductance (g_{max}) from 0.15 to 0.08 mS/cm^2, and did not alter the reversal potential (V_{rev}). The smooth curves through the data points were fitted with the equation:

$$I = \frac{g_{max} \cdot (V_{test} - V_{efa})}{1 + \exp(-(V_{test} - V_{1/2})/s)} \quad (1)$$

where I is the current, V_{efa} the potential where the extrapolated fully activated current (estimated by linear regression) reverses, $V_{1/2}$ the midpoint of activation, and s the slope factor. Two limitations have to be kept in mind when this equation is used: (i) only the linear part of the I/V gives a fair g_{max} value, and (ii) V_{efa} does not take into account an eventual rectification close to the true reversal potential.

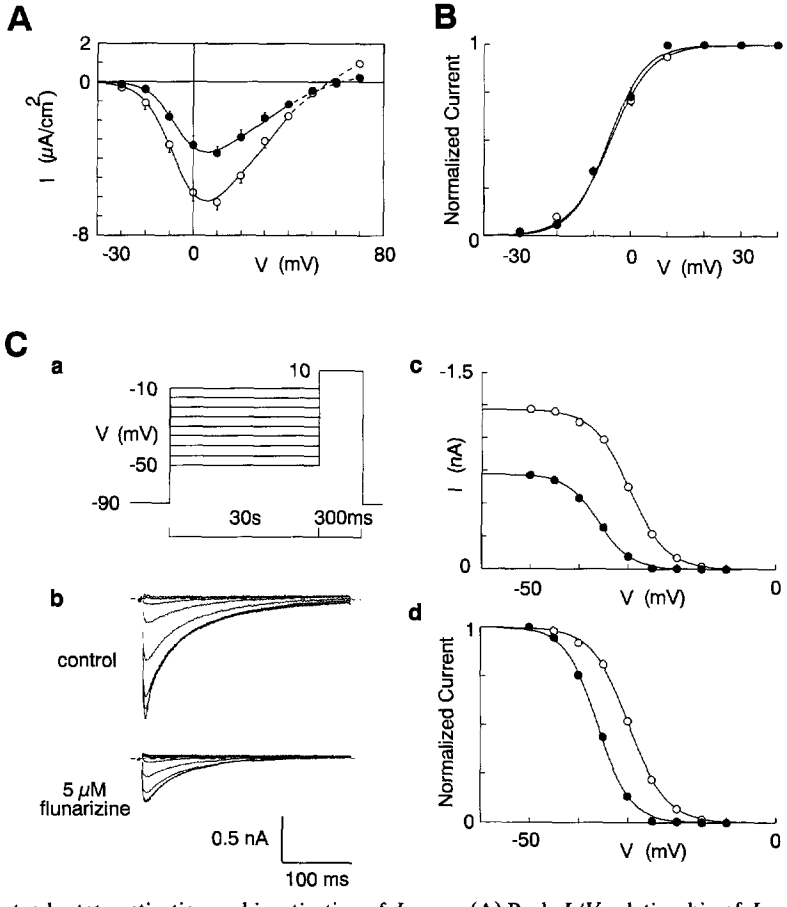

Fig. 2. Peak I/V relationship, steady state activation and inactivation of $I_{Ca2+,L}$. (A) Peak I/V relationship of $I_{Ca2+,L}$ density: 5 μM flunarizine (filled circles) reduced $I_{Ca2+,L}$ in the control (open circles) by about 41% at V_{test} +5 mV from V_{hold} −50 mV, without shifting the peak of the relationship along the voltage-axis. The drug reduced g_{max} from 0.15 to 0.08 mS/cm^2, but no significant effect on V_{rev} was observed. Smooth curves through the data points were fitted using equation (1). Current densities were averaged from 4 to 11 cells. (B) Steady state activation of $I_{Ca2+,L}$ with data points obtained from panel A. Smooth curves were fitted using equation (2). No significant effect of flunarizine is seen. (C) Steady state inactivation of $I_{Ca2+,L}$: currents in the control and in the presence of 5 μM flunarizine (b) were evoked during a test pulse to +10 mV, after 30-s prepulses ranging from −50 to −10 mV, from V_{hold} −90 mV (filtering 3 dB at 250 Hz, sampling at 1 kHz). The voltage protocol (a) was performed at 0.2 Hz. Panel c: flunarizine reduced $I_{Ca2+,L}$ at all potentials. Panel d: the presence of the drug shifted the inactivation curve significantly to hyperpolarized potentials. Smooth curves in panels c and d were fitted using equation (3), with $V_{1/2}$ values −29.7 and −36.1 mV under control and flunarizine conditions, respectively. Corresponding s values were 3.8 and 3.3 mV, respectively.

Fig. 2B shows the effect of flunarizine on the steady state activation of $I_{Ca2+,L}$. Dividing the currents in Fig. 2A in the range between −30 and +40 mV by the fully activated current, which was estimated by extrapolation of a linear regression through the currents between +10 and +40 mV, resulted in the steady state activation curve which was fitted with a Boltzmann function given by the equation:

$$I = \frac{I_{max}}{1 + \exp(-(V_{test} - V_{1/2})/s)} \quad (2)$$

In the control ($n = 11$), $V_{1/2}$ was -5.5 ± 0.6 mV and s was 5.5 ± 0.3 mV. In the presence of 5 μM flunarizine ($n = 4$), $V_{1/2}$ was -6.0 ± 1.3 mV, and s was 5.0 ± 0.2 mV. These results demonstrate that flunarizine does not significantly affect the steady state activation curve of $I_{Ca2+,L}$. Fig. 2C shows the effect of 5 μM flunarizine on the steady state inactivation, which was determined by measuring $I_{Ca2+,L}$ at V_{test} +10 mV from V_{hold} −90 mV after prepulses (V_{prep}) lasting for 30 s and ranging from −50 to −10 mV (panel a). Flunarizine reduced the current at all potentials (panel c), as can also be seen by comparing $I_{Ca2+,L}$ in the control and the presence of 5 μM flunarizine (panel b). The curves in panels c and d (the availability curve) were fitted with a Boltzmann function given by the equation:

$$I = \frac{I_{max}}{1 + \exp((V_{prep} - V_{1/2})/s)} \quad (3)$$

In the control ($n = 5$), $V_{1/2}$ and s were -28.6 ± 0.4 mV and 4.0 ± 0.2 mV, respectively. In the presence of 5 μM flunarizine ($n = 4$), $V_{1/2}$ and s were -36.3 ± 1.6 mV and 4.2 ± 0.3 mV. Therefore, flunarizine, in addition to decreasing the maximal conductance of $I_{Ca2+,L}$, shifts the inactivation curve towards more negative potentials without affecting the activation curve. A negative shift of the inactivation curve of Ca^{2+} channels has been interpreted as preferential binding of a drug to the inactivated state of the channel (Sanguinetti and Kass, 1984). Therefore, flunarizine can be considered as an inactive state blocker, also characterized by the properties of an active state blocker as described above.

Is block by flunarizine antagonized by extracellular Ca^{2+}? In their original work, Kohlhardt et al. (1972) proposed that the Ca^{2+} channel antagonists, verapamil and gallopamil, compete with Ca^{2+} for a common site. In 1983, Lee and Tsien compared the inhibitory effect of various blockers on $I_{Ca2+,L}$ in the presence of 3 and 30 mM extracellular Ca^{2+} (Lee and Tsien, 1983). In their experiments, elevation of external Ca^{2+} decreased significantly the percentage of total blocked Ca^{2+} channels. The competition with Ca^{2+} ions supported the common practice of designating the various

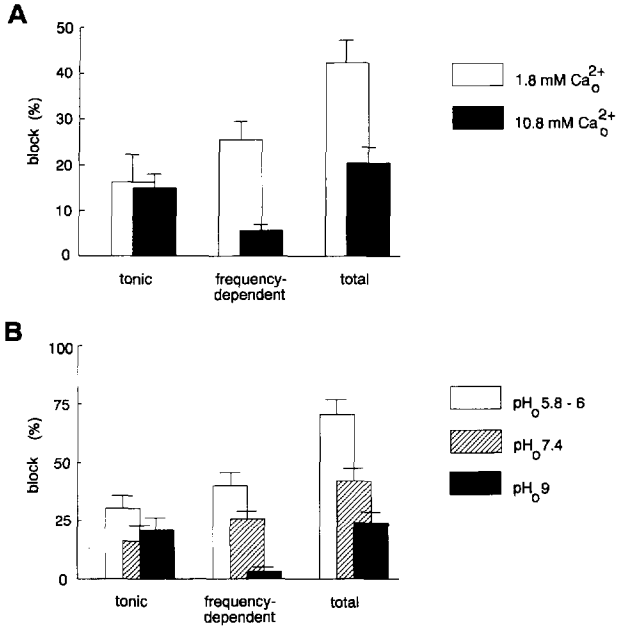

Fig. 3. Effect of Ca_o^{2+} and $proton_o$ concentration on tonic, frequency-dependent, and total block of $I_{Ca2+,L}$. (A) The ordinate shows the percentage of block induced by 5 mM flunarizine in the presence of 1.8 mM Ca_o^{2+} and 10.8 mM Ca_o^{2+}. Histogram bars indicate means for four to six observations. (B) The ordinate shows the percentage of block induced by 5 mM flunarizine obtained at pH_o 5.8–6, 7.4, and 9. Histogram bars indicate means for three to six observations.

compounds as Ca^{2+} antagonists. In order to check whether the tonic, frequency-dependent, and total block of $I_{Ca2+,L}$ by flunarizine is also Ca_o^{2+}-dependent, we performed experiments in 1.8 and 10.8 mM extracellular Ca^{2+}. Currents were evoked in the absence and presence of 5 μM flunarizine (after a 5-min period of drug equilibration at V_{hold} −50 mV) during a train of six pulses at 0.2 Hz, from V_{hold} −50 mV either to V_{test} +5 mV (1.8 mM Ca_o^{2+}), or +20 mV (10.8 mM Ca_o^{2+}), according to the maximum inward peak of the I/V at each Ca^{2+} concentration. In the absence of flunarizine, peak current magnitudes at V_{test} +20 mV in 10.8 mM Ca_o^{2+} were about 2.5 times greater than those measured at V_{test} +5 mV in 1.8 mM Ca_o^{2+}. The percentage of tonic block induced by flunarizine was not significantly changed on elevation of the extracellular Ca^{2+} from 1.8 to 10.8 mM (Fig. 3A). In contrast, frequency-dependent and total block by flunarizine were significantly reduced. These results point to Ca^{2+} antagonistic properties as observed for gallopamil, diltiazem and nitrendipine.

Next, we investigated whether tonic, frequency-dependent, and total block by flunarizine was affected by changes in pH_o. $I_{Ca2+,L}$ was evoked during a train of six pulses at 0.2 Hz from V_{hold} −50 mV either to V_{test} +10 mV at pH_o 5.8–6, to V_{test} +5 mV at pH_o 7.4, or to V_{test} 0 mV at pH_o 9, corresponding to the maximum

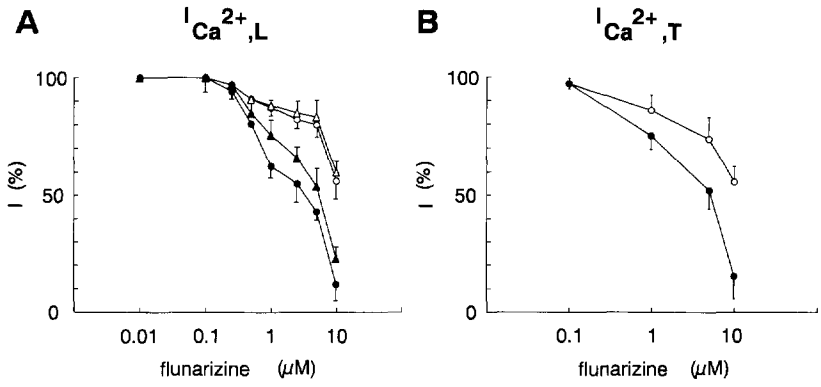

Fig. 4. Dose-response curve for $I_{Ca2+,L}$ and $I_{Ca2+,T}$. (A) The percentage $I_{Ca2+,L}$ left over during the 1st pulse (open symbols) and the 20th pulse (closed symbols) in a train from V_{hold} −50 (circles) or −80 mV (triangles) to V_{test} +10 mV at 0.8 Hz is shown in the presence of different concentrations of flunarizine. The results were obtained from 36 cells. (B) The percentage $I_{Ca2+,T}$ left over during the 1st (open symbols) and 20th pulse (closed symbols) in a train from V_{hold} −90 mV to V_{test} −30 mV at 0.8 Hz is shown in the presence of different concentrations of flunarizine. The results were obtained from four cells.

peak inward $I_{Ca2+,L}$ at each pH_o. In the absence of flunarizine, peak current magnitudes at pH_o 5.8–6 (V_{test} +10 mV) were about 60% of the size of those at pH_o 7.4 (V_{test} +5 mV). There was no significant difference between peak current magnitudes at pH_o 9 (V_{test} 0 mV) and pH_o 7.4 (V_{test} +5 mV). The percentage of tonic block induced by flunarizine was not significantly changed when the extracellular proton concentration was decreased from pH_o 5.8–6 to 9 (Fig. 3B). In contrast, frequency-dependent and total block were significantly suppressed upon alkalinization.

In order to check whether internal application of flunarizine was effective to block the channel, we studied the current density of $I_{Ca2+,L}$ in the presence of 5 μM flunarizine in the pipette solution. After pooling the results for different cells measured after 30- to 60-min perfusion, the current density of peak $I_{Ca2+,L}$ at V_{test} +5 mV was -5.2 ± 0.7 μA/cm^2 in the control ($n = 10$), and -5.5 ± 0.6 μA/cm^2 in the presence of 5 μM flunarizine ($n = 8$). This difference is not significant. Furthermore, neither the activation, nor the inactivation of the current was changed in the presence of intracellular flunarizine, and application of a train of depolarizations from V_{hold} −50 mV to V_{test} +5 mV did not induce a frequency-dependent block. These results do not favour an intracellular site of action of flunarizine on the cardiac L-type Ca^{2+} channel.

Van Nueten et al. (1978) have shown that flunar-

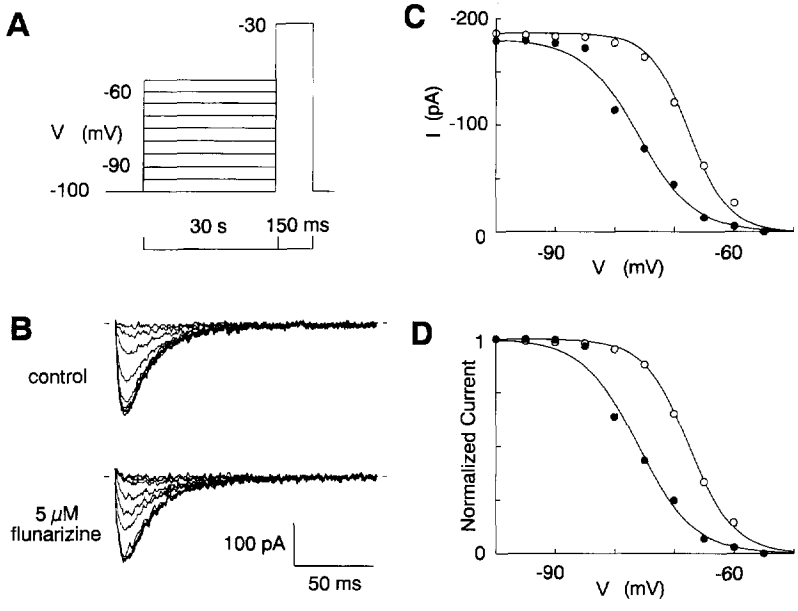

Fig. 5. Steady state inactivation of $I_{Ca2+,T}$. (A) The voltage protocol was performed at 0.2 Hz. (B) $I_{Ca2+,T}$ under control and 5 μM flunarizine conditions. (C) The effect of flunarizine as a function of voltage: control (open circles), flunarizine (filled circles). (D) Availability curves showing smooth curves through the data points fitted with equation (3): $V_{1/2}$ values −67.5 and −76.0 mV under control and flunarizine conditions, with s values 3.8 and 4.7 mV, respectively.

izine is only weakly active to antagonize Ca^{2+}-induced positive inotropic effects in cat papillary muscle (ED_{50} = 21 μM). The results in our study confirm this low blocking potency on $I_{Ca2+,L}$ in single ventricular cells. A train of 20 pulses (300-ms pulse duration; 0.8-Hz stimulation) from V_{hold} −50 or −80 mV to V_{test} +10 mV was applied in the presence of different concentrations of flunarizine (15-min drug equilibration). Fig. 4A illustrates the dose-response curve for the 1st and 20th pulse of the train. For the 1st pulse (V_{hold} −50 or −80 mV), the K_d was ≈ 11 μM. For the 20th pulse, the K_d was 3.3 μM and 5.2 μM for V_{hold} −50 and −80 mV, respectively.

3.2. Effect on the T-type Ca^{2+} current

To study the effect of flunarizine on $I_{Ca2+,T}$, we applied six repeated depolarizations from either V_{hold} −50 mV or −90 mV to V_{test} −30 mV (0.2 Hz stimulation) under control conditions and in the presence of 10 μM flunarizine after 30-min equilibration of the cell with the drug. The presence of flunarizine caused a tonic block of 33.5 ± 5.0% (n = 4), a marked frequency-dependent block of 47.0 ± 0.3% (n = 4), and a total block of 80.5 ± 0.3% (n = 4) (data not shown).

Steady state inactivation of $I_{Ca2+,T}$ (Fig. 5) was investigated using a voltage protocol similar to that described in Fig. 2 for $I_{Ca2+,L}$. Suppression of $I_{Ca2+,T}$ by 5 μM flunarizine was more pronounced at depolarized potentials, as can be seen in panel C and by comparing $I_{Ca2+,T}$ in the presence of flunarizine with the control (B). Panel D shows the availability curves for this experiment. The curves were fitted with equation (3): $V_{1/2}$ was −65.0 ± 2.1 mV (n = 5) in the control, and −76.3 ± 1.4 mV (n = 4) in the presence of 5 μM flunarizine. The respective s values were 4.7 ± 0.8 mV and 5.4 ± 0.7 mV. It could be concluded that flunarizine shifts the steady state inactivation curve significantly to hyperpolarized potentials. Tonic block was virtually absent in the protocol with 30-s-long prepulses in the range of −100 to −85 mV. This might indicate that tonic block is time- and voltage-dependent.

As for $I_{Ca2+,L}$, we have tested whether internal application of 5 μM flunarizine was effective to block $I_{Ca2+,T}$. After pooling the results for different cells measured after 30- to 60-min perfusion, the current density of peak $I_{Ca2+,T}$ at V_{test} −30 mV was −0.48 ± 0.03 $\mu A/cm^2$ in the control (n = 14), and −0.45 ± 0.07 $\mu A/cm^2$ in the presence of 5 μM flunarizine (n = 3). This difference is not significant. As observed for $I_{Ca2+,L}$, neither the activation, nor the inactivation of the current was changed in the presence of intracellular flunarizine, and application of a train of depolarizations to V_{test} −30 mV did not induce a frequency-dependent block. It is therefore concluded that flunarizine did not block $I_{Ca2+,T}$ from the inside.

The dose-response curve for flunarizine block of $I_{Ca2+,T}$ was obtained by using a train of 20 pulses (150-ms pulse duration; 0.8 Hz stimulation) from V_{hold} −90 mV to V_{test} −30 mV in the presence of different concentrations of flunarizine (15-min drug equilibration) (Fig. 4B). Although almost no $I_{Ca2+,L}$ was evoked from V_{hold} −50 mV to V_{test} −30 mV, we have always subtracted these currents from those evoked from V_{hold} −90 mV in order to obtain the pure $I_{Ca2+,T}$. For the 1st and 20th pulse, the K_d was ≈ 10 μM and 4.6 μM, respectively.

4. Discussion

Lee and Tsien (1983) have shown that organic inhibitors of Ca^{2+} entry prevent the inward as well as the outward current through the cardiac L-type Ca^{2+} channel, without affecting ionic currents at their reversal potential through the Ca^{2+} channel. As can be concluded from our first experiments with flunarizine, the drug 'plugged' the L-type Ca^{2+} channel in the same way as do other organic Ca^{2+} channel blockers.

Further investigations revealed that frequency-dependent block was found to be voltage-, time-, $proton_o$-, and Ca_o^{2+}-dependent. Tonic block was found to be voltage- and time-dependent. Taking into account flunarizine's pK_a value (7.71), the pH_o-dependence of frequency-dependent block correlates well with the fraction of drug molecules in the charged form at each particular pH_o. Therefore, it is plausible that there is strong competition between Ca^{2+} ions and the charged drug for a common binding site, explaining the pH_o- and Ca_o^{2+}-dependent differences in frequency-dependent block. Interestingly, Borgers et al. (1980) have shown that flunarizine interferes with the entry of Ca^{2+} at the plasma membrane level in vascular smooth muscle, but only under conditions of stimulated influx of the ion. Furthermore, Wermelskirchen et al. (1985) have shown that charged flunarizine displaces Ca^{2+} completely from phosphatidylserine monolayers, whereas the uncharged form is not very effective (Vogelgesang et al., 1988). More recently, Thomas (1990) has reported that uncharged flunarizine interacts with the cell membrane by virtue of the drug's hydrophobicity. At low pH_o values, a large fraction of flunarizine resides in the charged form, and therefore, the amphiphilic drug may concentrate at the membrane interface. Its blocking potency will now be determined, not only by its hydrophobicity (corresponding primarily with tonic block), but also by the fraction of molecules in the charged form (corresponding primarily with frequency-dependent block).

According to the hypothesis of Verkleij and Post

(1986), the results reported here may also indicate that charged flunarizine prevents Ca^{2+} binding to negatively charged phospholipids, which may protect the cell membrane from lipid reorganization and explain the anti-ischemic properties of the substance. However, we did not observe a flunarizine-induced shift in the midpoint of the activation process of I_{Ca2+}, resulting from surface charge effects near the channel. In contrast to the foregoing, flunarizine shifted the inactivation curve to more negative potentials. This result suggests (i) voltage-dependent binding, and/or (ii) preferential binding to the inactivated state of the channel, as described for nisoldipine (Sanguinetti and Kass, 1984). To our knowledge, the experiments with Ba^{2+} as charge carrier provide a new and clear-cut protocol to demonstrate active state block of the L-type Ca^{2+} channel. Since moderate depolarizations in the control resulted in non-inactivating currents, the apparent inactivation induced by flunarizine can be interpreted as a drug-dependent active state block.

It is known that flunarizine is much more active on smooth muscles than on cardiac muscles (Van Nueten and Janssen, 1973). Only minor changes in inotropy and chronotropy were observed at concentrations of flunarizine ranging from 0.5 to 25 μM. Van Nueten et al. (1978) have demonstrated that the action of flunarizine on peripheral vasoconstriction in vascular smooth muscle is characterized by a low ED_{50} value of 0.1 μM, and that it has a slow rate of onset with a long duration of action. Terada et al. (1987) reported that, for fragmented smooth muscle cells from the longitudinal muscle layer of the rabbit ileum, flunarizine inhibited $I_{Ca2+,L}$ in a frequency- and voltage-dependent manner. The same authors also demonstrated that intracellular, in contrast to extracellular, perfusion of 100 μM flunarizine did not modify $I_{Ca2+,L}$, and they reported a dose-dependent inhibition of $I_{Ca2+,L}$ (K_d 1.4 μM). Akaike et al. (1989a) have compared the actions of flunarizine on $I_{Ca2+,L}$ and $I_{Ca2+,T}$ in cultured rat aorta smooth muscle cells. The substance dose dependently reduced $I_{Ca2+,T}$ with a $K_d \approx 0.1$ μM, which represents a much more sensitive block than the one seen in cardiomyocytes, and its effect was strongly dependent on the external Ca^{2+} concentration. Differences in the blocking potency of $I_{Ca2+,L}$ and $I_{Ca2+,T}$ were found to be small, which our findings confirm. Wilhelm et al. (1989) reported that the effect of flunarizine on K^+-induced contractions in rat aorta showed a stronger inhibition at low pH_o, which was consistent with the observations in our pH experiments (Fig. 3B).

Akaike et al. (1989b) reported that, in isolated rat hypothalamic neurons, flunarizine blocked $I_{Ca2+,T}$ with a K_d of 0.7 μM, which is again much more sensitive than in cardiomyocytes. The block was found to be frequency- and voltage-dependent, developing with a slow time course and being only partly reversible. These results confirm the general features of the drug as observed in other cell types and in our experiments. With respect to the neuronal high-threshold N-type Ca^{2+} channel, experiments on hippocampal neurons (Tytgat et al., 1991) have revealed that flunarizine also blocks this channel with a K_d of 0.8 μM.

One hypothesis for the tissue selectivity is the different membrane potential of smooth muscle and cardiac cells under normal physiological conditions. However, our voltage clamp experiments performed at depolarized V_{hold} values of -40 and -50 mV, which mimic voltage conditions of smooth muscle cells, revealed that under these conditions also cardiac Ca^{2+} channels still are less sensitive to block by flunarizine than are smooth muscle Ca^{2+} channels. Moreover, the neuronal $I_{Ca2+,T}$ is more sensitive to flunarizine than the cardiac $I_{Ca2+,T}$, although they operate in the same negative voltage range (Akaike et al., 1989b). Therefore we conclude that tissue selectivity for flunarizine cannot be explained in terms of the membrane potential of the different cell types. A plausible and promising alternative for explaining tissue selectivity of Ca^{2+} channel antagonists is the different amino acid composition of the subunits forming the different Ca^{2+} channel types (see e.g. Godfraind et al., 1986). Since the functional expression of several types of cloned Ca^{2+} channels from different tissues is now being fully explored, we could expect a molecular approach to tissue selectivity of Ca^{2+} channels in the near future. An alternative possibility is that different ionic environments near the vestibule of the channel, based on differences in phospholipid composition and lipid interactions with the channel protein, or different local surface potentials and local concentrations of divalent ions, might also play a pivotal role in tissue selectivity.

Finally, it should be stressed that the action of flunarizine is not restricted to voltage-dependent Ca^{2+} channels. Evidence for inhibitory effects on neuronal Na^+ channels was provided by Pauwels et al. (1986). The drug also dose dependently inhibits K^+ current in smooth muscle cells and accelerates the inactivation of this current (Terada et al., 1987). Flunarizine might also interact with receptor-operated Ca^{2+} channels in rat and rabbit mesenteric arteries, as suggested by Godfraind and Miller (1983) and Itoh et al. (1987), with Ca^{2+}-calmodulin-activated processes (e.g. Kubo et al., 1984; Lugnier et al., 1984), with membrane systems and phospholipid physico-chemistry (Thomas, 1990; Vogelgesang and Scheufler, 1990), and with microsomal Ca^{2+}-activated ATPase of rat aorta (Morel and Godfraind, 1978). An intracellular site of action of flunarizine has been reported by Spedding (1983) and Cejalvo et al. (1993) for the contractile proteins of smooth muscle, and, as suggested by Vos et al. (1990), for the (oscillatory) Ca^{2+} release of the canine heart. Anti-histaminic, anti-arrhythmic, and anti-convulsant

effects of flunarizine have also been described (for a review see Holmes et al., 1984).

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