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Inhibition by mibefradil, a novel calcium channel antagonist, of Ca^{2+}- and volume-activated Cl^- channels in macrovascular endothelial cells

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1 We have studied the effects of mibefradil, a novel calcium antagonist, on the resting potential and ion channel activity of macrovascular endothelial cells (calf pulmonary artery endothelial cells, CPAE). The patch clamp technique was used to measure ionic currents and the Fura-II microfluorescence technique to monitor changes in the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$.

2 Mibefradil (10 μM) hyperpolarized the membrane potential of CPAE cells from its mean control value of -26.6 ± 0.6 mV ($n=7$) to -59.8 ± 1.7 mV ($n=6$). A depolarizing effect was observed at higher concentrations (-13.7 ± 0.6 mV, $n=4$, 30 μM mibefradil).

3 Mibefradil inhibited Ca^{2+}-activated Cl^- currents, $I_{Cl,Ca}$, activated by loading CPAE cells via the patch pipette with 500 nM free Ca^{2+} ($K_i=4.7\pm0.18$ μM, $n=8$).

4 Mibefradil also inhibited volume-sensitive Cl^- currents, $I_{Cl,vol}$, activated by challenging CPAE cells with a 27% hypotonic solution ($K_i=5.4\pm0.22$ μM, $n=6$).

5 The inwardly rectifying K^+ channel, IRK, was not affected by mibefradil at concentrations up to 30 μM.

6 Ca^{2+} entry activated by store depletion, as assessed by the rate of $[Ca^{2+}]_i$-increase upon reapplication of 10 mM extracellular Ca^{2+} to store-depleted cells, was inhibited by $17.6\pm6.5\%$ ($n=8$) in the presence of 10 μM mibefradil.

7 Mibefradil inhibited proliferation of CPAE cells. Half-maximal inhibition was found at 1.7 ± 0.12 μM ($n=3$), which is similar to the concentration for half-maximal block of Cl^- channels.

8 These actions of mibefradil on Cl^- channels and the concomitant changes in resting potential might, in addition to its effect on T-type Ca^{2+} channels, be an important target for modulation of cardiovascular function under normal and pathological conditions.

Keywords: Endothelium; patch clamp; mibefradil; calcium antagonists; Ca^{2+}-activated Cl^- channels; volume-activated Cl^- channels; inward-rectifier K^+ channels; CRAC

Introduction

Mibefradil (Ro 40-597) is a novel calcium-antagonist with a high bioavailability that causes well-tolerated antihypertensive and anti-ischaemic effects and prevents ischaemically induced ventricular fibrillation without negative inotropic effects (Clozel *et al*., 1990; Fang & Osterrieder, 1991; Veniant *et al*., 1995; Bernink *et al*., 1996; Billman *et al*., 1996; Roux *et al*., 1996; Braun *et al*., 1996). These properties have led to the organization of a large scale clinical trial. In addition, it may facilitate the effects of endothelium-derived NO, affect eicosanoid production, have antiproliferative effects on smooth muscle cells after vascular injury and exert a vasodilating action via protein kinase C (PKC)-inhibition (Kung *et al*., 1995; Schmitt *et al*., 1995; Chida *et al*., 1995; Hermsmeyer & Miyagawa, 1996). The obvious therapeutic efficacy of mibefradil is in contrast with the lack of information about its mechanism of action.

Most of these effects have been attributed to its rather selective block of cardiac and vascular smooth muscle T-type Ca^{2+} channels (Mishra & Hermsmeyer, 1994; Bakx *et al*., 1995; Roux *et al*., 1996), since a 10 fold higher concentration is required for an equi-effective block of L-, N-, Q- and R-type channels (Bezprozvanny & Tsien, 1995). T-type Ca^{2+} channels might be the major target of mibefradil, but given the multifunctional effects of mibefradil, it is rather unlikely that its action would be limited only to these channels. It has been shown that mibefradil affects vascular smooth muscle cells (Schmitt *et al*., 1995; Kung *et al*., 1995; Hermsmeyer & Miyagawa, 1996), and this action might be mediated by an effect of mibefradil on the underlying endothelial cells, which are important modulators of smooth muscle activity. The role of ion channels in regulating endothelial cell activity is now generally accepted including NO production, release of vasoactive compounds and control of cell proliferation (see Nilius *et al*., 1997b for a review). The driving force for Ca^{2+}-release-activated or store-operated Ca^{2+} entry (CRAC or SOC), which is extremely important for NO-signalling (for a review see Nilius & Casteels, 1996; Nilius *et al*., 1997b), is mainly controlled by K^+ and Cl^- channels (Groschner *et al*., 1994; Daut *et al*., 1994; Voets *et al*., 1996b). These channels might also be involved in the mitogenic activity of endothelial cells (Voets *et al*., 1995, 1996c; Nilius *et al*., 1996).

The purpose of the present experiments was to investigate the possible effects of mibefradil on resting membrane potential and ion channel activity in macrovascular endothelial cells. It was found that mibefradil efficiently inhibited both Ca^{2+}-activated and volume-activated Cl^- currents, $I_{Cl,Ca}$ and $I_{Cl,vol}$. The inwardly rectifying K^+ current, IRK, and the store depletion activated Ca^{2+} entry were only slightly affected. These effects of mibefradil on Cl^- channels and the concomitant hyperpolarization might modulate Ca^{2+}-signalling in endothelial cells and contribute to its complex cardiovascular actions. In addition, we showed that mibefradil efficiently inhibited proliferation of calf pulmonary artery endothelial (CPAE) cells.

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Methods

Cells from a bovine cultured pulmonary artery endothelial cell line (CPAE, ATCC CCL 209) were grown in DMEM containing 10% human serum, 2 mM L-glutamine, 2 μml^{-1} penicillin and 2 mg ml^{-1} streptomycin. Cultures were maintained at 37°C in a fully humidified atmosphere of 10% CO_2 in air. For electrophysiological measurements, only non confluent single endothelial cells were used.

Electrophysiological methods and Ca^{2+} measurements have been described in detail elsewhere (Nilius *et al*., 1994). Whole-cell membrane currents were measured with ruptured patches. Currents were monitored with an EPC-9 (List Electronic, Germany) patch clamp amplifier (2048 points per record, filtered at 500 or 100 Hz). The following voltage protocol was applied every 15 s from a holding potential of 0 mV: a step to −80 mV for 1.2 s, followed by a step to −150 mV for 0.4 s and a 5.2 s linear voltage ramp to +100 mV, thereafter a step to 0 mV. Current-voltage relationships were reconstructed from the ramp current, and the time course from the average current in a small voltage window around the potential of interest.

Cells were superfused with Krebs solution containing (in mM): NaCl 150, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, HEPES 10 and adjusted to pH 7.3 with 1 M NaOH. Tonicity as measured with a Wescor 5500 osmometer (Schlag Instruments, Gladbach, Germany) was 300 mOsmol. The standard internal pipette solution contained (in mM): KCl 40, K-aspartate 100, $MgCl_2$ 1, Na_2ATP 4, EGTA 0.1, HEPES 10, adjusted to pH 7.2 with 1 M KOH. Membrane potentials were measured in the current clamp mode (standard pipette solution and extracellular Krebs solution). For each cell, the mean membrane potential was calculated in the absence and presence of mibefradil from the Gaussian fits of the frequency distributions of all data points sampled during the whole experiments (sampling rate 2 Hz).

To active Ca^{2+}-sensitive Cl^- currents, $[Ca^{2+}]_i$ was increased by loading the cells via the patch pipette (see Nilius *et al*., 1997a, b) with a solution buffered at a Ca^{2+} concentration of 500 nM (5 mM EGTA, 3.79 mM $CaCl_2$ and 0.1 mM $MgCl_2$, pH = 7.2). During these measurements inwardly rectifying K^+ currents were blocked by substitution of K^+ by Cs^+ in the extracellular and intracellular solutions.

Volume-sensitive Cl^- currents, $I_{Cl,vol}$, were activated by exposing the cells to a 27% hypotonic extracellular solution. Before applying the hypotonic stimulus, the cells were superfused first with a modified isotonic Krebs solution (mannitol Krebs), that contained (in mM): NaCl 105, CsCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, HEPES 10, made isotonic with 90 mM mannitol, pH 7.3 with 1 M NaOH. Omitting mannitol from the above solution resulted in a hypotonic solution (HTS-Krebs) with a 27% reduced osmolarity (216 mOsmol) of the same ionic strength.

Inwardly rectifying K^+ currents, IRK, were measured with extracellular Krebs solution and the standard pipette solution as described. This current was rapidly, reversibly and com-

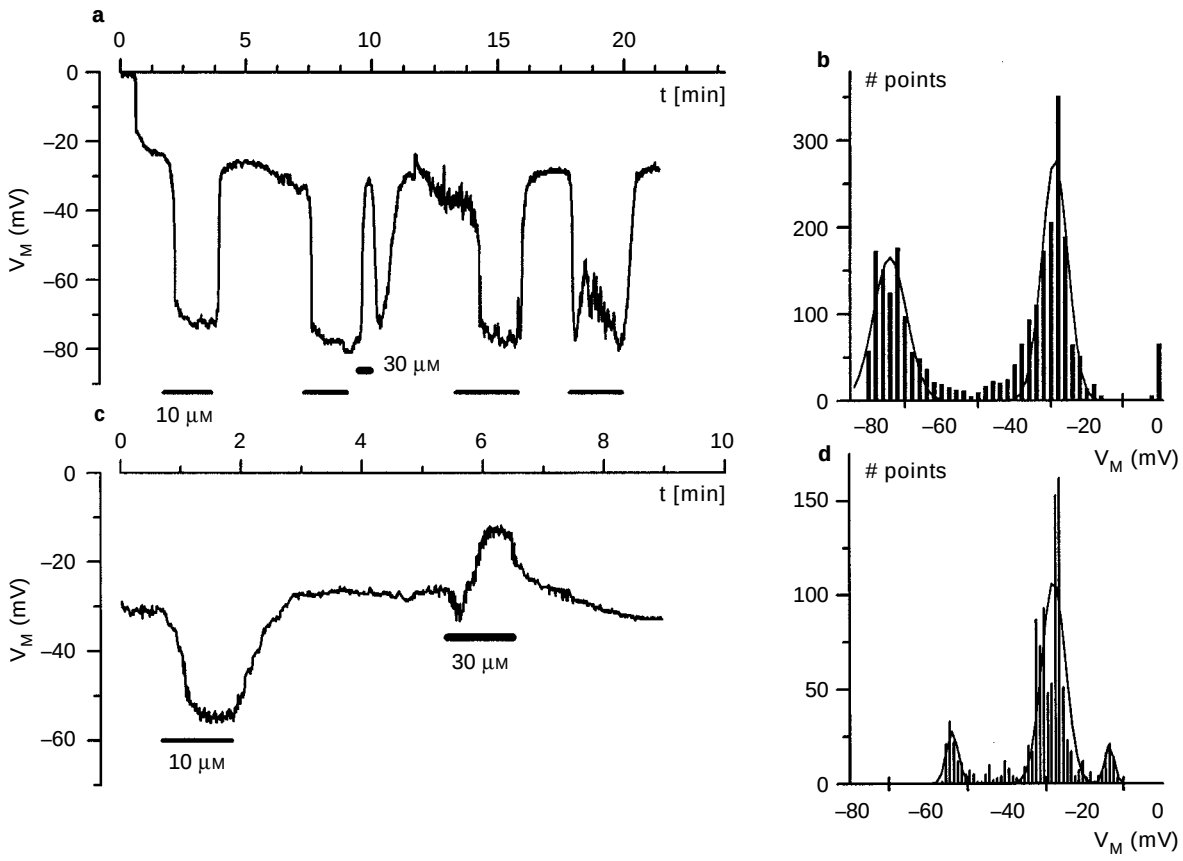

Figure 1 Effects of mibefradil on the membrane potential of calf pulmonary artery endothelial (CPAE) cells. (a) Membrane potential was measured in current clamp mode after the cell had been broken into. Mibefradil (10 μM) induced a fast and reversible hyperpolarization of the cells. Shown is a typical cell with a large Cl^- conductance and a resting potential fluctuating between −22 and −27 mV. At higher concentrations (30 μM and more) mibefradil depolarized the cell (used for the histograms in (b) and (c)). (b) Distribution of the membrane potentials sampled at 2 Hz from the cell shown in (a). The two peaks represent the membrane potential in the absence (control) and the presence of mibefradil. The mean values were taken from the Gaussian fits for the pooled data given in the text. Data obtained from the fits are: −28.8 mV, width 9.1 mV for control, −74.1 mV, width 7.2 mV for 10 μM mibefradil (bin width 2 mV). (c) Membrane potential from other CPAE cells during application of 10 and 30 μM mibefradil. The higher concentration clearly caused depolarization of the cell. (d) Distribution of the membrane potential measured from the cell in (c) (same method as in (b)). Data obtained from fits are: −28.1 mV, width 3.1 mV for the control (absence of mibefradil), −53.9 mV, width 3.1 mV for 10 μM mibefradil, −13.5 mV, width 2.5 mV for 30 μM mibefradil, (bin width 1 mV).

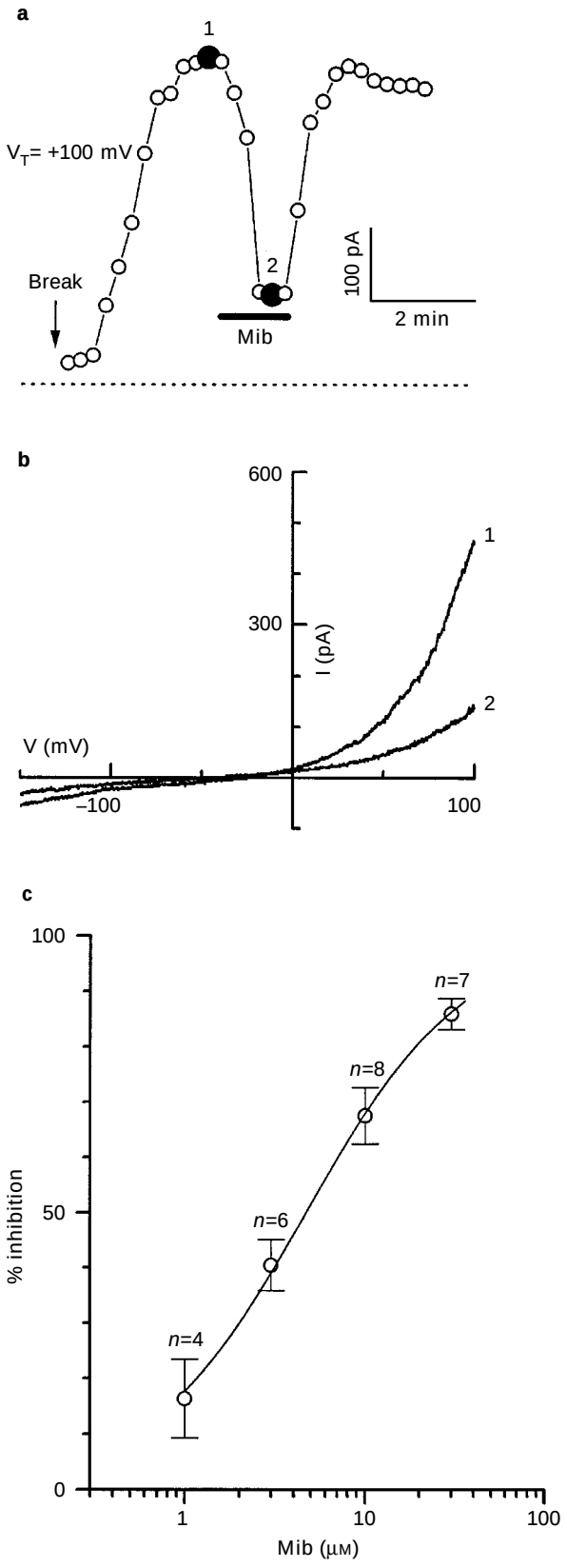

Figure 2 High-affinity block of Ca^{2+}-activated Cl^- channels by mibefradil. (a) A CPAE cell was loaded with a Ca^{2+}-solution buffered at 500 nM after breaking the membrane as indicated (the methods are described elsewhere, Nilius *et al*., 1997a). Elevation of $[Ca^{2+}]_i$ activated a Cl^- current, $I_{Cl,Ca}$. This current was probed by application of voltage ramps. From these ramps, the current can be measured at any potential. Shown is the current activation at +100 mV. Application of 10 μM mibefradil (Mib) induced a fast and reversible block of $I_{Cl,Ca}$. Potassium currents were blocked by extra- and intracellular Cs^+-solution (see Methods). (b) At the times indicated in (a), *I*-V curves were depicted; (1) shows the fully activated Cl^- current, (2) the blocked current after application of mibefradil. Note the strong outward rectification. The reversal potential of the current (approximately −26 mV), was close to the theoretical reversal Cl^- equilibrium potential ($E_{Cl} = -32$ mV). (c) Concentration-response curve of the inhibitory action of mibefradil. The curve is fitted by the Hill-equation

$$\text{Inhibition} = \frac{100}{1 + \left(\frac{K_i}{[\text{Mib}]}\right)^{n_H}}$$

with $K_i = 4.7 \pm 0.18$ μM, Hill coefficient $n_H = 0.99 \pm 0.03$.

pletely blocked by application of 1 mM $BaCl_2$. IRK was obtained from the difference of the control current and the current remaining after Ba^{2+} application.

For $[Ca^{2+}]_i$ measurements, cells were loaded with Fura-II by incubating them for 20 min at 37°C in Krebs solution containing 2 μM Fura-2/AM. The dye was excited at wave lengths of 360 and 390 nm via a rotating filter wheel. The fluorescence was measured at 510 nm and corrected for autofluorescence. Calibration and calculation of the apparent free Ca^{2+} concentration from the fluorescence ratio *R* have been described in detail elsewhere (Nilius *et al*., 1994):

$$[Ca^{2+}]_i = K_{eff} \cdot \frac{R - R_0}{R_1 - R} \quad (1)$$

where K_{eff} is the effective binding constant, R_0 the fluorescence ratio at zero calcium and R_1 that at high Ca^{2+}.

To deplete internal Ca^{2+} stores, cells were either incubated for 20 min in 1 μM thapsigargin (Scientific Marketing Ass., Herts, U.K.) or 10 μM BHQ (2,5-di(tert)-butyl-hydro-quinone, Aldrich) was added to the bathing solution. After either of these procedures, $[Ca^{2+}]_i$ strongly depends on the electrochemical Ca^{2+} gradient. To prevent effects of changes in membrane potential on Ca^{2+} entry, cells were incubated in a depolarizing Krebs solution in which Na^+ was substituted by K^+ (a high K^+ Krebs solution). Because of the K^+-sensitivity of the inwardly rectifying K^+ current (Voets *et al*., 1996b), this will stabilize the membrane potential at a value close to 0 mV. These thapsigargin or BHQ treated CPAE cells were then exposed to a nominal Ca^{2+}-free solution, which caused a further drop of $[Ca^{2+}]_i$ to values less than 30 nM. Stored-depletion activated Ca^{2+}-entry was then assessed from the initial rate of $[Ca^{2+}]_i$ increase induced by reapplication of 10 mM $CaCl_2$ to the bath solution.

Cell proliferation was assessed by counting the cells at day 1, 2, 3, 4 and 7 after plating them at a density of 20 000 cells/well. For counting, the cells were detached from the wells by washing in a 0.05% trypsin and 5 mM EDTA containing Ca^{2+} free solution. Thereafter, 1 ml normal culture medium was added to stop trypsinization. A 500 μl cell suspension to which 70 μl trypan blue was added was transferred into a BURKER chamber for cell counting. The number of cells is expressed μl^{-1}. Only cells which excluded trypan blue were counted. This enabled us to differentiate easily between reducing the cell proliferation rate and cell death.

Mibefradil (kindly provided by Dr J-P Clozel, Hoffmann-La Roche, Basel) was applied at concentrations between 1 and 30 μM from an aqueous 30 mM stock solution. We limited our studies to concentrations up to 30 μM, because higher concentrations caused depolarization and leakage currents in most of the cells together with an increase in $[Ca^{2+}]_i$. We found 30 μM to be a critical threshold concentration for measuring stable effects. Even this concentration induced cell depolarization, a possible indication of a non-specific leak conductance.

All experiments were performed at room temperature between 20 and 22°C.

Mean ± s.e.mean were calculated from pooled data. Significance was tested by means of Student's *t* test (level: $P < 0.05$). Inhibitory effects were defined as usual and given as % inhibi-

tion = 100* (1-($I_{+100,MIBEF}/I_{+100,CONT}$)), where $I_{+100,CONT}$ and $I_{+100,MIBEF}$ are the currents measured at +100 mV before and during application of mibefradil, respectively.

Results

Effects of mibefradil on the membrane resting potential

The membrane potential of CPAE cells was measured in current clamp mode by using standard pipette and extracellular Krebs solutions. Figure 1a shows a typical recording from a cell with a resting potential between −22 and −30 mV. Application of 10 μM mibefradil rapidly and reversibly hyperpolarized the membrane to potentials between −70 and −80 mV. However, if applied at a concentration of 30 μM, mibefradil rapidly depolarized the cell membrane (Figure 1c). Membrane potential was measured from the frequency distribution of all data points sampled during the experiment. The distributions were fit in the presence and absence of mibefradil. Mean values were obtained per cell from these Gaussian fits, which takes into account the membrane potentials during the whole experiment. Figure 1b and c give the respective examples for both cells shown in Figure 1a and c. By using this method, mean resting potentials were obtained in the absence of mibefradil of $-26-6\pm0.6$ mV ($n=7$), in the presence of 10 μM mibefradil of -59 ± 1.6 mV ($n=6$), and for 30 μM mibefradil -13.7 ± 0.6 mV ($n=4$). The large scattering in the measured values of the resting potential can be explained by different contributions of the inwardly rectifying K^+ current, the volume-sensitive Cl^- current and a non-selective cation current to the resting net current (Voets *et al*., 1996b). Mibefradil significantly shifted the membrane potential to more negative values. However, high concentrations of mibefradil depolarized the cell. This might be due to a non-specific leak that is generated by this high concentration. A similar indication for such a leak forming at high concentrations was seen in the Ca^{2+}-measurement experiment. These effects at high concentrations will obviously be without therapeutic significance because the concentrations for clinical trials are in the range of 300 ng ml^{-1} (0.51 μM) or even lower (Bakx *et al*., 1995; Clozel, personal communication).

Mibefradil inhibits Ca^{2+}-activated Cl^- currents ($I_{Cl,Ca}$)

We have recently shown that loading of CPAE cells with Ca^{2+} via the patch pipette activates a Cl^- current (Nilius *et al*., 1997a). Figure 2 shows an example of this current, analysed by the ramp voltage protocol: loading of the cell with Ca^{2+} after breaking the membrane under the sealed patch pipette activated a current. The time course of activation at +100 mV, as shown in Figure 2a, was reconstructed from the average current in a small voltage window around that potential recorded during subsequent ramp protocols. Two representative instantaneous *I*-V curves measured by the same protocol, as depicted in Figure 2b, illustrate the strong outward rectification of this current. Application of 10 μM mibefradil decreased the current at +100 mV by approximately 70% (Figure 2a, b). Inward currents were also decreased to a similar extent. Figure 2c shows a summary of the concentration-dependence of the blocking action of mibefradil on the Ca^{2+}-activated Cl^- current at +100 mV. Concentration-response curves were fitted by the Hill equation. From eight cells, a half-maximal block was observed at 4.7 ± 0.18 μM. The Hill coefficient, $n_H=0.99\pm0.03$, indicates a non-co-operative mode of inhibition by mibefradil.

Mibefradil inhibits volume-activated Cl^- currents ($I_{Cl,vol}$)

Volume-activated Cl^- currents, $I_{Cl,vol}$, activated by swelling CPAE cells at a constant $[Ca^{2+}]_i$, differ in many respects from

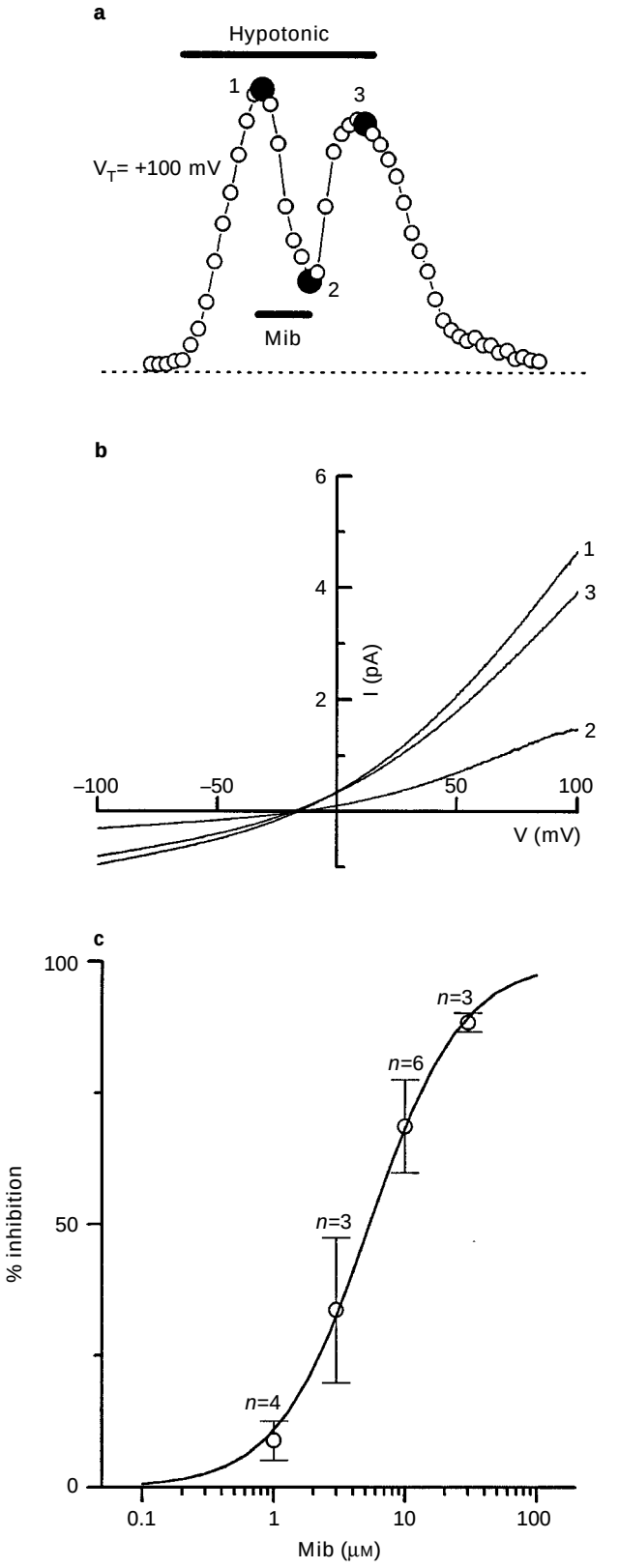

Figure 3 Mibefradil inhibited volume-activated Cl^- currents. (a) Activation of the volume-sensitive Cl^- current $I_{Cl,vol}$ (described in detail elsewhere, Nilius *et al*., 1994; 1997a for comparison between $I_{Cl,Ca}$ and $I_{Cl,vol}$) by challenging the cell with a 27% hypotonic solution (same protocol as in Figure 1). Mibefradil (Mib) - applied at the time indicated - induced a fast and reversible block of $I_{Cl,vol}$. K^+ currents were blocked by Cs^+. (b) At the times indicated, *I*-V curves were depicted. Reversal potential was approximately at the expected $E_{Cl}=-16$ mV (note the changed conditions under hypotonic stimulation, see Methods). (c) Concentration-response curve of the inhibitory action of mibefradil on $I_{Cl,vol}$. The curve was fitted by the Hill-equation with $K_i=5.4\pm0.22$ μM, Hill coefficient $n_H=1.25\pm0.03$.

the Ca^{2+}-activated Cl^- currents, $I_{Cl,Ca}$. The outward rectification of $I_{Cl,vol}$ is much less pronounced than that of $I_{Cl,Ca}$, and the kinetic behaviour of both currents is completely different. $I_{Cl,vol}$ also has a much larger amplitude than $I_{Cl,Ca}$ (Nilius *et al*., 1994; 1996; 1997a).

To investigate the effects of mibefradil on volume-activated Cl^- currents, cell swelling was induced by superfusing the cells with a hypotonic solution in which the osmolarity was decreased by 27%. The current activated by this procedure has been identified as the Cl^- current and characterized in detail (Nilius *et al*., 1994; Szücs *et al*., 1996). Figure 3a shows the time course of the current activated during a hypotonic challenge at +100 mV. The current activates rather slowly, but reaches a stationary value close to 5 nA, which is considerably higher than that of Ca^{2+}-activated currents at the same potential (134 pA/pF at +100 mV for $I_{Cl,vol}$, compared to 9 pA/pF for $I_{Cl,Ca}$ in Figure 1, for quantification see Nilius *et al*., 1997a). Some representative *I*-V curves are shown in Figure 3b, from which it is obvious that the outward rectification of $I_{Cl,vol}$ is less pronounced than for the Ca^{2+}-activated currents shown in Figure 2b. Application of 10 μM mibefradil reduced the outward current at +100 mV by approximatley 66%. This inhibition was almost completely reversible. Current inhibition was similar for inward and outward currents (Figure 3b). From 6 cells a concentration-response curve of the inhibition was constructed by using equation [2] and described by equation [3]. From the fit, a concentration for half-maximal inhibition of 5.4 ± 0.22 μM and a Hill coefficient of 1.25 ± 0.03 were obtained, indicating again a non-co-operative inhibition of $I_{Cl,vol}$ by mibefradil.

Mibefradil does not affect the inwardly-rectifying K^+ channel (IRK)

CPAE cells express inwardly rectifying K^+ channels, which have been described previously (Nilius *et al*., 1994; Voets *et al*., 1996a, b). This current can be dissected from other ionic currents in endothelial cells by calculating the difference current between control conditions and after application of 1 mM Ba^{2+}, which completely blocks IKR but leaves other currents unchanged (Voets *et al*., 1996a, b). Figure 4 shows an example of such an experiment. Voltage ramps were applied every 10 s. From these ramps *I*-V curves can be reconstructed and the current amplitudes can be measured at any potential. The current at −150 mV is mainly IRK, because it is completely and reversibly blocked by Ba^{2+}. However, at +100 mV this inhibition is almost absent (Figure 4a). These outward currents are carried by Cl^-, probably via volume-sensitive Cl^- channels and by K^+ via non-selective cation channels (Voets *et al*., 1996b). Application of 10 μM mibefradil induced only a small reduction of the current at negative potentials, but a clear inhibition of the outward current at positive potentials (Figure 4a, b). However, the Ba^{2+} sensitive current component which represents IRK was only slightly affected (Figure 3c). The current density of IRK at −150 mV was 11.6 ± 0.86 pA/pF under control conditions compared to 12.5 ± 1.23 pA/pF ($n = 9$) in the presence of 10 μM mibefradil. At a concentration of 100 μM, it was decreased to 9.1 ± 0.80 pA/pF ($n = 5$), i.e. an inhibition by 13 ± 2%. However, these latter effects were measured only 1 min after application of mibefradil, because the cells tend to get overloaded with calcium in the presence of these high concentrations of mibefradil.

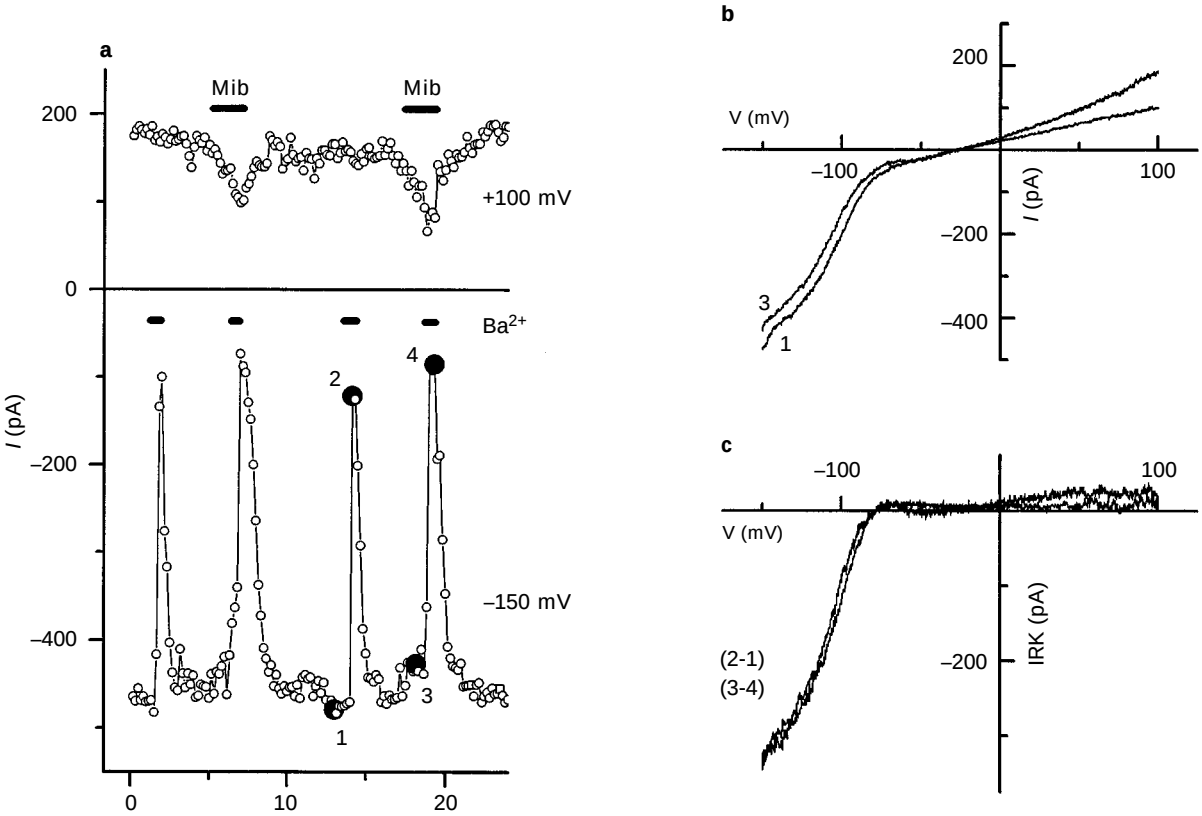

Figure 4 Mibefradil (Mib) did not inhibit the inwardly rectifying K^+ channel in CPAE cells. (a) By use of the same voltage protocol as in Figures 1 and 2, currents were measured at −150 and +100 mV in the absence of Cs^+. Inward rectification is clearly depicted. Application of 1 mM Ba^{2+} for 30–40 s, completely blocked the inwardly rectifying K^+ current, IRK (see also Voets *et al*., 1996a, b). Currents in Ba^{2+} were used as reference currents to obtain the true IRK as the difference between the net-current and the Ba^{2+}-resistant current. Outward currents were not affected by Ba^{2+}. Application of mibefradil (10 μM) inhibited the outward current but no clear effects were observed at negative potentials. (b) From the *I*-V curves it is obvious that mibefradil blocked an outward current but had a smaller effect on the inward current. The blocked outward current is mainly carried by $I_{Cl,vol}$ (see Voets *et al*., 1996b). (c) Difference currents in control (2–1) and in the presence of mibefradil (3–4) to obtain the *I*-V relationships show that IRK was not affected by mibefradil.

Effect of mibefradil on store-depletion operated Ca^{2+} entry

Ca^{2+}-entry evoked by release of intracellular Ca^{2+} is essential for generating sustained cytosolic Ca^{2+} signals in endothelial cells and for refilling Ca^{2+}-stores. To study the effects of mibefradil on this important mechanism, we depleted these stores by means of the thapsigargin and BHQ protocols, as described in the Methods section, and monitored the changes in $[Ca^{2+}]_i$ upon reapplication of extracellular Ca^{2+}. To prevent changes in resting potential which would modify the driving force for Ca^{2+}, Na^+ in the Krebs solution was substituted with K^+ to 'clamp' the membrane potential. Both procedures for store depletion gave similar results. Figure 5 shows two examples of the Ca^{2+}-signals observed in these cells. Figure 5a shows the protocol of the changes in $[Ca^{2+}]_e$ between nominally Ca^{2+}-free, 1.5 mM and 10 mM. Superfusion of the cells with nominally Ca^{2+}-free solution decreased $[Ca^{2+}]_i$. Reapplication of a 10 mM external Ca^{2+} induced a fast increase in $[Ca^{2+}]_i$ followed by a slow decay. These changes in $[Ca^{2+}]_i$ after variation in $[Ca^{2+}]_e$ do not occur if the intracellular stores are not previously depleted (Oike *et al*., 1994a, b). If mibefradil (10 μM) was applied before the reapplication of extracellular Ca^{2+}, it reduced the initial rate of rise in $[Ca^{2+}]_i$ during that reapplication. From seven cells, the mean rate of $[Ca^{2+}]_i$-elevation significantly decreased in the presence of 10 μM mibefradil from 3.4 ± 0.4 nM s^{-1} to 2.6 ± 0.3 nM s^{-1}, i.e. a reduction of $19.8 \pm 4.6\%$ ($n=7$). If applied during the decaying phase of $[Ca^{2+}]_i$ (or a plateau-like Ca^{2+}-level) mibefradil resulted in a reversible decrease of $[Ca^{2+}]_i$ (Figure 5b). This reduction was observed in 6 out of 8 cells. In the two remaining cells, mibefradil had no effect. $[Ca^{2+}]_i$ decreased during the late phase of Ca^{2+} reapplication by $17.6 \pm 6.5\%$ ($n=8$) or by $23.5 \pm 7.2\%$ ($n=6$) if only the responding cells were considered (for quantification see legend of Figure 5b). Because of the similarity of the effects on the Ca^{2+}-plateau and the rate measurements, this decrease is probably due to a small inhibition of Ca^{2+}-entry. Higher concentrations of mibefradil could not be used because they induced, in most of the cells, an increase in $[Ca^{2+}]_i$ probably by influx through non-specific leaks.

Effects of mibefradil on endothelial cell proliferation

We have recently shown that modulation of ion channels influences proliferation of different cell types (Nilius & Droogmans, 1994). Especially, inhibition of $I_{Cl,vol}$ by structurally completely different channel blockers inhibits cell proliferation (Voets *et al*., 1995). Also mibefradil induced a sensitive inhibition of the proliferation of CPAE cells. The presence of mibefradil in the culture medium significantly inhibited growth of CPAE cells at a concentration of 1 μM (Figure 6a), whereas 10 μM reduced the number of cells by more than 90% without inducing cell death. The cells did not survive if exposed to a concentration of 100 μM. Obviously, these high concentrations of mibefradil have cell-toxic effects, which is consistent with the non-specific effects on membrane permeability and Ca^{2+}-loading described above. From the concentration-response relationship constructed from the pooled data, we estimated a concentration for half-maximal inhibition of cell proliferation of approx-

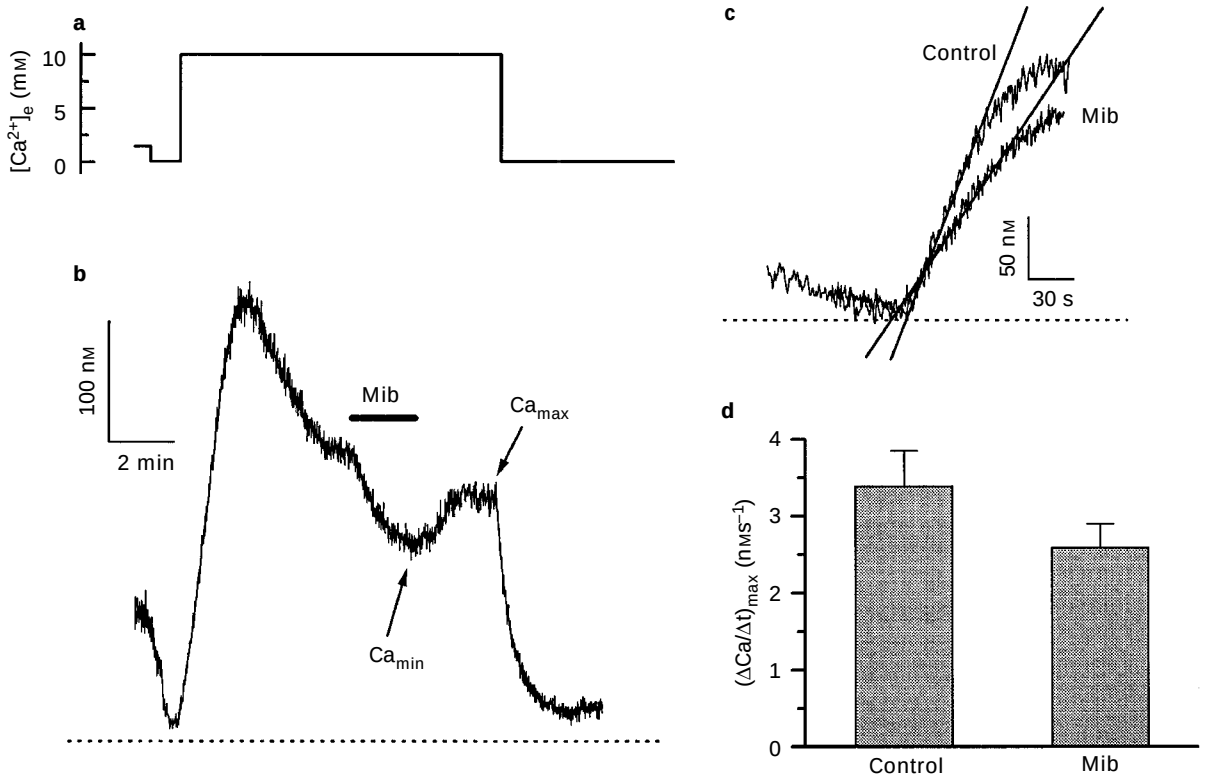

Figure 5 Mibefradil had only little effect on the store-depletion induced Ca^{2+} entry. (a) and (b) CPAE cells were pre-incubated with 1 μM thapsigargin (same effects were obtained with incubation with 10 μM BHQ) to induce depletion of intracellular Ca^{2+}-stores (Oike *et al*., 1994a, b). During changing of the perfusion from 1.5 mM Ca^{2+} (normal Krebs solution) to a nominally Ca^{2+}-free bath solution, $[Ca^{2+}]_i$ dropped to concentrations less than 20 nM. Re-application of 10 mM $[Ca^{2+}]_e$ induced an overshooting Ca^{2+}-transient followed by a slow decay. These signals represent Ca^{2+}-entry after store depletion. The change of $[Ca^{2+}]_e$ is shown in (a). Application of mibefradil (Mib, 10 μM) during the delayed phase of decay induced a small decrease in $[Ca^{2+}]_i$, indicating an inhibitory effect of the store-operated Ca^{2+}-entry. The reduction was quantified as $100.[1-(Ca_{max}-Ca_{min})/Ca_{min}]$. Pooled data from this analysis are given in the text. (c) Mibefradil (10 μM) also induced a small decrease in the rate of $[Ca^{2+}]_i$ elevation following re-application of external Ca^{2+}. The rate was measured from linear fits of the onset of $[Ca^{2+}]_i$ elevation which should be a valid estimation of the maximal value of dCa/dt (data from the same cell). (d) The maximal rate of Ca^{2+}-entry (in nM s^{-1}) was decreased in the presence of 10 μM mibefradil (results from seven cells); $P<0.05$.

imatley 2 μM (Figure 6b). This value fits very well with that for inhibition of $I_{Cl,vol}$ and supports the view that this channel might be involved in the control of proliferation of endothelial cells (Voets *et al*., 1995).

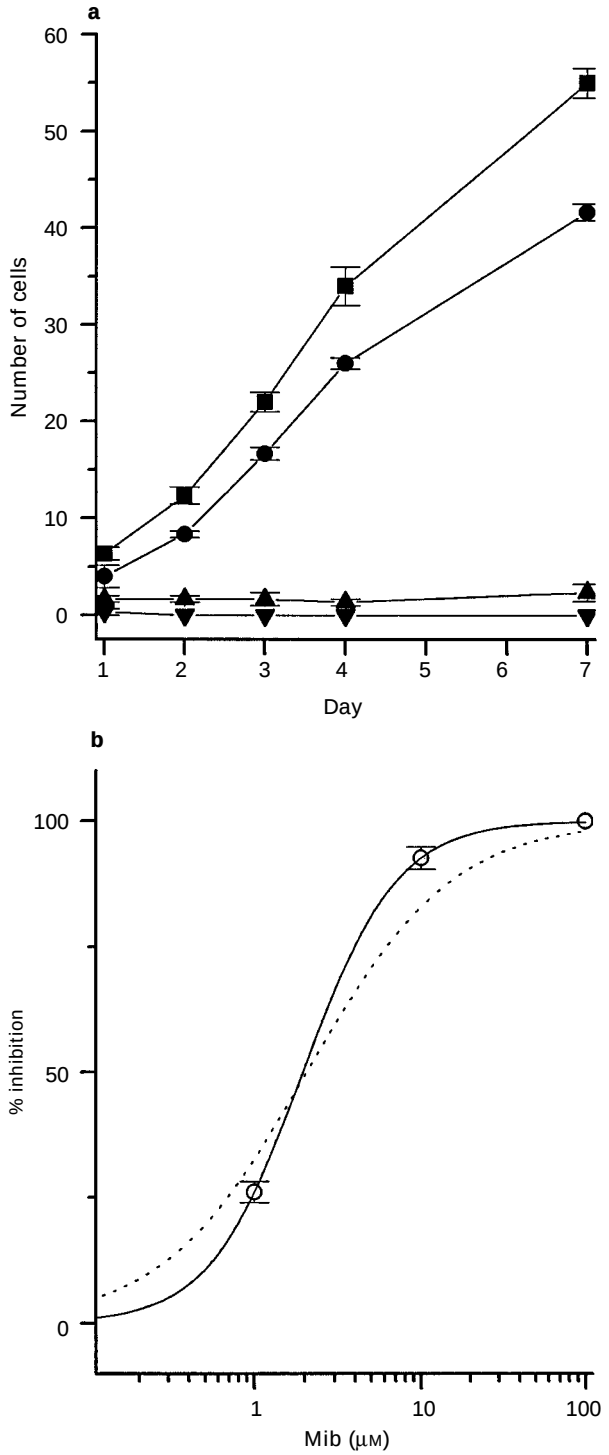

Figure 6 Mibefradil inhibited proliferation of CPAE cells. (a) Cells were seeded (three wells per day and per condition) as described in Methods in the absence (■, control) and the presence of (●) 1, (▲) 10 and (▼) 100 μM mibefradil (Mib). Inhibition of proliferation was significant at all concentrations. At 100 μM no living cells were present, at 10 μM adhesive cells could still be counted indicating a true anti-proliferative effect. Plotted are means and s.e.mean (vertical lines) at each day after seeding. (b) Inhibitory effects were pooled from day 2 to 7. Data were fitted by the Hill equation [3]. Dotted line represents the fit for a constant $n_H = 1$. Parameters of the fits are: $K_i = 1.9 \pm 0.01$ μM, $n_H = 1.5 \pm 0.01$, and $K_i = 2.1 \pm 0.5$ μM for constant $n_H = 1$.

Discussion

Macrovascular CPAE cells are characterized electrophysiologically by three ionic currents: an inwardly rectifying K^+ current, a volume-sensitive Cl^- current and a non-selective cation current which determine the resting membrane potential under control conditions (Voets *et al*., 1996b). During stimulation, at least two other currents are activated: a Ca^{2+}-dependent Cl^- current and a current that is responsible for Ca^{2+}-entry after store depletion (Oike *et al*., 1994b; Nilius *et al*., 1997a). These currents might be essential for normal endothelial cell function and are therefore used in this study to evaluate the effects of the novel Ca^{2+}-antagonist mibefradil.

Until now, the effects of mibefradil on endothelial cells have not been studied. It has been shown that this novel Ca^{2+}-antagonist induces, in addition to its direct myocardial effects, a variety of vascular responses, such as antihypertensive and antivasospastic effects, it interacts with endothelium-derived NO-effects, suppresses vascular consequences of hypertension without interfering with the renin-angiotensin system and has a potent antiproliferative effect on smooth muscle cells (Schmitt *et al*., 1995; Veniant *et al*., 1995; Kung *et al*., 1995; Roux *et al*., 1996; Bernink *et al*., 1996). At least some of these effects of mibefradil may be mediated via a modulation of endothelial cell function. Interestingly, mibefradil affects the atrial blood pressure by a mechanism that can be reversed by NO-synthase inhibitors (Gray *et al*., 1993). This improvement of endothelial cell function could be related to a positive feedback via a driving force induced by the hyperoplarization of the membrane potential. We were therefore interested in a possible modulation of the functionally most important channels in macrovascular endothelial cells by mibefradil.

Summary of findings

Mibefradil has been described as a Ca^{2+}-channel blocker with an approximately ten fold higher sensitivity to T-type Ca^{2+} channels than to other, cloned, L-, N-, R, P and Q-type channels (Bezprozvanny & Tsien, 1995). We show here for the first time that mibefradil is a potent inhibitor of endothelial Cl^- channels which are activated by changes in cell volume and those which are activated by an increase in $[Ca^{2+}]_i$. We have shown recently that $I_{Cl,vol}$ is partially activated in resting endothelial cells (Voets *et al*., 1996b), and that its inhibition induces hyperpolarization. Similar effects could now be observed in the presence of mibefradil. In contrast, K^+ inwardly rectifying channels are rather insensitive to mibefradil. This relative insensitivity is important for the hyperpolarizing effects after Cl^- channel inhibition. Store-depletion operated Ca^{2+}-entry was also only weakly, but significantly inhibited by mibefradil. Mibefradil was also efficient in inhibiting proliferation of the CPAE cells.

Possible functional impact

Cl^- channels have been described in a variety of endothelial cells and have triggered a number of speculations about their functional role: (1) a role in pH regulation (Groschner & Kukovetz, 1992), (2) an obvious role in the control of membrane potential (Voets *et al*., 1996b) and as shown here, (3) modulation of agonist-induced or store-depletion dependent intracellular $[Ca^{2+}]_i$ signals, including the regulation of Ca^{2+}-influx by controlling the membrane potential (Groschner *et al*., 1994; Hosoki & Iijima, 1994; Yumoto *et al*., 1995), (4) they may provide a transport pathway for amino acids (Strange & Jackson, 1995), and interestingly (5) they may play a role in cell proliferation (Voets *et al*., 1995; 1996c; Nilius *et al*., 1996; 1997a, b). We show here that mibefradil indeed efficiently blocked proliferation of the endothelial cells used. Half-maximal inhibition occurred in the same concentration range as the block of the Cl^- currents. This might further support the hypothesis that $I_{Cl,vol}$ could play a regulatory role in endothelial cell proliferation. Functionally, the modulatory role of Cl^-

channels in electrogenesis of the resting potential of endothelial cells and in their proliferation might be of interest because in other cell systems (smooth muscle cell proliferation after vascular injury, Schmitt *et al*., 1995) a similar effect has been described. We propose that these antiproliferative effects can be attributed to an inhibition of Cl^- channels rather than a modulation of T-type Ca^{2+} channels. Block of Cl^- channels could also be of functional importance for other signalling systems. During cell activation (increased shear stress, volume and cell shape changes, agonist application) which is usually accompanied by a rise in $[Ca^{2+}]_i$, both volume-sensitive and Ca^{2+}-activated Cl^- currents are activated and may regulate the inwardly driving force for Ca^{2+}-entry via store-operated Ca^{2+} channels (Hosoki & Iijima, 1994; Yumoto *et al*., 1995). Store-operated Ca^{2+}-influx mediating a long-lasting plateau-like elevation of $[Ca^{2+}]_i$ after stimulation by agonists might be essential for a variety of endothelial cell functions (e.g. production and release of vasoactive transmitters, such as nitric oxide, prostacyclin, endothelins; for a review see Nilius & Casteels, 1996). Inhibition of Cl^- channels ($I_{Cl,Ca}$ and/or $I_{Cl,vol}$) can shift the membrane potential towards negative values (Voets *et al*., 1996b) and enhance the driving force for Ca^{2+}-entry. Such a mechanism could explain the facilitating action of mibefradil on NO release (Gray *et al*., 1993).

In conclusion, we describe a selective inhibition of Cl^- channels in macrovascular endothelial cells by mibefradil. Although the concentrations used in our study are higher than those used therapeutically (Clozel, personal communication; Bakx *et al*., 1995), these effects may contribute to the beneficial effects of mibefradil on cardiovascular functions.

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References

BAKX, A.L., VAN DER WALL, E.E., BRAUN, S., EMANUELSSON, H. BRUSCHKE, A.V. & KOBRIN, I. (1995). Effects of the new calcium antagonist mibefradil (Ro 40-5967) on exercise duration in patients with chronic stable angina pectoris: a multicenter, placebo-controlled study. Ro 40-5967 International Study Group. *Am. Heart J*., **130,** 748–757.

BERNINK, P.J., PRAGER, G., SCHELLING, A. & KOBRIN, I. (1996). Antihypertensive properties of the novel calcium antagonist mibefradil (Ro 40-5967). A new generation of calcium antagonists? Mibefradil International Study Group. *Hypertension* **27,** 426–432.

BEZPROZVANNY, I. & TSIEN, R.W. (1995). Voltage-dependent blockade of diverse types of voltage-gated Ca^{2+} channels expressed in *Xenopus* oocytes by the Ca^{2+} channel antagonist mibefradil (Ro 40-5967). *Mol. Pharmacol*., **48,** 540–549.

BILLMAN, G.E. & HAMLIN, R.L. (1996). The effects of mibefradil, a novel calcium channel antagonist on ventricular arrhythmias induced by myocardial ischemia and programmed electrical stimulation. *J. Pharmacol. Exp. Ther*., **277,** 1517–1526.

BRAUN, S., VAN DER WALL, E.E., EMANUELSSON, H. & KOBRIN, I. (1996). Effects of a new calcium antagonist, mibefradil (Ro 40-5967), in silent ischemia in patients with stable chronic angina pectoris: a multicenter placebo-controlled study. The Mibefradil International Study Group. *J. Am. Coll. Cardiol*., **27,** 317–322.

CHIDA, M., VOELKEL, N.F., ONO, S., TANITA, T. & FUJIMURA, S. (1995). Leukocyte-mediated production of eicosanoids in rat lungs: modulation by a calcium antagonist. *Nippon Kyobu Shikkan Gakkai Zasshi*, **33,** 1198–1203.

CLOZEL, J.P., VENIANT, M. & OSTERRIEDER, W. (1990). The structurally novel Ca^{2+} channel blocker Ro 40-5967, which binds to the [^{3}H]-desmethoxyverapamil receptor, is devoid of the negative inotropic effects of verapamil in normal and failing rat hearts. *Cardiovasc. Drugs Ther*., **4,** 731–736.

DAUT, J., STANDEN, N.B. & NELSON, M.T. (1994). The role of the membrane potential of endothelial and smooth muscle cells in the regulation of coronary blood flow. *J. Cardiovasc. Electrophysiol*., **5,** 154–181.

FANG, L.M. & OSTERRIEDER, W. (1991). Potential-dependent inhibition of cardiac Ca^{2+} inward currents by Ro 40-5967 and verapamil: relation to negative inotropy. *Eur. J. Pharmacol*., **196,** 205–207.

GRAY, G.A., CLOZEL, M., CLOZEL, J.P. & BAUMGARTNER, H.R. (1993). Effects of calcium channel blockade on the aortic intima in spontaneously hypertensive rats. *Hypertension*, **22,** 569–576.

GROSCHNER, K., GRAIER, W.F. & KUKOVETZ, W.R. (1994). Histamine induces K^+, Ca^{2+}, and Cl^- currents in human vascular endothelial cells - Role of ionic currents in stimulation of nitric oxide biosynthesis. *Circ. Res*., **75,** 304–314.

GROSCHNER, K. & KUKOVETZ, W.R. (1992). Voltage-sensitive chloride channels of large conductance in the membrane of pig aortic endothelial cells. *Pflügers Arch. Eur. J. Physiol*., **421,** 209–217.

HERMSMEYER, K. & MIYAGAWA, K. (1996). Protein kinase C mechanism enhances vascular muscle relaxation by the Ca^{2+} antagonist, Ro 40-5967. *J. Vasc. Res*., **23,** 71–77.

HOSOKI, E. & IIJIMA, T. (1994). Chloride-sensitive Ca^{2+} entry by histamine and ATP in human aortic endothelial cells. *Eur. J. Pharmacol*., **266,** 213–218.

KUNG, C.F., TSCHUDI, M.R., NOLL, G., CLOZEL, J.P. & LUSCHER, T.F. (1995). Differential effects of the calcium antagonist mibefradil in epicardial and intramyocardial coronary arteries. *J. Cardiovasc. Pharmacol*., **26,** 312–318.

MISHRA, S.K. & HERMSMEYER, K. (1994). Selective inhibition of T-type Ca^{2+} channels by Ro 40-5967. *Circ. Res*., **75,** 144–148.

NILIUS, B. & CASTEELS, R. (1996). Biology of the vascular wall and its interaction with migratory blood cells. In *Comprehensive Human Physiology*. ed. Greger, R. & Windhorst, U. pp. 1981–1994. Berlin Heidelberg: Springer Verlag.

NILIUS, B. & DROOGMANS, G. (1994). A role for potassium channels in cell proliferation? *News in Physiological Sciences*, **9,** 105–109.

NILIUS, B., EGGERMONT, J., VOETS, T. & DROOGMANS, G. (1996). Volume-activate Cl^- channels. *Gen. Pharmacol*., **27,** 1131–1140.

NILIUS, B., OIKE, M., ZAHRADNIK, I. & DROOGMANS, G. (1994). Activation of a Cl^- current by hypotonic volume increase in human endothelial cells. *J. Gen. Physiol*., **103,** 787–805.

NILIUS, B., PRENEN, J., SZÜCS, G., WEI, L., TANZI, F., VOETS, T. & DROOGMANS, G. (1997a). Calcium-activated chloride channels in bovine pulmonary artery endothelial cells. *J. Physiol. (Cambr*.), **498,** 381–396.

NILIUS, B., VIANA, F. & DROOGMANS, G. (1997b). Ion channels in vascular endothelium. *Ann. Rev. Physiol*., **59,** 145–170.

OIKE, M., DROOGMANS, G. & NILIUS, B. (1994a). Mechanosensitive Ca^{2+} transients in endothelial cells from human umbilical vein. *Proc. Natl. Acad. Sci. U.S.A*., **91,** 2940–2944.

OIKE, M., GERICKE, M., DROOGMANS, G. & NILIUS, B. (1994b). Calcium entry activated by store depletion in human umbilical vein endothelial cells. *Cell Calcium*, **16,** 367–376.

ROUX, S, BUHLER, M. & CLOZEL, J.P. (1996). Mechanism of the antiischemic effect of mibefradil, a selective T calcium channel blocker in dogs: comparison with amlodipine. *J. Cardiovasc. Pharmacol*., **27,** 132–139.

SCHMITT, R., CLOZEL, J.P., IBERG, N. & BUHLER, F.R. (1995). Mibefradil prevents neointima formation after vascular injury in rats. Possible role of the blockade of the T-type voltage-operated calcium channel. *Arterioscler. Thromb. Vasc. Biol*., **15,** 1161–1165.

STRANGE, K. & JACKSON, P.S. (1995). Swelling-activated organic osmolyte efflux: A new role for anion channels. *Kidney Int*., **48,** 994–1003.

SZÜCS, G., BUYSE, G., EGGERMONT, J., DROOGMANS, G. & NILIUS, B. (1996). Characterisation of volume-activated chloride currents in endothelial cells from bovine pulmonary artery. *J. Membr. Biol*., **179,** 189–197.

VENIANT, M., GRAY, G.A., HEUDES, D., MENARD, J. & CLOZEL, J.P. (1995). Structural changes and cyclic GMP content of the aorta after calcium antagonism or angiotensin converting enzyme inhibition in renovascular hypertensive rats. *J. Hypertens.*, **13,** 731 – 737.

VOETS, T., DROOGMANS, G. & NILIUS, B. (1996a). Potent block of volume-activated chloride currents in endothelial cells by the uncharged form of quinine and quinidine. *Br. J. Pharmacol.*, **118,** 1869 – 1871.

VOETS, T., DROOGMANS, G. & NILIUS, B. (1996b). Ionic currents in non-stimulated endothelial cells from bovine pulmonary artery. *J. Physiol. (Cambr.)*, **497,** 95 – 107.

VOETS, T., SZÜCS, G., DROOGMANS, G. & NILIUS, B. (1995). Blockers of volume-activated Cl^- currents inhibit endothelial cell proliferation. *Pflügers Arch. Eur. J. Physiol.*, **431,** 132 – 134.

VOETS, T., WEI, L, DE SMET, P., VAN DRIESSCHE, W., EGGERMONT, J., DROOGMANS, G. & NILIUS, B. (1996c). Down-regulation of volume-activated chloride currents during muscle differentiation. *Am. J. Physiol.*, (in press).

YUMOTO, K., YAMAGUCHI, H. & OCHI, R. (1995). Depression of ATP-induced Ca^{2+} signalling by high K^+ and low Cl^--media in human aortic endothelial cells. *Jap. J. Physiol.*, **45,** 111 – 122.

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