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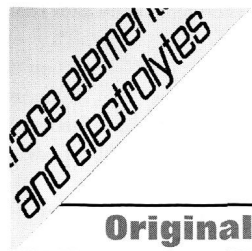
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Importance of calcium and potassium currents in human lens epithelial cells (hLEC) and the effect of the calcium channel blocker mibefradil

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Key words

lens epithelial cells –
calcium current – potassium current – mibefradil

Abstract. Background: To prevent posterior capsule opacification (PCO), we followed the hypothesis that calcium channel blockers (antagonists) interfere with integrin signaling and block cell adhesion in lens epithelial cells (LEC). In primary human LEC we found that the T channel antagonist mibefradil induces apoptosis which was accompanied with cell shape changes and loss of cell adhesion. Although T-type calcium channels are substantially present in membranes of freshly dispersed primary cultured hLEC and calcium currents are inhibited by mibefradil at concentrations of 10^{-8} M, the antiproliferative site of action of this drug remains unclear, since this feature is observed at concentrations 200-fold higher than that for calcium channel blockade. **Methods:** Epithelial cells of the human lens were dispersed by enzymatic treatment, recordings of membrane currents were performed using patch clamp technique in the whole cell configuration. Westernblot analysis was used for protein detection. **Results:** Total current elicited on depolarizing voltage steps from a holding potential of -80 mV was composed of inward (calcium) and outward (potassium) current. Outward current could be inhibited mostly by intracellular application of cesium ions. Currents in inward direction were activated fast (< 2 ms) and inactivated during the following 20 ms. They were characterized as calcium currents since the known calcium channel inhibitor nifedipine blocked these currents in a concentration-dependent manner. Using potassium in the pipette (145 mM) as main charge carrier, additionally a noninactivating potassium current and a voltage- and time-dependent potassium current which slowly inactivated (K_v) were observed (control). Adding mibefradil in concentrations from 10^{-6} M – 10^{-5} M to the bath solution, the inwardly directed and the non-inactivating current component were inhibited concentration-dependent. The K_v component was affected in a similar way, however, this component showed an increased inactivation behavior after applica-

tion of mibefradil in the named concentrations. **Conclusion:** The total effects of mibefradil are significant for the calcium homeostasis since calcium current itself is inhibited but, moreover, the membrane is permanently depolarized up to 20 mV by the drug. Both effects may contribute to the observed reduced cell adhesion during mibefradil treatment.

Introduction

Posterior capsule opacification (PCO) is the most common long-term complication with more than 25% overall incidence within 5 years after cataract surgery of the human eye [Bertelman and Kojetinsky 2001]. PCO is caused by activation of epithelial cells, which is accompanied with proliferation, migration and increased production of matrix [Nishi 1999, Saxby et al. 1998]. T-type calcium channels have been implicated in the proliferation of many cell types [Chemin et al. 2002, Kuga et al. 1995, McRory et al. 2001]. To prevent PCO, pharmacological therapies are used, but mostly the therapeutically effect of such drugs is accompanied with a cytotoxic behavior against other tissues [Beck et al. 2001]. One group of such pharmacological substances represents calcium channel blockers, which have different potency and selectivity for voltage-dependent calcium channels [Beck et al. 2001]. They block either L- or T-type calcium channels more or less selective. Mibefradil, a potent vasodilator, selectively blocks transient, low voltage-activated (T-type) calcium channels over long-lasting, high voltage-activated (L-type) calcium channels [Clozel et al. 1997]. L- and T-type channels are distinguished by differences in their electrophysiological and pharmacological properties [Bean

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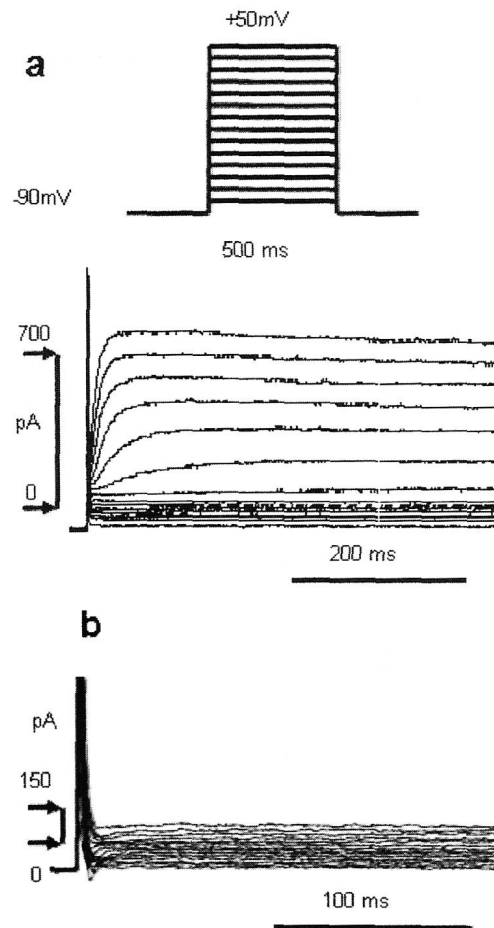


Figure 1. Size time course of whole-cell currents in HLE-B3 ($n = 10$) measured under control conditions (a) with potassium or (b) cesium as main charge carrier in pipette solution (VH -90 mV, VT -10 mV). Currents were mainly carried by calcium and potassium ions. Traces show (a) outwardly directed and (b) inwardly directed currents with a general voltage dependency.

1989] and in protein structure. The L-type channel opening depends on strong depolarization for activation, shows high sensitivity to dihydropyridines and inactivates slowly, whereas T-type calcium channels inactivate rapidly, are activated by weaker depolarizations; and commercially available L-type channel blockers show only little effect on their activity. T-type calcium channels are relatively sensitive to mibefradil.

Several studies have reported that calcium channel blockers also interact with voltage-gated potassium channels [Noack et al. 1990, Perchenet and Clement-Chomienne 2000]. Potassium channels play important roles in cell function like volume regulation, membrane potential maintenance, fluid secretion, proliferation and cell growth control [Lu et al. 2001]. Therefore, the aim of this

work was to identify calcium and potassium channels in human lens epithelial cells and to describe the effect of mibefradil on inward and outward currents in these cells.

Materials and methods

Cell culture

HLE-B3 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM containing 10% heat-inactivated fetal calf serum (FCS) and 1% gentamycin (Gibco, Grand Island, NY, USA) in a humidified incubator supplied with 5% CO_2 at 37 °C. Cells were passed at 10^6 cells/ml seeding density.

Calcium channel antagonists

T-type calcium channel blocker mibefradil (Hoffmann La Roche, Basel, Switzerland) and L-type calcium channel blocker verapamil (Sigma, St. Louis, MO, USA) were prepared in stock solutions diluted in water (Mib.) and DMSO (Vera.). Potassium channel blocker tetraethylammonium (TEA) and an antagonist for voltage-sensitive potassium channels 4-aminopyridine (4-AP) were used at concentrations between 1 and 5 mM.

Immunoblot

For isolation of membrane proteins, confluent HLE-B3 cells in culture flasks were used. Cell lysis was performed in buffer, containing 140 mM NaCl, 10 mM Tris, 5 mM EDTA, 1% Triton, 1 mM PMSF, 1 mM DTT (pH 7.3/HCl). For protease inhibition, 25 $\mu\text{g}/\text{ml}$ protease inhibitor cocktail (Roche, Basel, Switzerland) was added.

Cells were suspended in 1 ml buffer and centrifuged at 14,000 rpm for 30 min. Protein concentrations of supernatants were determined using a kit based on Bradford method (Biorad, Hercules, CA, USA).

Membrane proteins were separated by polyacrylamid gelelectrophoresis (10% polyacrylamid). Equal amounts of each sample were loaded in each lane of the gel (approximately 15 $\mu\text{g}/\text{lane}$).

After protein transfer to PVDF membrane (Hybond, Amersham, Buckinghamshire, UK),

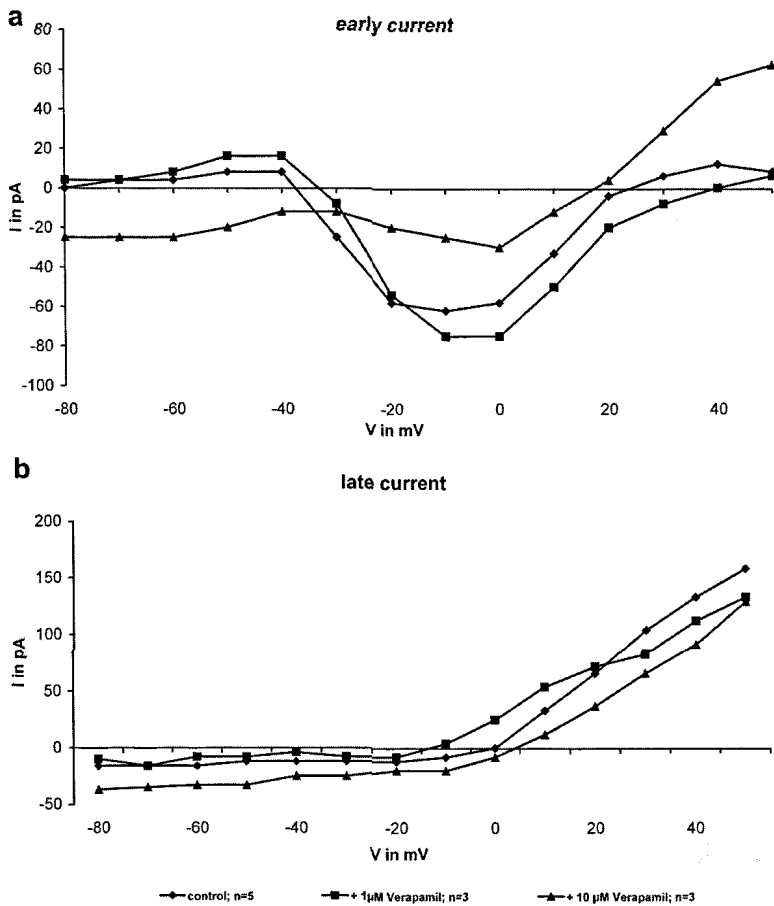


Figure 2. Current voltage curve of patch clamp experiments in HLE-B3 under control conditions and after application of different concentrations of the calcium channel blocker verapamil. a) $I-V$ curve was established by early currents (25 ms), 2.5 μM verapamil were necessary to produce 50% inhibition of the inwardly directed calcium current. b) $I-V$ curve was established by late currents (250 ms).

membranes were blocked with phosphate-buffered saline (PBS) + 2% Tween 80 (PBS-Tween) and 5% milk powder (Milasan, MalliB, Germany) for 1 h at room temperature. Antibodies were applied overnight at following dilutions: anti-Kv 1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5 and Kv1.6 (Alomone, Jerusalem, Israel) 1 : 200, anti-Cav3.1 and anti-Cav1.2 (Alomone, Jerusalem, Israel). For detection, Peroxidase-conjugated anti-rabbit IgG (Amersham, Buckinghamshire, UK) was employed at a dilution of 1 : 40,000.

Visualization was performed using an enhanced chemoluminescence (ECL) detection kit (Amersham, Buckinghamshire, UK).

Electrophysiology

Patch clamp experiments were performed in whole cell configuration at room tempera-

ture (20 °C). Cells were placed in perfusion chamber mounted on the stage of an inverted microscope (Wild) in bath solution, containing 141 mM NaCl, 4.7 mM KCl, 1.8 mM CaCl_2 , 1.2 mM MgCl_2 , 10 mM Hepes and 10 mM glucose (pH 7.4).

Patch pipettes with resistance 3 – 5 M Ohm were pulled from borosilicate tubes (World Precision Instruments, Sarasota, FL, USA). Pipette solution contained 127 mM CsCl, 11 mM glucose, 10 mM Hepes, 5 mM oxalacetate, 5 mM succinic acid, 5 mM pyruvic acid and 1.2 mM MgCl_2 for investigation of calcium currents. A similar solution (cesium ions changed equimolarly by potassium ions) was used to detect potassium currents.

Low-pass filtered signals were digitized at 5 kHz with Axolab 1,100 hardware and a microcomputer in conjunction with pClamp software (Version 5.5 and 8.1, Axon Instruments, Sunnyvale, CA, USA).

Results

Separation of ionic currents in hLEC

When the HLE cells were investigated using a normal intracellular medium (potassium as the main charge carrier), the whole-cell currents were mainly carried by calcium and potassium ions. Representative examples of a whole set of current recordings are shown in Figure 1a, which illustrates the typical size and time course of the whole-cell currents. The currents were elicited with 500 ms depolarizing voltage clamp pulse from a holding potential of -90 mV. Potentials in the range of -80 – $+50$ mV were tested in increments of 10 mV. At potentials from positive to -40 mV, the initial capacitive surge was followed by a transient net inward current that peaked in about 2 – 4 ms. This inward current then declined and reversed to become an outward current. The family of current traces shown in Figure 1a illustrates the general voltage dependency of the inward- and outward-directed current in this tissue. Since total current flow is composed of outward and inward currents, the total size and time course of the two current components can not be separated.

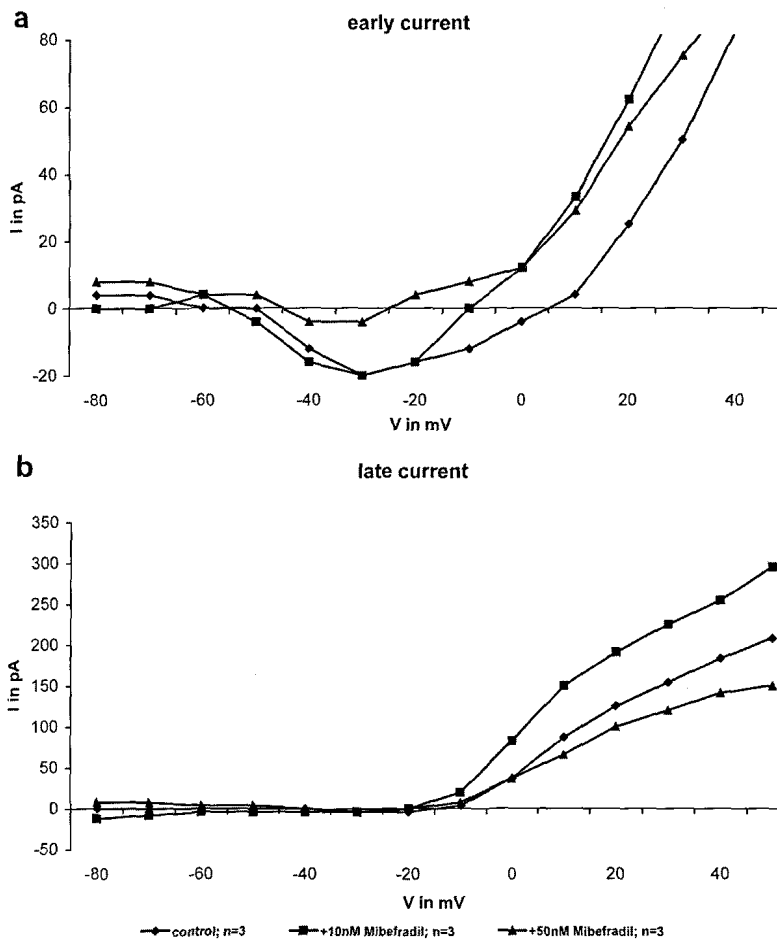


Figure 3. Current voltage curve of patch clamp experiments in HLE-B3 ($n = 5$) under control conditions and after application of different concentrations of the calcium channel blocker mibefradil. a) $I - V$ curve was established by early currents (25 ms), 10 nM mibefradil were necessary to produce 50% inhibition of the inwardly directed calcium current. b) $I - V$ curve was established by late currents (250 ms).

When cesium pipettes were used (see Methods) (this ion can only hardly pass potassium channels), most of the potassium current was inhibited (Figure 1b). Using this approach, inward-directed currents and their time dependency can be clearly identified. Representative records illustrating the size and time course of the whole-cell currents with a cesium-loaded pipette are shown in Figure 1b. Note the difference between the current calibration in Figure 1a and b. The current traces were elicited with 500 ms depolarizing voltage clamp pulses from a holding potential of -90 mV to test potentials of -80 to $+50$ mV in increments of 10 mV. At potentials positive at -60 mV, the initial capacitive surge was followed by a transient net inward current that peaked about 2 ms and then declined to a steady current level. This family of

current traces illustrates the voltage dependency of the inward current generated by this tissue and shows that the maximum inward current was obtained at test potentials between 0 mV and $+20$ mV.

Characterization of inward currents

To determine voltage dependency of inward currents, the peak inward current was measured at each test potential giving a current voltage relationship ($I - V$ curve) (Figure 2). This curve shows the typical shape with an inverted maximum close to 0 mV. The application of the known calcium channel inhibitor verapamil changes the time course and size of inward-directed current and the $I - V$ curve (Figure 2b). This effect of verapamil was concentration-dependent with a half inhibition of 2.5 μ M. In respect to inhibitory effect of verapamil on inward currents, the effect of this drug on outward (cesium) currents was smaller and only observed at higher concentrations (Figure 2). Using nifedipine as another calcium channel blocker, the effect on outward currents were in similar size compared with Verapamil. Within 3 min after adding mibefradil (1 nM – 10 μ M) to the bathing solution, inward-directed currents became smaller than under control conditions, whereas the outward currents elicited at each potential were only affected at the higher concentrations of the drug. Figure 3 shows the current voltage relationship for the inward-directed peak under control conditions and in the presence of 10 nM and 50 nM mibefradil. The outward peak current (cesium) and the effect of mibefradil is shown in Figure 3b: only high concentrations of this drug (> 1 μ M) inhibited outward current. If the effects of verapamil and mibefradil are compared on the time course of the total inward current, a difference can only be hardly detected at higher concentration; at lower concentrations (close to thresholds), the proportion of current being inhibited by the one but not by the other drug, however, might be present (Figure 4).

To verify the electrophysiological measurements, the presence of calcium channels in HLE-B3 was checked by Westernblot analysis. Therefore, lysates of membranes were produced and used for Immunoblot. With an-

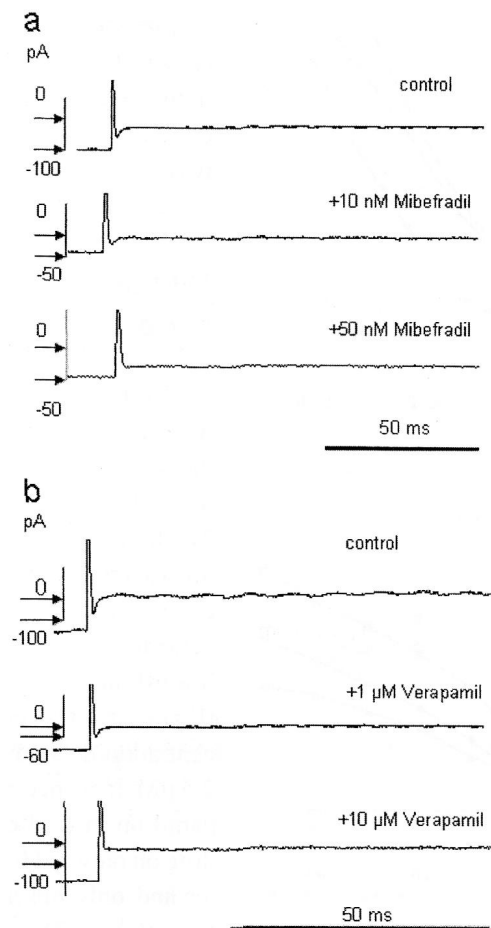


Figure 4. Inhibition of inwardly directed calcium currents by (a) mibefradil ($n = 5$) and (b) verapamil ($n = 4$) at different concentrations. a) Inward currents blocked by mibefradil: 10 nM mibefradil were necessary to reach 50% inhibition; at a concentration of 50 nM mibefradil, the inward current was completely blocked. b) Inward currents blocked by verapamil: 50% inhibition was produced at concentrations of 2.5 μM ; at concentrations between 10 μM and 12.5 μM , these currents were completely blocked.

tibodies against Cav 1.2 and Cav 3.1 L- and T-type calcium channels were detected in human lens epithelial cells (Figure 5).

Characterization of outward currents

When potassium was the main charge carrier in the pipette solution, outward currents recorded from hLEC cells were mainly potassium currents (Figure 1). The typical $I - V$ curve of the outward current (50 ms) is illustrated in Figure 6. The total currents were reduced when the holding potential was set to -10 mV. This indicates that inactivating, voltage-sensitive outward currents (Kv) are present in hLEC. The action of the known po-

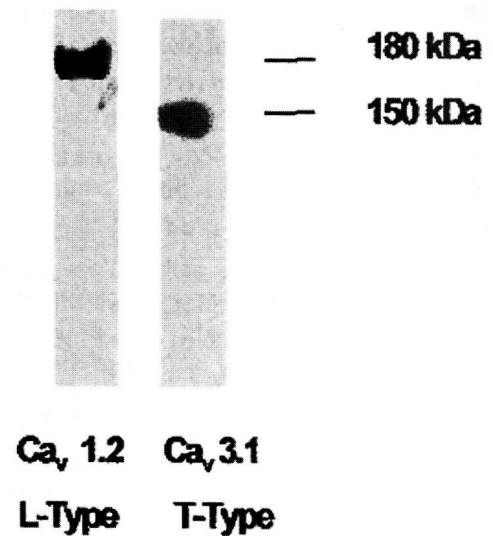


Figure 5. Expression of voltage-gated calcium channels in human lens epithelial cells. Western blot analysis was performed with membrane lysates of HLE-B3, detected with antibodies against Cav 1.2 and Cav 3.1.

tassium channel blocker 4-aminopyridine (4-AP) on both components is shown in Figure 6. Both components are partially inhibited by 5 mM 4-AP.

The effect of mibefradil on outward currents was evaluated with the same protocol as above. From concentrations of 1 μM mibefradil, clear effects on outward current components were detected (Figure 6b). The comparison of the time course of current in the presence of mibefradil with that under control does not show a significant change of time-dependent inactivation behavior of these channels (Figure 7).

The same membrane lysates as used in Immunoblot experiments for calcium channels were accomplished to verify the expression of Kv channels in membranes of HLE-B3. Representative results are shown in Figure 8: Kv1.2, Kv1.4, Kv1.5 and Kv1.6 were expressed in membranes in these cells.

Discussion

The calcium channel blocker mibefradil was described to show an approximately 10-fold higher sensitivity to T-type than to other types of calcium channels [Bezprozvanny and Tsien 1995]. The results described in this paper indicate that mibefradil blocks calcium currents in single, isolated human lens epithelial cells, which express two types of calcium

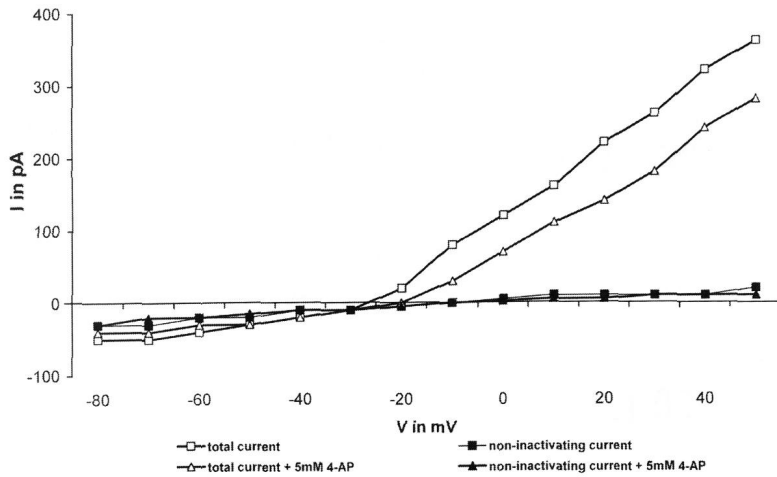


Figure 6. I–V curve of the different components of the outwardly directed current (total current and non-inactivating component) established from electrophysiological recordings of HLE-B3 under control conditions and after application of the potassium channel blocker 4-AP.

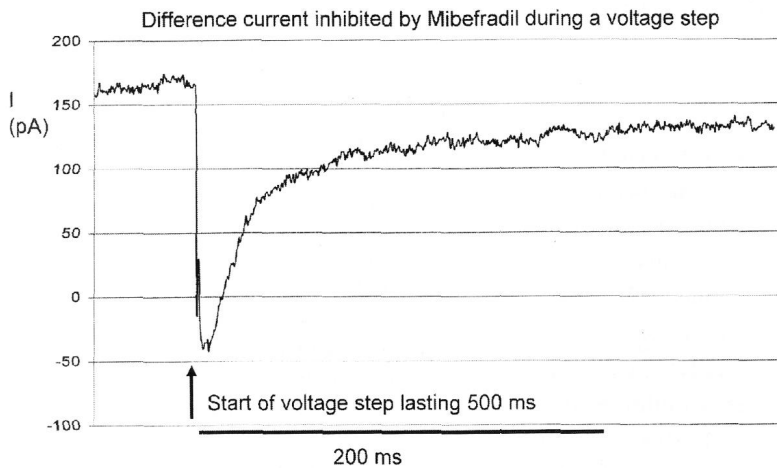


Figure 7. Time course (300 ms) of the mibefradil (10 μ M) inhibited time-dependent inactivating current ($n = 3$). Current recording demonstrates the downward deflected inward current and the inhibition of the upward deflected outward current which was a 30% inhibition of the total voltage-dependent outward current.

channels (L- and T-type). According to the hypothesis that the calcium channel modulator mibefradil interacts with potassium channels, potassium currents were examined by performing patch clamp experiments using a wide range of concentrations (10 nM, 1 μ M, 10 μ M mibefradil). The inhibition of voltage-gated potassium channels by mibefradil is characterized by a concentration-dependent reduction in current amplitude. This effect is similar to those found with other calcium channel blockers on Kv1.5, such as verapamil [Rampe et al. 1993] and nifedipine [Zhang et al. 1997].

Furthermore, the experiments have demonstrated that HLE-B3 cells exhibit potassium currents additional to calcium currents. As shown, mibefradil inhibited the outward potassium current in a concentration-dependent manner. However, this was only observed in much higher concentrations. The concentration of mibefradil necessary to produce 50% inhibition of the peak inward current was estimated to be approximately 10 nM, whereas about 2 μ M mibefradil were required for 50% inhibition of the outward potassium current. The T-type calcium channels might be the major target of mibefradil, but given the multifunctional effects of mibefradil, it is unlikely that its action would be limited only on these channels. It has been shown that mibefradil inhibits calcium-activated and volume-regulated chloride channels in microvascular cells [Nishi 1999]. Mibefradil also blocks in a concentration-dependent manner the cloned HERG potassium channel and Kv channels expressed in COS-7 cells [Chouabe et al. 1998]. The observed calcium inward currents were normally fast and showed a T-type character. In some cases, an additional slow component was present. This additional calcium current component had a high activation threshold and was much slower in inactivation, similar to L-type calcium currents. The T-type calcium channel blocker mibefradil inhibited both types of currents at concentrations between 10 nM and 1 μ M. The higher concentrations blocked the delayed rectifier potassium current. Furthermore, a study has shown that mibefradil interferes with myoblast fusion, suggesting that this drug exerts this effect by combined inhibition of L- and T-type calcium channels and a delayed rectifier potassium channel, an HERG potassium channel and an inward rectifying potassium channel [Liu et al. 2001].

In resume, these results show that in HLE-B3 cells 2 types of calcium channels are expressed (L- and T-type) beside 4 types of potassium channels (Kv2.2, Kv1.4, Kv1.5 and Kv1.6). At low concentrations, mibefradil blocks calcium inward currents ($IC_{50} \sim 10$ nM) and at higher concentrations ($IC_{50} \sim 2$ μ M) this substance blocks potassium outward currents. These results suggest that the impairment of hLEC adhesion by mibefradil, as in previous cell culture experiments [Beck et al. 2001, Nebe et al. 2004], may be related to

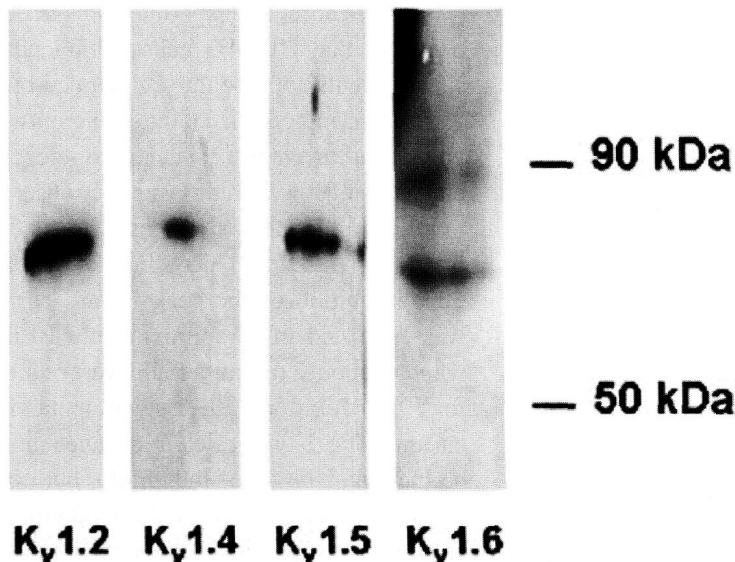


Figure 8. Expression of voltage-gated potassium channels in human lens epithelial cells. Western blot analysis was performed with membrane lysates of HLE-B3, detected with antibodies against Kv1.2, Kv1.4, Kv1.5 and Kv1.6.

block of ion channels. However, the question remains if the inhibition of ion currents by mibefradil is sufficient to explain its apoptotic/antiproliferative action. It is also conceivable that this drug simultaneously affects signal receptors or directly destroys cell structures crucial for cell adhesion. Because different apoptotic pathways exist, a multiple interference of a pharmacological substance with cells is very likely. The investigation of those multiple pathways have to be addressed in further experiments.

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