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Anja Meissner, Thomas Noack

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Proliferation of human lens epithelial cells (HLE-B3) is inhibited by blocking of voltage-gated calcium channels

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Abstract Calcium, as an integral part of a large number of cellular regulatory pathways, is selective in the control of specific cell functions like the start of G1 phase in cell cycle. Cell proliferation has been suggested to depend on increasing intracellular calcium levels. A major regulatory pathway for intracellular calcium is the calcium influx into the cell via voltage-gated calcium channels. T-type and L-type calcium channels are substantially present in human lens epithelial cell (hLEC), and total calcium currents are inhibited by mibefradil. Here, the hypothesis was tested if calcium influx via Ca_v channels regulates proliferation in epithelial cells. Cell proliferation was determined by cell culture assays using the L- and T-type Ca_v channel blockers mibefradil and verapamil as modulators for calcium influx. Calcium influx was investigated using the Manganese quench technique. Western blot experiments were accomplished under standard conditions using antibodies against MAPK 3. Mibefradil as well as verapamil impaired cell proliferation, but in different concentration ranges. Furthermore, the activation of MAPK 3 was reduced by both antagonists. Calcium influx was also reduced in the presence of both blockers. We conclude that the transmembrane influx of Ca^{2+} through Ca_v channels contributes to the regulation of hLEC proliferation, identifying Ca_v channel blockers as potential therapeutic substances in ocular diseases.

Keywords Voltage-dependent calcium channel · Ca^{2+} influx · T-type calcium channel · Protein phosphorylation · Epithelial cell

A. Meissner (✉) · T. Noack
Institute of physiology, University of Rostock,
Gertrudenstrasse 9,
Rostock 18055, Germany
e-mail: anja.meissner@web.de

Introduction

Posterior capsule opacification (PCO) is the most common complication of cataract surgery with an incidence of 35%. It is mainly caused by proliferation and migration of lens epithelial cells remaining in the capsule bag after cataract surgery [1]. As one of the main causes of PCO, proliferation could be a possible target in the prevention of PCO [2].

Cell proliferation has been suggested to depend on increasing intracellular calcium levels. Calcium is an integral part of a large number of cellular regulatory pathways and mandatory for the control of specific cell functions [3]: Calcium release from internal stores mediated by inositol-3-phosphat (InsP3) marks the start of the G_1 phase in cell cycle [2, 3]. Calcium also plays important roles in the differentiation program of keratinocytes by accumulating the products of calcium-activated genes as well as calcium-activated enzymes for proliferation and differentiation [3]. Thus, intracellular calcium levels are important for differentiation and proliferation [4].

Voltage-dependent calcium channels (Ca_v) are important routes for calcium influx into cells in response to membrane depolarisation regulating intracellular processes like contraction, secretion and gene expression in many different cell types [5, 6]. Five classes of Ca_v have been described based on their biophysical and pharmacological properties: L-, T-, N-, R- and P/Q-type calcium channels. High-voltage-activated calcium channels, like L-type Ca_v (Ca_v 1.1, 1.2, 1.3 and 1.4), require a strong depolarisation for opening, whereas low-voltage-activated (T-type) calcium channels (Ca_v 3.1, 3.2 and 3.3) require only weak depolarisation [6, 7]. L-type calcium channels have properties different from those of T-type channels: They activate at lower voltages, the currents are long lasting and can be blocked by dihydropyridines, phenylalkylamines and benzothiazepines. They are expressed in neurons and cardiac pacemaker cells and form the main calcium current in muscles [8, 9].

The expression of L- and T-type Ca_vs has been reported to change during various biological events, including differentiation and proliferation [10, 11]. Several studies suggested that T-type calcium channels are frequently expressed in proliferation phases [10, 12]. Kuga et al. demonstrated a cell-cycle-dependent expression of Ca_v: L-type calcium channels were expressed in all phases of the cell cycle, whereas T-type calcium channel expression was increased in proliferating cells. It has also been shown that some cells express only one type of Ca_v [11], whereas a large number of cells express both channel types [6].

In conclusion, calcium is an important regulator of intracellular events governing cell growth and differentiation. A major regulatory pathway for intracellular calcium is the calcium influx into the cell via voltage-gated calcium channels, the expression of which has been shown to be cell-cycle-dependent in several cells [10, 12]. Previous results have shown that the electrical properties of freshly isolated human lens epithelial cells were comparable to those, measured in HLE-B3 cells [13]. Although the existence of different Ca_v channels as well as the ability of substances like mibefradil and verapamil to block those channels concentration-dependently has been shown in human lens epithelial cells [13, 14]; their role in proliferation, one of the main causes of posterior capsule opacification, is unknown. Therefore, the hypothesis was tested that the modulation of calcium influx via Ca_v channels regulates proliferation in human lens epithelial cells.

Materials and methods

Cell culture

HLE-B3 cells were purchased from American Type Culture Collection (ATCC, Rockville MD). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) and 1% Gentamycin (GIBCO, Grand Island, NY) in a humified incubator supplied with 5% CO_2 at 37°C. Cells were passed at 10^6 cells seeding density.

Calcium channel modulators

T- and L-type calcium channel blocker mibefradil (Hoffmann La Roche, Basel, CH) and L-type calcium channel blocker verapamil (Sigma, St. Louis, MO, USA) were prepared in stock solutions containing 0.5% dimethyl sulfoxide (DMSO). BAY K 8644 (Sigma) was solved in DMSO.

Immunoblot

For isolation of membrane proteins, confluent HLE-B3 cells were solubilised 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 μg/ml protease inhibitor cocktail (Roche, Basel, CH) were added. Protein concentrations of supernatants were determined using a kit based on the Bradford method (Biorad, Hercules, CA, USA). Membrane proteins were separated by polyacrylamide gel electrophoresis. After protein transfer to polyvinylidene fluoride membranes (Amersham, Buckinghamshire, UK), the membranes were incubated with primary antibodies overnight at the following dilutions: anti-Ca_v3.1, anti-Ca_v3.3, anti-Ca_v1.2, anti-Ca_v1.3 (Alomone, Jerusalem, Israel) 1:200, anti-pERK1/2, anti-pERK1/2 and anti-p105 (Biomol, Hamburg, Germany) 1:1,000. For detection, peroxidase-conjugated anti-rabbit IgG (Amersham) was employed at a dilution of 1:40,000. The X-ray films were evaluated by scanning densitometry; the mean values of the mass peaks in control conditions and in the presence of the blockers were compared, and paired Student's t tests were performed. A level of $p<0.05$ was considered to be statistically significant.

Patch clamp experiments

Patch clamp experiments were performed in whole-cell configuration at room temperature. Cells were placed in perfusion chamber mounted on the stage of an inverted microscope in bath solution containing 141 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 10 mM glucose (pH 7.4). Patch pipettes with resistance 3–5 MΩ 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 succinate, 5 mM pyruvate and 1.2 mM $MgCl_2$ for calcium currents and 5 mM NaCl, 120 mM KCl, 10 mM HEPES, 11 mM glucose, 5 mM oxalacetate, 5 mM succinate, 2 mM pyruvate, 1.2 mM K-EGTA, 1.2 mM $MgCl_2$ and 1.2 mM K_2HPO_4 for potassium currents. Low passed filtered signals were digitised at 5 kHz with Axolab 1100 hardware and a microcomputer in conjunction with pClamp 8.1 software (Axon Instruments, Sunnywale, CA, USA).

Polymerase chain reaction

RNA from HLE-B3 cells was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). The RNA in the upper phase was precipitated with isopropanol and washed with

70% ethanol. RNA pellets were resuspended in RNase-free water. Complementary DNA synthesis was performed using a RT-PCR kit (Invitrogen). PCRs were performed with DNA oligonucleotides (Molbiol, Berlin, Germany) for the different L- and T-type Ca_v channels. The PCR products were separated on a 1.5% agarose gel and were analysed with BIOMETRA software.

Proliferation assay

Aliquots of 10^5 cells were suspended in 2 ml of DMEM medium supplemented as described above and allowed to adhere in individual wells of six-well cell culture plates. They were grown in the absence of any modulator for 48 h. Culture media was changed, and calcium channel modulators were added in different concentration ranges for 24 h. For analysis, cells from individual wells were detached from the plastic surface in 0.5 ml of 0.05% trypsin/0.02% EDTA (Biochrom, Berlin, Germany) for 2 min at 37°C before 1.5 ml of medium was added to restore the original volume. Aliquots of cell suspensions were counted using a Thoma chamber, and numbers of cells were extrapolated to the total volume of the culture.

Soft agar assay

A working solution (0.6%) of agar noble (DIFCO) and culture media was prepared, and 500 µl were filled in each well of a 24-well-plate. Cells were counted, a defined cell number was gently mixed with 500 µl agar working solution, and the polymerised bottom was coated with the cell suspension. After polymerisation, 500 µl culture media was added to each well. After 10 days, the cells were incubated with different calcium channel modulators. After the incubation period of 24 h, 10 µl of MTT solution (10 mg/ml) was added for 1 h and the cells were counted.

Measurement of cellular vitality

Cells were seeded on poly-lysine (Sigma) coated coverslips for 24 h, culture media was removed and replaced by physiological solution (PSS), and either 1 µM acridine orange solution (Sigma) for 1 h at 37°C and 5% CO_2 for vital cells or 10 µl (1 mg/ml) propidium iodide (Sigma) for 30 min at 37°C and 5% CO_2 for apoptotic cells was added. Acridine orange was excited at a wavelength of 420 nm, and the fluorescent signal was acquired at a wavelength of 520 nm. Propidium iodide was excited at a wavelength of 536 nm, and the fluorescent signal was acquired at a wavelength of 617 nm. Signals of vital or necrotic cells were recorded over 6 h with or without adding calcium channel modulator.

Calcium measurement

Cells were seeded on poly-L-lysine (Sigma) coated coverslips for 24 h, and culture media were removed and replaced by PSS (10 mM HEPES, 10 mM glucose, 4.7 mM KCl, 1.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 141 mM NaCl; pH 7.4) and 1 µM Fura-2 AM (Invitrogen) for 1 h at 37°C and 5% CO_2. Changes in intracellular calcium levels were measured by fluorescence microscopy with excitation wavelengths of 340 and 380 nm; the fluorescent signal was acquired at an emission wavelength of 520 nm by FELIX software. The standard procedure for calculating $[Ca^{2+}]$ from dual wavelength measurements of Fura-2 fluorescence involves the use of the following equation.

$$\left[Ca^{2+}\right] = K_d - \left[(R - R_{min})/(R_{max} - R)*\beta\right.$$

R is the ratio of the background corrected fluorescence of the sample at 340 and 380 nm; R_{max} and R_{min} represent the ratios for Fura-2 at the same wavelengths in the presence of saturating Ca^{2+} and in nominally zero Ca^{2+}, respectively; β is the ratio of fluorescence of Fura-2 at 380 nm in zero and saturating Ca^{2+}; K_d is the dissociation constant of Fura-2 for Ca^{2+}, assumed to be 224 nM at 37°C. An important assumption in this procedure for $[Ca^{2+}]$ calculation is that there are only two forms of fura-2 associated with the cell: Fura-2 bound to and free of Ca^{2+}.

Calcium influx was investigated using manganese chloride, which quenches the calcium signal by binding to Fura-2. Fluorescence was measured at the isobestic wavelength of Fura-2 (360 nm); the decrease of fluorescence shows the influx of manganese into the cell and is a qualitative indicator for calcium influx.

Statistics

All data are presented as mean±SEM unless otherwise stated. The concentration response curves were obtained by fitting the equation $y=100-(100 - y_{max})*[x^n/(x^n + EC50^n)]$ to the data points. Max is the highest concentration of the blocker added in every experiment. X represents each blocker concentration and n is the smallest concentration of the blocker added in each experiment. After fitting the results of every experiment, a mean for EC_{50} was calculated. The equation was calculated with all determined parameters. Direct comparisons between mean values in control conditions and in the presence of the blockers were performed by analysis of variance and the Bonferroni post hoc test. A level of $p<0.05$ was considered to be statistically significant.

Results

L- and T-type Ca_v channels are present in human lens epithelial cells

For the investigation of the effect of different calcium channel blockers on proliferation and vitality, at first, the possible existence of L- and T-type Ca_v channels in HLE-B3 cells was evaluated. Figure 1 shows the results of patch clamp experiments, PCR experiments and Western blot analyses in HLE-B3 cells.

Calcium currents in HLE-B3 cells were measured using patch clamp technique. Pipettes were loaded with caesium ions (instead of potassium ions) to inhibit larger parts of potassium outward currents. Cells were investigated using the whole-cell configuration. From holding potentials of −90 mV, cells were depolarised for 500 ms to different test potentials starting at −80 mV and in increments of 10 mV (14 steps). Typical current recordings are shown in Fig. 1a. At test potentials positive to −50 mV, an inward current which rapidly activated and inactivated was observed. This current was completely inactivated after 20 ms, its amplitude had a maximum between −20 and −10 mV. At more positive test potentials, a much slower activating and inactivating inward current was observed. The time course and voltage dependency of the first current component was similar for T-type calcium channel currents; the characteristics of the latter were similar to those of L-type calcium channel current. Larger depolarisations exhibited a third component which had characteristics of caesium current flow through voltage/time-dependent potassium channels. In a former study, we already described the calcium currents existing in HLE-B3 cells and native tissue as well as the inhibition of L- and T-type currents by calcium channel antagonists mibefradil and verapamil [14]. We found out that with mibefradil, the T- and L-type channel antagonist had strong concentration-dependent inhibitory effects on the inward currents; the substance was able to block T-type Ca_vs and at 100-fold higher concentrations and also L-type currents in our cell system.

The existence of the different L- and T-type channels was additionally determined by PCR amplification using proliferating HLE-B3 cells (passages 8–12). As shown in Fig. 1b, proliferating HLE-B3 express three different T-type Ca_v channels (Ca_v 3.1, 3.2 and 3.3) besides only one L-type Ca_v channel (Ca_v 1.2). To check the hypothesis that T- and L-type Ca_v channels are differently expressed depending on their growth behaviour, we investigated their expression in two proliferation stages (passages 9 and 16). In HLE-B3 cells, data suggest that proliferation changes in a cell-passage-dependent manner, which means that cells in passages 1–14 showed normal growth behaviour, whereas the growth of cells in passage 15 and 16 remained static. Further evidence was conducted from the different expression

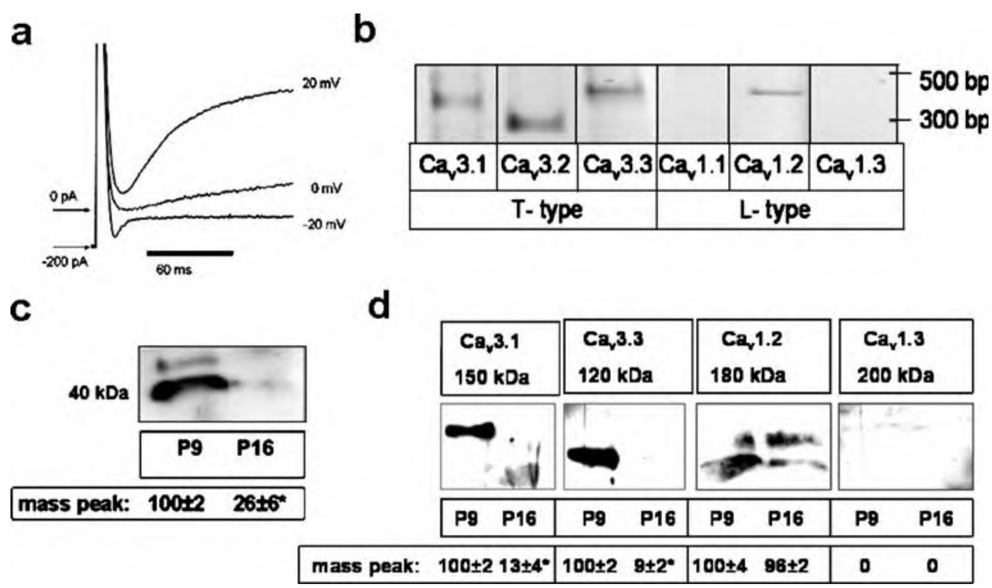


Fig. 1 Expression of voltage-gated calcium channels (*Cav*) in human lens epithelial cells (*HLEB3*). **a** Current traces elicited by voltage steps in an HLE-B3 cell, loaded with caesium ions, as indicated. The different time courses and activation thresholds indicated the existence of a least three different time/voltage-dependent current components (calcium T-type, L-type and caesium through Kdr channels). Note that neither background nor leakage currents were subtracted. **b** PCR experiment showing the expression of different L- and T-type calcium channels in HLE-B3 (n=3). **c** Western blot with antibody against proliferation-associated antigen (*p105*) in cells in passage 9 (*P9*) and cells in passage 16 (*P16*; n=3). **d** Western blot with antibodies against C_{av} 3.1 and 3.3 (T-type) and C_{av} 1.2 and 1.3 (L-type) in HLE-B3 cells in passage 9 (*P9*) and passage 16 (*P16*). C_{av} 3.2 and 1.1 were not investigated in Western blot experiments because no antibodies were provided from ALOMONE Labs. Western blot data are expressed as mass peak (*m.p.*) determined by scanning densitometry (n=5; *p<0.05)

of the proliferation associated antigen p105, which can be found either in a dimeric form (105 kDa) or monomeric form (41 kDa). Only the monomeric form of the protein was found in HLE-B3, and its expression was markedly depressed in HLE-B3 in passage 16: The expression of the proliferation associated antigen (p105) decreased from 100±2 (n=3) in cells of passage 9 to 26±6 (n=3; p<0.05) in cells of passage 16 (Fig. 1c). To check the hypothesis that T- and L-type Ca_v channels are differently expressed in well proliferating cells and cells in which growth remained static, Western blot experiments were performed. The expression of the L-type channel (Ca_v 1.2) seems to be similar in both states of growth, whereas the protein level of Ca_v 3.1 and 3.3 (T-type) changes during aging of the cells (Fig. 1d): The expression of Ca_v 3.1 decreased from 100±2 in passage 9 to 13±4 in passage 16 (n=3; p<0.05), and the expression of Ca_v 3.3 was reduced to 9±2 in passage 16 (n=3; p<0.05), whereas the expression of L-type calcium channel Ca_v 1.2 did not change significantly within the different states of growth. The mass peak of 92±4 in passage 9 and the mass peak of 98±4 (n=3; p=0.83) in passage 16 show that the protein levels remained the same in both states of growth. Further experiments were performed using cells from low passage numbers showing under control conditions a high grade of proliferation to make sure that both, L- and T-type channels, are expressed in the cell model.

Mibefradil and verapamil inhibit proliferation

To examine the effect of mibefradil and verapamil on the growth behaviour of HLE-B3 cells, we performed proliferation assays in cell culture. The dose–response curve in Fig. 2a shows that mibefradil and verapamil inhibited cell proliferation in a dose-dependent manner. The EC_{50} value for mibefradil was $1.0 \pm 0.2 \times 10^{-7}$ M (n=10; p<0.05), whereas verapamil inhibited proliferation in the same cells with an EC_{50} value of $2.7 \pm 0.7 \times 10^{-6}$ M (n=10; p<0.05). As mibefradil and verapamil strongly inhibited cell proliferation, we investigated their effects on cellular vitality using MTT staining to make sure that the investigated effects are not due to cytotoxic effects. MTT assay is a

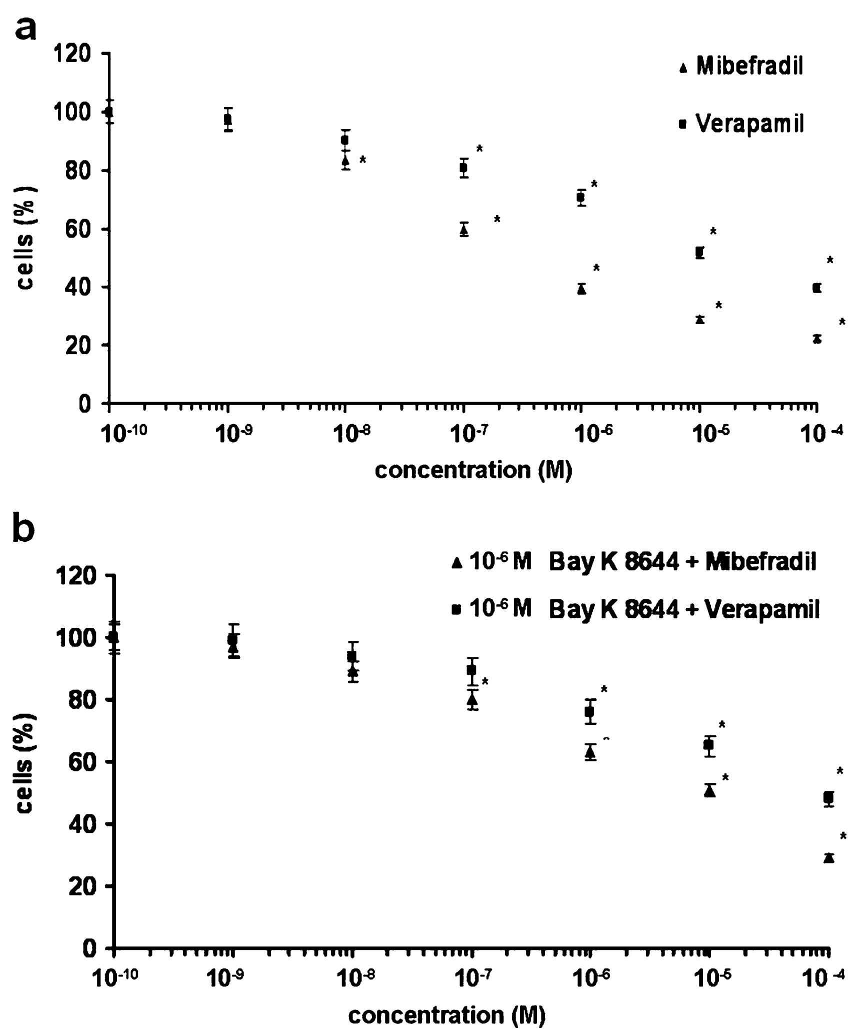


Fig. 2 Influence of mibefradil and verapamil on growth of HLE-B3 cells. Concentration response curves for the effect of mibefradil and verapamil (**a**) and the antagonists together with 10^{-6} M Bay K 8644, a calcium channel agonist (**b**), on cell number. The number of cells was normalised with respect to control (100%). The curves were obtained by fitting the equation $y = y_{max} \times [x^n/(x^n + EC50^n)]$ to the data points. **a** EC_{50} value of mibefradil is $1 \pm 0.2 \times 10^{-7}$ M (n=10; p<0.05), whereas EC_{50} value of verapamil is about $2.7 \pm 0.7 \times 10^{-6}$ M (n=10; p<0.05). **b** After an incubation with 10^{-6} M of the calcium channel agonist Bay K 8644, only 20% of the cells were affected by concentration of $1 \pm 0.2 \times 10^{-7}$ M mibefradil and 38% of cells were affected by concentrations of 10^{-5} M verapamil

laboratory test and a standard colorimetric assay for determining cellular proliferation. Yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore, conversion can be directly related to the number of viable cells. At the concentration, mentioned above, mibefradil inhibited 50% of cellular growth; the antagonist reduced the vitality of HLE-B3 only about 24±6% (data not shown). Verapamil had similar effects on the vitality of HLE-B3 cells. At 10^{-6} M verapamil, cellular vitality was reduced to 23±3%.

The L-type calcium channel agonist Bay K 8644 was shown to be a potent activator of calcium influx and to affect proliferation [15]. When HLE-B3 cells were incubated with 10^{-6} M Bay K 8644 for 24 h, an increase in cellular growth was monitored: The cell number increased about 20±8% compared to untreated cells. In addition to the incubation with the different calcium channel antagonists, the inhibitory effect on cellular growth of both, mibefradil and verapamil, was reduced in the presence of Bay K 8644 (Fig. 2b). At a concentration of 10^{-6} M, mibefradil alone inhibited cell growth about 79±3%; the blocker reduced cell growth by only 29±6% (n=6; p<0.05) in the presence of 10^{-6} M Bay K 8644. The effect of Bay K 8644 on verapamil-induced growth inhibition was more pronounced. At a concentration of 10^{-6} M, verapamil alone inhibited about 44±6% of cell growth; the blocker reduced cell growth by only 19±4% (n=6; p<0.05) in the presence of 10^{-6} M Bay K 8644. Cellular vitality was also checked under Bay K conditions: The co-incubation with Bay K 8644 also affected cellular vitality by reducing the effect of Mibefradil: At 10^{-6} M mibefradil, cellular vitality was reduced to 24±6%, whereas mibefradil in the presence of 10^{-6} M Bay K 8644 reduced it only about 16±3% (data not shown). Bay K 8644 showed the same effects in verapamil-incubated cells.

To mimic tissue-like cell structures, soft agar assays were performed. There, cells were incubated with different calcium channel inhibitors in the same concentration ranges and spaces of time they affected proliferation behaviour. As shown in Fig. 3, the total number of cells decreased with rising concentrations of the blocker substances: Under control condition, a confluent cell layer is visible and 52±6 (n=5) cells per visual field were visible. After treatment with 10^{-7} M mibefradil, the number of cells per visual field decreased to 29±3 (n=5; p<0.05), and in the presence of 10^{-6} M mibefradil, the number of cells was reduced to 21±4 (n=5; p<0.05; Fig. 3a). The experiments with verapamil were performed under the same conditions: 10^{-7} M verapamil reduced the number of cells in the visual field from 52±6 (n=5) to 49±2 (n=3; p<0.05), 10^{-6} M

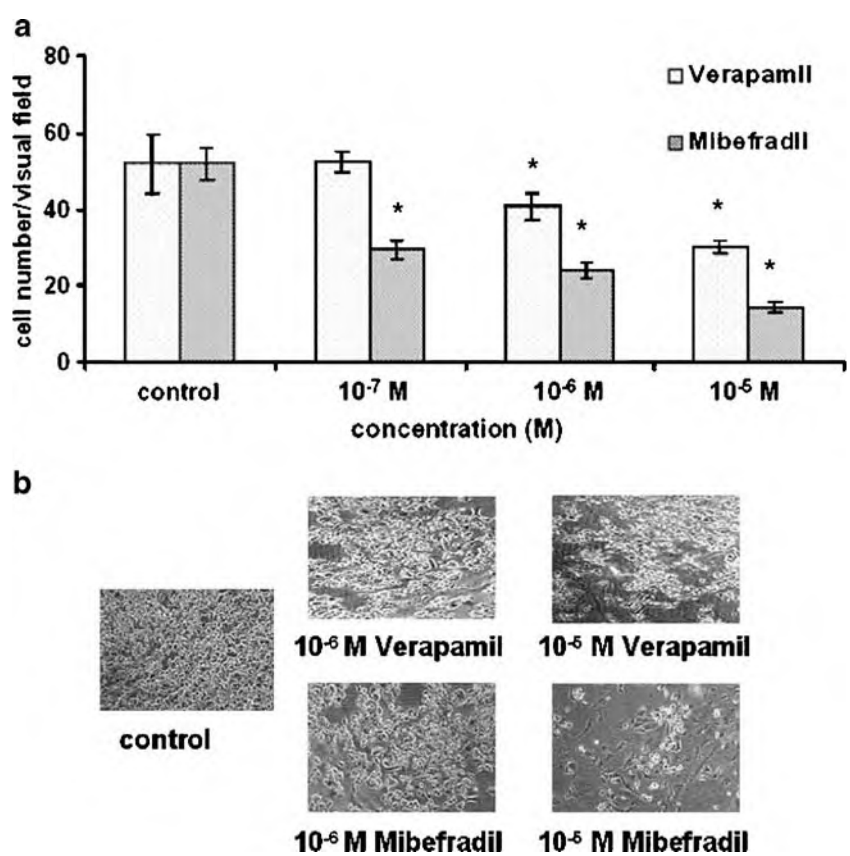


Fig. 3 Influence of mibefradil and verapamil on cell growth of HLE-B3. **a** Analysis of counted cells per visual field after staining with MTT solution in the presence of different concentrations of mibefradil and verapamil (n=5). **b** Original photographs of cells in control and in the presence of different blocker concentrations representative for this kind of experiment

verapamil to 42±3 (n=3; p<0.05) and 10^{-5} M verapamil to 37±5 (n=3; p<0.05; Fig. 3b). The calcium channel agonist Bay K 8644 was also tested in these experiments. The same effect on cellular growth as shown in the proliferation assays was monitored under this conditions. Bay K 8644 increased the number of HLE-B3 cells in contrast to untreated cells and reduced the effects of the calcium channel antagonists (Fig. 3a and b).

To obtain additional evidence for the effect of mibefradil and verapamil on the growth behaviour of HLE-B3, we studied their effect on the phosphorylation of ERK1/2. Phosphorylation of mitogen-activated protein kinase 3 (ERK) leads to activation of cyclin D1, which promotes cell cycle in proliferating cells. In proliferating HLE-B3 cells, ERK (p42 and p44) is active; ERK phosphorylation decreased in mibefradil- and verapamil-treated cells. Figure 4a shows the inactivation of MAPK 3 by treatment with 10^{-7} M, 10^{-6} M and 10^{-5} M mibefradil and verapamil (24 h), respectively (n=5). In the presence of mibefradil, a stronger inhibitory effect was observed compared to verapamil-treated cells. Mibefradil (10^{-6} M) reduced the mass peaks, showing the phosphorylation intensity from 80±4 (p44) and 110±2 (p42; n=5) to 54±6 (p44) and 16±2 (p42; n=5; p<0.05). Verapamil reduced the mass peaks at the same concentration to 67±6 (p44) and 54±2 (p42; n=5; p<0.05). In this regard, other protein kinases, which were described to be involved in proliferation processes and seem to be upstream activators of ERK1/2 [16–18] were examined due to their phosphorylation after applying the calcium channel antagonists mibefradil and verapamil. As shown in Fig. 4b, the mass peaks of Raf-1 and CaMKII were reduced after incubating HLE-B3 cells with different concentrations of mibefradil: 10^{-7} M mibefradil reduced the mass peaks, showing the phosphorylation intensity from 120±6 (Raf-1) and 100±2 (CaMKII; n=5) to 54±4 (Raf-1) and 62±2 (CaMKII; n=5; p<0.05). Higher concentrations reduced the mass peaks of both kinases abundantly clear. The L-type antagonist verapamil had similar effects, but at higher concentrations (data not shown). In addition, the gene expression level of the proliferation associated antigen (p105), a marker for cell growth, was determined (Fig. 4c). The mass fits show that the expression level decreased after application of 10^{-6} M mibefradil from 112±5 (n=3) to 32±4 (n=3; p<0.05); also by incubating the cells with 10^{-6} M verapamil, the mass peaks were reduced from 112±5 (n=3) to 45±6 (n=3; p<0.05).

To make sure that the previously shown effects of mibefradil and verapamil are not due to cytotoxic actions, fluorescence microscopy using dyes for staining vital and death cells were performed. Propidium iodide (PI) is membrane-impermeant and generally excluded from viable cells. Acridine orange (AO) itself, as a membrane permeable vital staining dye, allows the differentiation of live

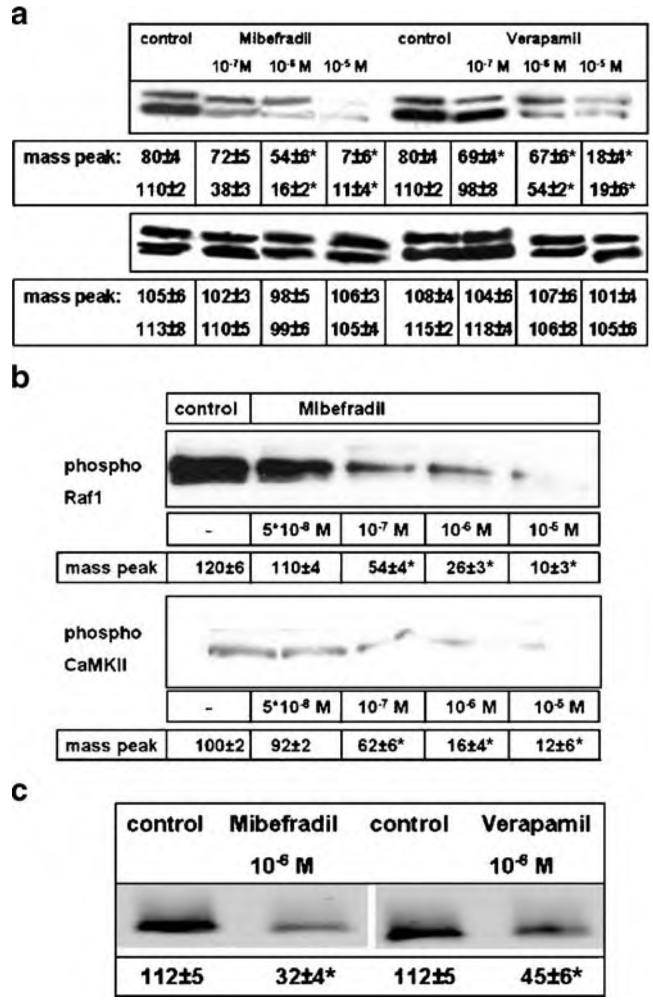


Fig. 4 Influence of mibefradil and verapamil on expression of proliferation associated proteins by: **a** Western blot of extracellular regulated kinase (*ERK1/2*) in the presence of different concentrations of mibefradil and verapamil (n=5). **b** Western blot CaMKII and Raf-1 in the presence of different concentrations of mibefradil and verapamil (n=5). **c** Western blot experiments of proliferation-associated antigen (*p105*), a marker for cell growth, in the presence of mibefradil and verapamil (n=3). Data are expressed as mass peak (*m.p.*) determined by scanning densitometry (n=3; *p<0.05)

cells from those undergoing apoptosis. As shown in Fig. 5a, the AO fluorescence signal decreased depending on the exposure time and on the concentration of mibefradil. Measurements at each concentration were performed with an untreated sample of cells which were exposed to the antagonist either 60 or 360 min: After incubating the cells 60 min with 10^{-6} M mibefradil, the signal was reduced about 17±5% (n=5; p<0.05). Applying this concentration over 360 min, the signal decreased about 49±6% (n=5; p<0.05). Compared to the decrease after applying 10^{-6} M mibefradil, the reduction of the AO signal after incubation with 10^{-7} M mibefradil for 360 min appeared small and possibly negligible. More than ever, you have a look at the

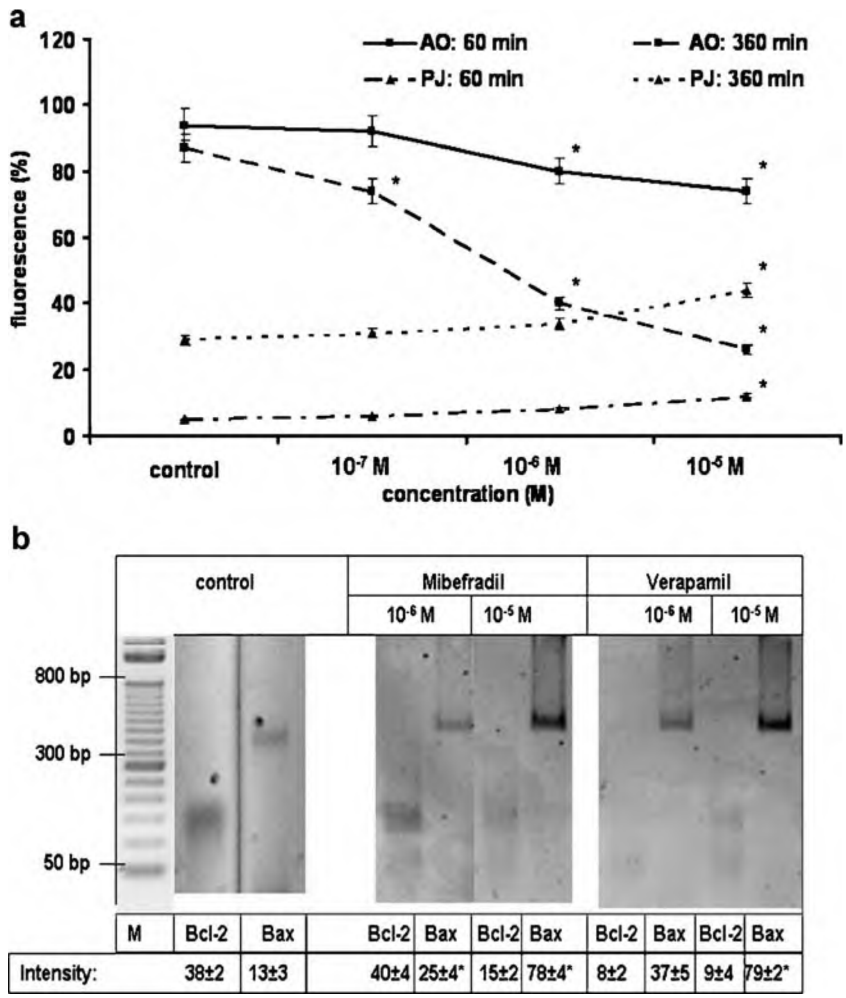


Fig. 5 Influence of mibefradil on cellular vitality. Fluorescence microscopy using AO and PI as dyes for staining either viable or death cells were accomplished in using different concentration of the blocker for either 60 or 360 min apoptotic behaviour of HLE-B3 cells was tested by determining the expression levels of Bax and Bcl-2 in untreated cells and in cells incubated with different concentrations of Mibefradil. **a** Mibefradil reduced the signal of AO in mibefradil (10^{-6} M) treated HLE-B3 about 17±5% (n=5; p<0.05) after 60 min and about 49±6% (n=5; p<0.05) after 360 min. The PI signal increased about 16±6% (n=5; p<0.05) after an incubation with 10^{-6} M mibefradil for 360 min. **b** Bax and Bcl-2 expression levels shown after incubating HLE-B3 cells with different concentrations of mibefradil for 48 h

other two curves showing the PI fluorescence signals. The PI fluorescence signals were evaluated with fresh cell samples under comparable conditions as above for AO. The PI signal increased about 16±6% (n=5; p<0.05) after an incubation with 10^{-6} M mibefradil for 360 min and to 8±4% (n=5; p<0.05) after an incubation with 10^{-7} M mibefradil for 360 min. Furthermore, we checked out the expression levels of Bcl-2 and Bax after incubating the cells with different concentrations of mibefradil and verapamil (Fig. 5b). Figure 5b shows the increase of Bax expression, an indicator for cell death, and the decrease of the levels of Bcl-2, which is mainly accompanied with cellular growth. The Bax–Bcl ratio is a good marker for these events. Increased Bax/Bcl-2 ratio was shown to up-regulate caspase-3 and increases apoptosis [19]. Whereas control cells showed ratios of 0.31±0.08 (n=5), HLE-B3 cells treated with 10^{-5} M mibefradil showed ratios of 5.17±0.42 (n=5; p<0.05). HLE-B3 cells incubated with only 10^{-6} M mibefradil had Bax/Bcl-2 ratios of 0.59±0.06 (n=5; p=1.24). The ratios after treating the cells with 10^{-6} M verapamil or 10^{-5} M verapamil rose up to either 4.46±0.23 (n=5; p<0.05) or 8.57±0.19 (n=5; p<0.05) compared to untreated cells.

Mibefradil decreases calcium inward movements

To show the role of calcium channels (L- and T-type Ca_v) for calcium influx and homeostasis in HLE-B3 cells, calcium imaging using Fura-2 was performed. Calcium influx was investigated using the manganese quench technique. As illustrated in Fig. 6a, calcium influxes decreased when mibefradil was added to the extracellular media. Under control conditions, the addition of manganese quenched the fluorescence signal rapidly: After 1 min, fluorescence was reduced to 57±3% (n=3), after 4 min to 21±4% (n=3). However, in the presence of 10^{-5} M mibefradil, the signal decreased only to 67±2% (n=3; p<0.05) after 1 min and to 50±4% (n=3; p<0.05) after 4 min. Verapamil also impaired the calcium influx, but less than mibefradil (Fig. 6b): In the presence of 10^{-5} M verapamil, the signal decreased to 62±2% (n=3; p<0.05) after 1 min and to 43±3% (n=3; p<0.05) after 4 min.

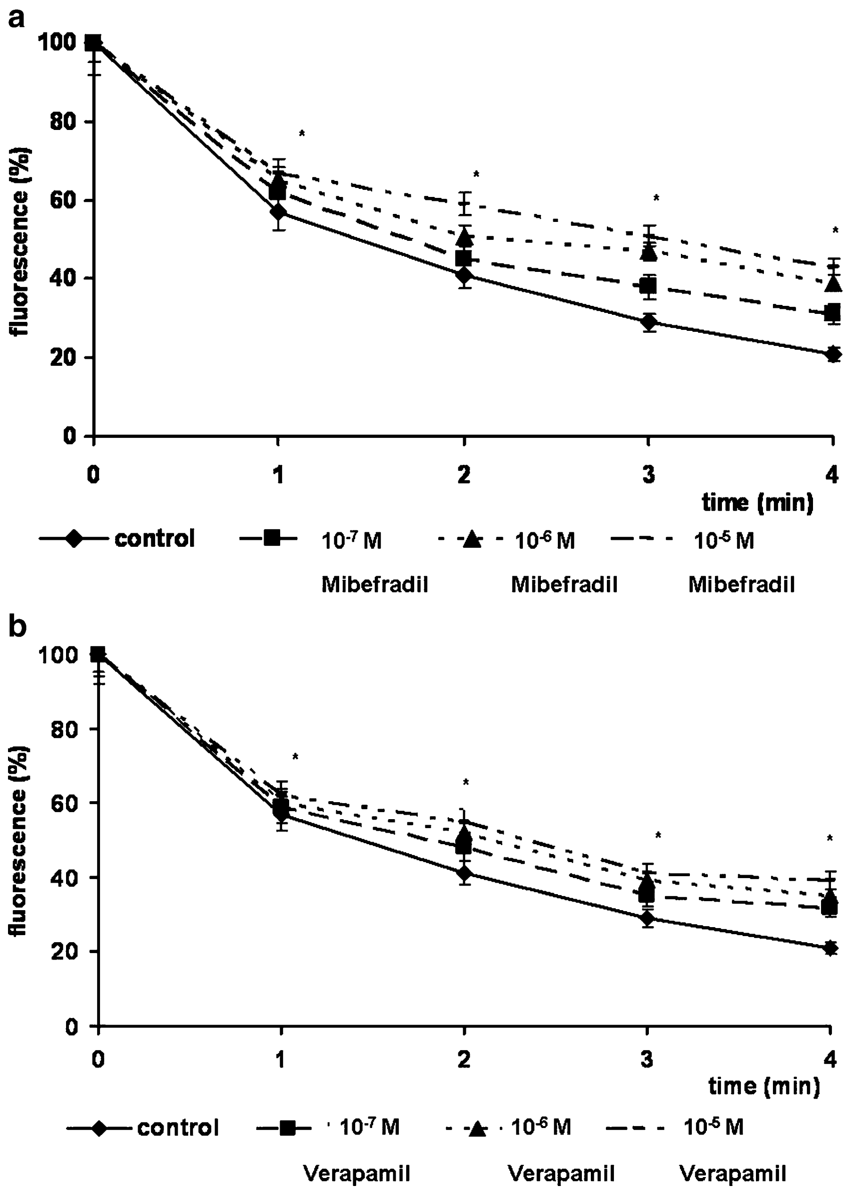
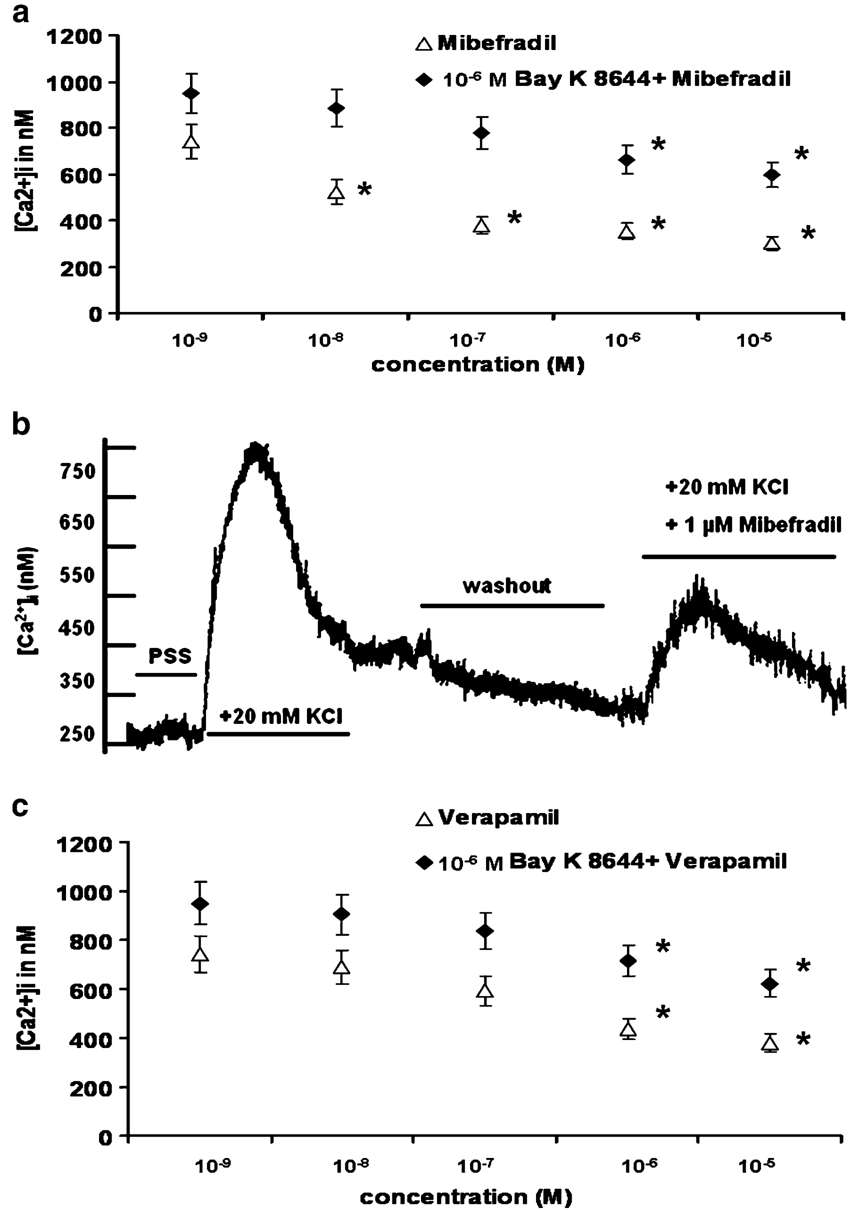


Fig. 6 Influence of mibefradil and verapamil on calcium influx. The time-dependent change of fluorescence is shown in response to manganese at different concentrations of mibefradil (**a**) and verapamil (**b**)

To study changes in the intracellular calcium concentrations resulting from calcium influx, 20 mM KCl was added to the extracellular media to induce membrane depolarisation, activation of voltage-gated calcium channels and a subsequent influx of extracellular calcium.

HLE-B3 cells responded to 20 mM KCl with an increasing calcium concentration as illustrated in Fig. 7a. Under resting conditions, intracellular calcium was 250± 50 nM (n=6). In response to KCl, intracellular calcium concentrations rose up to 750±50 nM (n=6; p<0.05). Mibefradil inhibited the KCl-induced calcium change in a dose-dependent manner; at a concentration of 10^{-7} M, the intracellular calcium rose up to only 400±50 nM (n=6; p< 0.05) in response to 20 mM KCl. Figure 7b shows a representative original registration of a Fura-2 signal under the described conditions: Under resting conditions, cells had an intracellular calcium concentration of about 250 nM. After applying 20 mM KCl, the signal rose up to approximately 850 nM. Mibefradil, as shown in this figure, is able to reduce this KCl-induced increase of intracellular calcium. The 10^{-6} M Bay K 8644-incubated cells reacted more sensitively to a depolarisation by KCl. The intracellular calcium concentration rose up to 950±50 nM (n=6; p<0.05) under these conditions, whereas a co-incubation with 10^{-7} M mibefradil decreased the response to 20 mM KCl to 800±50 nM (n=6; p<0.05). Verapamil also reduced the intracellular calcium concentrations; at a concentration of 10^{-7} M verapamil, the intracellular calcium rose up to only 625±50 nM (n=6; p<0.05) in response to 20 mM KCl (Fig. 7c).

Fig. 7 Influence of mibefradil and verapamil on KCl-induced alteration of intracellular calcium concentrations. Concentration-dependent changes of the fluorescence are shown in the presence of **a** mibefradil with or without application of 10^{-6} M Bay K 8644 and in the presence of **c** verapamil with or without application of 10^{-6} M Bay K 8644. An original representative registration of a Fura-2 signal (calculated to the absolute intracellular calcium concentrations) in response to 20 mM KCl and the reduced response to this depolarisation after applying mibefradil is shown in **b**



To examine to what extent store-operated calcium channels (SOCs) are involved in the raise of the intracellular calcium level after depolarisation of the membrane, 5×10^{-7} M 2-APB were applied to the extracellular media. 2-APB did not significantly reduce the KCl-induced change of the intracellular calcium level, which supposed that SOCs are not involved in the effect of KCl. The reaction of 2-APB-incubated cells did not significantly change in response to 20 mM KCl; intracellular calcium level rose up to 730±50 nM (n=3; p>0.05). Only after applying 100 nM mibefradil and 50 nM 2-APB in common did the intracellular calcium response change: The intracellular calcium level reached about 450±50 nM (n=3; p<0.05).

Discussion

In this study, we observed the influence of L- and T-type Ca_v channels on calcium influx, intracellular calcium concentration, proliferation and vitality in human lens epithelial (HLE-B3) cells by blocking these channels with two different calcium channel antagonists. Our findings show that HLE-B3 cells express both L- and T-type calcium channels (Ca_v 3.1, 3.2, 3.3 and Ca_v 1.2). We ascertained that T-type calcium channels (Ca_v 3.1 and 3.3) are only expressed in well proliferating cells, whereas the L-type calcium channel (Ca_v 1.2) is expressed also in slower growing cells. By blocking T- and L-type Ca_v via mibefradil and verapamil, a reduced calcium influx into the cells and a clearly decreased raise in intracellular calcium after membrane depolarisation was observed. Our results also show that mibefradil as well as verapamil have dose-dependent antiproliferative effects in HLE-B3 cells. Besides the reduction of the cell number, the activation of the extracellular activated kinase (ERK1/2) was also impaired by both blockers.

The ubiquitous intracellular second messenger Ca^{2+} is an integral part of several pathways controlling different phases of the cell cycle. Accordingly, cell proliferation has been suggested to depend on increases in intracellular Ca^{2+}. In many cell types, Ca^{2+} levels can be effectively controlled via regulation of transmembrane Ca^{2+} influx through L- and T-type voltage-gated calcium channels (Ca_v channels) [20, 21]. Our findings show that HLE-B3 cells express both types of voltage-gated calcium channels, and a large component of the Ca^{2+} influx into these cells is mediated by those channels. A possible connection between Ca^{2+} influx via L- and T-type channels and cellular growth behaviour seems to be the cell-passage-dependent expression of the T-type channels. HLE-B3 cells of a late passage (p16) showed a significantly decreased expression of the proliferation-associated nuclear antigen (p105), which is localised in interchromatin granules of the cell nucleus and whose level increases just before the entrance to S phase [22]. In the same cells, the expression of the T-type channels Ca_v 3.1 and 3.3 was significantly reduced. Although the biological role of T-type calcium channels in cells has yet to be fully described, the expression of these calcium channels has been implicated in both cell differentiation and proliferation [11, 23, 24]. T-type calcium channels seem to be preferentially expressed during development with a decrease in expression after differentiation [10, 12]. Therefore, a possible way to affect cellular growth in a variety of pathological phenomena could be the modulation of these T-type calcium channels.

There is a variety of agents that can affect L- and T-type Ca_v with different degrees of specificity. In this study, we used verapamil, a phenylalkylamin that inhibits L-type in general and does not inhibit T-type calcium channels at therapeutic concentrations, and mibefradil, a tetralol derivate that blocks T-type channels as well as L-type calcium channels, but the inhibition of T-type Ca_v was reported to be stronger than that of L-type Ca_v [25]. The calcium channel blocker mibefradil was described to show an approximately tenfold higher sensitivity to T-type than to other types of calcium channels [25, 26]. Although mibefradil was reported to interact with a variety of other ion channels, we can rule out these possibilities in the used concentration ranges. In several studies, the antagonist was described to block voltage-gated sodium channels, potassium channels and even chloride channels, but all effects occurred at 5- to 500-fold higher concentrations used to block Ca_v and cell proliferation [27–32].

With this regard, we tested the effect of Ca_v channels blockers on the transmembrane influx of Ca^{2+}, intracellular calcium concentration and growth behaviour of HLE-B3 cells. We could show a rapidly decreased influx of Ca^{2+} after incubation with mibefradil, whereas verapamil impaired the influx of the ion less than the T-type blocker. As shown in this study, there is a change in intracellular calcium levels in HLE-B3 cells, which is due to membrane depolarisation and activation of voltage-gated calcium channels. A blockade of T- and L-type Ca_v via mibefradil and verapamil showed a clearly decreased raise in intracellular calcium after membrane depolarisation. Several studies support these findings by determination of the involvement of Ca_v-induced Ca^{2+} influx and Ca^{2+} signalling in cancer cells [23, 33, 34].

As intracellular calcium levels seem to play an important role in cellular functions like proliferation and differentiation [35, 36] and the growth of tumour or non-tumour cells is mainly regulated by Ca^{2+} as second messenger [3, 37, 38], its ability to activate or inhibit a variety of cellular proteins could be a possible target to regulate cellular functions like proliferation and differentiation. Intracellular Ca^{2+} concentrations are regulated by various mechanisms including T- and L-type Ca_v channels. Several studies show that the expression of T-type Ca_v affects cell division [39], and others recommend that silencing of these channels blocks proliferation [36]. Our findings show that mibefradil as well as verapamil have dose-dependent antiproliferative effects in HLE-B3 cells. As 50% of proliferation was inhibited by 10^{-7} M of mibefradil, the decreasing calcium influx under these conditions could be a possible explanation for the findings. Whereas mibefradil was described to block calcium influx and tumour cell proliferation, in a variety of cells, verapamil also impaired both effects, but in a concentration beyond physiological range, which seemed to be cytotoxic [37]. The concentration ranges we used for our experiments were used as therapeutic doses, and the described effects cannot be addressed as cytotoxic. The results of fluorescence microscopic experiments with acridine orange and propidium iodide as well as the studies on Bax and Bcl-2 expression levels support this proposition, as both antagonists seemed to induce apoptosis in higher concentration ranges and longer incubation periods (48 h). Based on the findings that mibefradil blocks T-type calcium currents at concentrations of 10^{-7} M and L-type currents at higher concentrations as well as the matter of fact that the L-type channel blocker verapamil affected cellular growth only at much higher concentrations, we suggest that only a block of T- and L-type channels leads to an antiproliferative effect in HLE-B3 cells. The ability of mibefradil to block L-type channels seems to be an important fact in this context because HLE-B3 cells reacted less conspicuously to specific T-type channel antagonists like ethosuximide.

Extracellular activated kinase (ERK1/2), one of the most important pathways leading to proliferation or cell cycle arrest [40], was also affected by mibefradil and verapamil. The phosphorylation of this protein was reduced after application of both blockers in the same concentration ranges, as they inhibited proliferation in cell culture. Both antagonists were also able to reduce the phosphorylation of CaMKII and Raf-1, two important upstream regulators in the ERK1/2 signalling cascade. In pancreatic cancer cells, a pharmacological inhibition of RAF-MEK-ERK pathway also leads to cell cycle arrest [41]. As activation of ERK1/2 and JNK using calcium activation by depolarisation has already been demonstrated, Ca_v could have a very important role in the regulation of calcium-dependent cellular signalling. Our findings support the hypothesis that the modulation of cellular free calcium via Ca_v channels is an important regulator for proliferation, which seems to be mediated via the CaMKII, Raf-1 and ERK1/2 signalling pathway.

To find an additional evidence for the influence of Ca^{2+} and L- and T-type calcium channels, we observed the effect of the calcium channel agonist Bay K 8644 on cellular events like cell growth and vitality. It was shown that the agonist, which acts via L-type Ca_v [42], promotes cell growth and minimised the effects of both antagonists at the investigated EC_{50} values. Only higher concentrations of both blockers were able to reach similar effects on proliferation and vitality. That supports our suggestion that only L- and T-type Ca_v blockade leads to inhibition of proliferation and vitality.

In conclusion, we suggest that mibefradil, as a potent T-type calcium channel blocker, inhibits proliferation of HLE-B3 cells; this effect is possibly due to a common L- and T-type Ca_v blockade. Mibefradil could be a possible target as a therapeutic substance in the prevention of diseases due to proliferation processes like PCO. In this special case, an encapsulation of the substance in the capsule bag could be conceivable to inhibit the proliferation of lens epithelial cells remaining in the capsule bag after cataract surgery.

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