Anterior lens capsule as a tool to study the physiology of human lens epithelial cells

Sprednja lečna ovojnica kot orodje za študij fiziologije lečnih epitelnih celic pri človeku

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Abstract

Background: Anterior lens capsules excised during routine cataract surgeries are a potential source of the human lens epithelium. The purpose of the work was to check the possibility of using this material to study the physiology and pathophysiology of lens epithelial cells, mainly the homeostasis of the intracellular calcium concentration ($[Ca^{2}+]_i$) which serves as an universal indicator of cellular function.

Methods: Capsules were isolated during the cataract operations of patients of both genders aged between 35 and 98. Approximately a third of the capsules used were stained during the operation by gentian violet. We monitored the changes in $[Ca^{2}+]_i$ with the fluorescent dye Fura-2. The standard stimulus applied was acetylcholine. We measured the temporal and spatial changes of the Fura-2 fluorescence in epithelial cells in experiments done using the system consisting of the inverted fluorescent microscope, the computer controlled light filter changer and the cooled digital CCD camera.

Results: Lens epithelial cells typically responded to acetylcholine application with an increase in $[Ca^{2}+]_i$ expressed as the 360/380 ratio of fluorescence. Generally, the responses were strongest in capsules with best morphological appearance. Not all the cells responded simultaneously in acetylcholine responsive capsules. We observed linked responses of local cell networks. Both the capsules that were stained during the surgery with gentian violet and the non-stained ones responded to acetylcholine. In approximately ¼ of the capsules epithelial cells contracted in response to acetylcholine application.

Conclusions: We found that anterior capsules of the human lens isolated during the cataract surgery are an adequate source for the studies of lens epithelial cells function and can potentially provide important insights into the pathophysiology of the cataract.

Izvleček

Izhodišča: Operacije katarakte, pri katerih operater redno izreže sprednjo lečno ovojnico so lahko vir človeškega lečnega epitela. Namen našega dela je bilo ugotoviti ali je možno ta material uporabiti za študij fiziologije in patofiziologije lečnih epitelnih celic, predvsem homeostaze znotrajcelične koncentracije kalcija ($[Ca^{2}+]_i$), ki predstavlja splošni indikator delovanja celic.

Metode: Poskuse smo izvedli s ovojnicami izoliranimi pri operacijah katarakte bolnikov obeh spolov in starosti med 35 in 98 let. Približno tretjina uporabljenih kapsul je bila pri operaciji obarvana z barvilom gentiana violet. Spremembe $[Ca^{2}+]_i$ smo spremljali s pomočjo fluorescentnega barvila Fura-2, kot standardni dražljaj pa smo uporabili aplikacijo acetilholina. Poskuse v katerih smo merili časovne in prostorske spremembe fluorescence Fura-2 v epitelnih celicah smo izvedli na merilnem sistemu, sestavljenem iz invertnega fluorescentnega mikroskopa, računalniško krmiljenega menjalnika svetlobnih filtrov in hlajene digitalne CCD kamere.

Rezultati: Tipičen odgovor lečnih epitelnih celic na acetilholin je bil zvišanje $[Ca^{2}+]_i$ kar je izražalo razmerje fluorescenc pri ekscitacijah s 360 in 380nm. Na splošno so bili najmočnejši odgovori pri ovojnicah, ki so tudi morfološko izgledale najbolje. Pri vseh ovojnicah, ki so odgovorile na acetilholin, smo opazili, da ne odgovarjajo vse celice hkrati, opazili pa smo povezano odgovarjanje lokalnih omrežij celic. Na acetilholin so odgovarjale tako celice, ki so bile pri operaciji katarakte obarvane z gentiano violet kot nebarvane. Pri približno ¼ ovojnic je aplikacija acetilholina povzročila kontrakcijo lečnih epitelnih celic.

Zaključki: Ugotovili smo, da so sprednje ovojnice človeške leče izolirane pri operacijah katarakte primeren vir za študij delovanja lečnih epitelnih celic ter da lahko pomenijo pomemben model za študij patofiziologije nastanka katarakte.

Introduction

The loss of transparency during cataract development severely impairs the ability of the lens to focus an image on the retina, and is the leading cause of blindness worldwide. The cataract is a result of the functional impairment of the two types of cells that comprise the human lens, epithelial and fibre cells. The cells in the lens epithelium have typical epithelial morphology. They are cuboid and are tightly packed in a single layer with very little intercellular space. This single layer of cells acts as a metabolic engine that sustains the physiological health of the tissue. Because of important role in functioning of the lens and its accessibility, it is a subject of numerous studies.[1-4]

As in most cells, intracellular calcium activity ($[Ca^{2}+]_i$) is a key mediator of signalling within lens cells. The loss of calcium homeostasis in epithelial cells has been implicated in the loss of transparency and cataract formation.[3,5]

In human lens epithelial cells acetylcholine (ACh) binds to M1 muscarinic receptors, which belong to the superfamily of G-protein coupled receptors that are predominant in freshly isolated lens anterior epithelial cells.[6,7] Only the anterior lens epithelial cells respond to Ach.[8] Although the lens itself is not innervated, extracellular application of acetylcholine has been shown to induce a rise in $[Ca^{2}+]_i$.[9] The physiological significance of ACh in adult lens is not known while there are many potential sources of ACh in the tissues surrounding the lens, including the ciliary body, retina and iris or more precisely the nerve endings therein.[10] The binding of ACh to M1 receptors triggers the release of $Ca^{2}+$ from intracellular $Ca^{2}+$ stores. The pharmacological stimulation induced by application of ACh can also be a tool to study the events involved in cell--to-cell calcium signalling between the lens epithelial cells, since lens epithelial cells are connected to each other with gap junctions.[11]

In order to study the role of the lens epithelial cells in the development of the cataract formation and the mechanisms of calcium regulation in these cells, we have set up the preparation of a human lens capsule. Anterior lens capsules are routinelly removed during the cataract surgery and discarded. However, immediate use of this material allows studies of the physiology of living capsule epithelial cells. The functional property we monitored was $[Ca^{2}+]_i$ and its homeostasis. This was investigated using the calcium sensitive fluorescent dye Fura-2 and time-resolved fluorescence microscopy. The agonist we used to probe the intracellular $Ca^{2}+$ homeostasis was ACh.

The quality of the material obtained from surgery material also had to be assessed in for estimation of the relevance of the results of experiments. The testing criteria we used were the amount of capsule covered with the epithelium, the responsiveness of the epithelium to ACh stimulation and the contractions of epithelial cells in response to stimulation.

Methods

Experiments were done on the anterior lens capsule preparation consisting of the monolayer of anterior lens epithelial cells lying on the basal lamina. The preparation was obtained routinely during the cataract surgery at the Eye Hospital, University Medical Centre (UMC), Ljubljana, Slovenia. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia and all patients signed informed consent before the operation.

The capsules were from the patients of both genders aged 35 to 98 years. Depending on the visibility, some capsules were stained during the surgery with gentian violet (crystal violet) for the intraocular usage: 0.1ml 0.5 % gentian violet (made by the UMC Ljubljana pharmacy) aspirated trough 0.2 mm filter (Sartorius, Minisart) + 4.9 ml BSS aspirated trough 0.2 mm filter. After the surgery, each capsule was stored in Minimal Essential Medium Eagle Sigma + newborn calf serum heat inactivated, MEM, and transported to the laboratory where it was kept until utilization on 37° C, 5 % CO_2 in Incubator Innova CO-48 (New Brunswick Scientific, USA).

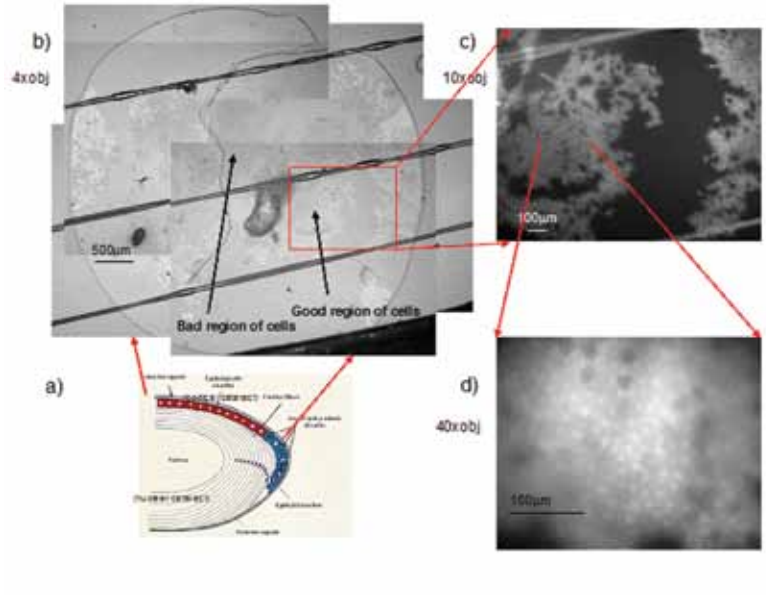

Figure 1: The preparation–Human lens anterior capsule with the epithelial cells, stained by Fura-2. a) Schematic presentation of the lens morphology. b) Reconstruction of the image of the lens as isolated after the operation and stained by Fura-2. c) Selected part of the capsule with well preserved cells. d) Smaller selected part of the capsule, as seen during acquisition.

The $[Ca^{2}+]_i$ time courses were imaged using the fluorescent $Ca^{2}+$-sensitive dye Fura-2. The dye has two excitation (absorption) peaks (340 and 380 nm), an isosbestic point at 360 nm and one emission peak at 510 nm. Its absorption and fluorescent properties change in accordance with $Ca^{2}+$ binding (low $[Ca^{2}+]_i$ – high absorption at 380 nm, high $[Ca^{2}+]_i$ – high absorption at 340 nm while the absorption is not $Ca^{2}+$-dependent at the isosbestic point of 360 nm). For $[Ca^{2}+]_i$ monitoring the capsules were loaded with the AM ester of Fura-2 (Fura-2 AM; Invitrogen – Molecular Probes, USA). The loading was done in the incubator at 37°C with 2mM of the Fura-2 AM in 3ml MEM for 30 min. The capsules were washed after loading twice for 7 min with MEM. Capsules were then transferred to the glass bottom Petri dishes (Mattek Corp., USA), filled with the bath solution consisting of (in mM): NaCl 131.8, KCl 5, $MgCl_2$Î6 H_2O 2, NaH_2PO_4 0.5, $NaHCO_3$ 2, $CaCl_2$ 1.8, HEPES 10, Glucose 10), pH 7.24. There they were immobilized by a grid, similar to the one used for experiments with rodent brain slices, so that the addition of the agonist solution would not displace them. The grid was made of platinum wire bent in U shape with the nylon filaments taut across it to form a harp-like shape. Grid, in addition to immobilizing the capsule, also flattened it so that the cells were more or less in the same focal plane, as is necessary for optical recording. The orientation of the capsule was with the basal lamina on the bottom, so that the agonist could be applied and easily diffused to the cells without having to cross the barrier of the basal lamina. The agonist used was acetylcholine (ACh; Sigma, USA) in 10mM concentration. The capsule fixed by the grid to the bottom of the glass dish, was put under the inverted microscope Zeiss Axiovert S 100 (Carl Zeiss, Germany). Acquisitions were done with the 12 bit cooled CCD camera SensiCam (PCO Imaging, Germany). The software used for acquisition was WinFluor (developed by J. Dempster, University of Strathclyde, Glasgow, U.K.). Objectives used were: 4x/0.10 AchroPlan; 10x/0.3 Plan-NeoFluar Zeiss; 40x/0.75 Plan-NeoFluar Zeiss; 63x/1.25 oil Plan-NeoFluar Zeiss.

The absorptive properties of Fura-2 allow the use of ratio imaging (either 340/380 or 360/380 ratio), which considerably reduces the effects of uneven dye loading, leakage of the dye, and photobleaching, as well as problems associated with measuring $[Ca^{2}+]_i$ in cells of unequal thickness. In order to perform $[Ca^{2}+]_i$ ratio imaging using Fura-2, we used the excitation filters mounted on a Lambda LS-10 filterwheel (Sutter Instruments Co., USA): either 360 nm or 340 (in initial experiments) and 380 nm (all by Chroma, USA). The 360/380 nm excitation ratio allowed visualization of the calcium concentration changes in the cytoplasm while excitation with the 360 nm filter alone (close to Fura-2 isosbestic point) allowed the observation of the changes in the concentration of the dye and the cell morphology. The image acquisition, the timing and filterwheel operation were all controlled by Winfluor software via a PCI6229 interface card (National Instruments, USA) together with the BNC-2090A rack-mount connector board.

The light source used was Zeiss XBO--75W xenon arc lamp. The light intensity was attenuated when necessary with Chroma grey filters with 0.5, 1and 2 optical densities (30 %, 10 % and 1 % transmittance). The experimental protocol consisted of the time before stimulation, the stimulation, usually by the ACh, and the subsequent recovery time. In one set of experiments, gentian violet, the same as the one used during the

surgery, was added during the acquisition, on capsules stained by Fura-2 or not stained at all, neither by gentian during the surgery. In the experiments where the capsules were exposed to the strong light for a period of 10s-20s, it was the white light of its maximal intensity and the CCD camera was closed during that time.

Results

Patients and surgeons

Average age for all the patients with Fura-2 staining of capsule was 75+/-10. Average age for all the patients with ACh response was 73+/-12 and for all the patients with no ACh response was 76+/-11 years, indicating that the age is not the discriminative factor for the Ach responsiveness.

5 different surgeons provided the capsules. More than 50 % of capsules provided by each surgeon responded to ACh.

The preparation

Figure 1 presents a typical anterior lens capsule preparation obtained after surgical operation and subsequent Fura-2 AM staining. As seen from Figure 1b, three regions of the capsule can be identified. The regions we were recording from (as seen from Figure 1c and 1d) are the regions where the cells are in a good physiological state. We did notice that cells that take up dye well and appear as very bright when excited at 380 nm also tend to exhibit "healthy" morphology when observed under phase contrast. The same cells have 360/380 (340/380) ratios associated with low $[Ca^2+]_i$ and in most cases also respond by $[Ca^2+]_i$ transients to ACh application. In our opinion these are indicators of a "good physiological state" and viability. The other two types are the regions where no cells could be seen and the regions where the cells were not in a good state. Such cells had poor morphology, indicated by grainy appearance of the cytoplasm and an often discernible nucleus on a phase-contrast image. They have only partially or not at all taken up the dye and in consequence were poorly or non-fluorescent.

In total, 46/63 (73 %) of the capsules stained by Fura-2 had the cells visible under the microscope, and they were recorded in average time 02:35h+/-01:08h after the surgery.

On raw (non-ratio) images, nuclei appeared bright with the surrounding cytoplasm dimmer.

Resting $[Ca^2+]$ of the cells

In some resting cells the ratio signal proportional to $[Ca^2+]_i$ exhibited temporal fluctuations. The fluctuations of the ratio before any application of the ACh were visible in 36/63 capsules.

ACh response

Figure 2 shows a typical response of the epithelium cells from a good region of the capsule. It shows the spatial and as well as the temporal profile of the fluorescent and the ratio response. Not all the cells responded at the same time to the ACh application but rather exhibited responses of cells that appeared to be linked like local cell networks – the sub-network responses. Such sub-network response may be an indicator of similar functional properties of the cells involved as well as of preferential coupling of certain cells via gap-junctions.

In total, 38/63 (60 %) capsules responded to ACh, 8/63 (13 %) did not respond to ACh and 17/63 (27 %) had no fluorescent cells. All 38/38 (100 %) capsules responding to ACh exhibited the sub-network response upon the application of ACh.

When analysis was made from 30 capsules stained with Fura-2 and all 30 capsules were classified in the groups according to the time between the isolation of the capsule and the time of the experiment, we saw that the response to ACh was always present if the period was smaller than 1 h and half (5/30 (17 %) capsules), and longer than 2h and half, (12/30 (40 %) capsules). From the period between, which included 13/30 (43 %) capsules, 5/13 (38 %) didn't respond to ACh.

For 16 capsules, application of ACh was repeated and all responded to ACh the second time as well.

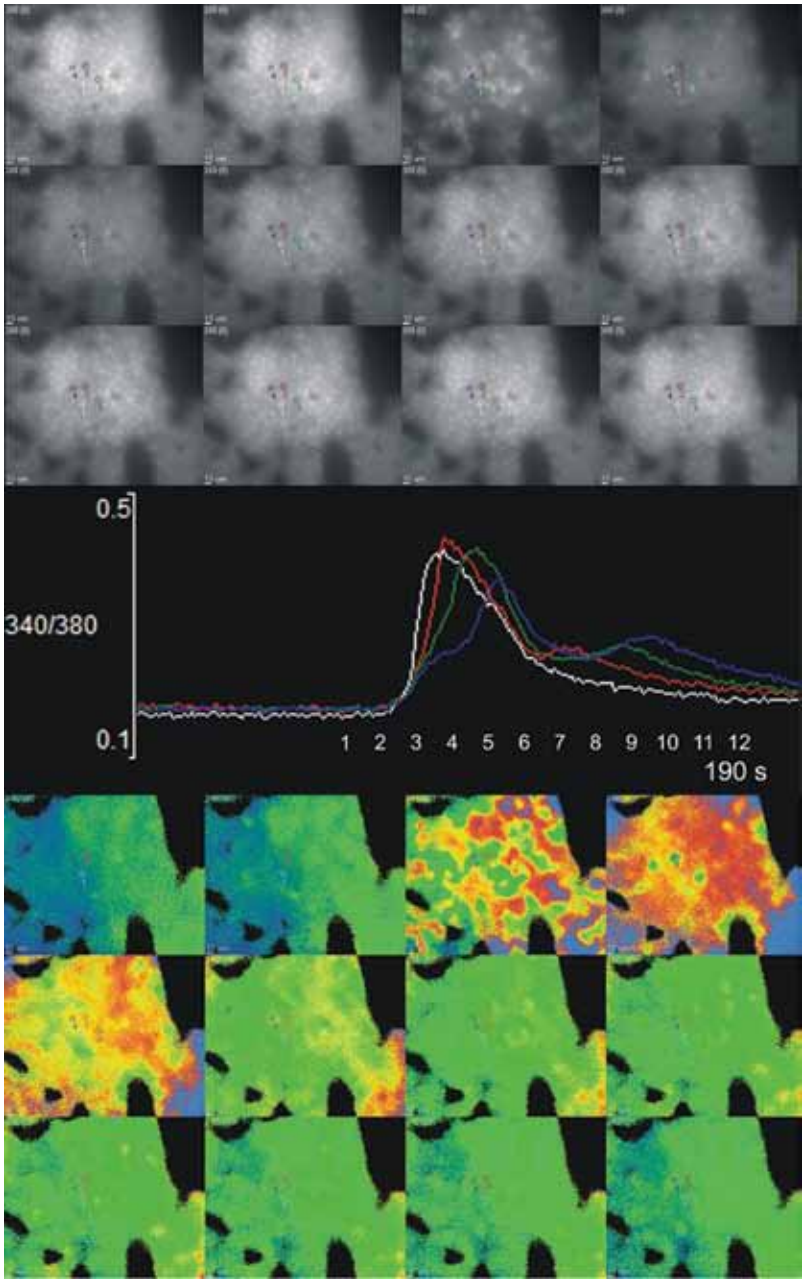

Figure 2: An example of a typical spatial and temporal profile of the response of the lens epithelium to ACh application. The top (b&w) montage shows a series of raw images of Fura-2 fluorescence when the preparation was excited at 380 nm. The middle panel shows the time courses of the 340/380 ratio proportional to $[Ca^{2+}]_i$, while the bottom panel shows a montage of 340/380 ratio images from the same time points as the top montage. The values are color coded with blue/green representing low ratio values and yellow/red representing high ratios. The time points are indicated by numbers on the middle panel and beginning of the ACh application coincided with number 1 (of 12). The four traces on the middle panel correspond to regions of interest (ROI) the size of individual cells and are indicated on both montage panels by circles of matching colours.

Gentian violet

Gentian violet is a water soluble dye and often used during surgical procedure to visualize the capsule. As it may possibly have cytotoxic and phototoxic effects, we wanted to explore whether the dye itself affected the $[Ca^{2+}]_i$ regulation in these cells and whether this can be observed on a time scale of our experimental procedures. We wanted to make sure that both groups of capsules could be used in our work and that the results were not affected by Gentian violet.

Both stained and non stained capsules responded to ACh. 14 capsules were stained by gentian violet during the surgical isolation: 6/14 (43 %) had ACh response, 3/14 (21 %) not, and 5/14 (36 %) had no fluorescent cells. 26/37 (70 %) capsules that were not stained by gentian responded to ACh, 2/37 (5 %) not and 9/37 (24 %) had non fluorescent cells. For remaining 12 capsules, we had no records on staining.

3/3 (100 %) capsules were stained by gentian violet alone in the laboratory (without Fura-2) and observed on several wavelengths. The gentian violet fluorescence is visible best using a 63x oil immersion objective and 570nm excitation light. With 360 and 380 nm excitation, there was hardly any fluorescence present.

Autofluorescence of the capsule was checked with 40x objective and 3/3 (100 %) capsules showed small and not significant autofluorescence on all 3 wavelengths: 360nm, 380nm and 570 nm.

Adding the gentian during the experiment, showed the increase of the fluorescence on 570 nm in 4/4 (100 %) capsules registered and no response on 360 and 380 nm, consistent with the optical properties of the gentian violet. Cells were visible both on 570 nm and 380 nm when the 63x oil immersion objective was used.

To evaluate the possible acute phototoxic effects of gentian violet we did a series of experiments where we applied gentian violet. After the fluorescence, due to the applied dye excited at 570 nm, reached a steady state, the capsule was exposed to a very strong white light for 10–20 sec after which the recording was resumed (during the li-

ght exposure, the recording was halted to prevent the damage to the camera). In 7/9 (78 %) capsules, the 360/380 ratio signal did not change much after the white light exposure, showing that there is no immediate phototoxic effect of the light/gentian violet combination on $[Ca^{2}+]_i$, at least not in the time window of 2–3min of exposure and recording.

Contraction of the cells

In 12/51 (23 %) capsules the cells contracted upon ACh application. Figure 3 shows an example of such preparation. In 16 cases, we repeated the ACh application: 5/16 (31 %) of the capsules contracted during both applications of ACh; 2/16 (12 %) contracted during the first but not second ACh application; 1/16 (6 %) capsules contracted only during the second application of ACh and 8/16 (50 %) capsules didn't contract during either applications of ACh.

When analysis was made from 30 capsules stained with Fura-2 and all 30 capsules were classified in the groups according to the time between the isolation of the capsule and the time of the experiment, contraction was not observed in the period longer than 3 h (8/30 (27 %) capsules). In the period shorter than 3h, from the 22/30 (73 %) capsules, contraction was observed in 9/22 (41 %).

Discussion

In our work we studied the human lens epithelium present on the anterior lens capsule extracted during cataract surgery with the special attention to the role of calcium in physiology of lens epithelium cells. The role of calcium was brought to the forefront of cataract research in 1975 by paper of Duncan and Bushell,[12] and is enjoying renewed scientific attention.[3]

There are many advantages of using this particular preparation. Firstly, the capsules are regularly excised and normally discarded during cataract surgery, so there is a steady supply of human lens capsule material. Secondly, the lens capsule preparation has the advantage of preserving the epithelium in a fairly "intact" configuration, i.e. all, or at least most, of the connections between neighbouring cells are preserved and if the preparation was not mishandled they should behave as they do in their normal environment. This is not the case with cells separated from each other during primary culturing procedure or with cloned epithelial cells from cell culture collections. There was also the difference in receptor subtype expression found between native and cultured cells. In the human lens cell line HLE-B3, it is the M3 subtype that predominates and not M1 as in the native epithelium.[6] Many of the physiological studies were until now done on cultured lens epithelial cells[13] and animal lens epithelium.[14-16] Only few were done on intact human lenses.[7] Regarding animal models, no single animal species is a complete model of the human lens. Even when the same agonist induces responses in different species, there are differences in receptor subtype expression. In the case of muscarinic receptors, the native human lens cells express the M1 subtype, while rat and rabbit express the M3 as the dominant subtype.[17] As the experimental conclusions obtained on animal species can not be directly applied to the humans, working on the human preparation obtained during the surgery, allows direct studies of the tissue of interest in different forms of cataract and the results obtained are directly applicable to enhance our understanding of the cataract pathophysiology.

The quality of surgical material

We assessed the quality and the applicability of the surgical material for physiological experiments. In all cases we found that sufficient amount of the material obtained form cataract surgery was of good enough quality to allow subsequent studies. We also found that neither the age of patients nor the surgical practices of particular surgeons were considerably affecting the results.

Responses to ACh

As was found previously,[7, 9, 13] the lens epithelial cells respond to the bath application of ACh with a rise in $[Ca^{2}+]_i$. The physiological significance of this response is not

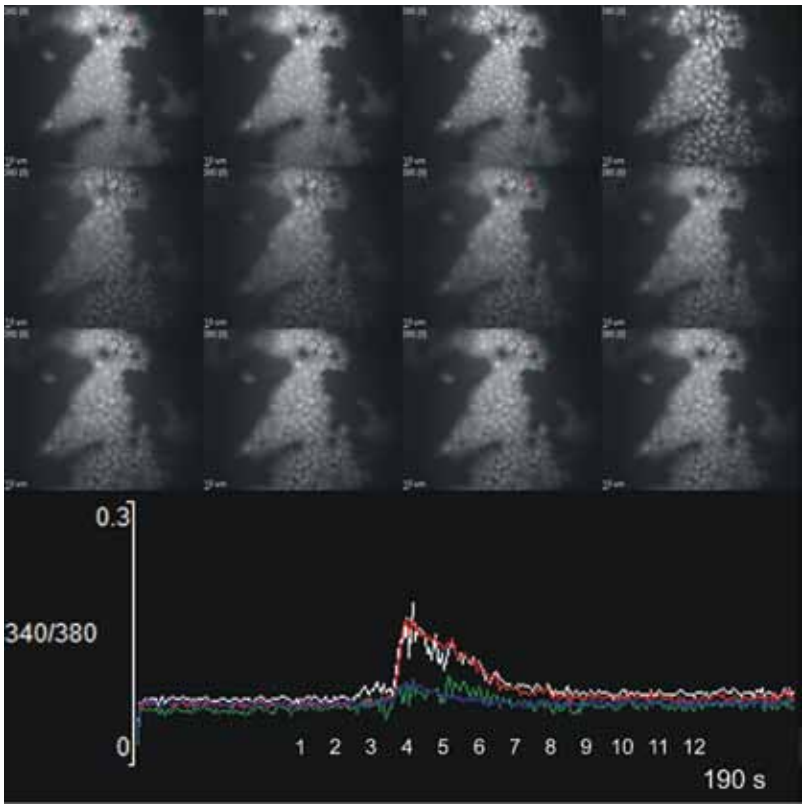

Figure 3: Example of the contractions of the epithelial cells as a response to the application of ACh. The figure is arranged in the same way as Figure 2, only without the ratio montage added at the bottom. Note the pronounced contraction of individual cells, especially pronounced in montage subpanels 4–8, corresponding to the highest rise in $[Ca^{2}+]_{i}$.

known, yet we have shown it is present even in epithelial cells coming from patients aged 70 years and more. There were no responses to ACh application only in 13 % of capsules, which we otherwise considered to be still in relatively good shape and where the cells have loaded Fura-2. The reasons for this are not known but may range from errors in the post-surgical handling and preparation procedures to long term cataract-associated changes. We have nevertheless found that good responses of $[Ca^{2}+]_{i}$ to ACh application normally coincide with good epithelial morphology and are very likely also an indicator of good functional status of the epithelium. This may be also due to the fact that in order to get a good response to ACh application, there had to be more cells connected to each other. Solitary or lone couples of cells did not respond to ACh application. The working (culturing) time, at least up to 7h, did not seem to affect the ACh responses, as have also not repetitive ACh applications, providing that preparations were washed in between applications and that time lapse was sufficiently long (typically we used 10 min). In all we believe that the response to ACh is a good functional test of the quality of the lens capsule epithelium.

Contraction of epithelial cells

The fact that isolated lens epithelial cells sometimes contract has been known for some time.[18] As with ACh application the functional significance of this phenomenon is also not known. However, if this actually does occur *in situ*, then gaps forming in between the epithelial cells would very likely seriously impair the normal function of the lens epithelium, which is to provide the driving force for the ionic gradients and the fluid circulation within the lens. In our experiments we found this phenomenon in approximately 1/4 of the preparations, where cells contracted in response to ACh application. Although actin cytoskeleton was described in lens epithelial cells,[13] we do not have an explanation for this, also due to its rather inconsistent occurrence. In some cases we did double applications of ACh and we observed all possible combinations: the contraction occurred in response only to the first application of ACh, in response to both and in response only to the second one, as well as no contraction to either application.

The use of gentian (crystal) violet

Gentian violet is often used in cataract surgery in order to visualize the lens capsule. It preferentially stains living cells. It has been shown to mainly partition into mitochondria and can actually also be used to stain these organelles as well as act as an uncoupler of oxidative phosphorylation.[19-21] On the other hand gentian violet is also used as a therapeutic agent for fungal and protozoan infections[22, 23]. It is effective mainly due to its phototoxicity and the toxic effects on mitochondria. Due to its absorption properties (very low absorption in the 330–420 nm region) we were able to monitor $[Ca^{2}+]_{i}$ with Fura-2 in combination with gentian violet present in the tissue. In our work we have used both stained and non-stained capsules and we could not establish any obvious differences in the $[Ca^{2}+]_{i}$ homeostasis between the two groups, which doesn't mean that more subtle differences still exist.

Moreover we also applied gentian violet to the unstained preparation and subsequently illuminated it with bright white light that should have had a phototoxic effect. However we have not been able to illicit any rise in $[Ca^2+]_i$ that could be attributed to gentian violet. Our conclusion is that, at least acutely and in concentrations used in surgery, gentian violet does not have detectable cytotoxic effects on $[Ca^2+]_i$ homeostasis in lens epithelial cells.

In conclusion, at Eye Hospital, University Medical Centre Ljubljana, we have developed our own methodology to study phisiology of human lens epithelial cells which is getting renewed attention in research of mechanisms of cataractogenesis. Our results show that most capsule specimens were of sufficient quality, that the results are reproducible and that the preparation and the techniques are adequate to study the mechanism of calcium homeostasis in different forms of cataract, which is of our future interest.

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