Calcium Activates SK Channels in the Intact Human Lens

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PURPOSE. Apamin-sensitive, calcium-activated SK potassium channels have been implicated in schizophrenia and myotonic dystrophy (MD), and both conditions carry an increased risk of cataract. The presence and functional activity of SK channels were therefore investigated in the human lens.

METHODS. The expression of all three members of the SK channel family was quantified by PCR. Their functional activity was investigated by inserting a single electrode into the intact human lens, and changes in intracellular calcium were recorded simultaneously after fura-2 incorporation.

RESULTS. Expression of all three SK family members was detected in both anterior and equatorial lens epithelial cells. Application of either G-protein (e.g., adenosine triphosphate (ATP)) or tyrosine kinase (EGF) receptor agonists induced a hyperpolarization of lens voltage that was accompanied by an increase in intracellular calcium. The calcium ionophore ionomycin also induced a rapid hyperpolarization. The hyperpolarizing responses were abolished by apamin and trifluoperazine and were accentuated by the SK channel activator 1-ethyl-2-benzimidazolizolone (1-EBIO).

CONCLUSIONS. SK channels are an integral part of the G-protein and tyrosine kinase calcium signaling mechanisms in the human lens, and their activation is inhibited by certain antipsychotic drugs. These findings help explain why a change in channel activity, whether by abnormal gene expression or by drug intervention, can lead to cataract. (Invest Ophthalmol Vis Sci. 2003;44:3927–3932) DOI:10.1167/iovs.03-0013

Cataract is mainly associated with old age in the developed world, but its prevalence can be increased by certain diseases and clinical conditions. For example, myotonic dystrophy (MD) carries a greatly increased risk of cataract, with most carriers showing opacities by age 40. Indeed, in many individuals the early onset of cataract is the only indication of the disease.1 In a study of more than 300 individuals from 9 MD families all affected members above 20 years of age had cataracts.2 In the initial stages MD cataract is characterized by white, light-scattering opacities in the outermost regions of the lens. The later stages show evidence of cortical swelling along the suture lines, and this finally progresses to a mature cataract in which, in slit lamp examination, “an iridescent display of scintillating colors” (Ref. 1) can often be seen. Recently, specific changes in the lens epithelium have been identified in patients with MD.3 Exposure to a variety of stresses, including radiation, oxidation, and several drug therapies, also accelerates the onset of many forms of cataract. For example, phenothiazine treatment for schizophrenia4 carries an increased risk of cataract. There are also some reports that the disease itself may be associated with an increased incidence of cataract.5 Although the mechanisms underlying MD and schizophrenia are not completely understood, changes in the genes encoding for the Ca2+-activated SK family of channel proteins are believed to play a role in both conditions.6–9 The only Ca2+-activated K+ channel type that has been unequivocally identified in the human lens is the large-conductance Ca2+-activated K+ (BK) channel,10 but no association with cataract development has been made. K+ channels in general play an obviously important role in determining the overall membrane potential of the lens, and there has also been one report of the involvement of K+ channels (of unspecified type) in controlling lens growth in early chick lens development.11 In fact a disturbance of Ca2+-homeostasis itself has long been known to play a critical role in cortical cataract formation.12

In the present study, we show that the human lens does indeed possess members of the SK family and that activation of either G-protein or tyrosine kinase (TK) Ca2+ signaling systems produces a hyperpolarization of the membrane potential measured in the intact human lens. This hyperpolarization is abolished by exposure of the lens to apamin and to the phenothiazine derivative trifluoperazine (TFP).

METHODS

Tissues and Reagents

1-Ethyl-2-benzimidazolizolone (1-EBIO) and charybdotoxin were obtained from Tocris Cookson, Ltd. (Northampton, UK). All other chemicals were from Sigma-Aldrich Co. Ltd. (Poole, UK), unless stated otherwise. Human donor eyes were obtained from the East Anglian Eye Bank (Norwich, UK) and used 24 to 48 hours after enucleation. As no donor details, apart from age, sex, and cause of death were released, this research followed the tenets of the Declaration of Helsinki. Donors were between the ages of 50 and 85 years old. The lens was dissected through the anterior of the eye and placed into artificial aqueous humor (AAH)3 at 35°C before use.

Membrane Potential Recordings

After dissection, the lenses were placed into an acrylic chamber (1.7 mL) and perfused with AAH (35°C) at 1 mL minute⁻¹. AAH has the following composition (mM): NaCl, 130; KCl, 5; NaHCO3, 5; CaCl2, 1; MgCl2, 0.5; glucose, 5; HEPES, 20; pH (35°C) was adjusted to 7.25 with NaOH. Agonists and inhibitors were added to the perfusate. Lens membrane potential (Vm) was measured as previously described.13,14 A glass micropipette filled with 2 M KCl (1–6 MΩ) inserted into a cortical fiber through the posterior of the lens measured Vm with reference to a low-resistance electrode in the bath. Lens cells are electrically very well coupled14,15 and so Vm is a measure largely of the voltage across the plasma membrane of the outermost cells. Electrical measurements were recorded with a 2-channel high-impedance amplifier (Firbank Electronics, Norwich, UK) in conjunction with an analogue-to-digital converter (Handyscope; TiPie Engineering, Leeuwarden, The Netherlands) for storage and analysis on computer.

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Intracellular Ca\(^{2+}\) Measurement

Intracellular Ca\(^{2+}\) levels were monitored using methods that have been described in detail elsewhere.\(^{16}\) Briefly, the intact lens was loaded with 3 \(\mu\)M fura-2 (fura-2/AM) in AAH for 40 minutes at 35°C. The lens was washed for a further 20 minutes in AAH and placed anterior surface downward in an acrylic chamber (Perspex) and perfused with AAH (35°C). The solution turnover time for the chamber was approximately 10 seconds. Ratiometric imaging of cytosolic Ca\(^{2+}\) was performed on the stage of an epifluorescence microscope (TE-200; Nikon, Tokyo, Japan). Cells were excited alternately with light of 340 and 380 nm wavelength. The resultant fluorescent emissions were collected by a charge-coupled device (CCD) camera at 510 nm and sampled every 2 seconds. After background subtraction the fluorescence ratios were recorded. No fluorescence signal was obtained from the lens posterior region or lens nucleus, but stable signals were obtained from the anterior and equatorial cells. Lens voltage was monitored simultaneously by inserting a glass micropipette into a cortical cell through the posterior of the lens, as previously described. Both the resting membrane potential of the lens and the characteristics of the voltage responses appeared to be unaffected by the simultaneous measurement of intracellular calcium and were similar to those recorded in separate experiments in this and other studies.\(^{17}\)

Quantitative Real-Time PCR

There are three subtypes of apamin-sensitive K\(^+\) channel (SK1–3) that have been identified in other human tissues,\(^{18}\) and the molecular basis for this diversity has been determined by the cloning of three genes encoding SK channels from mammalian tissue.\(^{19}\) We used quantitative real-time PCR molecular techniques to determine which subtypes are present in the lens.

For RNA extraction the lens epithelium was dissected into the anterior and equatorial regions. Total RNA was isolated from each region using a kit (RNEASY with the RNase-free DNase set; Qiagen, Valencia, CA). Two hundred nanograms of RNA were used to make cDNA in a total reaction volume of 20 \(\mu\)L with a commercial system (Superscript II RNase H\(^-\) reverse transcriptase; Invitrogen-Life Technologies, Rockville, MD). PCR (TaqMan) was performed with a sequence-detection system (Prism 7700; Applied Biosystems, Foster City, CA) to identify and quantify the expression of SK1, SK2, and SK3 channels in the regions of the lens, according to the manufacturer’s protocol. Each reaction was performed in a total volume of 25 \(\mu\)L and contained the equivalent of 5 ng human cDNA (1 ng for the 18S analyses), 50% 2X PCR master mix (TaqMan Master Mix; Applied Biosystems, Inc.), 100 nM each of forward and reverse primer, and 200 nM probe. Conditions for the PCR reaction were 2 minutes at 50°C and 10 minutes at 95°C, and then 40 cycles, each consisting of 15 seconds at 95°C and 1 minute at 60°C. The primer and probe sequences used (TaqMan; Applied Biosystems, Inc.) were those described by Rimini et al.,\(^{20}\) and were designed from human sequences for SK1, SK2, and SK3. Probes were FAM labeled at their 5’ end. Data were analyzed using the software provided (TaqMan; Applied Biosystems, Inc.). \(C_0\) values were converted to arbitrary units, and differences between the samples were normalized with 18S mRNA by having a standard curve on each plate. Absolute quantification is possible if the standard curve uses a synthetic RNA corresponding to the target gene; however, in this case the standard was based on total RNA from lens epithelium and so the analysis provided only relative quantification.

Capsular Bags

To elucidate any possible effect of apamin inhibition on the expression of SK channels in human lens cells, we used the capsular bag system developed in this laboratory.\(^{21}\) A sham cataract operation was performed on donor lenses, and the resultant capsular bag was then dissected from the zonules and secured to a plastic Petri dish by pins that retained its original circular shape. The capsular bags were maintained in a nonsupplemented (serum-free; SF) Eagle’s minimum essential medium (EMEM) at 35°C in a 5% CO\(_2\) atmosphere. The medium was replaced at 4-day intervals. Previous work from this laboratory has shown that native lens cells residing on their original capsular matrix continue to grow over long periods in SF medium.\(^{22}\) Experiments were performed in a matched-pair format in which apamin was added to the medium of the capsular bag from one lens while the corresponding bag from the same donor served as the control. When epithelial cells had completely covered the posterior capsule (~10–15 days) RNA was extracted and PCR performed as described earlier.

RESULTS

Changes in Lens Voltage in Response to Ca\(^{2+}\)-Mobilizing Agonists

The lens represents a well-coupled electrical syncytium, so that the voltage measured at any point in the lens monitors the net effect of channel activity, largely in the membranes of the surface epithelium and superficial fibers.\(^{14,15,23}\) Exposure to 100 \(\mu\)M adenosine triphosphate (ATP) induced an initial hyperpolarization, ranging from 0.5 to 4 mV in amplitude (\(n = 10\)), and this was followed by a more prolonged depolarization phase (Fig. 1A). On some occasions, small oscillations were apparent in the hyperpolarization phase of the response (see Figs. 1A, 2B). The same two phases in the response were also apparent when the lens was exposed to epidermal growth factor (EGF; 10 ng/mL; Fig. 1B), although the initial hyperpolarization was less rapid and much smaller in amplitude (0.1–
0.2 mV, n = 3). A number of other G-protein coupled receptor (GPCR) agonists (histamine, acetylcholine and uridine triphosphate [UTP]) were also found to induce the hyperpolarization phase and their amplitudes of response and time course were similar to those found with ATP. Note that not only were the responses to the various agonists reproducible in different preparations but subsequent applications on the same preparation gave similar responses providing sufficient time (t > 15 minutes) was allowed between applications.

Recently, techniques have been developed to measure intracellular Ca\(^{2+}\) changes in the intact lens using the fluorometric reporter dye fura-2. Figure 2A shows intracellular Ca\(^{2+}\) and lens voltage data obtained simultaneously from the same lens, and in this case, the fura-2 signals, in response to histamine (100 \(\mu M\)), were recorded from equatorial epithelial cells of an intact lens. Histamine induces a typical biphasic voltage response, whereas the Ca\(^{2+}\) response shown is monotonic in form, which was typical of all the GPCR agonists used. However, a second phase is in fact embedded in the Ca\(^{2+}\) signal, because the Ca\(^{2+}\) influx blocking agent La\(^{3+}\) greatly reduced the duration of the Ca\(^{2+}\) signal. The La\(^{3+}\) data were generated in a separate series of experiments and are shown in Figure 2A (dashed line). Note that La\(^{3+}\) had no effect on the rising phase of the Ca\(^{2+}\) response, which is due to a rapid release of Ca\(^{2+}\) from inositol-trisphosphate-sensitive stores. Exposure to La\(^{3+}\) had no effect on the initial hyperpolarization but greatly reduced the depolarization phase of the response (Fig. 2B). The increase in intracellular Ca\(^{2+}\) in response to EGF was significantly slower than for the GPCR agonists (data not shown) and this was reflected in the dynamics of the hyperpolarization (Fig. 1B). Another study has also shown that the Ca\(^{2+}\) response to EGF is smaller in magnitude than that induced by GPCR agonists, such as ATP.

**Selective Inhibition–Activation of the Voltage Response**

A series of selective K\(^+\) channel inhibitors were applied to the lens to assess their ability to inhibit the hyperpolarization phase of the response. The SK-specific inhibitor apamin totally abolished the hyperpolarization at nanomolar concentrations (IC\(_{50}\) 600 pM; data not shown) and had no inhibitory effect on the depolarization. In fact, the depolarization phase of the response was slightly larger in the presence of apamin (Fig. 3A). When apamin was removed, the hyperpolarization phase gradually recovered on subsequent exposure to ATP (Fig. 3A) and the rate of recovery depended greatly on the apamin concentration. Note that apamin had no effect on the unstimulated membrane potential of the lens. Charybdoxin (100 nM) had no effect on the voltage response (data not shown).

1-EBIO is an activator of SK channel activity, and in the human lens greatly accentuated the hyperpolarization phase of the ATP response without increasing the depolarization phase (Fig. 3B). The hyperpolarization in the presence of 1-EBIO was also blocked by apamin (data not shown).

**Hyperpolarization Phase Specifically Induced by Ca\(^{2+}\)**

Some GPCR agonist responses are due in part to a specific interaction of the agonist with a membrane channel and so it was important to show in the lens whether an increase in intracellular Ca\(^{2+}\) alone could induce a hyperpolarization in the membrane voltage. This was indeed found to be the case, because ionomycin, a Ca\(^{2+}\) ionophore, induced a marked hyperpolarization in the presence of 1 mM external Ca\(^{2+}\). In contrast, removing external Ca\(^{2+}\) in the presence of ionomycin depolarized the lens, whereas adding 5 mM external Ca\(^{2+}\) caused a very large reversible hyperpolarization (Fig. 3C). The ionomycin-induced hyperpolarization was blocked by apamin (data not shown). Note that although ionomycin remained in the bath for only approximately 5 minutes, it rapidly and reversibly incorporated into membranes and is in fact used as a means of equilibrating internal and external Ca\(^{2+}\) levels in lens cells and a range of other tissues.

In the intact human lens prior exposure to TFP (100 \(\mu M\)) totally abolished the large ionomycin-induced hyperpolarization (Fig. 3D). TFP also blocked the hyperpolarization induced by ATP (data not shown).

**Quantitative Real-Time PCR Analysis of SK Channel Expression**

The data in Figure 4A show that, although all three SK channel family members were present in the lens, they were not uniformly distributed between the two cell types. In general, they were more abundant in the anterior epithelial cells than they are in the equatorial cells. The asymmetry in distribution is most pronounced for SK1 (Fig. 4A) which showed between...
3.7- and 11-fold greater expression in the anterior epithelium than the equatorial cells \((n = 3)\). The three SK family members were also found to be expressed in the human lens cell line FHL-124 (data not shown).

**Apamin-Induced Changes in Channel Expression**

The human lens capsular bag provides a convenient means of culturing lens cells on their natural matrix where cells continue to grow for many weeks under SF conditions. Exposure to apamin \((1 \mu M)\) induced an upregulation in the expression of SK1 and SK3 relative to control in every case \((n = 4)\), but there appeared to be little effect on the expression of SK2 (Fig. 4B). Visually, exposure to apamin \((7 \text{ days})\) appeared to have little effect on the capsular bags apart from a small growth retardation, relative to the match-paired control.

**DISCUSSION**

The Ca\(^{2+}\)-activated K\(^+\) channel family exists as a heterogeneous association of three subfamilies: BK, IK, and SK channels. The large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK) channels are characterized by having a large unit conductance \((\sim 250\ pS)\) which is activated by both voltage and Ca\(^{2+}\) and blocked by charybdotoxin and iberiotoxin. The voltage-independent intermediate conductance (IK) channel subfamily has unit conductance in the range of 20 to 80 pS and is sensitive to block by charybdotoxin. The gating of small-conductance Ca\(^{2+}\)-activated K\(^+\) (SK) channels by Ca\(^{2+}\) is mediated by calmodulin, which is stably bound as an obligatory subunit of the channel complex. IK channels share related amino acid sequences with the SK channels and also use calmodulin binding to confer their Ca\(^{2+}\) sensitivity. SK channels have a unit conductance in the range of 4 to 14 pS and are also voltage independent. They are insensitive to charybdotoxin but sensitive to apamin. They are activated by submicromolar intracellular Ca\(^{2+}\) concentrations with a half-maximum activation in the range of 400 to 800 nM in neuronal cells. The calibrated Ca\(^{2+}\) changes in the epithelial cells of the whole lens after GPCR activation indicate that the Ca\(^{2+}\) increased from \(100\ nM\) to \(1 M\). This Ca\(^{2+}\) increase occurred at precisely the time that lens membrane hyperpolarization occurs (Fig. 3A), indicating that the two processes are closely linked. In neurons there is an apamin-sensitive after-hyperpolarization (AH) that also parallels the increase and decrease in Ca\(^{2+}\). In our study, hyperpolarization in the lens was induced by an increase in intracellular Ca\(^{2+}\), whatever the source (Fig. 3C).

Because the voltage response represents the net activity of all the channels in the lens, it is important that a change in perfusate be experienced by all surface receptors at the same time. This is especially true for a response that has both hyperpolarizing and depolarizing phases. Previous studies on the human lens have focused on the depolarizing phase of the response, and we have found that a relatively rapid perfusion is required to reveal the full extent of the hyperpolarization.

The depolarization phase of the normal voltage response undoubtedly arises from the activity of the store-operated or capacitative Ca\(^{2+}\) entry pathway (Fig. 1), as this phase is
in all cases.

Additional pharmacological evidence that SK channels are re-
unaffected by La$^{3+}$, confirming that it is caused by the rapid
release of Ca$^{2+}$ from intracellular stores.

In the human lens, the hyperpolarization was totally inhib-
by La$^{3+}$. It is interesting in the lens that the entry
pathway is only activated after a significant delay.$^{16,25,24}$ The
delay allows for a separation in time between the increase in
intracellular Ca$^{2+}$ due to release from the Ca$^{2+}$ stores and the
subsequent depolarization due to the opening of the Ca$^{2+}$
entry pathway. This in turn allows for the rapid initial hyper-
polarization to be quite separate from the later depolarization,
and this separation would not occur in those cell types where
there is no, or only a very brief, delay between Ca$^{2+}$
and this separation would not occur in those cell types where
polarization to be quite separate from the later depolarization,
subsequent depolarization due to the opening of the Ca$^{2+}$
entry. In the present study the hyperpolarization was
unaffected by La$^{3+}$, confirming that it is caused by the rapid
release of Ca$^{2+}$ from intracellular stores.

In the human lens, the hyperpolarization was totally inhib-
inated by nanomolar concentrations of apamin, indicating that
neither BK nor IK channels mediated the response. Further-
more, the hyperpolarization was insensitive to charybotoxin.
Because neither apamin nor 1-EBIO induced a change in the
unstimulated lens membrane potential, it is likely that SK
channels do not contribute to the steady state resting potential
of the lens. This also appears to be the case in neuronal tissue
in which apamin sensitivity is seen only during the AH phase.$^{18}$
Additional pharmacological evidence that SK channels are
responsible for the hyperpolarization phase of the response was
shown by the potentiation effect of 1-EBIO on the hyperpolariza-
tion (Fig. 3B). 1-EBIO can activate both IK and SK channels
by increasing the channel open probability without affecting
the affinity for Ca$^{2+}$ although activation is strictly Ca$^{2+}$ de-
dendent.$^{25,31}$ In the lens the enhanced hyperpolarization in the
presence of 1-EBIO was blocked by apamin, confirming that it
acted on SK and not IK channels.

The GPCRs for ATP and histamine are present in both
anterior and equatorial epithelial cells, whereas acetylcholine
receptors are present only in the anterior epithelium. In con-
trast, EGF stimulates only the equatorial cells.$^{16}$ Because both
acetylcholine and EGF induce hyperpolarizations in the whole
lens, it is possible to conclude that, both anterior and equato-
rial epithelial cells can contribute to the hyperpolarization
phase of the response. It is to be expected, however, that
activation of GPCRs and tyrosine kinase receptors (TKRs) will
not contribute equally to the overall voltage response for a
number of reasons. TKR agonists (e.g., EGF and platelet-de-
derived growth factor [PDGF]) only initiate Ca$^{2+}$ responses in
equatorial cells.$^{16}$ and this represents only a small subpopula-
tion of the total epithelial cells. Furthermore, the TK Ca$^{2+}$
response from equatorial cells is slower in time course and
smaller in amplitude than the corresponding GPCR responses
(see Ref. 16 for discussion). Because of the slow nature of the
TKR response the Ca$^{2+}$ release and capacitative entry phases
are not separated in time with the same clarity as the GPCR
responses, and so the overall voltage response, even in the ini-
tial changes, contains both hyperpolarizing and depolarizing
phases. Because the superficial fiber cells do not appear to
accumulate fura-2, it is not possible to assess any potential
contribution from this source. However, in the double-cham-
ber system used to isolate voltage responses in the rabbit lens
to acetylcholine, there was no response from the posterior
fibers.$^{5,2}$ The posterior fibers certainly contain K$^+$ channels,
but because most have lost their endoplasmic reticulum it is
unlikely that agonist-induced Ca$^{2+}$ signals can be induced in
this population.

An indication of the importance of SK channel activity to
the lens is shown by the fact that all three family members
were found to be present in the same cell type (Fig. 4A). The
SK channel subtypes, at least SK1 and SK2, were found to be
present in the same cell type (Fig. 4A). The
SK1 and SK2 channel densities are similar suggesting that
both SK1 and SK2 contribute to the initial hyperpolarizing
phase. SK3, however, is expressed to a significantly lesser
density (including TFP) block SK channels with micromolar activity.

Changes in SK channel activity have also been associated
with schizophrenia.$^{9,9,53,54}$ and cataract has been attributed
to both the disorder and the antipsychotic drug therapy.$^{4}$
Direct exposure to the phenothiazines TFP and chlorproma-
azine in animal models has been shown to induce cataract.$^{35,36}$
and alter the activity of unspeci-
identify SK as the channel type responsible, because a general Ca\(^{2+}\)-activated hyperpolarization induced by ionomycin was abolished both by apamin and TFP (Fig. 3D).

SK channel activation should now be seen as an integral part of calcium cell signaling in the lens, and it is possible that they participate in a range of functions. For example, recent studies have shown that SK channel activation occurs after osmotic stress \(^{40-41}\) and lens swelling is one characteristic of cortical cataract.\(^{42}\) It is interesting that there was an upregulation in the expression of SK1 and SK3 mRNA in cells grown in the presence of apamin (Fig. 4B), suggesting a dynamic role for these channels in the lens.

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