Molecular and Functional Expression of High Conductance Ca^{2+} Activated K^{+} Channels in the Eel Intestinal Epithelium

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Key Words

BK channels • Intestine • RVD • Eel • Cell volume regulation

Abstract

Several types of K^{+} channels have been identified in epithelial cells. Among them high conductance Ca^{2+} -activated K^{+} channels (BK channels) are of relevant importance for their involvement in regulatory volume decrease (RVD) response following hypotonic stress. The aim of the present work was to investigate the functional and molecular expression of BK in the eel intestine, which is a useful experimental model for cell volume regulation research. In the present paper using rat BK channel-specific primer, a RT-PCR signal of 696 pb cDNA was detected in eel intestine, whole nucleotide sequence showed high similarity (83%) to the α subunit of BK channel family. BK channel protein expression was verified by immunoblotting and confocal microscopy, while the functional role of BK channels in epithelial ion transport mechanisms and cell volume regulation was examined by electrophysiological and morphometric analysis on the intact tissue. BK_{Ca} channels appeared to be localized along all the plasma membrane of the

enterocytes; the apical part of the villi showed the most intense immunostaining. These channels were silent in basal condition, but were activated on both membranes (apical and basolateral) by increasing intracellular Ca^{2+} concentration with the Ca^{2+} ionophore ionomycin (1 μM). BK_{Ca} channels were also activated on both membranes by hypotonic swelling of the epithelium and their inhibition by 100 nM iberiotoxin (specific BK_{Ca} inhibitor) abolished the Regulatory Volume Decrease (RVD) of the intestinal cells after hypotonic swelling. In conclusion, our results demonstrated the molecular and functional expression of high conductance Ca^{2+} -activated K^{+} channels in eel intestine; the physiological role of these channels is mainly related to the RVD response of the epithelial cells following hypotonic swelling.

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Introduction

The Ca^{2+} -activated K^{+} channels (K_{Ca}) are ubiquitously expressed in both excitatory and non-excitatory cells where they integrate changes in

intracellular Ca^{2+} concentrations with membrane potential, thus contributing to various and diverse physiological outcomes.

K_{Ca} subfamily can be classified into three main groups based on their biophysical characteristics, single channel conductance, and their pharmacological footprints. The big conductance (BK) (100-300 pS) group includes the K_{Ca} 1.1 channel [1], the intermediate - conductance (IK) (25-100 pS) consists of the K_{Ca} 3.1 channel [2-4] and the small - conductance (SK) (2-25 pS) group comprises the K_{Ca} 2.1, K_{Ca} 2.2 and K_{Ca} 2.3 channels [5, 6].

All K_{Ca} open in response to an increase in intracellular Ca^{2+} in the micromolar range [7] while the BK channel is the only one that can also be activated by membrane depolarization. These two properties link BK channels to Ca^{2+} permeable cation channels as their triggering mechanism since activation of these will depolarize the cell and result in increased Ca^{2+} influx. BK channels are rather selectively inhibited by (scorpion) iberiotoxin. The functional channel of all the K_{Ca} family members is a multimeric protein composed of four pore-forming α - subunits; in the case of the BK channel four auxiliary β subunits are also present [8, 9]. The BK is also unique in its topology which includes seven transmembrane domains with an extracellular N-terminus [10, 11]. The β subunit has both N and C terminus inside the cell [7].

The BK channel was first characterized in *Drosophila* [12] as the slowpoke channel (Slo) and later identified in mouse [13] and humans [14, 15].

In spite of the fact that native currents of the BK channel with very different properties are known in different cell types, only one gene corresponding to the pore-forming alpha subunit (KCNMA1) has been identified. This gene is remarkably conserved among different species in mammals (mammalian maxi- K_{Ca} channel α -subunits have almost identical amino acid sequences among different species, >97%), suggesting that there is an evolutionary pressure to maintain its optimized function [15, 16].

BK channels are abundant in smooth muscle and in neurons, but they have been also detected in absorptive and secretory epithelial cells in the apical membrane [7], such as kidney tubular cells [17-20], male reproductive tract [21, 22], oviducts [23], gall bladder [24], vestibular dark cells [25], colon [26-30], acinar cells from salivary glands [31] and frog skin [32]. However, little is known about the functional role and molecular expression of BK $_{\text{Ca}}$ channels in epithelia; in particular, the most information available come from cell-free systems, non attached cells,

cell lines cultured or heterologous expression systems, but very little comes from intact epithelia. Hypotonic activation of BK channels is reported from several kidney cell preparations, a cell line from rat thick ascending limb, and in cells from rat and rabbit collecting duct (see [33, 34] for references). In some cells they seem to be directly stretch activated but in the majority of cells the activation has been found to be secondary to an increase in Ca^{2+} (see e.g. [35], in some cases coupled to an autocrine/paracrine ATP release (see [33])).

The aim of the present work was to investigate the molecular expression and the physiological role of BK in a native salt transporting epithelium, the intestine of the teleost fish *Anguilla anguilla*, which is a useful model system for functional studies of epithelia that perform near-isosmotic fluid absorption. The mechanisms accounting for ion and water transport in the eel middle intestine have been characterized and considerable information is also available on regulation of the transport (for reviews see [36-38]).

Briefly, the eel intestine develops a transepithelial Cl^- absorption, measurable as transepithelial potential (V_{te}) and short circuit current (Isc), sustained by the operation of the luminal $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter, in series with a basolateral Cl^- conductance and in parallel with an apical K^+ conductance. The $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ on the basolateral membrane, by generating an inwardly directed electrochemical gradient for Na^+ , provides the driving force for the $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransporter and, thus, for the active intracellular accumulation of Cl^- [39, 40]. This model is basically identical to the model published for the mammalian renal cortical thick ascending limb (cTAL) (see e.g. [41]).

The eel intestinal epithelium is physiologically exposed to anisosmotic conditions because of the euryhaline nature of the eel. As previously demonstrated [42, 43] this epithelium is sensitive to the osmolarity of the extracellular medium and shows a dynamic regulation of cell volume with a RVD response after hypotonic stress, making this tissue a good physiological model for epithelial cell volume regulation research. As previously demonstrated by short circuit current measurements [44] hypotonicity induces a Ca^{2+} -dependent biphasic decrease of V_{te} and Isc, that was attributed to the hypotonicity induced activation of several ion channels including iberiotoxin-sensitive K^+ channels [44].

In the present paper using rat BK channel-specific primer, a RT-PCR signal of 696 pb cDNA was detected in eel intestine, whole nucleotide sequence showed high similarity (83%) to the alpha subunit of BK channel fam-

ily. BK channel protein expression was verified by immunoblotting and confocal microscopy, while the functional role of BK channels in epithelial ion transport mechanisms and cell volume regulation was examined by electrophysiological and morphometrical analysis on the intact tissue. To our knowledge it is the first time that a molecular and functional expression of BK was performed in an epithelium of a lower vertebrate.

Materials and Methods

Sea water acclimated yellow eels (*Anguilla anguilla*) (weighing about 200g) were purchased from a commercial source (Agroittica, Lesina-Foggia, Italy). They were kept unfed in seawater aquaria under conditions of controlled photoperiod cycles and water temperature (18–20 °C) for at least a week before sacrifice. The animals were anaesthetized with 2-phenoxyethanol (0.3 ml/dm³) prior to sampling. All experiments were carried out in accordance with the European Committee Council Directive (86/609/EEC).

All chemicals were reagent grade. Cacodylate buffer, glutaraldehyde, OsO₄ and Epon 812 were purchased from Fluka BioChemika; all the other chemicals were purchased from Sigma (St. Louis, U.S.A.).

Iberiotoxin stock solution (100 µM in distilled water) was prepared and kept at -20°C until use. Unless otherwise noted solutions were freshly prepared.

Transepithelial electrophysiological analysis

The middle intestine of seawater acclimated eels was removed, stripped of longitudinal and circular layers with two pairs of fine forceps and mounted vertically in a modified Ussing chamber (CHM6, World Precision Instruments) (membrane area: 0.6 cm²), where it was perfused on both sides by isotonic Teleost Ringer solution (NaCl mM 133, KCl mM 3.2, NaHCO₃ mM 20, MgCl₂ mM 1.4, CaCl₂ mM 2.5, KH₂PO₄ mM 0.8, glucose mM 20; osmolarity: 315 mOsm/Kg). The Ringer solution was bubbled with a mixture of 1% CO₂ and 99% O₂ to yield a pH of 8.0 (closed to the pH of the teleost plasma).

The preparations were kept “open circuited” through the time-course of the experiments except for a few seconds every 5 min for recording of the short-circuit current (I_{sc}). Tissues were connected to an automatic short-circuit current device (WPI’s DVC-1000) by four Ag/AgCl electrodes (two voltage electrodes and two current electrodes) which made contact with the bathing solutions via agar-Ringer filled cartridges. Transepithelial voltage (V_{te}) was measured with respect to the mucosal bath (grounded); I_{sc} was measured by passage of sufficient current through Ag/AgCl electrodes to reduce the spontaneous V_{te} to zero automatically (resistance of the chamber fluid was subtracted automatically). The I_{sc} is referred as negative when current flows across the tissue from the apical membrane to the basolateral membrane. Transepithelial resistance (R_{te}) was measured by pulsed current injection (33 µA cm⁻², 500 ms) through the tissue. This injected current

produces a voltage deflection (ΔV_{te}) from which R_{te} was calculated.

In the hypotonic stress experiments the osmolarity of the teleost Ringer solution was bilaterally decreased to 175 mOsm in two steps: first the concentration of NaCl was reduced to 66.5 mM at unaltered osmolarity by mannitol replacement; then the hypotonic stress was applied by removing mannitol. This experimental maneuver was necessary to avoid the superimposing of diffusion potential in the unstirred layers (due to the NaCl reduction) on the hypotonic stress effect.

Iberiotoxin treatments were performed by 1h incubation of the tissue with the drug (0.1µ) before removing mannitol. The same time protocol was respected in the corresponding control sample. In the drug test the drug was still present during the hypotonic stress application.

In hypotonic stress experiments Ringer osmolarity was decreased only once in each experiment. The osmolarity of solutions was measured using a DELCON (Arcore, ITALY) vapour pressure osmometer 5520. In all Ussing chamber experiments the temperature of the perfusing Ringer solution was kept constant at 18°C.

Retrotranscription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the scraped mucosa of the whole eel intestine, using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) [45]. 1 µg of total RNA isolated from eel intestinal mucosa was subjected to RT-PCR by using the GeneAmp RNA-PCR kit (PE Applied Biosystems, Foster City, CA, USA) in accordance to the manufacturer’s protocol. Briefly, reverse transcription was performed for 12 min at 42°C in the presence of oligo(dT)₁₆ primer and the resulting cDNA was subjected to PCR by using primers designed on the basis of the mouse [15] (NM_010610), rat [46] (AF135265) and human [47] (NM_002247) BK channel sequences (Forward primer: 5'- TGA ATA TCC TTA AAA CAA GTA ACT CC -3', starting at nucleotide 946, and reverse primer: 5'- TGT TGA GCA GAT GGG CCT TGT T -3', starting at nucleotide 1668 of the mouse BK channel cDNA). PCR amplification was performed for 35 cycles with a denaturation at 95°C for 1 min, an annealing at 46°C for 1 min, an extension at 72°C for 1 min, followed by a final synthesis at 72°C for 7 min. RT-PCR products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide (1 mg l⁻¹) and visualised under UV light using the Gel-Doc System (Bio-Rad Laboratories, Hercules, CA, USA). The PCR product was subcloned in a TOPO TA cloning vector (Invitrogen, CA, USA), and subjected to sequencing. Sequence alignment was performed by using the Clustal W program (EMBL-EBI).

Immunoblotting

Approximately 1 g of intestinal mucosa was collected into glass homogenizers with 2 ml of homogenization buffer containing 10 mM Tris-HCl, pH 7.4, 250 mM sucrose and 1 mM PMSF plus 10 µg/ml leupeptin, 1 µg/ml pepstatin and 1 µg/ml aprotinin. The tissues were homogenized for 3 min on ice and cell debris was spun down and removed at 2000 x g for 5 min at 4°C. The supernatants were collected, and the proteins were separated by SDS-PAGE. SDS-PAGE was performed according

Fig. 1. (A,B). (A) Expression of BK channel-related mRNA in eel intestine by RT-PCR. The BK channel isoform was detected in the intestine of eel (lane 2). The identity of isoform was confirmed by PCR product size (696 bp) and sequencing (GenBank accession number EU267177). Lane 1: markers; lane 3: RT-PCR control (RNA without reverse transcriptase). (B) Comparison of partial primary amino acid sequences of eel BK channel with human (NP_002238), rat (AAD34786), and mouse (Q08460) homologous proteins. Putative transmembrane segments (S5 and S6) and the pore-forming loop (P-loop) are denoted by solid lines based on hydropathy analysis of BK channel (Wallner et al., 1996 [11]). Asterisks indicate identical amino acids; colons, conserved substitution (small, acidic, basal or hydroxyl + Amine+Basic, etc.); stops, semi-conserved substitution.

to the method of Laemmli on one-dimensional 8% polyacrylamide gel with the Bio-Rad Mini-Protean II cell [48]. After SDS-PAGE, proteins were blotted onto a nitrocellulose membrane at 200 mA for 60 minutes and blocked for 1 hour in PBS/0.1% Tween-20 (PBST) with 5% non-fat milk. The membrane was incubated overnight with the monoclonal rabbit anti-BK_{Ca} antibody (SIGMA, P8357) diluted 1:300 with 5% milk/PBST, targeted against amino acids of the “tail region” of the maxi-K channel α subunit located in the carboxy-terminal domain [anti- $\alpha_{(1098-1196)}$] and then washed three times with PBST for 10 minutes each. The horseradish peroxidase-conjugated anti-rabbit antibody was used as the secondary antibody (1:2000 dilution). Visualization of immunoreactive bands was accomplished by chemiluminescence and exposure of the blots to luminescence detection film (ECL Western Blotting detection reagents and Hyperfilm-ECL, Amersham). No non-specific bands were observed.

The molecular mass of the eel BK channel was calculated from a calibration curve obtained by plotting the relative mobility of standard proteins on the gel against the log of their respective molecular mass obtained in three different SDS-gel electrophoreses.

Confocal immunofluorescence microscopy

Small segments of eel middle intestine, stripped from their outer layers, were fixed in paraformaldehyde (4% in PBS containing: 0.1 M sodium phosphate, 0.1 M NaCl (pH 8) for 2 h and paraffin-embedded. Sections of 10 μ m thickness were cut

along planes perpendicular to the luminal epithelium surface, placed on poly-L-lysine coated glass slides and rehydrated in a xylene-graded alcohol scale. Section were incubated with the monoclonal rabbit anti-BK_{Ca} antibody (SIGMA, P8357) diluted 1:1000 overnight. Alexa fluor 488 goat anti-rabbit IgG (1:1500) were utilized as secondary antibody. The slides were viewed using the 488 nm Argon laser line of a C1 NIKON confocal laser scanning unit coupled to a NIKON TE300 microscope with a 100X/1.30 oil objective (NIKON).

Tissue morphometric analysis

Morphometric analysis of the epithelium height was performed as previously described [44]. Briefly, small segments of the eel middle intestine, stripped from their outer layers, perfused with Ringer solution (315 mOsm), were fixed before (control), after 5 min and after 45 min exposure to hyposmotic Ringer solution (160 mOsm) respectively and embedded in Epon 812. Semithin (0.5 μ m) sections were cut along planes perpendicular to the luminal epithelium surface and were stained with 1% toluidine blue. Sections were placed on an optic microscope, Axiolab, (Zeiss, Oberkochen, Germany) (objective utilized: 100x oil immersion), and the images obtained from a video camera (Polaroid Digital Microscope Camera DMC 1, CCD, 1600x1200 pixels) were digitalized using NIH Image 1.62; each image was calibrated with respect to the overall magnification and in each specimen epithelium thickness was measured in 80 points, where the plane of section appeared to be exactly perpendicular to the basal membrane.

Fig. 2. Immunoblot analysis of BK channel in eel intestine. Increasing amounts of intestinal proteins (50, 100, 200 and 250 μ g) were separated by 6% SDS-PAGE under reducing conditions. Immunoblotting was performed with antibody recognizing BK channel α subunit. Channel protein was recognized as specific band with expected molecular mass of $\sim$ 60 kDa. No non-specific bands were detected in the immunoblot. Molecular masses for the standard lane are given in kDa.

Fig. 3. BK localization in eel intestinal epithelium. 10 μ m transverse section of the apical part of a villous from the eel intestine. The section was incubated with the monoclonal rabbit anti-BK_{Ca} antibody and Alexa fluor 488 goat anti-rabbit IgG (as secondary antibody) and visualized using confocal laser scanning microscopy as described (see Methods) at 100X objective.

Statistics

Values are given as the mean $\pm$ S.E. of the mean.

Statistical tests utilized to evaluate statistical significance of differences were: paired Student's *t*-test, one way ANOVA test and Keuls-Newman Multiple Comparison Test as indicated in legends to figures. * = $P < 0.05$; ** = $P < 0.01$.

Results

Detection of a BK channel product in eel intestine by specific primers

Using specific primers designed on the basis of the mouse, rat, and human potassium channel sequences, a BK channel-related RT-PCR product of 696 base pairs (bp) was amplified from the total RNA isolated from the eel intestine (Fig. 1A). The partial nucleotide sequence of the resulting cDNA (GenBank accession number EU267177) showed a similarity higher than 82% to human, mouse and rat BK channel sequences and presumably encoded for an amino acid sequence of the eel BK

channel showing a 97% of similarity to the corresponding protein sequences of the above reported mammalian channels (Fig. 1B)

Immunological detection of a BK channel in eel intestine

The existence of a BK channel in eel intestine was also investigated by a Western blot analysis performed with a monoclonal antibody obtained by using a highly purified GST fusion protein of a C-terminal part of the mouse Slo (mSlo) α subunit as immunogen (Fig. 2). The immunoblot analysis revealed a well resolved single band of approximately 60 kDa, as reported for the purified α -subunit of BK channel from bovine tracheal smooth muscle (62 kDa; [49]).

Detection of BK channels by confocal immunofluorescence microscopy

The precise localization of BK channels was determined by confocal immunofluorescence microscopy on

Fig. 4. (A,B,C). Changes in V_{te} and I_{sc} in response to ionomycin in the presence of 0.1 μ M iberiotoxin in the mucosal (B) or the serosal bathing solution (C). Fig. A shows the only ionomycin treatment. V_{te} = transepithelial voltage; I_{sc} = short circuit current; the “-” sign of V_{te} refers to the mucosa (grounded), the “-” sign of I_{sc} indicates current flowing from mucosal to serosal side. V_{te} time-course represents the registered trace, while I_{sc} time-course was performed by keeping preparation “short circuited” every 5 min. Representative time-courses of $n=3$.

10 μ m sections cut along planes perpendicular to the luminal epithelium surface, using the monoclonal anti-BK channel antibody targeted against amino acids of the “tail region” of the maxi-K channel α subunit. In Fig. 3 the upper part of a villus is shown. BK appeared localized on all the plasma membrane of the enterocytes; the brush border region showed the most intense immunostaining.

Functional expression of BK channel

After having demonstrated the molecular expression of BK channels in eel intestine, we addressed their functional expression and physiological role in the intact epithelium by using the selective blocker iberiotoxin [8] in Ussing chamber mounted tissue. Iberiotoxin, a toxin purified from the scorpion *Buthus tamulus*, is a 37 amino acid peptide which binds specifically to the channel with high affinity [8]. When 0.1 μ M iberiotoxin was applied in the Ringer bathing solutions it did not exert any significant effect on the basal electrophysiological parameters

neither when applied in the luminal nor in the serosal bathing solution, suggesting that these channels are inactive in unstimulated cells. (Fig.4B,C). Then, we addressed the Ca^{2+} induced activation of these channels in the eel intact epithelium by increasing intracellular Ca^{2+} concentration with the calcium ionophore ionomycin (1 μ M). The drug induces a depolarization of V_{te} and a decrease of I_{sc} (Fig. 4A) by about 30%. The exact mechanism underlying such changes in the electrophysiological parameters was not explored because it goes outside the scope of this paper. Most likely the ionomycin induced increase in the intracellular calcium concentration interferes somehow with the transepithelial Cl^- absorption, which requires a given basal level of intracellular Ca^{2+} to be performed as reported by Schettino and Lionetto [36]. Interestingly, when 0.1 μ M iberiotoxin was present in the luminal (mucosal) Ringer solution, the ionomycin induced depolarization of V_{te} was significantly increased (Fig.4B, Fig.5A), on the contrary in the presence of 0.1 μ M iberiotoxin in

Fig. 5. (A,B). (A) Ionomycin induced depolarization under control condition and after pre-treatment with 0.1 μ M iberiotoxin from the mucosal side (Ibtx, m) or the serosal side (Ibtx, s). ΔV_{te} was calculated after 15 min 1 μ M ionomycin incubation as follows: $\Delta V_{te} = V_{te \text{ before ionomycin}} - V_{te \text{ after ionomycin}}$. (B) BK dependent I_{sc} current in eel intestine. The ionomycin induced iberiotoxin sensitive current was calculated as the difference between the ionomycin induced ΔI_{sc} measured after pre-treatment with iberiotoxin, either in the serosal or the mucosal Ringer solution, and the ΔI_{sc} value measured in the absence of iberiotoxin. Calculation was performed after 15 min ionomycin incubation. Data are expressed as mean $\pm$ S.E.M. of four separate experiments.

Fig. 6. (A,B). Changes in V_{te} and I_{sc} in response to hypotonic stress in the presence of 0.1 μ M iberiotoxin in the mucosal (A) or the serosal bathing solution (B). Details as in Fig. 4.

the basolateral (serosal) solution, the ionomycin induced depolarization of V_{te} was significantly decreased (Fig. 4C, Fig. 5 A). The same behaviour was observed for I_{sc} (Fig. 4 B, C), while the transepithelial resistance (mean value in control condition: $24.6 \pm 4.8 \Omega \cdot cm^2$) did not show any significant change during the time-course of the experiment. The choice of 1 μ M ionomycin has been made on the basis of preliminary dose-response experiments and comes from a balance between the need to obtain the maximal effect of iberiotoxin on V_{te} and I_{sc} and the need to minimize the effects of the ionophore on the basal V_{te} and I_{sc} .

The obtained results can be explained by a Ca^{2+} induced activation of BK either on the apical or the basolateral membrane of the epithelium and the opposite effect of iberiotoxin added to the luminal or serosal bathing solution on the electrophysiological parameters can be understood considering that K^+ efflux through the basolateral membrane produces a depolarization of V_{te} .

Fig. 7. Hypotonicity induced activation of BK on mucosal (empty symbols) or serosal membrane (filled symbols). The BK dependent I_{sc} current was calculated as follows: $\Delta I_{sc} = \Delta I_{sc_{tot}} - \Delta I_{sc_{ibtx}}$, where $\Delta I_{sc_{tot}}$ was calculated in the control tissue as the differences between the I_{sc} values after hypotonic stress application and the value before every 5 minutes during the time-course of the exposure, while $\Delta I_{sc_{ibtx}}$ was calculated in the same way but following iberiotoxin ($0.1 \mu M$) incubation from the serosal or the mucosal side. Data are expressed as mean $\pm$ S.E.M. of four separate experiments.

Fig. 8. Effect of iberiotoxin ($0.1 \mu M$) on the RVD response in eel intact epithelium. Epithelium height was measured by morphometric analysis of semithin ($0.5 \mu m$) sections of fixed intestine cut along planes perpendicular to the luminal epithelium surface. Sections were stained with 1% toluidine blu and observed by optical microscopy (100-magnification). Hypotonic stress was performed by decreasing Ringer osmolarity from 315 to 175 mOsm by NaCl concentration decrease. Data are expressed as mean $\pm$ S.E. of three separate experiments. The statistical analysis of data was performed by one-way ANOVA and Newman-Keuls multiple comparison post test.

(therefore its inhibition shows a decrease of the ionomycin induced depolarization of V_{te}), while the K^+ efflux through the apical membrane produces a hyperpolarization of V_{te} (therefore its inhibition shows an increase of the ionomycin induced depolarization of V_{te}).

Overall, the ionomycin induced and iberiotoxin sensitive I_{sc} was equivalent either on the luminal or the serosal membrane as it is possible to observe in Fig.5B, where it was calculated as the difference between the I_{sc} value before and after 15 min of ibtx incubation either in the serosal or the mucosal Ringer solution.

Role of BK channels in RVD

After having evidence of the activation of BK channels in the intact epithelium following a $[Ca^{2+}]_i$ increase signal, we addressed the role of BK in osmotic cell

swelling, a known cellular event involving an increase in $[Ca^{2+}]_i$ mainly in epithelia [50]. As previously demonstrated [44] the eel intestinal epithelium responded to a hypotonic challenge with a Ca^{2+} -dependent biphasic decrease in V_{te} and I_{sc} (Fig. 6, control trace) which is correlated with a regulatory volume decrease (RVD) response. When the epithelium was preincubated with $0.1 \mu M$ iberiotoxin in the mucosal Ringer solution the hypotonicity induced depolarization of V_{te} was significantly increased, on the contrary when the epithelium was preincubated with $0.1 \mu M$ iberiotoxin in the serosal Ringer solution, the hypotonicity induced depolarization of V_{te} was significantly decreased. The same behaviour was observed for I_{sc} (Fig. 6 A,B), clearly indicating the hypotonicity induction of BK either on the apical or the basolateral membrane. Fig. 7 shows the hypotonicity in-

duced iberiotoxin sensitive Isc on either the apical or the basolateral membrane as a function of time. As it is possible to observe on both membranes, the current variation showed an increase in the first minutes of exposure reaching the maximal value in 5 min; the observed time-course was approximately the same on either the apical or the basolateral membrane.

The physiological role played by BK in the eel intestine RVD response was directly investigated by morphometric analysis of the epithelium height during hypotonic stress exposure.

After eel intestinal epithelium was exposed to hypotonic solution (performed by decreasing the Ringer osmolarity from 315 to 175 mOsm), the mean epithelium height promptly increased, due to cell swelling. In fact, since cells in a epithelium have very little space for lateral expansion, it is reasonable to observe an epithelium height increase in a hyposmotic medium. After about 30 min exposure, the mean epithelium height gradually recovered (Fig.8), clearly indicating the presence of an RVD response. Interestingly, the RVD response was inhibited by incubation with 100 nM iberiotoxin, suggesting that the BK activation represents one of the main physiological volume recovery mechanism after hypotonic swelling in this tissue.

Discussion

K⁺ channels can contribute to several aspects of epithelial physiology such as the maintenance of the resting cell membrane potential as well as ion transport processes and cell volume regulation. Several types of K⁺ channels have been identified in epithelial cells, and their functional role is just starting to emerge. In many cases the molecular identity of the different K⁺ channels associated with different cellular functions remains unknown.

In the present work we demonstrated the presence of the BK channel in the intestine of the European eel by a molecular and functional approach on the native tissue and we addressed its physiological role in cell volume regulation.

Reverse transcription of mRNA extracted from eel intestinal mucosa cells and subsequent PCR amplification led to a sequence of 696 bp cDNA showing an 83% of similarity to human, mouse and rat BK channels. It is known that the BK channel proteins are structurally related to tetrameric voltage-gated K1 channels by a homology within an S1-S6 region that contains the pore-forming domain, S5-P-S6, and an S4 voltage-sensing

element [13]. The partial amino acid sequence encoded by the mRNA fragment amplified in eel intestine showed 97% similarity to human, mouse and rat BK channels and should correspond to a part of the pore-forming motif of BK channel isoforms consisting of the P-loop, which bears the receptor for the binding of the pore blocker iberiotoxin, and the S6 domain, thought to be the gate for the potassium-selective pore [51]. The expression of a BK channel-related protein in the absorbing epithelium of the eel was also demonstrated by immunoblotting experiments where the monoclonal rabbit anti-BK_{Ca} antibody, targeted against amino acids of the "tail region" of the maxi-K channel α subunit, recognised a well resolved single band of approximately 60 kDa in accordance with results found by Garcia-Calvo et al [49] in bovine tracheal and smooth aortic muscle. The discrepancy with a size of 120 KDa, predicted for the α subunit on the basis of the known slo coding region [47], could be explained by a reproducible proteolysis decay of the α subunit within the long C-terminal tail domain during sample preparation, as it was previously demonstrated by Knauss et al. [47] with the use of sequence-directed antibodies.

After having demonstrated the existence of mRNA coding for BK channels and BK protein expression in eel intestine, we addressed the physiological role played by these channels in this native epithelium. BK channels seem to be inactive in unstimulated intestinal cells, according to data previously obtained with clonal kidney cells (Vero cells) by Hafting and Sand [52]. BK channels seem, therefore, not to contribute to the basal ion transport mechanisms of the eel intestine, which, as previously demonstrated, mainly require a Ba²⁺ sensitive K⁺ conductance on the apical membrane, that permits recycling of K⁺ into the lumen and contributes to the operation of the luminal Na⁺-K⁺-2Cl⁻ [39, 53], and a Ba²⁺ inhibitable K⁺ conductance on the basolateral membrane [53].

On the contrary the BK channels are activated when a [Ca²⁺]_i increase signal occurs, as indicated by ionomycin experiments in Ussing chamber mounted epithelium. Since the early work by Wong and Chase [54] it is known that an increase in [Ca²⁺]_i is a most constant feature of cell swelling induced by hypotonic stress in epithelial cells. In eel intestine a previous study [44] clearly indicated a strong dependence on the extracellular Ca²⁺ in the V_{te} and Isc response to hypotonic stress. Considering that the BK channels in general are not regulated by cell volume changes *per se* [34] it is, therefore, reasonable to assume that in eel intestine swelling activated influx of Ca²⁺ increases cytosolic Ca²⁺, thereby activating BK chan-

nels on both membranes. This event in turn leads to the corrective K^+ efflux and the swelling induced hyperpolarization in either apical or basolateral membrane. The effective volume regulation requires a parallel efflux of cation and anions through the membrane; therefore, the BK induced changes of membrane electrical potential can in turn influence Cl^- efflux on both membranes, allowing the cells to recover their initial volume after hypotonic swelling. As previously demonstrated in eel intestine the main cation electrodiffusive pathway in basal condition is a Ba^{2+} sensitive K^+ conductance on the apical membrane that permits recycling of K^+ into the lumen and contributes to the operation of the luminal $Na^+-K^+-2Cl^-$ [39, 40]. If we consider that this basal K^+ conductance is not involved in the electrogenic response to hypotonic stress (as previously demonstrated by Lionetto et al, [44]), we can suggest that eel intestinal epithelial cells use specific type of K^+ channels for cell volume regulation purpose. In fact, the RVD mechanisms is of essential importance for epithelial cells which are physiologically exposed to osmotic stress resulting in alteration of cell volume in several aspects of their functioning. In fact, epithelia, being interfaces between the internal and the external environment of the organism, can experience changes of extracellular osmolarity. Moreover epithelial transport, accomplished by the entry or extrusion of osmotically active substances at the two cellular membranes, represents a continuous challenge to cell volume constancy, since slight changes in the large apical or basolateral fluxes could lead to rapid changes in cell volume.

An interesting aspect emerging from the analysis of the BK activation dependent Isc is that the iberiotoxin sensitive Isc induced by hypotonic stress is considerably higher than the one induced by $1\mu M$ ionomycin. No increase was observed in the iberiotoxin sensitive Isc by increasing the ionomycin concentration to $5\mu M$ (data not shown) suggesting that $1\mu M$ ionomycin was able to evoke the maximal Ca^{2+} induced stimulation of BK in eel intestine. It is possible to argue that in swelling cells other mechanisms could contribute to the BK activation following a hypotonic stress and may affect the extent of BK current activation. It is known that BK channel sensitivity to voltage and calcium is potently modified by reversible protein phosphorylation of serine/threonine or tyrosine residues within the channel, or closely associated regulatory proteins [55, 56]. Thus the dynamic interactions between competing protein kinases and protein

phosphatases at the channel complex provide an important mechanism to tune BK channel function and behaviour. Protein phosphorylation events have been widely reported to play a pivotal role for in the activation of volume-regulatory ion transport mechanisms following osmotic stress in several cell types [57] and, in particular, several serine/threonine protein kinases have been found to be involved in the V_{te} and Isc response of eel intestine to hypotonic stress (unpublished results). Therefore, the activation of BK in eel intestine during hypotonic stress could be modulated by the complex signalling phosphorylation pathways associated with cell swelling.

Little is known about the precise localization of BK channels in epithelia. In the eel intestine electrophysiological data suggested the localization of these channels on both membrane, and this was confirmed by confocal immunofluorescence microscopy. In most other epithelial cells such as renal epithelium [18, 20], oviduct [23], epididymus [21], gallbladder [24], and vestibular dark cells [25] BK channels were exclusively found in the apical membrane, whereas a basolateral localization has been reported in acinar glands [31] and sea bass gill cells [58]. In the above-mentioned studies the localization has been deduced from electrophysiological measurements, and determination of the precise localization of BK channels in most of these tissues awaits immunohistochemical studies. To our knowledge the colonic surface epithelium is the only other example of targeting of BK channels to both epithelial membranes [30].

In conclusion, our results demonstrated the molecular and functional expression of high conductance Ca^{2+} -activated K^+ channels in eel intestine and demonstrated that their physiological role is mainly related to the RVD response of the epithelium following a hypotonic swelling. The eel intestinal epithelium showed to be a useful model for functional studies of BK in epithelia permitting the functional analysis of these channels in an integrated system such as a freshly isolated living tissue with intact tight junctions, retaining the functional characteristics of the native epithelium *in vivo*.

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