Role of BK Channels in the Apoptotic Volume Decrease in Native Eel Intestinal Cells

Maria Giulia Lionetto, Maria Elena Giordano, Antonio Calisi, Roberto Caricato, Else Kay Hoffmann¹ and Trifone Schettino

Dept. of Biological and Environmental Sciences and Technologies, University of Salento, ¹Section of Cell and Developmental Biology, Department of Biology, University of Copenhagen

Key Words

Apoptosis • Cell volume • K⁺ channel • Intestine • Epithelial cell • Anoikis • Calcium

Abstract

High conductance Ca²⁺-activated K⁺ channels (BK channels) have previously been demonstrated in the eel intestine. They are specifically activated following a hypotonic stress and sustain Regulatory Volume Decrease (RVD). The aim of the present work was to address the possible role of these channels in the Apoptotic Volume Decrease (AVD) of isolated eel enterocytes, and the possible interaction between BK channels and the progression of apoptosis. The detection of apoptosis was performed by confocal microscopy and annexin V and propidium iodide labelling; cell volume changes were monitored by video imaging. Within a few hours after isolation, enterocytes underwent anoikis (apoptosis induced by detachment from the extracellular matrix). They showed an early normotonic volume decrease (AVD) preceding the appearance of annexin V positivity. AVD occurred in correspondence with an increase in the [Ca²⁺]_i, measured with Fura-2. When the cells were resuspended in high K⁺ solution or treated with

iberiotoxin, AVD was completely abolished. In addition, treatment with high K⁺ or iberiotoxin significantly inhibited apoptosis progression. It was demonstrated for the first time in native enterocytes that BK channels, which are involved in RVD in these cells, plays also a crucial role in the AVD process and in the progression of apoptosis.

Copyright © 2010 S. Karger AG, Basel

Introduction

Apoptosis is programmed cell death in response to extrinsic and intrinsic cues such as death receptor ligands or various forms of cellular stress. Apoptosis is crucial in growth, maintenance, and normal development of tissues and there are a growing number of pathologies linked to defective apoptotic processes.

The apoptotic process is associated with a distinct set of biochemical and physical changes involving the cytoplasm, nucleus and plasma membrane [1]. These changes include cell shrinkage, formation of apoptotic bodies, chromatin condensation, and DNA degradation

[2]. In recent years much has been learned about the molecular underpinnings of apoptosis; however, despite the universal prominence of cell volume loss in cells undergoing apoptosis, the relationship between this event and ensuing cell death remains uncertain. Among the proposed signalling events in cell shrinkage induced apoptosis are: (i) cell shrinkage activates the small GTP binding protein Rac, and p38 MAPK followed by phosphorylation and nuclear translocation of p53 [3]; (ii) cell shrinkage inhibits PDGF receptor mediated signalling with reduced MEK1/2 and ERK 1/2 activity and reduced Akt/PKB activity [4] which results in reduced BAD phosphorylation; (iii) cell shrinkage induces death receptor CD95 trafficking to the plasma membrane [5] (for a review see [6]).

The apoptotic cell shrinkage occurs in two distinct stages: the first phase starting before cell fragmentation or formation of the apoptotic body and the second phase associated with cell fragmentation [7]. The early-phase shrinkage is termed Apoptotic Volume Decrease (AVD) [8] and corresponds to an isotonic shrinkage of the cell. A throughout description of the volume and ion changes during the two stages of apoptotic shrinkage has been given in Ehrlich ascites tumour cells [9]. Recently apoptotic cell shrinkage has been associated with alterations of cell volume regulation [10]. Most cells have active volume regulatory mechanisms to maintain a constant volume in response to environmental osmotic challenges. The mechanisms for the control of cell volume are highly conserved and principally similar in cells from various tissues as well as between evolutionary distant species [6, 11]. Following a hypotonic cell swelling, they consist in the release of osmolytes, mainly K⁺ and Cl⁻, followed by loss of osmotically obliged water and are termed Regulatory Volume Decrease (RVD); following a hypertonic shrinkage they consist in the uptake of osmolytes followed by gain of osmotically obliged water and are termed Regulatory Volume Increase (RVI) [6, 12]. Interestingly, and in contrast to the volume changes and responses described above, the loss of cell volume during apoptosis occurs under normotonic conditions, suggesting that AVD is triggered independent of changes in the extracellular osmotic environment. Although AVD occurs under normotonic conditions, Maeno et al. [8] reported in HeLa and U937 cells that loss of cell volume during programmed cell death may share some of the ionic mechanisms that participate in inherent volume regulatory responses, thus linking AVD to RVD. As in RVD, the changes in cell volume during AVD are the consequence of an exit of Cl⁻ and K⁺ from the cells [for a

review see 6, 9, 13]. With respect to Cl⁻ channels the volume regulated anion channel (VRAC) has been shown to be involved in AVD [9, 14]. With respect to the molecular identity of the K⁺ channel involved in both AVD and RVD different candidates have been proposed depending on the tissue under study [6, 9, 13, 15-19] or the stimulus used [20]. Moreover, it is still uncertain whether the K⁺ currents in AVD and RVD are driven by the same type of channels. In T lymphocytes Elliott and Higgins [21] suggested that the same IKCa channel is involved in RVD and in AVD and in Ehrlich cells it seems that a chlofilium sensitive TASK-2 channel is involved in both RVD and AVD [9]. In a previous work Lionetto et al. [22] demonstrated the molecular and functional expression of high conductance Ca²⁺-activated K⁺ channels (BK channels) in the eel (*Anguilla anguilla*) intestinal epithelium. The partial amino acid sequence encoded by the mRNA fragment amplified in eel intestine showed 97% similarity to human, mouse and rat BK channels. The authors reported the activation of these channels on both membranes (apical and basolateral) as one of the main physiological volume recovery mechanism after hypotonic swelling in this epithelium which represents a good physiological model for epithelial cell volume regulation research. In fact it shows a dynamic regulation of cell volume in anisomotic conditions with a RVD response after hypotonic swelling and a RVI response after hypertonic shrinkage [23, 24]. Based on these observations, the aim of the present work was to address (i) the possible role of BK channels in the AVD process and (ii) the possible interaction between BK channel activation and the progression of apoptosis.

We tested our hypothesis on isolated eel enterocytes kept in suspension in serum free medium. We demonstrated that within a few hours after isolation the cells undergo anoikis, i.e apoptosis induced by detachment from the extracellular matrix. We utilized these cells as an experimental model for studying the role of cell volume regulation mechanisms in the apoptotic process directly in native epithelial cells.

To our knowledge this is the first time that the AVD process and its underlining mechanisms are investigated in native enterocytes.

Materials and Methods

Sea water acclimated yellow eels (*Anguilla anguilla*) (weighing about 200 g) 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°C–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. Alexa Fluor® 488 annexin V, propidium iodide, and Fura-2 AM were purchased from Invitrogen - Molecular Probes (Carlsbad, CA, USA). All the other chemicals were purchased from Sigma (St. Louis, U.S.A.).

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

Isolation of intestinal cells

Enterocytes were isolated according to Vilella et al [25]. Briefly, the intestine was removed, stripped of longitudinal and circular layers with two pairs of fine forceps, and cut in small segments. The segments were placed in a sodium citrate buffer containing (in mM): Na-citrate 27, NaCl 96, NaH₂PO₄ 5.6, KCl 1.5 adjusted to pH 7.9 with Tris and gently stirred with a glass rod for 10 min to facilitate cells detachment. This procedure was repeated twice. The released cells were filtered through a 225, 100, and 50 µm nylon mesh and centrifuged at 100 g for 10 min with a J-25 Beckman centrifuge. The resulting pellet containing the cells was resuspended in fish physiological saline solution containing (in mM): NaCl 101, KCl 3.2, MgCl₂ 1.4, CaCl₂ 2.5, KH₂PO₄ 0.8, NaHCO₃ 20, glucose 20 mM, L-glutamine 2, Hepes 30 mM, buffered at pH 7.9 with Tris (osmolality: 300 mOsm/Kg) at a cytocrit of 4%. Cells were kept in suspension for 6–7 min in a humid chamber under shaking in a saturated atmosphere of CO₂ 1% and O₂ 99%.

In the experiments with high [K⁺], K⁺ was increased by 10 times (32 mM final concentration) by equimolar replacement of Na⁺. The cells were resuspended in high-[K⁺] fish physiological saline solution containing (in mM): NaCl 72.2, KCl 32, MgCl₂ 1.4, CaCl₂ 2.5, KH₂PO₄ 0.8, NaHCO₃ 20, glucose 20 mM, L-glutamine 2, Hepes 30 mM, buffered at pH 7.9 with Tris (osmolality: 300 mOsm/Kg) at a cytocrit of 4% and kept as reported above.

Unless otherwise indicated, experiments were carried out at room temperature.

Confocal microscopy detection of Apoptosis

One of the earliest indications of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane [26]. Once exposed to the extracellular environment, binding sites on PS become available for Annexin V, a 36 kDa, Ca²⁺-dependent, phospholipid binding protein with a high affinity for PS [27]. Isolated eel enterocytes were double marked with Alexa Fluor® 488 annexin V and propidium iodide which binds to nucleic acids, but can only penetrate the plasma membrane when membrane integrity is breached, as occurs in dying cells. Therefore, the combined use of the two markers allows the detection of appearance and progression of apoptosis.

10 µl of cell suspension were dispensed on a poly-L-lysine coated slide and incubated in a humid chamber for 2 min. The cells were incubated with annexin V conjugated and propidium iodide solution (Alexa Fluor® 488 annexin V diluted to 5% in fish physiological saline solution containing 0.15 mM propidium iodide) for 15 min in a humid chamber in the dark. Then, the cells were viewed using a 100X NA plan apochromat objective mounted on a NIKON TE300 inverted microscope coupled to a NIKON C1 confocal laser scanning unit (Nikon, Tokyo, Japan). Alexa Fluor® 488 annexin V and propidium iodide were excited using the Argon 488-nm and Elio-Neon 543-nm laser lines, respectively. Photomultiplier (PMT) and laser intensity settings and emission wavelengths were optimized for minimal bleed through between the two channels. Live cells, early apoptotic, apoptotic and dead cells were distinguished on the basis of annexin V and propidium iodide positivity by setting different fluorescence threshold values for different regions of interest (ROIs), each containing a cell. Unlabeled preparations were found to exhibit no fluorescence under the conditions used.

At least a minimum of 100 cells/slide were analysed.

Estimation of changes in cell volume

Isolated eel enterocytes appear as spherical shaped cells. Cell volume changes in different apoptotic stages were monitored by morphometric analysis of annexin V and propidium iodide double marked cells observed by confocal microscopy. Vertical cross-sectional images of the cells were visualized by using repetitive z-optical scanning with a step distance of 0.3 µm. The estimation of cell volume changes was performed by measuring the cell area of the equatorial 2-D image of each cell. The C1 NIKON image analysis software was utilized.

The RVD response was measured by morphometric analysis of freshly isolated eel enterocytes observed by an optic microscope (Eclipse E600, Nikon, Tokyo, Japan). Briefly 10 µl of cell suspension were dispensed on a poly-L-lysine coated slide and incubated in a humid chamber for 1 min. Then, a cover-slide was applied and the slide was ready to be observed under the microscope. The images were obtained from a video camera (TK-C1381, JVC, Yokohama, Japan) and were digitalised and analyzed using the LUCIA images analysis software (Nikon, Tokyo, Japan).

At least a minimum of 100 cells/slide were analysed.

Measurement of the [Ca²⁺]_i

The cytoplasmic Ca²⁺ concentration of eel enterocytes in suspension was determined using the ratiometric indicator Fura-2. Cells were suspended to a concentration of 10⁶ cells/ml and incubated with 6 µM fura-2 acetoxymethyl ester (fura-2/AM) (stock solution: 1 mM fura-2/AM in DMSO) for 45 min at room temperature in the dark. After incubation, the cells were washed three times with the fish physiological saline solution free of fura-2/AM. The fluorescence intensity emitted by the suspended cells was recorded every 30 min for 4 hours at alternating excitation wave length 340 (F₃₄₀) and 380 nm (F₃₈₀, 5-nm slit for each) (emission 510 nm, 5-nm slit) using a Jasco FP-750 spectrofluorometer. Then, the [Ca²⁺]_i-dependent fluorescence ratio ($R = F_{340}/F_{380}$) was calculated. Ratio (R) values were con-

Fig. 1. (A,B) (A) Representative confocal image (objective utilised: 100x oil immersion) of isolated eel enterocytes: cell population analyzed included not apoptotic cells (double negative), early apoptotic cells (weakly annexin V positive), apoptotic cells (annexin V positive), dead cells (double positive). (B) Annexin V and propidium iodide positive cell percentage was monitored through the time. The measurements were performed immediately after the isolation (time 0) and every one hour for 6 hours from the isolation respectively. Data are expressed as mean $\pm$ SEM of three independent experiments. The statistical significances of differences (each time vs time 0) was analyzed by Dunnett's test. * = $P < 0.05$.

verted to an estimate of $[Ca^{2+}]_i$ using the Grynkiewicz formula [28]: $[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R) \times S_{fb}$, where R_{min} is 340/380 ratio under Ca^{2+} free condition, R_{max} is 340/380 ratio under Ca^{2+} saturating conditions, S_{fb} is the ratio of baseline fluorescence (380 nm) under Ca^{2+} free and Ca^{2+} saturating conditions. A K_d of 224 nM was assumed. The R_{max} and R_{min} values were determined by the addition of Triton X-100 (1% final concentration) and EGTA (10 mM final concentration) respectively.

Statistics

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

Statistical tests utilized to evaluate statistical significance of differences were: Dunnett's test, Student's t-test, one Way ANOVA and Newman Keules post test. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

Results

Apoptotic Volume Decrease in isolated eel enterocytes

It is known that epithelial cells normally undergo apoptosis in response to disruption of cell adhesions in a process called anoikis [29]. Eel enterocytes, isolated from the entire epithelium and kept in suspension in a serum

free medium, were monitored for the possible appearance of apoptotic characteristics over time. The cells were double marked with Alexa Fluor® 488 annexin V and propidium iodide and observed by confocal microscopy. As reported in the Method section, annexin V and propidium iodide can be used together to monitor the progression of apoptosis: from cell viability to early-stage apoptosis, and finally to late-stage apoptosis and cell death. As shown in Fig. 1A, live cells remained negative for both dyes, early apoptotic cells showed weak positivity to annexin V, apoptotic cells showed marked positivity to annexin V, while dead cells (or permeabilized cells) were double positive. Early apoptotic, apoptotic and dead cells increased their frequency through the time course of the experiment. A significant ($P < 0.05$, Dunnett's test) increase was observed after four hours from the isolation for early apoptotic cells and one hour later for apoptotic and dead (or permeabilized cells) cells (Fig. 1B). This result clearly indicated that eel isolated enterocytes kept in suspension undergo apoptosis within few hours from isolation.

In parallel to the apoptotic signal detection, cell volume changes were monitored by morphometric analysis of the area of 2-D digitised images of annexin V and

Fig. 2. Cell size in different apoptotic stages. The area of 2D-D confocal images of annexin V and propidium iodide double marked cells immediately after isolation is reported beside the relative cell dimension as percentage versus live cells. Data are reported as mean $\pm$ S.E.M of three experiments. The statistical significance of differences was analyzed by one way Analysis of Variance and Newman Keules post test. * $P<0.05$, *** $P<0.001$ vs time 0.

Fig. 3. Time-course of cell volume changes in isolated eel enterocytes in different apoptotic stages. As described in the Method section, the morphometric analysis was performed on 2D images of annexin V and propidium iodide double marked cells observed by confocal microscopy. Objective utilised: 100x (oil immersion). Data are reported as mean $\pm$ S.E.M of three experiments. The statistical significance of differences (for each stage each time vs time 0) was analyzed by Dunnett's test. Double negative cells showed a significant ($P<0.01$) normotonic shrinkage after three hours from isolation. No significant variation in the dimension of early apoptotic, apoptotic, and dead cells was observed during the course of the experiment.

propidium iodide double marked cells. In Fig. 2 the area of 2D confocal images of the cells immediately after isolation is reported beside the relative cell dimension as percentage versus double negative cells. One way Analysis of variance and Newman Keules post test revealed significant differences in the dimension of the cells in different stages. Early apoptotic cells showed a 25% decrease of cell size with respect to double negative cells, while apoptotic cells showed a further decrease (about 15%) in the cells size. This reduction was maintained in permeabilized cell (propidium iodide positive).

Interestingly, when the dimension of the cells in different stages was monitored during the course of the

experiment, annexin V and propidium iodide double negative cells showed a significant ($P<0.01$) normotonic shrinkage after three hours from isolation, reaching the dimension of early apoptotic cells (Fig. 3). On the other hand no significant variation in the dimension of early apoptotic, apoptotic, and apoptotic dead cells was observed during the course of the experiment. This result clearly demonstrated that the observed normotonic cell volume decrease in annexin and propidium iodide negative cells can be ascribed to the Apoptotic Volume Decrease (AVD). It represents one of the first events of apoptosis and precedes the appearance of apoptotic characters.

Fig. 4. Effect of neutralization of K^+ gradient across the cell membrane on AVD in eel enterocytes. The cells were incubated in high- $[K^+]$ (32 mM) physiological saline solution. Cell volume changes were measured as changes in the area of 2-D confocal digitised images of annexin V and propidium iodide negative cells. Data are reported as mean $\pm$ S.E.M of three experiments. *** = $P < 0.001$ high K^+ vs ctrl. The dashed line across the time denotes the initial level of cell area.

Fig. 5. RVD response in isolated eel enterocyte measured in control and following exposure to 0.1 μM ibertoxin. Cell volume changes were measured as changes in the area of 2-D digitised images of enterocyte exposed to hypotonic stress. Hypotonic stress was applied by reducing the osmolarity of the saline solution from 315 mOsm to 175 mOsm by NaCl concentration decrease. The cells were visualised by optical microscopy. Objective utilised: 40x. Data are reported as mean $\pm$ S.E.M. The statistical significance of differences (between control and ibtx treated cells at each time) was analyzed by Student's t test. * = $P < 0.05$ vs ctrl, ** = $P < 0.01$ vs ctrl; *** $P < 0.001$ vs ctrl. The dashed line across the time versus area graphs denotes the initial level of area.

The further decrease in the cell volume observed in apoptotic and permeabilized cells could be ascribed to the cytoplasmic condensation due to blebbing of the plasma membrane and/or apoptotic body formation occurring later during the apoptosis.

Effect of K^+ gradient neutralization on AVD in eel enterocytes

It is known that the changes in cell volume during AVD are the consequence of an exit of Cl^- and K^+ from the cells [for a review see 6, 9, 13]. The involvement of K^+ efflux in the annexin and propidium iodide negative cells was addressed by monitoring cell volume changes when the K^+ gradient across the cell membrane was neu-

tralized. As reported in Fig. 4, cells incubated in high- $[K^+]$ physiological solution failed to show the characteristic isotonic shrinkage appearing in control enterocytes. This result clearly demonstrated that K^+ efflux represents one of the main mechanisms underlying AVD in eel enterocytes.

High conductance Ca^{2+} activated K^+ channels in RVD and AVD

In order to identify the specific potassium channels responsible for K^+ efflux in AVD in eel enterocytes, the role of Ca^{2+} activated K^+ channels, whose molecular and functional expression has been recently demonstrated in these cells [22], was addressed. Lionetto et al. [22]

Fig. 6. Effect of 0.1 μM iberiotoxin on AVD in eel enterocytes. Cell volume changes were measured as changes in the area of 2-D confocal digitised images of annexin V and propidium iodide negative cells. Data are reported as mean $\pm$ S.E.M of three experiments. *** = $P < 0.001$ ibtx vs ctrl. The dashed line across the time denotes the initial level of cell area.

Fig. 7. Time-course of the intracellular Ca^{2+} concentration of isolated eel enterocytes during six hours from isolation. Cells were charged with 6 μM fura-2/AM. The fluorescence ratio ($R = F_{340}/F_{380}$) values were converted to an estimate of $[\text{Ca}^{2+}]_i$ using the Grynkiewicz formula (see Methods). Representative experiment of $n=3$. Data are reported as mean $\pm$ S.E. The statistical significances of differences (each time vs time 0) was analyzed by Dunnett's test.

previously demonstrated in the intact tissue that the physiological role of these channels sensitive to iberiotoxin is mainly related to the RVD response of the epithelial cells following hypotonic swelling.

In the present work it was first tested if isolated enterocytes maintained the functional role of BK channels in the RVD response as observed in the intact epithelium. When the cells were exposed to a hypotonic stress (performed by decreasing the incubating solution osmolarity from 315 to 175 mOsm by decrease of the NaCl concentration) cell volume initially increased for 2-3 min, due to the osmotic influx of water. Thereafter, the cells were able to activate a RVD response, as indicated by the decrease of cell volume towards the initial value although the stress condition was still persisting (Fig. 5, control trace). When, however the cells were preincubated with 0.1 μM iberiotoxin, a specific inhibitor

of BK channels [30], the initial swelling was not followed by a RVD response (Fig. 5, ibtx trace). This result confirms that BK channels maintain their key role in the RVD response in the isolated enterocytes.

In order to address if BK channels contribute to the reduction in cell volume observed in the AVD process, we monitored cell volume changes in annexin V and propidium iodide double negative cells over six hours from the isolation in control and iberiotoxin incubated cells. As reported in Fig. 6, iberiotoxin did not induce any changes in the cell volume in the first hours of incubation, but it was able to completely abolish the characteristic isotonic shrinkage appearing later. This result demonstrated the key role of BK channels in AVD.

As previously demonstrated in the intact tissue BK channels are silent in basal condition and are activated when a $[\text{Ca}^{2+}]_i$ increase signal occurs [22]. Therefore,

Fig. 8. (A, B, C). Annexin V and propidium iodide positive cell percentage was monitored through the time in control, high K^+ and ibtx exposed cells. Details as in Fig. 1B. The statistical significance of differences (between control and ibtx treated cells and high K^+ treated cells at each time) was analyzed by one Way Anova and Newman Keules post test. No statistical difference was observed between high K^+ and ibtx treated cells at any times. On the other hand the differences were statistically significant between control cells and ibtx or high K^+ treated cells from the fourth hour of experiment for early apoptotic cells (A) and from the sixth hour for apoptotic (B) and dead cells (C). Data are expressed as mean $\pm$ S.E.M. of three experiments.

in parallel to the morphometrical analysis of the cells, $[Ca^{2+}]_i$ was spectrofluorimetrically monitored in suspended cells during the period 1.5 to 6 hours after isolation. The reason why $[Ca^{2+}]_i$ monitoring was started after 1.5 hours after the cell isolation is the Fura-2 AM charging procedure.

As reported in Fig. 7, a 10 time increase in the $[Ca^{2+}]_i$ (from ~ 60 nM to ~ 600 nM) was observed between the second and the third hour of experiment, in correspondence with the characteristic isotonic shrinkage observed at the third hour from isolation on double negative cells (Fig. 3). This result suggests that the inducer of the K^+ efflux through BK channels is an increase in the $[Ca^{2+}]_i$ when the cells detach. A further increase in the $[Ca^{2+}]_i$ was also observed following the fifth hour of experiment, indicating a multistep increase in the $[Ca^{2+}]_i$ in eel enterocytes undergoing anoikis.

High conductance Ca^{2+} activated K^+ channels in the progression of apoptosis

After having demonstrated that the BK channels blockade inhibits the AVD process, the possible relationship between K^+ efflux through BK channels and the progression of apoptosis was addressed by confocal microscopy. As reported in Fig. 8 (A,B,C) the time-dependent increase in the frequency of early apoptotic, apoptotic and permeable dead cells, which was observed in the control cells, was completely inhibited in either high K^+ or iberiotoxin treated cell. In all cases the effect was statistically significant as stated by one way ANOVA and Newman Keules post test. Therefore, the inhibition of K^+ efflux through BK channels was able to inhibit the onset of the phosphatidylserin translocation that occurs during anoikis, as measured by the annexin V measure-

ment, and also to inhibit the much later occurring cell membrane disruption as measured by propidium iodide.

Discussion

K^+ channels play a key role in several aspects of epithelial physiology such as the maintenance of the resting cell membrane potential as well as fluid transport processes and cell volume regulation. Several types of K^+ channels have been identified in epithelial cells. Among them high conductance Ca^{2+} -activated K^+ channels (BK channels) have been shown to be involved in regulatory volume decrease (RVD) response following hypotonic

stress in several epithelial cell types [see 6]. The cell volume regulation is fundamental 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 [see 6 for a review].

The present study focused on BK channels in the intestinal epithelium of the euryhaline teleost European eel. A previous study [22] demonstrated the presence of BK channel sensitive to iberiotoxin in this epithelium and addressed the role of these channels in cell volume regulation. As demonstrated by the authors, BK channels do not contribute to the basal ion transport mechanisms in the intact tissue of the eel intestine, but they are specifically activated following a hypotonic stress and allow the recovery of cell volume after swelling [22]. The present paper for the first time disclosed a novel functional role of these channels in native intestinal cells, their contribution to the apoptotic process. Apoptosis is fundamental for maintaining tissue homeostasis and this is of particular relevance for the intestinal epithelium which is characterized by a high cellular turnover ensured by a high division rate of stem cells and a corresponding high apoptotic rate of mature cells [31].

In the present paper an experimental model for studying the role of these channels in the apoptotic process directly in native intestinal cells was developed. It was represented by freshly isolated eel enterocytes maintained in suspension in a serum free medium. The isolated enterocytes maintained the ability to perform a RVD response following a hypotonic swelling by the activation of BK channels, as previously demonstrated in the intact epithelium [22]. These cells underwent apoptosis following the detachment from the extracellular matrix (phenomenon known as anoikis) as indicated by confocal microscope observations of annexin V and propidium iodide labelled cells. Moreover, these cells showed a multistep decrease of their volume during the progression of apoptosis: an early normotonic volume decrease, first observed in annexin V and propidium iodide negative cells, and a further cell volume decrease observed in strong positive cells and in permeable dead cells. While the last cell volume reduction can be attributed to the

cytoplasmic condensation due to blebbing of the plasma membrane and apoptotic body formation, the first cell volume reduction can be ascribed to AVD, which represents a first event in the apoptotic process. Such an AVD during early apoptosis is in agreement with what is seen in essential all cells investigated [6]. In Ehrlich ascites tumour cells it was clearly shown that the early AVD precedes activation of Caspase 3 [9], in isolated eel enterocytes the AVD was shown to precede a key hallmark of apoptosis such as externalization of membrane phosphatidylserine.

When the cells were resuspended in high K^+ saline solution the characteristic AVD was completely abolished. The same result was obtained with iberiotoxin incubation, strongly suggesting that K^+ efflux through BK channels is a crucial event for the AVD process in eel enterocytes. Involvement of K^+ currents in apoptosis has been previously demonstrated in neurons [32–34], lymphocytes [21, 35], smooth muscle cells [36], cardiac cells [37], Ehrlich ascites tumour cells [9], and various cell lines [38], including IEC-6 cells, a line of cultured intestinal epithelial cells [39].

The identity of K^+ channels involved in AVD has been ascribed to 4-AP and TEA-sensitive K_v channels in cortical neurons [34], pulmonary artery myocyte [36, 40], T-leukemic cells [19], cardiomyocytes [37], or to quinine- and Ba^{2+} -sensitive K^+ channels in human epithelial HeLa, human lymphoid U937, mouse neuroblastoma 3 rat glioma hybrid NG108-15, and rat pheochromocytoma PC12 cells [8] and the two-pore K^+ channels that have been implicated in RVD in multiple cell types [6, 17]. Nietsch et al. [41] reported the Ca^{2+} dependence of the activation of K^+ conductance in HTC cell death, but a clear evidence of the involvement of iberiotoxin sensitive high conductance K^+ channels in AVD has to date only been reported by Krik et al [36, 42] in vascular smooth muscle cells. Our results demonstrated for the first time in native intestinal cells that the AVD process depends on BK channel activation and that the same type of K^+ channels responsible for cell volume regulation is also used by the cells for the AVD process.

Bortner and Cidlowski [20] pointed out that distinct potassium channels or potassium transport mechanisms may be involved to achieve the apoptotic loss of intracellular potassium depending on the cell type or stimulus used to induce apoptosis. In the present paper the apoptotic stimulus was detachment of the cells from the basal lamina in the process known as anoikis. It is known that epithelial and endothelial cells when lack attachment to the extracellular matrix undergo death known as anoikis

[29]. Anoikis mediates cell death to maintain homeostasis in tissues with high cyclic renewal rates [43]. In the intestine it is thought to be normally involved in the shedding of enterocytes at the tip of intestinal villi [44-46]. Anoikis is under the control of signalling evoked by integrins and is mediated by several tyrosine kinases, including focal adhesion kinases (FAK). It presents morphological and biochemical markers of classical apoptosis, such as cell shrinkage, PS translocation, nucleus condensation, caspase activation and DNA laddering, and proceeds by hetero-oligomerization of Bax and Bid and activation of caspase 9 and 3 [47].

In the present paper for the first time the ionic mechanisms underlying AVD were explored during anoikis in native intestinal cells. We demonstrated that BK channel blockade completely inhibits the onset of AVD in the anoikis process as indicated by the inhibition exerted by high K^+ and ibtx on the increase of early apoptotic cells observed in control condition. AVD precedes important apoptotic events such as PS translocation, suggesting that BK activation is one of the earliest events in the anoikis program. In addition, anoikis progression appeared significantly attenuated after treatment with high K^+ or ibertoxin, as indicated by the high K^+ and ibtx inhibition of the apoptotic and late apoptotic cell frequencies observed in control. These results suggest that the activation of BK channels is an essential step in the anoikis process in the eel enterocytes.

As previously demonstrated in the intact tissue BK channels are silent in basal condition and are activated when a $[Ca^{2+}]_i$ increase signal occurs. Since the early work by Wong and Chase [48] it is known that an increase in $[Ca^{2+}]_i$ is a most constant feature of cell swelling induced by hypotonic stress in epithelial cells. In addition, it is known that an increase in the intracellular calcium concentration by influx through plasma membrane channels or by release from the endoplasmic reticulum is a requisite for apoptosis in many cases [49, 50]. In eel enterocytes a two-step increase in $[Ca^{2+}]_i$ was observed following detachment from the basal lamina. The first $[Ca^{2+}]_i$ increase occurred before the third hour from cell isolation and was in correspondence with the onset of the BK dependent isotonic shrinkage observed in double negative cells. This results strongly suggest that the inducer of the K^+ efflux through BK channels and in turn of AVD is an increase in the $[Ca^{2+}]_i$. On the other hand the second $[Ca^{2+}]_i$ increase occurred between the fourth and fifth hour from cell detachment. It is known that vari-

ous apoptotic stimuli may affect the $[Ca^{2+}]_i$, and, in turn, Ca^{2+} signals can trigger apoptosis and lead to the activation of caspases [49, 50]. Once activated, the caspases may impair key components of the Ca^{2+} homeostasis, such as Ca^{2+} -ATPase, Na^+ - Ca^{2+} exchanger [50]. The impairment of the calcium homeostasis could explain the later strong increase of the $[Ca^{2+}]_i$ observed in our experimental model.

Considering that the inhibition of K^+ efflux through BK channels inhibit the PS translocation, which is known to be Ca^{2+} dependent [50], and the progression of apoptosis, it is possible to argue that BK channels represent one of the first target of $[Ca^{2+}]_i$ increase in the apoptotic effector machinery playing a crucial role in the apoptosis induction and progression.

Moreover, it is known that BK channel sensitivity to voltage and calcium is potentially modified by reversible protein phosphorylation of serine/threonine or tyrosine residues within the channel, or closely associated regulatory proteins [51, 52]. 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. In a previous paper [22] it was suggested that phosphorylation events are involved in the modulation of BK channel activation in the RVD response in eel intestine. Although the signal transduction mechanisms underlying AVD activation in the eel intestine will be the objective of future work, it is possible to argue that signalling pathways responsible for AVD and RVD could share common downstream signals in BK channel activation. In turn, BK channel activation could represent a key event in the complex mechanisms of cell volume regulation and cell survival. This is of particular relevance for a tissue like the intestinal epithelium where cell volume regulation and apoptosis represent fundamental process of the cellular physiology.

Due to the importance of anoikis in the normal physiological development of the body, as well as in disease states such as cancer [53] where cells harbour anoikis resistance allowing spread to occur, the understanding of the early mechanisms of anoikis in epithelia has advanced clinical implications in cancer and cell therapy research.

Acknowledgements

This work was supported by Fondazione Cassa di Risparmio di Puglia.

References

- 1 Lawen A: Apoptosis - an introduction. *Bioassays* 2003;25:888-896.
- 2 Cohen GM: Caspases: the executioners of apoptosis. *Biochem J* 1997;326:1-16.
- 3 Friis MB, Friberg CR, Schneider L, Nielsen MB, Lambert IH, Christensen ST, Hoffmann EK: Cell shrinkage as a signal to apoptosis in NIH3T3 fibroblasts. *Am J Physiol* 2005;294:C1552-C1565.
- 4 Nielsen MB, Christensen ST, Hoffmann EK: Effects of osmotic stress on the activity of MAPKs and PDGFR-beta-mediated signal transduction in NIH-3T3 fibroblasts. *Am J Physiol* 2008;294:C1046-C1051.
- 5 Reinehr R, Häussinger D: Hyperosmotic activation of the CD95 system. *Methods Enzymol* 2007;428:145-160.
- 6 Hoffmann EK, Lambert IH, Pedersen SF: Physiology of cell volume regulation in vertebrates. *Physiol Rev* 2009;89:193-277.
- 7 Benson RSP, Heer S, Dive C, Watson AJM: Characterization of cell volume loss in CEM-C7A cells during dexamethasone-induced apoptosis. *Am J Physiol* 1996;270:C1190-1203.
- 8 Maeno E, Ishizaki Y, Kanaseki T, Hazama A, Okada Y: Normotonic cell shrinkage because of disordered volume regulation is an early prerequisite to apoptosis. *Proc Natl Acad Sci* 2000;97:9487-9492.
- 9 Poulsen KA, Andersen EC, Klausen TK, Hougaard C, Lambert IH, Hoffmann EK: Deregulation of Apoptotic Volume Decrease and ionic movements in Multidrug Resistant Tumor Cells: Role of the chloride permeability. *Am J Physiol* 2010;298:C14-25.
- 10 Okada Y, Maeno E, Shimizu T, Dezaki K, Wang J, Morishima S: Receptor-mediated control of regulatory volume decrease (RVD) and apoptotic volume decrease (AVD). *J Physiol* 2001;532:3-16.
- 11 Lang F, Busch GL, Volkl H: The Diversity of Volume Regulatory Mechanisms. *Cell Physiol Biochem* 1998;8:1-45.
- 12 Hoffmann EK, Dunham PB: Membrane mechanisms and intracellular signalling in cell volume regulation. *Int Rev Cytol* 1995;161:173-262.
- 13 Okada Y, Maeno E: Apoptosis, cell volume regulation and volume-regulatory chloride channels. *Comp Biochem Physiol A* 2001;130:377-383.
- 14 Okada Y, Maeno E, Shimizu T, Manabe K, Mori S, Nabekura T: Dual roles of plasmalemmal chloride channels in induction of cell death. *Pflugers Arch* 2004;448:287-295.
- 15 Burg ED, Remillard CV, Yuan JXJ: K⁺ channels in apoptosis. *J Membr Biol* 2006;209:3-20.
- 16 Remillard CV, Yuan JX: Activation of K⁺ channels: an essential pathway in programmed cell death. *Am J Physiol* 2004;286:L49-L67.
- 17 Trimarchi JR, Liu L, Smith PJ, Keefe DL: Apoptosis recruits two-pore domain potassium channels used for homeostatic volume regulation. *Am J Physiol* 2002;282:C588-C594.
- 18 Lu L: Stress-induced corneal epithelial apoptosis mediated by K⁺ channel activation. *Prog Retin Eye Res* 2006;25:515-538.
- 19 Bock J, Szabo I, Jekle A, Gulbins E: Actinomycin D-induced apoptosis involves the potassium channel Kv1.3. *Biochem Biophys Res Commun* 2002;295:526-531.
- 20 Bortner CD, Cidlowski JA: Cell shrinkage and monovalent cation fluxes: role in apoptosis. *Arch Biochem Biophys* 2007;462:176-188.
- 21 Elliott JL, Higgins CF: IKCa1 activity is required for cell shrinkage, phosphatidylserine translocation and death in T lymphocyte apoptosis. *EMBO* 2003;4:189-194.
- 22 Lionetto MG, Rizzello A, Giordano ME, Maffia M, De Nuccio F, Nicolardi G, Hoffman EK, Schettino T: Molecular and functional expression of high conductance Ca²⁺ activated K⁺ channels in the eel intestinal epithelium. *Cell Physiol Biochem* 2008;21:361-37.
- 23 Lionetto MG, Giordano ME, Nicolardi G, Schettino T: Hypertonicity stimulates Cl⁻ transport in the intestine of freshwater acclimated eel, *Anguilla anguilla*. *Cell Physiol Biochem* 2001;11:41-54.
- 24 Lionetto MG, Pedersen SF, Hoffmann EK, Giordano ME, Schettino T: Roles of the cytoskeleton and of protein phosphorylation events in the osmotic stress response in eel intestinal epithelium. *Cell Physiol Biochem* 2002;12:163-17.
- 25 Vilella S, Zonno V, Lapadula M, Verri T, Storelli C: Characterization of plasma membrane Na/H exchange in eel (*Anguilla anguilla*) intestinal epithelial cells. *J Exp Zool* 1995;271:18-26.
- 26 van Engeland M, Kuijpers HJH, Ramaekers FCS, Reutelingsperger CPM, Schutte B: Plasma membrane alterations and cytoskeletal changes in apoptosis. *Exp Cell Res* 1997;235:421-30.
- 27 van Engeland M, Nieland LJW, Ramaekers FCS, Schutte B, Reutelingsperger CPM: Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. *Cytometry* 1998;31:1-9.
- 28 Gryniewicz G, Poenie M, Tsien RY: A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol Chem* 1985;260:3440-3450.
- 29 Meredith Jr, Fazeli B, Schwartz MA: The extracellular matrix as a survival factor. *Mol Biol Cell* 1993;4:953-961.
- 30 Kaczorowski GJ, Knaus HG, Leonard RJ, Mcmanus OB, Garcia ML: High conductance calcium-activated potassium channels; structure, pharmacology, and function. *J Bioenerg Biomembr* 1996;28:255-267.
- 31 Potten CG: Epithelial cell growth and differentiation: intestinal apoptosis. *Am J Physiol* 1997;273:G253-G257.
- 32 Nadeau H, McKinney S, Anderson DJ, Lester HA: ROMK1 (Kir1.1) causes apoptosis and chronic silencing of hippocampal neurons. *J Neurophysiol* 2000;84:1062-1075.
- 33 Pal S, Hartnett KA, Nerbonne JM, Levitan ES, Aizenman E: Mediation of neuronal apoptosis by Kv2.1-encoded potassium channels. *J Neurosci* 2003;23:4798-4802.
- 34 Yu SP, Yeh CH, Sensi SL, Gwag BJ, Canzoniero LM, Farhangrazi ZS, Ying HS, Tian M, Dugan LL, Choi DW: Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science* 1997;278:114-117.
- 35 Hughes FM Jr, Bortner CD, Purdy GD, Cidlowski JA: Intracellular K⁺ suppresses the activation of apoptosis in lymphocytes. *J Biol Chem* 1997;272:30567-30576.
- 36 Krick S, Platoshyn O, Sweeney M, Kim H, and Yuan JX: Activation of K⁺ channels induces apoptosis in vascular smooth muscle cells. *Am J Physiol* 2001;280:C970-C979.
- 37 Ekhterae D, Platoshyn O, Zhang S, Remillard CV, Yuan JX: Apoptosis repressor with caspase domain inhibits cardiomyocyte apoptosis by reducing K⁺ currents. *Am J Physiol* 2003;284:C1405-C1410.
- 38 Yu SP: Regulation and critical role of potassium homeostasis in apoptosis. *Prog Neurobiol* 2003;70:363-386.
- 39 Grishin A, Ford H, Wang J, Li H, Salvador-Recatala V, Levitan ES, Zaks-Makhina E: Attenuation of apoptosis in enterocytes by blockade of potassium channels. *Am J Physiol* 2005;289:G815-G821.
- 40 Krick S, Platoshyn O, Sweeney M, McDaniel SS, Zhang S, Rubin LJ, Yuan Jason XJ: Nitric oxide induces apoptosis by activating K_p channels in pulmonary vascular smooth muscle cells. *Am J Physiol* 2002;282:H184-H193.

- 41 Nietsch HH, Roe MW, Fiekers JF, Moore AL, Lidofski SD: Activation of potassium and chloride channels by tumor necrosis factor. Role in liver cell death. *J Biol Chem* 2000;275:20556-20561.
- 42 Krick S, Platoshyn O, Sweeney M, McDaniel SS, Zhang S, Rubin LJ, Yuan JXJ: Nitric oxide induces apoptosis by activating K⁺ channels in pulmonary vascular smooth muscle cells. *Am. J. Physiol* 2002;282:H184-H193.
- 43 Fouquet S, Lugo-Martinez VH, Faussat AM, Renaud F, Cardot P, Chambaz J, Pincon-Raymond M, Thenet S: Early loss of e-cadherin from cell-cell contacts is involved in the onset of anoikis in enterocytes. *J Biol Chem* 2004;279:43061-43069.
- 44 Gordon JI, Hermiston ML: Differentiation and self-renewal in the mouse gastrointestinal epithelium. *Curr Opin Cell Biol* 1994;6:795-803.
- 45 Mayhew T M, Myklebust R, Whybrow A, Jenkins R: Epithelial integrity, cell death and cell loss in mammalian small intestine. *Histol Histopathol* 1999;14:257-267.
- 46 Grossmann J, Walther K, Artinger M, Kiessling S, Scholmerich J: Apoptotic signaling during initiation of detachment-induced apoptosis ("anoikis") of primary human intestinal epithelial cells. *Cell Growth Differ* 2001;12:147-155.
- 47 Valentijn AJ, Zouq N, Gilmore AP: Anoikis. *Biochemical Society Transactions* 2004;32:421-425.
- 48 Wong SMF, Chase HS: Role of intracellular calcium in cellular volume regulation. *Am J Physiol* 1986;250:C841-C852.
- 49 Mattson MP, Chan SL: Calcium orchestrates apoptosis. *Nature Cell Biol* 2003;5:1041-1043.
- 50 Orrenius S, Zhyvotovskyy B, Nicotera P: The regulation of cell death: the calcium-apoptosis link. *Nat Cell Biol* 2003;4:552-565.
- 51 Levitan IB: Modulation of ion channels by protein phosphorylation – how the brain works. *Adv Sec Mess Phosphoprotein Res* 1999;33:3-2.
- 52 Calderone V: Large-conductance Ca²⁺-activated K⁺ channels: function, pharmacology and drugs. *Curr Med Chem* 2002;9:1385-1395.
- 53 Shanmugathan M, Jothy S: Apoptosis, anoikis and their relevance to the pathobiology of colon cancer. *Pathol Int* 2000;50:273-279.