Induced TRPC1 expression sensitizes intestinal epithelial cells to apoptosis by inhibiting NF- κ B activation through Ca^{2+} influx

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Apoptosis occurs within crypts and at the intestinal luminal surface and plays a critical role in mucosal homeostasis. NF- κ B (nuclear factor- κ B) is the central regulator of the transcription of genes involved in apoptosis, and its activity is highly regulated in the intestinal mucosa. We have recently demonstrated that TRPC1 (transient receptor potential canonical-1) is expressed in IECs (intestinal epithelial cells) and functions as a Ca^{2+} permeable channel activated by Ca^{2+} store depletion. The present study tests the hypothesis that TRPC1 channels are implicated in the regulation of apoptosis by inhibiting NF- κ B through the induction of TRPC1-mediated Ca^{2+} influx in the IEC-6 line. The expression of TRPC1 induced by stable transfection of IEC-6 cells with the wild-type *TRPC1* gene (IEC-TRPC1 cells) increased Ca^{2+} influx after Ca^{2+} store depletion and repressed NF- κ B transactivation, which was associated with an increase in susceptibility to apoptosis induced by exposure to TNF α (tumour necrosis factor- α) plus CHX (cycloheximide) (TNF- α /CHX), or

STS (staurosporine). By contrast, the induction of endogenous NF- κ B activity, by the depletion of cellular polyamines, promoted resistance to apoptosis, which was prevented by the ectopic expression of the I κ B α super-repressor. Furthermore, inhibition of TRPC1 expression by transfection with siRNA (small interfering RNA) targeting TRPC1 (siTRPC1) decreased Ca^{2+} influx, increased NF- κ B transactivation, and prevented the increased susceptibility of IEC-TRPC1 cells to apoptosis. Decreasing Ca^{2+} influx by exposure to a Ca^{2+} -free medium also induced NF- κ B activity and blocked the increased susceptibility to apoptosis of stable IEC-TRPC1 cells. These results indicate that induced TRPC1 expression sensitizes IECs to apoptosis by inhibiting NF- κ B activity as a result of the stimulation of Ca^{2+} influx.

Key words: capacitative calcium entry (CCE) mechanism, I κ B, mucosal homeostasis, polyamine, programmed cell death, store-operated Ca^{2+} channel (SOC).

INTRODUCTION

The epithelium of mammalian intestinal mucosa has the most rapid turnover rate of any tissue in the body, and maintenance of its integrity depends on a complex interplay between processes involved in cell proliferation, differentiation, migration and apoptosis [1]. Under physiological conditions, undifferentiated epithelial cells continuously replicate in the proliferating zone within the crypts and differentiate as they migrate up the luminal surface of the colon and to the villous tips of the small intestine [2]. To maintain stable numbers of enterocytes, cell division must be dynamically counterbalanced by apoptosis, a fundamental biological process involving selective cell deletion to regulate tissue homeostasis [2,3]. Apoptosis occurs in the crypt area, where it maintains the critical balance in cell number between newly divided and surviving cells, and at the luminal surface of the colon and villous tips in the small intestine, where differentiated cells are lost [1–3]. It is now well established that IEC (intestinal epithelial cell) survival and death are subject to differentiation-state-specific control mechanisms, and that apoptosis in the crypt area and the luminal surface of the intestine is regulated by distinct cellular signalling pathways [4,5]. Although an imbalance between epithelial cell survival and apoptosis alters mucosal homeostasis and has significant pathological consequences, the exact mechanisms involved in this process remain unclear.

Several lines of evidence indicate that $[\text{Ca}^{2+}]_{\text{cyt}}$ (cytosolic free Ca^{2+} concentration) regulates apoptosis and that increased $[\text{Ca}^{2+}]_{\text{cyt}}$ can induce or block apoptosis, depending upon cell-type and other factors [6–9]. To carry out cellular regulatory functions, Ca^{2+} signals need to be flexible yet precisely regulated. At the cellular level, $[\text{Ca}^{2+}]_{\text{cyt}}$ is derived from two sources, both external and internal. Although mobilization from intracellular stores results in a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$, a sustained increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ depends on extracellular Ca^{2+} influx [10–12]. Ca^{2+} entry due to store depletion is referred to as CCE (capacitative Ca^{2+} entry), and is mediated by Ca^{2+} -permeable channels termed SOCs (store-operated Ca^{2+} channels). It is clear that CCE through SOCs contributes to maintaining a sustained increase in intracellular $[\text{Ca}^{2+}]_{\text{cyt}}$, and the refilling of Ca^{2+} into the stores [7,13]. Although the participation of voltage-gated K^{+} channels is critical for the control of Ca^{2+} influx through regulation of the membrane potential that governs the driving force for Ca^{2+} influx in IECs and other non-excitable cells [11,12], the exact channel proteins that participate in forming SOCs and that mediate CCE have not yet been completely defined.

TRPC (transient receptor potential canonical) channels are mammalian homologues of the *Drosophila* photoreceptor channel, TRP. To date, seven short mammalian cDNA homologues of *Drosophila* TRP channels have been characterized, termed TRPC1–TRPC7 [14–16]. It has been shown that the expression

Abbreviations used: $[\text{Ca}^{2+}]_{\text{cyt}}$, cytosolic free Ca^{2+} concentration; CCE, capacitative calcium entry; CHX, cycloheximide; cIAP, cellular inhibitor of apoptosis protein; CPA, cyclopiazonic acid; C-siRNA, control siRNA; DFMO, α -difluoromethylornithine; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FBS, foetal bovine serum; fura 2-AM, fura 2 acetoxymethyl ester; IAP, inhibitor of apoptosis protein; IEC, intestinal epithelial cell; I_{SOC} , store-operated Ca^{2+} current; I κ BSR, I κ B α super-repressor; NF- κ B, nuclear factor- κ B; p-NA, p-nitroanilide; siRNA, small interfering RNA; SOC, store-operated Ca^{2+} channel; STS, staurosporine; TNF- α , tumour necrosis factor- α ; TRPC1, transient receptor potential canonical 1; XIAP, X-chromosome-linked IAP.

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of TRPC genes in oocytes or mammalian cells results in the formation of Ca^{2+} -permeable cation channels, which are implicated in store-operated Ca^{2+} entry [17–19,50]. Inhibition of TRPC gene expression attenuates the I_{soc} (store-operated Ca^{2+} current) and prevents the increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to CCE [20,21]. TRPCs have been proposed as molecular candidates for SOCs in non-excitable cells for many years, but an increasing body of evidence suggests that only a few TRPC gene products meet the functional criteria of an SOC [19,20]. TRPC1 is one of the most-studied TRPC channels and contains a short hydrophobic sequence that is believed to be involved in forming the pore of the cation channels [22,23]. TRPC1 is broadly, but not ubiquitously expressed across mouse, rat and human cell types, with evidence of quantitatively differential expression [15,22,23]. Recently, we have demonstrated that normal IECs express TRPC1 and TRPC5 and display typical I_{soc} and CCE after Ca^{2+} store depletion [24]. The expression of TRPC1 induced by stable transfection with the *TRPC1* gene increases Ca^{2+} influx after Ca^{2+} store depletion, indicating that TRPC1 is a candidate protein for the SOCs, and regulates $[\text{Ca}^{2+}]_{\text{cyt}}$ homeostasis in IECs.

Studies from our laboratory [3,25,26] and others [27–29], have also shown that NF- κ B (nuclear factor- κ B) integrates various intracellular and extracellular signals in IECs and is an important biological regulator of apoptosis. The current study tested the hypothesis that TRPC1 channels are implicated in the regulation of apoptosis by inhibiting NF- κ B through the induction of TRPC1-mediated Ca^{2+} influx in normal IECs. First, we sought to determine whether increased TRPC1 expression, by stable transfection with the *TRPC1* gene, affected cell survival in IECs. Second, we examined whether TRPC1 regulated apoptosis, by repressing NF- κ B signalling. Third, we wanted to determine whether the induced expression of TRPC1 inhibited NF- κ B by increasing Ca^{2+} influx. Some of these data have been published previously in abstract form [30].

MATERIALS AND METHODS

Chemicals and supplies

Disposable culture-ware was purchased from Corning Glass Works (Corning, NY, U.S.A.). Tissue culture media and dFBS (dialysed foetal bovine serum) were purchased from Invitrogen (Carlsbad, CA, U.S.A.), and biochemicals were obtained from Sigma Chemical (St. Louis, MO, U.S.A.), and DFMO (α -difluoromethylornithine) was purchased from Ilex Oncology (San Antonio, TX, U.S.A.). The affinity-purified rabbit polyclonal antibody against TRPC1 was purchased from Alomone Laboratories (Jerusalem, Israel), and antibodies against NF- κ B subunits p65, p52 or p50 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL, U.S.A.).

Cell culture

The IEC-6 line was purchased from the American Type Culture Collection at passage 13. The cell line was derived from normal rat intestinal crypt cells and was developed and characterized by Quaroni et al. [31]. Stock cells were maintained in T-150 flasks in DMEM (Dulbecco's modified Eagle medium) supplemented with 5% heat-inactivated FBS, 10 $\mu\text{g}/\text{ml}$ insulin, and 50 $\mu\text{g}/\text{ml}$ gentamicin. Flasks were incubated at 37°C in a humidified atmosphere of 90% air/10% CO_2 , and passages 15–20 were used in experiments. There were no significant changes of biological function and characterization of IEC-6 cells at passages 15–20 [32,33].

The stable TRPC1-transfected IEC-6 cells (IEC-TRPC1) were developed and characterized by Rao et al. as described in our recent publication [24]. The IEC-6 cells were transfected with the pcDNA3.0(+) expression vector containing the full-length cDNA of human TRPC1 with the cytomegalovirus promoter (pcDNA-TRPC1) or the pcDNA3.0(+) vector containing no TRPC1 cDNA by using a LipofectAMINE™ kit. The transfected cells were selected for TRPC1 integration by incubation with the selection medium containing 0.6 mg/ml of G418, and clones resistant to the selection medium were isolated, cultured and screened for TRPC1 expression by RT (reverse transcriptase)-PCR using a specific TRPC1 primer, and by Western blot analysis using a specific anti-TRPC1 antibody.

RNA interference

The siRNA (small interfering RNA) specifically targeting TRPC1 mRNA (siTRPC1) was designed and synthesized by Sequitur Inc (Natick, MA, U.S.A.) [24]. The siTRPC1 was screened against the TRPC1 mRNA coding region in the GenBank® database (accession number U31110), and no matches were found to other non-targeted genes. The scrambled C-siRNA (control siRNA) with no homology to any known gene was used as the control. For each 60 mm cell culture dish, 15 μl of the 20 μM stock siTRPC1 or C-siRNA was mixed with 300 μl of Opti-MEM medium (Invitrogen, Carlsbad, CA, U.S.A.). This mixture was gently added to a solution containing 15 μl of LipofectAMINE™ 2000 in 300 μl of Opti-MEM medium. The solution was incubated for 20 min at room temperature and gently overlaid on to the monolayer of cells in 3 ml of medium, and cells were harvested for various assays after 24 or 48 h of incubation.

Recombinant adenovirus construction and infection

The recombinant adenoviral vector expressing $\text{I}\kappa\text{BSR}$ ($\text{I}\kappa\text{B}\alpha$ super-repressor; mutant $\text{I}\kappa\text{B}\alpha$) was constructed using the Adeno-X Expression system (Clontech) according to the protocol recommended by the manufacturer. Briefly, the $\text{I}\kappa\text{BSR}$ cDNA (S32A/S36A) was cloned into the pShuttle by digesting pCMV- $\text{I}\kappa\text{B}\alpha\text{M}$ with BamHI/HindIII and ligating the resulting fragments into the XbaI site of the pShuttle vector, as described in our previous publications [25,26]. The pAdeno-X/ $\text{I}\kappa\text{BSR}$ ($\text{AdI}\kappa\text{BSR}$) was constructed by digesting pShuttle constructs with PI-SceI/I-CeuI and ligating the resulting fragments into the PI-SceI/I-CeuI sites of the pAdeno-X adenoviral vector. Recombinant adenoviral plasmids were packaged into infectious adenoviral particles by transfecting HEK (human embryonic kidney)-293 cells using LipofectAMINE™ PLUS reagent. The adenoviral particles were propagated in HEK-293 cells and purified by cesium chloride ultracentrifugation. The titre of the adenoviral stock was determined by a standard plaque assay. Recombinant adenoviruses were screened for expression of the gene that was introduced by Western blot analysis using an anti- $\text{I}\kappa\text{B}\alpha$ specific antibody. pAdeno-X, which was the recombinant replication-incompetent adenovirus without an $\text{I}\kappa\text{BSR}$ cDNA insert, was grown and purified as described above and served as a control adenoviral vector. Cells were infected with various concentrations of the $\text{AdI}\kappa\text{BSR}$ or control vector, and cell samples were collected for various measurements 48 h after the infection.

Western blot analysis

Cell samples, placed in SDS sample buffer, were sonicated and centrifuged (12000 rev./min) at 4°C for 15 min. The supernatant from cell samples was boiled for 5 min and then subjected to SDS/(7.5%) PAGE according to the method of Laemmli [34].

After the transfer of protein on to nitrocellulose filters, the filters were incubated for 1 h in 5 % non-fat dried milk in $1 \times$ PBS-T [15 mM NaH_2PO_4 , 80 mM Na_2HPO_4 , 1.5 M NaCl (pH 7.5) and 0.5 % (v/v) Tween-20]. Immunological evaluation was performed for 1 h in 1 % FBS/PBS-T buffer containing 1 $\mu\text{g}/\text{ml}$ TRPC1, p65, p52, p50 or caspase-3 specific antibodies. The filters were subsequently washed with $1 \times$ PBS-T and incubated for 1 h with a horseradish-peroxidase-conjugated secondary antibody. After extensive washing with $1 \times$ PBS-T, the immunocomplexes on the filters were reacted for 1 min with chemiluminescence reagent (NEL-100, DuPont NEN). Finally, the filters were placed in a plastic sheet protector and exposed to autoradiography film for 30 or 60 s.

Measurement of $[\text{Ca}^{2+}]_{\text{cyt}}$

Details of the digital imaging methods employed for measuring $[\text{Ca}^{2+}]_{\text{cyt}}$ have been described in our previous publications [11,12,24]. Briefly, cells from either the control IEC-6 or stable IEC-TRPC1 lines were plated on to 25 mm cover slips and were incubated in culture medium containing 3.3 μM fura 2-AM (fura 2 acetoxymethyl ester) for 30–40 min at room temperature (22–24°C) under an atmosphere of 10 % CO_2 . Fura 2-AM-loaded cells were then superfused with standard bath solution for 20–30 min at 22–24°C to wash away extracellular dye and permit intracellular esterases to cleave cytosolic fura 2-AM into active fura 2. Images of fura 2 fluorescence in the cells, and background fluorescence were viewed using a Nikon Diaphot microscope equipped for epifluorescence. Fluorescent images were obtained using a microchannel plate image intensifier (Amperex XX1381; Opelco, Washington DC, U.S.A.) coupled by fibre optics to a Pulnix charge coupled device (Stanford Photonics, Stanford, CA, U.S.A.). Image acquisition and analysis were performed using the Metamorph Imaging System (Universal Imaging). The $[\text{Ca}^{2+}]_{\text{cyt}}$ was calculated from fura 2 fluorescent emission spectra, excited at 380 and 360 nm using the ratio method [11,12,24].

Preparation of nuclear protein, and EMSAs (electrophoretic mobility shift assays)

Nuclear proteins were prepared via the procedure described previously [3,25], and the protein content in nuclear preparations was determined according to the method of Bradford [35]. The double-stranded oligonucleotides used in these experiments included 5'-AGTTGAGGGGACTTCCAGGC-3', which contains a consensus NF- κ B binding site (underlined). These oligonucleotides were radioactively end-labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T_4 polynucleotide kinase (Promega, Madison, WI, U.S.A.). For EMSAs, 0.035 pmol of ^{32}P -labelled oligonucleotides (approx. 30000 c.p.m.) and 10 μg of nuclear protein were incubated in a total volume of 25 μl in the presence of 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT (dithiothreitol), 5 % glycerol and 1 μl of poly(dI-dC). The binding reactions were allowed to proceed at room temperature for 20 min. Thereafter, 2.5 μl of Bromophenol Blue (0.1 % in water) were added and the protein–DNA complexes were resolved by electrophoresis on non-denaturing 4 % polyacrylamide gels and visualized by autoradiography. The specificity of binding interactions was assessed by competition assay with an excess of unlabelled double-stranded oligonucleotide of identical sequence.

Measurement of caspase-3 activity

The caspase-3 activity was measured by using the Caspase-3 Colorimetric Assay kit (R&D Systems, Inc. Minneapolis, MN, U.S.A.) and performed according to the protocol recommended

by the manufacturer. Briefly, cells were treated with TNF (tumour necrosis factor)- α and CHX (cycloheximide) for 4 h, washed with ice-cold Dulbecco's-PBS, and scraped from the dishes. The collected cells were washed with Dulbecco's-PBS and then lysed in ice-cold cell lysis buffer [50 mM Hepes (pH 7.4), 0.1 % CHAPS, 1 mM DTT, 0.1 mM EDTA and 0.1 % Nonidet P40]. The assay for caspase-3 activity was carried out in a 96-well plate. In each well, there was 50 μl of cell lysate (approx. 150 μg of total protein), 50 μl of reaction buffer [50 mM Hepes (pH 7.4), 0.1 % CHAPS, 100 mM NaCl, 10 mM DTT and 1 mM EDTA], 5 μl of caspase-3 colorimetric substrate, and a caspase-specific peptide that is conjugated to a chromogen, *p*-NA (*p*-nitroanilide). The 96-well plate was incubated at 37°C for 90 min, during which the caspase-3 in the sample presumably cleaved the chromophore, *p*-NA, from the substrate molecule. Absorbance readings at 405 nm were carried out after the incubation, with the caspase-3 activity directly proportional to the colour of the reaction. The protein level of each sample was determined by the method of Bradford [35].

Determination of the amount of apoptosis

After various experimental treatments, cells were photographed with a Nikon inverted microscope before fixation. Annexin V staining for apoptosis was carried out by using a commercial apoptosis detection kit (Clontech Laboratories, Inc. Palo Alto, CA, U.S.A.) and performed according to the protocol recommended by the manufacturer. Briefly, cells were rinsed with $1 \times$ binding buffer, and resuspended in 200 μl of $1 \times$ binding buffer. Annexin V (5 μl) was added on to the slide and incubated at room temperature for 10 min in the dark. Annexin-stained cells were visualized and photographed under a fluorescence microscope using a dual filter, set for FITC and rhodamine, and the percentage of 'apoptotic' cells was determined.

To determine the amount of DNA fragmentation, cells were lysed with 1.0 ml of digestion buffer and incubated at 50°C for 18 h. Samples were extracted twice with 1 volume of phenol/chloroform/isoamyl alcohol and precipitated with 7.5 M ammonium acetate and 100 % ethanol, and resuspended in 10 mM Tris/HCl. Samples were then treated with RNase (40 $\mu\text{g}/\text{ml}$) in the presence of 0.1 % SDS for 1 h at 37°C. Samples were re-extracted, precipitated and resuspended a second time as described above. DNA (2 μg) was loaded into each well and electrophoresed in 1.5 % agarose gel. Gels were visualized by UV fluorescence, and photographed with a Polaroid camera system.

Measurement of NF- κ B-dependent transcriptional activity

The NF- κ B-dependent luciferase reporter gene construct containing the synthetic sequence with four tandem copies of NF- κ B-binding elements was purchased from Clontech. Transient transfection was performed by using the LipofectAMINE™ kit as recommended by the manufacturer (Invitrogen). Cells were collected 48 h after transfection, and luciferase activity from individual transfections was normalized to the β -galactosidase activity of a co-transfected pCMV- β -galactosidase plasmid. The experiments were carried out in triplicate and luciferase activity is reported as the ratio of mean relative light units $\pm$ S.E.M. over β -galactosidase activity.

To determine the amount of NF- κ B-motif-binding activity, nuclear extracts were isolated and a commercial TransAM NF- κ B kit (Active Motif) was used to specifically measure the binding of activated NF- κ B to its consensus sequence attached to a micro-well plate. Briefly, nuclear extracts (5 μg) were added to the wells, immobilized with oligonucleotides containing the NF- κ B consensus site (5'-GGGACTTCC-3'). An anti-NF- κ B (p65)

antibody that recognizes p65 only when it's activated and bound to its target DNA was used in conjunction with a horseradish-peroxidase-conjugated secondary antibody to provide a sensitive colorimetric readout of NF- κ B (p65) activation quantified spectrophotometrically.

Statistics

All data are expressed as the means $\pm$ S.E.M. from six dishes or three separate experiments. Immunoblotting and autoradiographic experiments were repeated three times. The significance of the difference between means was determined by ANOVA. The level of significance was determined using Duncan's multiple-range test [36].

RESULTS

Ectopic expression of the TRPC1 gene in IECs increased sensitivity to apoptosis

To determine the extent of involvement of TRPC1 in the regulation of apoptosis in IECs, we used the stable TRPC1-transfected IEC-6 line (IEC-TRPC1) recently developed and characterized in our laboratory [24]. These stable IEC-TRPC1 cells highly express TRPC1, and the level of TRPC1 protein was approx. 4-fold greater than the level in control IEC-6 cells that were transfected with the empty vector which did not contain TRPC1 cDNA (Figure 1A, panel a). Consistent with our previous studies [24], these stable IEC-TRPC1 cells exhibited a significant increase in Ca^{2+} influx through SOCs after depletion of stored Ca^{2+} by CPA (cyclopiazonic acid). As shown in Figure 1(A), panel b, exposure of IEC-6 cells or stable IEC-TRPC1 cells to CPA induced a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ because of leakage of Ca^{2+} from the endoplasmic reticulum to the cytosol in the absence of extracellular Ca^{2+} . The transient CPA-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in cells bathed in Ca^{2+} -free solution declined back to the baseline level upon the depletion of the store Ca^{2+} . Under these conditions, restoration of $[\text{Ca}^{2+}]_o$ (extracellular Ca^{2+} concentration) induced a rise in $[\text{Ca}^{2+}]_{\text{cyt}}$, which was apparently mediated through SOCs. Although there were no differences in resting $[\text{Ca}^{2+}]_{\text{cyt}}$ and transient Ca^{2+} release from the store depletion between control IEC-6 cells and stable IEC-TRPC1 cells, sustained Ca^{2+} influx after Ca^{2+} store depletion was increased by approx. 2-fold in stable IEC-TRPC1 cells.

Using these stable IEC-TRPC1 cells, the following two experiments were performed. First, we examined changes in spontaneous apoptotic cell death without any challenge from apoptotic stimulators, and demonstrated that forced expression of the TRPC1 gene in IEC-6 cells failed to directly induce apoptosis. There was no apparent difference in cell viability between clone-1 (C1) of stable IEC-TRPC1 cells and control IEC-6 cells as measured by a Trypan Blue staining assay. Furthermore, there were no typical morphological features of apoptosis, as measured by Annexin-A staining (Figures 1B, panel a, and 1C, panel a) and DNA fragmentation (Figure 1D) identified in C1 of the stable IEC-TRPC1 cells. To determine the effect of clonal variation on apoptosis, three other clones, C2, C3 and C4 of the stable IEC-TRPC1 cells were also examined and the results were similar to those observed using C1 (results not shown).

Second, we examined changes in the susceptibility of stable IEC-TRPC1 cells to apoptotic cell death after exposure to apoptotic stimulators, TNF- α /CHX or STS. These two apoptotic models were chosen in this study based on the following reasons: (i) TNF- α /CHX-induced apoptosis is a form of programmed cell death induced by a biological apoptosis inducer [26,37,38],

Figure 1 Apoptotic response to TNF- α in combination with CHX in control IEC-6 and stable IEC-TRPC1 cells

(A) Representative findings of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes measured in peripheral areas of IEC-6 cells and stable IEC-TRPC1 cells. The Ca^{2+} store was depleted by treatment with CPA in the absence of extracellular Ca^{2+} (0 Ca^{2+}). Three experiments were performed that showed similar results. (B) Changes in the level of apoptotic cell death in control IEC-6 (left panel) and IEC-TRPC1 cells (right panel) after exposure to TNF- α /CHX: panel a, cells treated without TNF- α /CHX; panel b, cells exposed to TNF- α /CHX for 2 h; and panel c, cells exposed to TNF- α /CHX for 4 h. Original magnification, $\times 150$. (C) Images of ApoAlert Annexin-V stained cells described in (B). (D) Change in the amount of DNA fragmentation in cells described in (B). (E) Percentage of apoptotic cells in control IEC-6 cells and IEC-TRPC1 cells after exposure to TNF- α /CHX. Values are the means $\pm$ S.E.M. for data from 3 experiments. *, $P < 0.05$ compared with control IEC-6 cells exposed to TNF- α /CHX for the same amount of time.

and (ii) STS-induced cell death is a widely accepted model that represents exogenous-chemical-induced apoptosis [3,28]. Increased expression of TRPC1 by stable transfection significantly enhanced the susceptibility of IEC cells to TNF- α /CHX-induced apoptosis. The amount of the morphological features typical of apoptosis increased dramatically when stable IEC-TRPC1 cells were exposed to TNF- α /CHX (Figures 1B and 1C). Morphological assessment of apoptosis was confirmed by

Figure 2 Changes in caspase-3 activity in control IEC-6 and stable IEC-TRPC1 cells as described in the legend of Figure 1

(A) Representative Western immunoblots of procaspase-3 and caspase-3: panel a, control IEC-6 cells; panel b, stable IEC-TRPC1 cells. Cells were exposed to TNF- α /CHX, and whole-cell lysates were harvested at different time points after the treatment. (B) Quantitative analysis of caspase-3 immunoblots by densitometry from cells described in 2(A). Values are the means $\pm$ S.E.M. for data from 3 separate experiments; the relative level of caspase-3 was corrected for loading, as measured by the densitometry of actin. *, $P < 0.05$ compared with control IEC-6 cells exposed to TNF- α /CHX for the same amount of time. (C) Change in caspase-3 activity in the cells described in 2(A). Values are the means $\pm$ S.E.M. from 6 samples. *, $P < 0.05$ compared with control IEC-6 cells.

measurement of internucleosomal DNA fragmentation. The classic DNA 'ladder' in stable IEC-TRPC1 cells was observed as early as 2 h after exposure to TNF- α /CHX, whereas control IEC-6 cells showed significantly less DNA fragmentation (Figure 1D). The percentage of apoptotic cells was increased from approx. 38 % in control IEC-6 cells to approx. 79 % in C1 of the stable IEC-TRPC1 cells, 4 h after exposure to TNF- α /CHX (Figure 1E).

Consistently, C1 cells exhibited markedly increased caspase-3 activation, as indicated by an increase in level of active caspase-3 protein (Figures 2A and 2B) and its enzymatic activity (Figure 2C), as compared with control IEC-6 cells after treatment with TNF- α /CHX. The activation of caspase-3 occurred more

Figure 3 Apoptotic response to STS treatment in control IEC-6 and stable IEC-TRPC1 cells as described in the legend of Figure 1

Cells were exposed to 1 μ M STS, and apoptotic cell death was examined 1 and 2 h after treatment with STS. (A) Images of STS-induced apoptosis in control IEC-6 cells (left panel) and stable IEC-TRPC1 cells (right panel): panel a, cells treated without STS; panel b, cells treated with STS for 1 h; and panel c, cells treated with STS for 2 h. Original magnification $\times 150$. (B) Percentage of STS-induced apoptotic cell death in the cells described in 3(A). Values are the means $\pm$ S.E.M. for data from 3 experiments. *, $P < 0.05$ compared with control IEC-6 cells exposed to STS for the same amount of time.

quickly and to a greater extent than in IEC-TRPC1 cells compared with control IEC-6 cells when they were exposed to TNF- α /CHX. The increased susceptibility to TNF- α /CHX-induced apoptosis in C1 cells does not result simply from clonal variation, because identical results were obtained when C2, C3 or C4 of the IEC-TRPC1 cells were exposed to TNF- α /CHX (results not shown).

In addition, stable IEC-TRPC1 cells also exhibited increased susceptibility to STS-induced apoptosis (Figure 3). The percentage of apoptotic cells was increased from approx. 39 % in control IEC-6 cells to approx. 73 % in C1 cells 1 h after exposure to STS. There were similar observations regarding STS-induced apoptosis when C2, C3 or C4 cells were examined. These results indicate that TRPC1 is implicated in the regulation of apoptosis in IECs and that increased TRPC1 expression sensitizes IEC cells to apoptosis.

Increased TRPC1 expression was associated with inactivation of NF- κ B transcriptional activity

To determine the possibility that induced TRPC1 expression increases susceptibility to apoptosis by inhibiting NF- κ B activity,

Figure 4 NF-κB activity in response to treatment with TNF-α in control IEC-6 and stable IEC-TRPC1 cells

(A) Representative autoradiograms of sequence-specific NF-κB DNA-binding activity. After cells were exposed to TNF-α (20 ng/ml), nuclear proteins were isolated at different time points. An EMSA was performed using 10 µg of nuclear protein and 0.035 pmol of ³²P-end labelled NF-κB oligonucleotides containing a single NF-κB binding site. Positions of the specifically bound DNA-protein complexes are indicated. Three experiments were performed that showed similar results. (B) Effect of the unlabelled NF-κB oligonucleotide and the specific antibody against NF-κB p65 subunit on NF-κB binding activity. Competitive inhibition experiments (left panel) were performed in the presence of unlabelled NF-κB oligonucleotides at a concentration of × 50. In the supershift assays (right panel), nuclear proteins were initially incubated with the ³²P-end-labelled NF-κB oligonucleotides for 20 min. The antibody against the NF-κB p65 subunit (Anti-NF-κB AB) was then added to the binding reaction mixture, and the reaction was allowed to proceed for an additional 30 min. No-AB, lane with nuclear extracts and no antibody.

we examined changes in the levels of NF-κB DNA binding activity and NF-κB-dependent transcriptional activity in control IEC-6 cells and stable IEC-TRPC1 cells. In control IEC-6 cells, treatment with TNF-α, a potent stimulator of NF-κB [37,39], resulted in a marked induction of NF-κB binding activity, which occurred 2 h after treatment with TNF-α and remained elevated for an additional 4 h (Figure 4A, panel a). To evaluate the specificity of the binding reaction shown in Figure 4(A), competitive inhibition experiments were performed which showed that NF-κB binding activity in cells exposed to TNF-α was completely inhibited when the unlabelled NF-κB oligonucleotide was added to the binding reaction mixture at a concentration of × 50 (Figure 4B, left panel). Furthermore, the anti-NF-κB (p65) specific antibody, when added to the binding reaction mixture, dramatically supershifted the NF-κB complexes present in cells exposed to TNF-α (Figure 4B, right panel). This increase in NF-κB binding activity in cells exposed to TNF-α was associated with an induction of NF-κB transcriptional activity as measured by an NF-κB-dependent promoter luciferase reporter gene assay (Figure 5A, left panel) and a NF-κB-motif-binding assay (Figure 5B, left panel).

Figure 5 Changes in NF-κB-dependent transcriptional activity after treatment with TNF-α in IEC-6 and IEC-TRPC1 cells

(A) Effect of TNF-α on NF-κB-dependent transcriptional activity as measured by NF-κB-dependent promoter luciferase reporter gene assay. **a.** structure of luciferase (Luc) reporter structure: control pTAL-Luc and pNF-κB-Luc constructs with four NF-κB binding sites. **b.** level of NF-κB-dependent promoter activity. Cells were transfected with either pNF-κB-Luc or pTAL-Luc by the LipofectAMINE™ technique. After cells were transfected for 45 h, they were exposed to TNF-α. The luciferase activity was measured 2 and 4 h after administration of TNF-α. Data for the NF-κB-dependent promoter activity were normalized to β-galactosidase activity from co-transfection of pRSV β-galactosidase. Values are the means ± S.E.M. for data from 6 dishes. *, *P* < 0.05 compared with control IEC-6 cells exposed to TNF-α for the same amount of time. (B) Levels of NF-κB-motif-binding activity in the cells described in 5(A). Nuclear extracts were isolated, and the level of endogenous NF-κB (p65) transcriptional activation was examined by using a TransAM NF-κB kit. The specificity of NF-κB activation was verified by 3 experimental conditions (right): no external NF-κB oligonucleotide; mutant NF-κB oligonucleotide; and wild-type (WT)-oligonucleotide. Values are the means ± S.E.M. from 6 dishes. *, *P* < 0.05 compared with control IEC-6 cells exposed to TNF-α for the same amount of time. Mut-oligo, mutant oligonucleotide; WT-oligo, wild-type oligonucleotide.

By contrast, stable IEC-TRPC1 cells exhibited a significant decrease in the level of NF-κB transcriptional activity. When exposed to TNF-α, the extent of NF-κB binding activity that was induced in stable IEC-TRPC1 cells was much less than that observed in control IEC-6 cells (Figure 4, panel b). This decreased level of NF-κB binding activity was associated with a dramatic decrease in NF-κB transcriptional activity (Figure 5). The level of NF-κB-dependent promoter activity in stable IEC-TRPC1 cells was decreased by approx. 50 % at 2 h and approx. 80 % at 4 h after exposure to TNF-α, as compared with the level

in control IEC-6 cells (Figure 5A). The level of NF- κ B-motif-binding activity in IEC-TRPC1 cells was decreased by approx. 38 % at 2 h and approx. 44 % at 4 h after treatment with TNF- α (Figure 5B). The specificity of NF- κ B-motif-binding activity was confirmed by studies in which either no oligonucleotide, a mutated oligonucleotide or a wild-type oligonucleotide was added. As shown in Figure 5(B) (right panel), the control groups with no oligonucleotide or with a mutated oligonucleotide had no detectable change in NF- κ B-motif-binding activity. However, when the competing wild-type NF- κ B oligonucleotide was used, NF- κ B-motif-binding activity was dramatically inhibited. These results indicate that increased TRPC1 expression inactivates NF- κ B signalling in IECs.

Inactivated NF- κ B increases susceptibility to apoptosis

To determine the relationship between inactivated NF- κ B and the increased sensitivity of IEC-TRPC1 cells to apoptosis after the increased expression of TRPC1, we examined the effect of increased endogenous NF- κ B activity on TNF- α /CHX-induced apoptosis by depletion of cellular polyamines. As reported in our previous publications [3,25], treatment with DFMO (α -difluoromethylornithine, a specific inhibitor for polyamine biosynthesis) for 6 days almost completely depleted cellular polyamines spermidine, spermine and their precursor putrescine in control IEC-6 cells (results not shown). Polyamine depletion also increased NF- κ B activity as indicated by an increase in the level of nuclear p65 and p52 proteins (Figure 6A) and NF- κ B-dependent promoter activity (Figure 6B). An increased level of NF- κ B in polyamine-deficient cells was associated with increased resistance to TNF- α /CHX-induced apoptosis (Figure 6C). Interestingly, infection with the adenoviral vector encoding the non-degradable $\text{I}\kappa\text{BSR}$ cDNA not only prevented the induction of NF- κ B activity but also blocked the increased resistance to TNF- α /CHX-induced apoptosis in polyamine-deficient cells. These results indicate that activation of endogenous NF- κ B promotes cell survival in IECs, suggesting that increased susceptibility to apoptosis in stable IEC-TRPC1 cells results, at least partially, from the inhibition of NF- κ B activity.

Inhibition of TRPC1 expression by specific siRNA decreases Ca^{2+} influx and results in the activation of NF- κ B in stable IEC-TRPC1 cells

To examine the possibility that increased TRPC1 expression inhibits NF- κ B activity by increasing Ca^{2+} influx, the siRNA nucleotides complementary to TRPC1 mRNA (siTRPC1) were used to specifically block endogenous TRPC1 in stable IEC-TRPC1 cells. These specific siTRPC1 nucleotides were designed to cleave rat TRPC1 mRNA by activating endogenous RNase H, and to have a unique combination of specificity, efficacy and reduced toxicity [24,40]. Initially, we determined the transfection efficiency of the siRNA nucleotides in stable IEC-TRPC1 cells and demonstrated that more than 95 % of cells were positive when they were transfected with a fluorescent FITC-conjugated siTRPC1 for 24 h (results not shown). As shown in Figure 7(A), exposure to the siTRPC1 nucleotides inhibited the expression of TRPC1 in stable IEC-TRPC1 cells. The level of TRPC1 protein was decreased by approx. 90 % when cells were exposed to the siTRPC1 nucleotides for 24 or 48 h. Transfection with the control siRNA (C-siRNA) at the same concentration showed no inhibitory effect on TRPC1 expression. In addition, neither siTRPC1 or C-siRNA affected cell viability as measured by Trypan Blue staining (results not shown).

Figure 6 Effects of either increased endogenous NF- κ B by depletion of cellular polyamines or inactivation of NF- κ B by ectopic expression of the $\text{I}\kappa\text{BSR}$ gene on TNF- α /CHX-induced apoptosis

(A) Representative immunoblots of nuclear NF- κ B subunits p65 and p52 in polyamine-deficient cells. Cells were grown in the control culture or cultures containing 5 mM DFMO for 4 days and were then infected with either the recombinant adenoviral vector encoding human $\text{I}\kappa\text{BSR}$ cDNA (AdI κ BSR) or an adenoviral vector lacking $\text{I}\kappa\text{BSR}$ cDNA (Adnull) at a multiplicity of infection of 25 plaque-forming units per cell for 48 h in the presence of DFMO. The levels of nuclear p65 and p52 were assayed by Western blot analysis. (B) Change in NF- κ B transcriptional activity as measured by NF- κ B-dependent promoter activity in the cells described in 6(A). After cells were treated with DFMO for 4 days, they were transfected with a NF- κ B-dependent promoter luciferase reporter plasmid and the luciferase activity was measured 48 h after transfection. Values are the means $\pm$ S.E.M. for data from 6 dishes. * and +, $P < 0.05$ compared with control cells or cells treated DFMO and infected with Adnull respectively. (C) Apoptotic response to TNF- α /CHX treatment in the cells described in 6(A). The level of apoptosis was measured 4 h after exposure to TNF- α /CHX. Values are the means $\pm$ S.E.M. for data from 3 separate experiments. * and +, $P < 0.05$ compared with control cells or cells exposed to DFMO and infected with Adnull respectively.

A decreased level of TRPC1 expression due to the transfection with siTRPC1 substantially decreased Ca^{2+} influx after Ca^{2+} store depletion in stable IEC-TRPC1 cells (Figures 7B and 7C). The increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ due to CPA-induced Ca^{2+} influx was decreased by approx. 60 % in stable IEC-TRPC1 cells treated with siTRPC1, although there was no significant change in the level of resting $[\text{Ca}^{2+}]_{\text{cyt}}$. The inhibition of TRPC1 expression and the subsequent decrease in Ca^{2+} influx by siTRPC1, activated NF- κ B, as indicated by an increase in the level of its DNA binding activity (Figure 7D, panel a) and NF- κ B-motif-binding activity (Figure 7D, panel b). The NF- κ B transcriptional activity in stable IEC-TRPC1 cells transfected with

Figure 7 Effect of specific inhibition of TRPC1 expression by siRNA targeting of the TRPC1 mRNA coding region (siTRPC1) on Ca^{2+} influx, NF- κ B activity and apoptotic sensitivity in IEC-TRPC1 cells

(A) Representative immunoblots of TRPC1 protein. Stable IEC-TRPC1 cells were transfected with either control siRNA (C-siRNA) or siTRPC1, and the level of TRPC1 protein was measured by Western immunoblotting. Actin immunoblotting of the stripped blots was performed as an internal control for equal protein loading. Three separate experiments were performed that showed similar results. (B) Representative findings of $[Ca^{2+}]_{\text{cyt}}$ changes measured in peripheral areas of stable IEC-TRPC1 cells transfected with either siTRPC1 or C-siRNA for 48 h. CPA, cyclopiazonic acid; $0Ca^{2+}$, Ca^{2+} -free solution. (C) Summarized data showing resting $[Ca^{2+}]_{\text{cyt}}$ concentration (left) and the amplitude of CPA-induced Ca^{2+} influx (right) in the cells described in 7(B). Data were expressed as the means $\pm$ S.E.M. ($n = 30$). *, $P < 0.05$ compared with cells transfected with C-siRNA. (D) Change in NF- κ B transcriptional activity in the cells described in 7(A): panel a, representative autoradiograms of sequence-specific NF- κ B DNA-binding activity; and panel b, NF- κ B-motif-binding activity. Values are the means $\pm$ S.E.M. for data from 6 dishes. *, $P < 0.05$ compared with cells transfected with C-siRNA. (E) Percentage of apoptotic cell death in the cells described in 7(A). Apoptosis was examined 4 h after exposure to TNF- α /CHX. Data were expressed as the means $\pm$ S.E.M. for 3 separate experiments. *, $P < 0.05$ compared with cells transfected with C-siRNA.

siTRPC1 for 48 h was approx. 2.2-fold greater than in cells transfected with C-siRNA. The C-siRNA had no effect on either Ca^{2+} influx induced by CPA (Figure 7B) or NF- κ B activity (Figure 7D). Furthermore, NF- κ B activity increased by inhibiting TRPC1 expression using siTRPC1, protected stable IEC-TRPC1 cells against TNF- α /CHX-induced apoptosis (Figure 7E). The percentage of apoptotic cell death in IEC-TRPC1 cells transfected with siTRPC1 was decreased to approx. 40 % from approx. 84 % in cells transfected with C-siRNA. These findings indicate that the inhibition of TRPC1 expression decreases Ca^{2+} influx, activates NF- κ B, and represses apoptosis in IECs.

Removal of extracellular Ca^{2+} activates NF- κ B and inhibits apoptosis in stable IEC-TRPC1 cells

The results presented in Figure 8 show that decreasing Ca^{2+} influx by exposing cells to Ca^{2+} -free medium dramatically promoted NF- κ B nuclear translocation, as indicated by an increase in the level of nuclear NF- κ B proteins (subunits p65 and p52). This was paralleled by a dramatic increase in the induction of sequence-specific NF- κ B binding activity and NF- κ B-dependent transcriptional activity (Figure 8A). A significant increase in NF- κ B activation occurred at 1 h, and peaked between 2 and 4 h after exposure to the Ca^{2+} -free medium. The maximal increase in nuclear NF- κ B protein level and its DNA binding activity was approx. 5-fold greater than the pretreatment level, whereas the level of NF- κ B-motif-binding activity was more than twice that of the control value. Activated NF- κ B, by removal of extracellular Ca^{2+} , also protected stable IEC-TRPC1 cells against TNF- α /CHX-induced apoptosis (Figure 8B). The level of caspase-3 activity was decreased by approx. 35 % at 2 h and approx. 52 %

at 4 h after exposure to TNF- α /CHX in the Ca^{2+} -free medium, as compared with the level in cells exposed to TNF- α /CHX in normal DMEM medium (with Ca^{2+}). The percentage of apoptotic cell death declined from approx. 48 % at 2 h and approx. 71 % at 4 h in cells cultured in normal DMEM medium after exposure to TNF- α /CHX, to approx. 30 % and approx. 52 % in cells cultured in the Ca^{2+} -free medium respectively. These results clearly show that decreasing $[Ca^{2+}]_{\text{cyt}}$ by blocking Ca^{2+} influx activates NF- κ B and inhibits apoptosis in IECs.

DISCUSSION

An increasing body of evidence has shown that the members of the TRPC isoform family provide the structural basis for SOCs in mammalian cells [15], although evidence to the contrary has also been reported [50]. Studies have found that the pattern of expression of these TRPC isoforms differs among various tissues and is cell-type-dependent [14]. For example, almost all types of TRPC isoforms are highly expressed in neural tissue, whereas only TRPC1, TRPC4 and TRPC6 are expressed in endothelial cells [14,41]. We have recently demonstrated that in normal IECs (IEC-6 line) TRPC1 and TRPC5 are expressed as the predominant isoforms, and that induced TRPC channel activity increases early epithelial restitution after injury, as a result of TRPC-mediated Ca^{2+} influx [24]. Our results further suggest that TRPC1 is a candidate protein for the SOC mechanism in IECs based on the following evidence: (i) passive store depletion by CPA induces I_{SOC} and CCE in IEC-6 cells; (ii) inhibition of TRPC1 expression by specific siRNA targeting of TRPC1 decreases the amplitude of whole-cell I_{SOC} and CCE; (iii) overexpression of TRPC1 in IEC-6 cells significantly increases store depletion-mediated CCE;

Figure 8 Removal of extracellular Ca²⁺ by exposure to Ca²⁺-free medium and effect on NF-κB activity and apoptotic response to TNF-α/CHX treatment in IEC-TRPC1 cells

(A) Changes in NF-κB activity after exposure to Ca²⁺ free medium: panel a, representative immunoblots of nuclear NF-κB subunit proteins p65 and p52; panel b, representative autoradiograms of NF-κB binding; panel c, NF-κB transcriptional activity as measured by NF-κB-motif-binding activity. Stable IEC-TRPC1 cells were cultured in standard DMEM medium for 4 days, and the level of NF-κB activity was measured at different time points after exposure to Ca²⁺-free medium. Analysis of immunoblots and gel-shift assays was performed 3 times, and showed similar results. In studies dealing with NF-κB transcriptional activity, values are the means $\pm$ S.E.M. for data from 6 dishes. *, $P < 0.05$ compared with cells exposed to Ca²⁺-free medium for 0 h. (B) Changes in caspase-3 activity (a) and the percentage of apoptotic cell death (b) in the cells described in 8(A). Caspase-3 activity and apoptosis were measured 2 and 4 h after exposure to TNF-α/CHX plus Ca²⁺-free medium. Data were expressed as the means $\pm$ S.E.M. for 6 samples. *, $P < 0.05$ compared with cells exposed to TNF-α/CHX alone.

and (iv) inhibition of TRPC1 expression by siRNA prevents the enhancement of CCE in differentiated IEC-Cdx2L1 cells highly expressing endogenous TRPC1. The present studies have extended our previous observations by demonstrating that TRPC1 plays an important role in the regulation of cell survival and apoptosis in IECs.

Data from the current studies clearly indicate that TRPC1 has a pro-apoptotic role in intestinal undifferentiated/crypt cells. Although an increased level of TRPC1 by ectopic expression of the *TRPC1* gene does not directly induce apoptosis, stable IEC-TRPC1 cells exhibited a significant increase in susceptibility to apoptosis when they were exposed to TNF-α/CHX (Figures 1 and 2) or STS (Figure 3). The inhibition of TRPC1 expression by transfection with specific siTRPC1 prevented this increase in susceptibility to apoptosis (Figure 7). Consistent with our current findings, several previous studies also have shown that TRPCs are implicated in the regulation of apoptosis, but the role of activated TRPCs in apoptotic pathways has been rather controversial, and is dependent upon cell type, other factors and death stimulus used [42–44]. In human androgen-sensitive LNCaP prostate cancer cells, forced expression of Ca²⁺ permeable TRP-like channels by infection with a recombinant adenovirus enhances Ca²⁺ influx across the plasma membrane after Ca²⁺ store depletion and induces decreased cell survival [42]. In CHO (Chinese-hamster ovary) cells, increased TRPC2-mediated Ca²⁺ influx accelerates apoptosis, while the inhibition of TRPC2 activity decreased Ca²⁺ influx and increases cell survival [44], suggesting that

activation of TRPCs favours apoptosis. On the other hand, it has been reported that activation of TRPC1 can inhibit degenerative apoptotic signalling in human SH-SY5Y neuronal cells [43]. Overexpressing the TRPC1 gene, by infection with the adenoviral TRPC1 expression vector, or increased TRPC1 activity by treatment with thapsigargin or carbachol decreases MPP⁺ (1-methyl-4-phenylpyridinium)-induced neurotoxicity and provides neuroprotection against Parkinson's disease-inducing agents [43].

The present study further demonstrates that increased TRPC1 expression sensitizes IECs to apoptosis, at least partially, by inhibiting NF-κB signalling. As shown in Figures 4 and 5, the levels of induced NF-κB nuclear translocation and transcriptional activity in stable IEC-TRPC1 cells were much lower than those observed in control IEC-6 cells after treatment with TNF-α. This inactivation of NF-κB in stable IEC-TRPC1 cells was associated with an increased sensitivity to apoptosis induced by TNF-α/CHX or STS. By contrast, increased endogenous NF-κB activity through depletion of cellular polyamines induced an increased resistance to TNF-α/CHX-induced apoptosis, whereas the specific inhibition of NF-κB by infection with the *IκBSR* prevented this tolerance to cell death in polyamine-deficient cells (Figure 6). Furthermore, decreasing TRPC1 expression by siTRPC1 not only resulted in the activation of NF-κB but also prevented the increased sensitivity to apoptosis of stable IEC-TRPC1 cells (Figure 7). NF-κB is an inducible transcription factor, and many of the genes that are activated in the initiation of apoptosis are targets of NF-κB [3,25,27]. Under non-stress

conditions, NF- κ B is sequestered in the cytoplasm by binding to its inhibitory protein, I κ B. In response to a host of stimuli, I κ B proteins are phosphorylated and then degraded, allowing free NF- κ B to translocate to the nucleus to activate the transcription of specifically targeted genes [28]. In addition to regulation by inhibitory proteins, NF- κ B activity is also influenced differently by other factors [8,9,28,29]. We have recently demonstrated that NF- κ B activation is induced by increased FAK (focal adhesion kinase) activity or polyamine depletion, resulting in a significant increase in the expression of IAPs (inhibitor of apoptosis proteins) such as cIAP (cellular IAP) and XIAP (X-chromosome-linked IAP) [25,26]. Because NF- κ B-mediated cIAP and XIAP are shown to be the potent natural suppressors of apoptosis in IECs [25], it is suggested that inactivation of NF- κ B in stable IEC-TRPC1 cells induces sensitivity to apoptosis through the down-regulation of IAPs.

The results of the present study also provide evidence that increased TRPC1 expression inhibits NF- κ B activity by increasing Ca^{2+} influx in IECs. Stable IEC-TRPC1 cells exhibited increased Ca^{2+} influx after Ca^{2+} store depletion, which was associated with a decrease in induced NF- κ B activation after exposure to TNF- α (Figures 4 and 5). By contrast, decreased TRPC1 expression by specific siTRPC1 decreased Ca^{2+} influx and induced activation of NF- κ B (Figure 7). Furthermore, decreased $[\text{Ca}^{2+}]_{\text{cyt}}$ by removal of extracellular Ca^{2+} through exposure to the Ca^{2+} -free medium also promoted NF- κ B nuclear translocation and increased NF- κ B-dependent transcriptional activity in stable IEC-TRPC1 cells (Figure 8). NF- κ B has been identified as one of the major downstream effectors of Ca^{2+} for many years, but the regulatory effects of increased $[\text{Ca}^{2+}]_{\text{cyt}}$ on NF- κ B activity are paradoxical and depend on cell type and the nature of the stimuli [45–49]. In some cases, activated Ca^{2+} -sensitive effectors such as calcineurin and protein kinase C after increased $[\text{Ca}^{2+}]_{\text{cyt}}$, mediate NF- κ B activation in T lymphocytes and monocytic cells [45,46]. Similarly, a transient elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by treatment with RANK (receptor activator of NF- κ B) ligands also accelerates NF- κ B nuclear translocation and promotes cell survival in osteoclasts [47]. In other cases, increased $[\text{Ca}^{2+}]_{\text{cyt}}$, by depletion of mitochondrial DNA or exposure to mitochondrial-specific inhibitors, increases the level of cytoplasmic I κ B α and inhibits NF- κ B nuclear translocation in C2C12 rhabdomyocytes and human pulmonary A549 cells [48]. Antonsson et al. [49] have previously shown that Ca^{2+} -loaded calmodulin can directly interact with NF- κ B subunits, c-Rel and RelA, after their release from I κ B and that this interaction inhibits the transport of c-Rel, but not RelA, to the nucleus, suggesting that Ca^{2+} and its sensitive effectors differentially regulate the activation of NF- κ B after cell stimulation. In addition, it remains controversial at present whether NF- κ B regulation and apoptosis are triggered by Ca^{2+} store depletion or by subsequent Ca^{2+} influx through CCE in some cases. Clearly, more studies are needed to define the exact mechanisms by which NF- κ B activity is regulated by Ca^{2+} influx through SOC in different cell types and after various stimuli.

In summary, these results indicate that TRPC1 proteins function as Ca^{2+} permeable channels in IECs and play a critical role in the regulation of apoptosis. Forced expression of the *TRPC1* gene increases the susceptibility of IECs to apoptosis, whereas inhibition of TRPC1 by its specific siRNA promotes cell survival. Our studies further demonstrate that increased TRPC1 sensitizes IECs to apoptosis by inhibiting NF- κ B signalling. Decreased NF- κ B activity in stable IEC-TRPC1 cells is associated with increased susceptibility to apoptosis, whereas inhibition of TRPC1 expression induces NF- κ B activation and protects IECs against apoptosis. Importantly, this study provides new evidence suggesting that increased TRPC1 inactivates NF- κ B signalling by

increasing Ca^{2+} influx. Stable IEC-TRPC1 cells exhibit increased Ca^{2+} influx after Ca^{2+} store depletion, which is associated with inactivation of NF- κ B. By contrast, decreased Ca^{2+} entry by either specific inhibition of TRPC1 expression or removal of extracellular Ca^{2+} induces NF- κ B activation. Because TRPC1 is endogenously present in normal IECs and its channel activity is highly regulated under physiological conditions [24], the observation that TRPC1 is implicated in the regulation of apoptosis is of biological significance. These findings suggest that increased TRPC1 channel activity is critical for apoptotic cell death in the intestinal mucosa *in vivo* and contributes to the maintenance of mucosal homeostasis.

The present study was supported by a Merit Review Grant from the Department of Veterans Affairs and by National Institutes of Health Grants DK-57819, DK-61972 and DK-68491. J.-Y.W. is a Research Career Scientist, Medical Research Service, U.S. Department of Veterans Affairs.

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Received 19 January 2006/17 March 2006; accepted 22 March 2006

Published as BJ Immediate Publication 22 March 2006, doi:10.1042/BJ20060124