Non-Selective Cation Channel-Mediated Ca^{2+} -Entry and Activation of Ca^{2+} /Calmodulin-Dependent Kinase II Contribute to G_2/M Cell Cycle Arrest and Survival of Irradiated Leukemia Cells

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Key Words

Ionizing radiation • Calcium signaling • Vanilloid transient receptor potential • Calcium/calmodulin - dependent kinase II • Ratiometric Ca^{2+} fluorescence imaging • Patch-clamp recording

Abstract

Genotoxic stress induces cell cycle arrest and DNA repair which may enable tumor cells to survive radiation therapy. Here, we defined the role of Ca^{2+} signaling in the cell cycle control and survival of chronic myeloid leukemia (CML) cells subjected to ionizing radiation (IR). To this end, K562 erythroid leukemia cells were irradiated (0-10 Gy). Tumor survival was analyzed by clonogenic survival assay and cell cycle progression via flow cytometry. Plasma membrane cation conductance was assessed by patch-clamp whole-cell recording and the cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was measured by fura-2 Ca^{2+} imaging. Nuclear activity of Ca^{2+} /calmodulin-dependent kinase II (CaMKII) was defined by Western blotting. In addition, the effect of IR (5

Gy) on the cation conductance of primary CML cells was determined. The results indicated that IR (10 Gy) induced a G_2/M cell cycle arrest of K562 cells within 24 h post-irradiation (p.i.) and decreased the clonogenic survival to 0.5 % of that of the control cells. In K562 cells, G_2/M cell cycle arrest was preceded by activation of TRPV5/6-like nonselective cation channels in the plasma membrane 1-5 h p.i., resulting in an elevated Ca^{2+} entry as evident from fura-2 Ca^{2+} imaging. Similarly, IR stimulated a Ca^{2+} -permeable nonselective cation conductance in primary CML cells within 2-4 h p.i.. Ca^{2+} entry, into K562 cells was paralleled by an IR-induced activation of nuclear CaMKII. The IR-stimulated accumulation in G_2 phase was delayed upon buffering $[\text{Ca}^{2+}]_i$ with the Ca^{2+} chelator BAPTA-AM or inhibiting CaMKII with KN93 (1 nM). In addition, KN93 decreased the clonogenic survival of irradiated cells but not of control cells. In conclusion, the data suggest that IR-stimulated cation channel activation, Ca^{2+} entry and CaMKII activity participate in control of cell cycle progression and survival of irradiated CML cells.

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Introduction

Cell cycle progression of proliferating cells requires Ca^{2+} signals to enter and accomplish the S and the M phase of the cell cycle [1, 2]. At the same time, Ca^{2+} signals may also trigger programmed cell death. Fast proliferating tumor cells have been postulated to overcome this dilemma by remodeling their Ca^{2+} signalosome: Possibly hazardous Ca^{2+} signals generated by bulky release of Ca^{2+} from the ER (followed by mitochondrial Ca^{2+} buffering, store-dependent Ca^{2+} entry and ER refilling) are replaced by signals arising from slower Ca^{2+} entry and extrusion across the plasma membrane. These plasma membrane-born signals are generated by store-independent Ca^{2+} channels and the activity of Ca^{2+} pumps or antiporters, respectively [3]. Along those lines, androgen-independent advanced prostate cancer has been demonstrated to up-regulate Ca^{2+} -permeable TRPV6 channels in the plasma membrane that maintain a high proliferation rate by simultaneously preventing apoptosis [4]. In addition, TRPV6 is strongly up-regulated in breast adenocarcinoma [5] suggesting a tumor growth-promoting function of TRPV6 in different tumor entities. A similar function has been attributed to T-type Ca^{2+} channels which are up-regulated in several cell types during neoplastic transformation of epithelial cells into cancer cells [2].

Cell proliferation of primary chronic myelogenous leukemia (CML) cells has been demonstrated to critically depend on Ca^{2+} signaling, involving the Ca^{2+} /calmodium-dependent kinase gamma (CaMKIIgamma) [6]. In addition, expression of TRPV5/6 channels has been reported in a CML cell line [7]. CML is commonly defined by the presence of the Philadelphia chromosome, a reciprocal translocation of chromosomes 9 and 22. The resultant Bcr-Abl (breakpoint cluster region/Abelson) oncoprotein, a constitutively active tyrosine kinase, leads to uncontrolled cell proliferation. Tyrosine kinase inhibitors are the treatment of choice for patients with Philadelphia chromosome-positive CML [8]. While tumor growth is controlled by imatinib or second generation tyrosine kinase inhibitors, allogeneic hematopoietic stem cell transplantation (HSCT) is still regarded as the only curative treatment option [9]. Total-body irradiation (TBI) is used in myeloablative or intensity-reduced conditioning regimens prior to HSCT [10].

DNA damages caused by ionizing radiation induce an arrest in cell cycle progression and activate the DNA repair machinery. Whereas residual DNA damages of radiosensitive tumor cells compromise their clonogenic survival, radioresistant cells which accomplish to repair

their DNA damages may re-enter cell cycling and restore the tumor. In myeloid leukemia, relapse incidences of around 8-15 % after myeloablative TBIs (8-12.6 Gy) have been reported [11-13].

Until now, the role of Ca^{2+} signaling in the response of irradiated leukemia cells to genotoxic stress has not been defined. Therefore, the aim of the present study was to explore the modulation of the Ca^{2+} signaling through TRPV5/6 and CaMKII by ionizing radiation. The erythroleukemic cell line K562 originally isolated from a patient with CML [14] was subjected to ionizing radiation, and cell survival, Ca^{2+} channel activity, CaMKII activity, and cell cycle control were analyzed by colony formation, whole-cell patch clamp recording and fura-2 Ca^{2+} imaging, Western blotting, and flow cytometry, respectively. In addition, whole-cell recordings were performed in control and irradiated primary chronic myeloid leukemia cells. The data suggest that ionizing radiation stimulates Ca^{2+} -entry through TRPV5/6-like channels as well as activation of CaMKII, which in turn fosters G_1/S transition, S progression and accumulation in G_2 phase of cell cycle, resulting in an increased probability of clonogenic survival of the leukemia cells.

Materials and Methods

Cell Culture

K562 human erythroleukemia cells and primary chronic myeloid leukemia (pCML) cells (donors gave informed consent, and procedures were performed according to the declaration of Helsinki with local ethical committee approval) were cultivated in RPMI 1640 medium containing L-glutamine (Gibco, Karlsruhe, Germany) supplemented with 10% FCS and penicillin (100 U/ml) / streptomycin (100 µg/ml). Ionizing X-ray radiation (single dose of 0, 1, 5 or 10 Gy) was applied by a linear accelerator (LINAC SL25 Philips) at a dose rate of 4 Gy/min at room temperature. Following irradiation, cells were post-incubated in supplemented RPMI 1640 medium for 1-48 h.

Colony formation assay

To test for clonogenic survival, irradiated (0 or 10 Gy) cells were sequentially diluted in supplemented RPMI 1640 medium, seeded (0.4 - 25,000 cells in 200 µl per well) into a 96-well plate and incubated for 4 weeks. K562 cells adhered to the wells' bottom and formed round colonies which could be easily counted up to a number of 12 per well. The plating efficiency was defined by dividing the number of colonies by the number of plated cells. Survival fraction was calculated by dividing the plating efficiency of the treated cells by that of the untreated controls.

For assessing the effect of the CaMKII inhibitor KN93, irradiated (0 or 10 Gy) cells were pretreated (0.5 h), irradiated, and post-incubated (24 h) with vehicle or KN93 (1 nM).

Thereafter, cells were diluted for clonogenic survival. The survival fraction was calculated by dividing the plating efficiency of the KN93- and vehicle-treated irradiated cells by that of the vehicle-treated non-irradiated controls (Fig. 8D). The specific survival fraction was calculated by dividing the plating efficiency of the KN93- and vehicle-treated irradiated cells by that of the respective KN93- and vehicle-treated non-irradiated controls (Fig. 8E). If not stated otherwise, all chemicals were obtained from Sigma-Aldrich, Deisenhofen, Germany.

Patch-clamp recording

Whole-cell currents were evoked by 11 voltage pulses (700 ms each) from -50 mV holding potential to voltages between -100 mV and +100 mV (20 mV increments). Steady state and peak current values were analyzed 1-5 h p.i. The liquid junction potentials between the pipette and the bath solutions were estimated according to [15], and data were corrected for the estimated liquid junction potentials. Applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. Inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces.

Cells were superfused at 37°C temperature with NaCl solution (in mM: 125 NaCl, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 KCl, 5 D-glucose, 1 MgCl₂, 1 CaCl₂, titrated with NaOH to pH 7.4). Upon GΩ-seal formation and entry into the whole-cell recording mode, cells were recorded with Ca-D-gluconate₂ bath solution (in mM: 100 Ca-D-gluconate₂, 10 HEPES, titrated with tris(hydroxymethyl)aminomethane (Tris) to pH 7.4), Na-D-gluconate bath solution (in mM: 150 Na-D-gluconate, 10 HEPES, 1 Ca-D-gluconate₂, titrated with NaOH to pH 7.4) or EGTA-buffered Na-D-gluconate bath solution (in mM: 150 Na-D-gluconate, 10 HEPES, 3 ethylene glycol-bis(-aminoethyl ether)-N,N',N'-tetraacetic acid, (EGTA), titrated with NaOH to pH 7.4). The K-D-gluconate pipette solution contained (in mM): 140 K-D-gluconate, 5 HEPES, 5 MgCl₂, 1 K₂-EGTA, 1 K₂-ATP, titrated with KOH to pH 7.4.

Fura-2 Ca²⁺ imaging

Fluorescence measurements were performed using an inverted phase-contrast microscope (Axiovert 100; Zeiss, Oberkochen, Germany). Cells were excited alternatively at 340 or 380 nm and the excitation and emission light was deflected by a dichroic mirror into either the objective (Fluar x40/1.30 oil; Zeiss) or transmitted to the camera (Proxitronic Imaging GmbH, Bensheim, Germany). Emitted fluorescence intensity was recorded at 505 nm, and data acquisition was performed by employing specialized computer software (Metafluor, Universal Imaging, Downingtown, PA, USA). The 340/380 nm fluorescence ratio was used as a measure of cytosolic free Ca²⁺ concentration ([Ca²⁺]_i), the slope of the increase in the 340/380 nm-ratio as a measure of Ca²⁺ entry into the cells.

The cells were irradiated (0 or 10 Gy) and loaded with fura-2/AM (2 μM for 30 min at 37°C; Molecular Probes, Goettingen, Germany) in supplemented RPMI medium. [Ca²⁺]_i

was determined 2-7 h p.i. at 37°C during superfusion with NaCl solution, during extracellular Ca²⁺ removal in EGTA-buffered NaCl solution (in mM: 125 NaCl, HEPES, 5 KCl, 5 D-glucose, 1 MgCl₂, 1 EGTA, 0.001 or 0 thapsigargin, titrated with NaOH to pH 7.4), and Ca²⁺ re-addition (in NaCl solution containing 0 or 0.001 mM thapsigargin).

Flow cytometry

For cell cycle analysis, cells were pre-incubated (30 min), irradiated and incubated for further 24 h in supplemented RPMI 1640 medium containing either 0 or 10 μM BAPTA-AM (Calbiochem; Bad Soden, Germany), 0 or 1 μM ionomycin/0 or 3 mM EGTA or 0 or 1 nM KN93 (in the control situation the corresponding volume of DMSO or a 1:1 mixture of DMSO and ethanol was added to the medium). Cells were permeabilized and stained (30 min at room temperature) with propidium iodide (PI) solution (containing 0.1% Na-citrate, 0.1% triton X-100, 50 μg/ml PI in PBS), and the DNA amount was analyzed by flow cytometry (FACS Calibur, Becton Dickinson, Heidelberg, Germany) in fluorescence channel FL-3 (488 nm excitation and >670 nm emission wavelength).

For simultaneous determination of phosphatidylserine exposure and [Ca²⁺]_i, cells were washed in annexin-binding buffer (NaCl solution additionally containing 5 mM CaCl₂) and double-stained with annexin-V-APC (1:20 dilution; BD Biosciences, Heidelberg, Germany) and fluo-3-AM (2 μM) Calbiochem, Bad Soden, Germany). After 30 min of staining, samples were diluted 1:5 in annexin-binding buffer. Annexin-V-APC binding and the [Ca²⁺]_i-specific fluo-3 fluorescence were measured by flow cytometry in fluorescence channels FL-1 (488 nm excitation and 530 nm emission wavelength) and FL-4 (635 nm excitation and 661 nm emission), respectively. As loading control for fluo-3, cells were incubated with the Ca²⁺ ionophore ionomycin (1 μM for 10 min) prior to analysis by flow cytometry.

To test for DNA synthesis by bromodeoxyuridine (BrdU) incorporation, the cells were resuspended in supplemented RPMI 1640 medium containing BrdU (20 μM) and BAPTA-AM (0 or 10 μM), irradiated with 0 or 10 Gy, post-incubated for 8 h at 37°C, washed, and further incubated for 16 h in RPMI 1640 medium continuously containing BAPTA-AM (0 and 10 μM, respectively). After 24 h of total incubation time, cells were fixed with 70% ethanol and consecutively treated with RNase A (0.1 mg/ml in phosphate-buffered saline (PBS) for 10 min at 37°C), pepsin (0.5 mg/ml in 0.05 N HCl for 10 min at 37°C), and 2 N HCl (for 10 min at room temperature). For immunolabelling, cells were incubated (30 min at room temperature) with a monoclonal mouse anti-BrdU antibody (1:67 dilution in PBS / 1% bovine serum albumin (BSA), Becton Dickinson, Pharmingen, Freiburg, Germany) and post-incubated (30 min at room temperature) with a fluoresceinisothiocyanat (FITC)-conjugated rabbit anti-mouse IgG antibody (1:100 in PBS / 1% BSA, DAKO, Hamburg, Germany). Thereafter, cells were stained (15 min at 4°C) with PI solution (25 μg/ml PI and 20 μg/ml RNase A in PBS / 1% BSA). BrdU- and PI-specific fluorescence was analyzed by flow cytometry in fluorescence channels FL-1 (530 nm) and FL-3 (>670 nm), respectively (488 nm excitation).

Fig. 1. Effect of ionizing radiation (IR) on cell cycle, Ca^{2+} signaling and survival of K562 cells. A. Histogram recorded by flow cytometry showing DNA staining with propidium iodide (PI, Nicoletti protocol) of K562 cells grown for 0, 24 and 48 h post-irradiation (p.i.) with 10 Gy. Cells in the G_1 , S, and G_2 phase of the cell cycle as well as dead cells in the sub G_1 -population are indicated in the left histogram. Inserts give the mean percentage of cells ($n = 6$) in the indicated populations. Percentages refer to the total population including dead cells (sub G_1 population) and cells with higher DNA content (hyper G_2 population which is not shown). B. Relative clonogenic survival of control (0 Gy, open bar) and irradiated K562 cells (10 Gy, closed bar; $n = 6$). C. Time-dependent increase in the cell number of K562 cultures irradiated at day 0.25 with 0 Gy (open circles) or 10 Gy (closed triangles; $n = 3$). D. Dot blots showing the fluo-3 and annexin-V-APC fluorescence as a measure of $[\text{Ca}^{2+}]_i$ and breakdown of the phospholipids asymmetry in the plasma membrane, respectively. K562 cells were recorded by flow cytometry 48 h p.i. with 0 (left) and 10 Gy (right). E. Mean fluo-3 fluorescence ($n = 19$) of Ca^{2+} -permeabilized (1 μM ionomycin) and non-permeabilized (control) K562 cells 48 h p.i. with 0 (open bars) and 10 Gy (closed bars, left) as recorded in (D). E, right. Mean fluo-3 fluorescence ratio (fluo-3 ratio = $\text{fluo-3}_{\text{control}}/\text{fluo-3}_{\text{ionomycin}}$) of 0 Gy- (open bar) and 10 Gy-irradiated (closed bar) cells as calculated from the data in (E, left) by dividing the fluo-3 fluorescence of non-permeabilized cells by that of the Ca^{2+} permeabilized cells. F. Mean percentage of annexin-V-APC binding cells ($n = 19$) 48 h p.i. with 0 (open bar) and 10 Gy (closed bar). G. Mean fluo-3 $_{\text{control}}/\text{fluo-3}_{\text{ionomycin}}$ fluorescence ratio ($n = 19$) of the annexin-V-negative (left) and positive (right) fractions (gated as in D) of K562 cells 48 h p.i. with 0 (open bars) and 10 Gy (closed bars).

Western Blotting

For enrichment of nuclei, cells were swollen (30 min on ice) in hypotonic KCl solution (in mM: 100 KCl, 10 HEPES 0.1 Na-EGTA, 0.1 Na-EDTA, titrated with NaOH to pH 7.4 and supplemented with protease (Complete Protease Inhibitor Cocktail) and phosphatase inhibitors (PhosStop, both from Roche Applied Science, Mannheim, Germany). Plasma membranes were then lysed by adding 0.7% (final concentration) NP40 detergent, and nuclei were pelleted for 10 min at 14,000 rpm and 4°C in an Eppendorf centrifuge. The pelleted nuclei were lysed in 62.5 mM Tris/HCl (pH 6.8), 2% (w/v) sodium dodecylphosphate, 10% (v/v) glycerol, 50 mM dithiothreitol 0.01% (w/v) bromphenol blue, separated by SDS-PAGE, blotted on a PVDF membrane and probed against phospho-CaMKII and lamin A/C, using rabbit anti-phospho-CaMKII (Thr286) antibody (Cell Signaling #3361, New England Biolabs, Frankfurt, Germany) and anti-lamin A/C antibody (Cell Signaling #2032), respectively. Both antibodies were used in a 1:1000 dilution, incubated at 4°C overnight and detected with a horseradish peroxidase-linked goat anti-rabbit IgG antibody (Cell Signaling #7074; 1:2000 dilution, incubated at room temperature for 1 h) and the ECL-kit (Amersham, Braunschweig, Germany).

Statistics

Data are expressed as means $\pm$ SE and statistical analysis was made by normal or Welch-corrected two-tailed t-test (Figs. 1F, 3-5, and 8E) or ANOVA using Tukey's test as post hoc test (Figs. 1G, 2, 6, 7, and 8C-D). *, **, and *** indicate $p \leq 0.05$, 0.01 and 0.001, respectively.

Results

K562 cells subjected to ionizing irradiation (IR, 10 Gy) accumulated within 24 h post-irradiation (p.i.) in G_2 phase of the cell cycle as evident from flow cytometry analysis of Nicoletti staining (Fig. 1A, left and middle). Within 48 h p.i., almost 20% of the cells were dead (sub G_1 fraction in Fig 1A, right). Further 10% of the cells were released from G_2/M arrest and underwent mitosis as illustrated by a re-increase of the G_1 population (Fig 1A, right). In clonogenic survival assays, only about 0.5% of all irradiated (10 Gy) K562 cells remained clonogenic when normalized to the clonogenic survival of non-

Fig. 2. IR stimulates Ca^{2+} -permeable channels in K562 cells. A. Whole-cell current tracings from a control (0 Gy, left) and irradiated (10 Gy, right) K562 cell. Records were obtained with K-D-gluconate pipette combined with Ca-D-gluconate_2 bath solution. The drawing on the left shows the applied voltage pulse protocol. For simplification, only the current tracings elicited by the voltage square pulses to -100, -60 and -40 mV are depicted. The zero current value is indicated by the red line. B. Relationship between mean current densities and voltage of control (open circles; $n = 13$) and irradiated K562 cells (10 Gy, closed circles; $n = 8$) recorded as in (A). C. Mean conductance density of control (0 Gy, open bar; $n = 15$) and irradiated K562 cells (1, 5, or 10 Gy, closed bar; $n = 5-8$) determined 1-5 h p.i.. The conductance densities were calculated for the inward currents between -40 and -100 mV by linear regression as shown in (B) by blue and red lines.

Fig. 3. IR stimulates an inwardly rectifying nonselective cation current which is inhibited by external Ca^{2+} . A. Whole-cell current tracings recorded from an irradiated (10 Gy) K562 cell with K-D-gluconate pipette solution. Records were obtained with 1 mM Ca^{2+} -containing Na-D-gluconate bath solution (left) and after removal of external Ca^{2+} by superfusing a Ca^{2+} -free Na-D-gluconate bath solution containing 3 mM EGTA (middle). The inset on the right shows the Ca^{2+} sensitive current fraction ($\Delta I_{\text{EGTA-Ca}}$) as obtained by subtracting the right from the middle tracings. B. The mean external Ca^{2+} -sensitive fraction of the current densities is plotted against the voltage for control (0 Gy, open circles; $n = 6$) and irradiated K562 cells (10 Gy, closed triangles; $n = 6$). The current density fractions were recorded 1.5-4 h p.i. as shown in (A). C. Mean Ca^{2+} -sensitive conductance density of control (0 Gy, open bar) and irradiated K562 cells (10 Gy, closed bar). The conductance densities were calculated from the data in (B) for the inward currents between -40 and -100 mV by linear regression (blue and red line).

irradiated control cells (Fig. 1B). These cells were capable to rebuild the tumor cell mass with a delay of about 3 weeks compared to non-irradiated controls (Fig. 1C). The IR-induced cell cycle arrest and death of K562 cells were paralleled by an elevated $[\text{Ca}^{2+}]_i$ 48 h p.i., as suggested by a higher fluo-3 fluorescence monitored by flow cytometry (Fig. 1D, E). In addition to the increase of $[\text{Ca}^{2+}]_i$, irradiated K562 cells exhibited a larger population with broken-down phospholipid asymmetry of the plasma membrane (Fig. 1D, F). Comparison of $[\text{Ca}^{2+}]_i$ between cells with intact and broken-down phospholipid asymmetry (see Fig. 1D) suggested an IR-stimulated increase in $[\text{Ca}^{2+}]_i$ predominantly in the population with lost asymmetry (Fig. 1G).

The observed modulation of $[\text{Ca}^{2+}]_i$ at 48 h p.i. might be an epiphenomenon of the IR-induced clonogenic inactivation of K562 - it might, however, be also part of the signaling complex leading to clonogenic inactivation.

Therefore, we tested for early Ca^{2+} signaling events by recording whole-cell Ca^{2+} currents in irradiated (1-10 Gy) and control cells at 1-5 h p.i.. As shown in Fig. 2, IR (10 Gy) significantly increased the whole cell currents at negative voltages when recorded with Ca^{2+} -gluconate in the bath and K-gluconate in the pipette. In particular, an increase in inward currents at voltages of -60 mV or below, as well as of outward currents at -10 mV, was observed (indicated by asterisks in Fig. 2B) suggestive of an IR-stimulated nonselective cation conductance. This conductance exhibited a relative Ca^{2+} permeability of $P_{\text{Ca}}/P_{\text{K}} = 0.13$ as deduced from the -35 mV reversal potential of the IR-stimulated current fraction (Fig. 2B). The absence of any IR-stimulated outward currents at positive voltages suggested strong inward rectification of the cation conductance. Fig. 2C illustrates the IR dose-dependency of the conductance that generated the Ca^{2+} influx into K562 cells.

Fig. 4. IR stimulates Ca^{2+} -permeable channels in primary chronic myeloid leukemia (pCML) cells. A. Whole-cell current tracings from a control (0 Gy, left) and irradiated (5 Gy, right) pCML cell. Records were obtained with K-D-gluconate pipette combined with Ca-D-gluconate_2 bath solution. The drawing on the left shows the applied voltage pulse protocol. B. Relationship between mean current densities and voltage of control (open circles; $n = 6$) and irradiated pCML cells (5 Gy, closed circles; $n = 4$) recorded as in (A) 2–4 h p.i.. Shown are the peak (upper plot) and the steady state currents (lower plot). C, D. Mean reversal potential (C) and mean conductance density (D) of control (0 Gy, open bar) and irradiated pCML cells (5 Gy, closed bar). Reversal potentials and conductance densities were calculated from the steady state currents in (B). Conductances were analyzed for the inward currents between -60 and -100 mV by linear regression (blue and red line).

Fig. 5. IR stimulates Ca^{2+} entry into K562 cells. A. Time course of the mean ratio between the 340 nm- and the 380 nm-excited fura-2 fluorescence as a measure of the cytosolic free Ca^{2+} concentration. Ratios were recorded 2–7 h p.i. in control (0 Gy, open circles; $n = 13$ –19) and irradiated cells (10 Gy; $n = 15$) during removal and re-addition of external Ca^{2+} in the presence (left) or absence (right) of Ca^{2+} store depletion by the SERCA pump inhibitor thapsigargin (1 μM) as indicated. B. Mean slope of ratio increase upon Ca^{2+} re-addition. Slopes for the linear course of ratio increase (indicated by blue and red lines in A) upon Ca^{2+} re-addition were calculated from the data in (A) for store-depleted (+thapsigargin, protocol as in A, left) and non-store-depleted (-thapsigargin, protocol as in A, right) control (open bars) and irradiated K562 cells (closed bars).

The high relative Ca^{2+} permeability and the strong inward rectification resemble properties of TRPV5/6 channels. Monovalent cation currents through these channels have been demonstrated in K562 cells to be effectively inhibited by micromolar concentrations of extracellular Ca^{2+} [7]. We therefore recorded control and irradiated K562 cells with K-gluconate pipette and Na-gluconate bath solution and modified the extracellular free Ca^{2+} concentration. As a result, removal of extracellular Ca^{2+} activated a strongly inwardly rectifying nonselective

cation conductance which was much higher in irradiated than in control cells (Fig. 3), suggesting IR-induced activation of TRPV5/6 channels in K562 cells.

We next tested for IR-stimulated Ca^{2+} -permeable channels in pCML cells by performing whole-cell recordings with Ca^{2+} -gluconate in the bath and K-gluconate in the pipette solution. As shown in Fig. 4A, pCML cells expressed time-dependently inactivating voltage-gated K^{+} channels. IR (5 Gy) stimulated a significant increase in inward currents within 2–4 h (Fig.

Fig. 6. IR induced G_1 /S transition, S progression and accumulation in the G_2 phase of the cell cycle is partially mediated by Ca^{2+} signals. A. Flow cytometry histograms of propidium iodide (PI)-stained K562 cells (Nicoletti protocol) 24 h p.i. Cells were pre-incubated (0.5 h), irradiated and post-incubated in the absence (vehicle, left) or presence (right) of the membrane-permeable Ca^{2+} chelator BAPTA-AM (10 μ M). B-C. Mean percentage ($n = 16-18$ in B and $n = 6$ in C) of control (0 Gy) and irradiated cells (10 Gy) residing in G_1 (left), S (middle) or G_2 phase of the cell cycle 24 h p.i. (B) Cells were pre-incubated (0.5 h), irradiated and post-incubated with 0 μ M (vehicle, open bars) or 10 μ M BAPTA-AM (closed bars) or (C) with 0 μ M ionomycin and 0 mM EGTA (vehicle, open bars), with 1 μ M ionomycin and 0 mM EGTA (grey bars), or with 1 μ M ionomycin and 3 mM EGTA in the medium (closed bars).

4A and B) which were carried by Ca^{2+} influx. The current-voltage relationships of the control and IR-stimulated currents converged at around 0 mV voltage (Fig. 4B), suggesting a high relative Ca^{2+} permeability in the range of $P_{Ca}/P_K = 0.75$ for the IR-stimulated conductance. This is also deduced from the IR (5Gy)-induced shift of the current reversal potential towards more positive potentials (i.e., towards the Ca^{2+} equilibrium potential, Fig. 4C). The

Fig. 7. Intracellular Ca^{2+} chelation delays irradiation-induced accumulation in the G_2 phase of cell cycle. A. Dot plots showing the DNA-synthesis (repair) by bromodeoxyuridine (BrdU) incorporation and the DNA amount by propidium iodide (PI) staining after incubation for 24 h following irradiation with 0 Gy (control, top) and 10 Gy (bottom). BrdU-positive and -negative cells in the S-phase of the cell cycle are outlined in turquoise, cells which reside in G_1 and G_2 and which did not incorporate BrdU (low BrdU) are outlined in violet and yellow, respectively. The BrdU-positive cells (high BrdU) in G_1 and G_2 are indicated by the red and grey box, respectively. B. Mean percentage ($\pm$ SE, $n = 3-4$) of control (0 Gy) and irradiated (10 Gy) cells residing in the G_2 low BrdU, G_1 low BrdU, S, G_2 high BrdU, or G_1 high BrdU populations as defined in (A). Cells were irradiated and post-incubated (24 h) in the absence (open bars) or presence (closed bars) of the Ca^{2+} chelator BAPTA-AM (10 μ M).

current- voltage relationships in Fig. 4B and the conductance densities in Fig. 4D indicate that pCML exhibited an about ten-time higher basal conductance density as compared to K562 cells. IR (5 Gy) stimulated comparable increases of the Ca^{2+} inward currents in K562 and pCML cells (compare Fig. 2C with Fig. 4D).

To assess the functional impact of the IR-activated cation conductance on Ca^{2+} signaling, control and irradiated (10 Gy) K562 cells were loaded with fura-2, and $[Ca^{2+}]_i$ was determined 2-7 h p.i. by ratiometric fluorescence imaging applying a Ca^{2+} removal-and-re-addition protocol in the presence and absence of the SERCA pump inhibitor thapsigargin (1 μ M). Fig. 5 shows that IR stimulated Ca^{2+} entry into the K562 cells which was not dependent on Ca^{2+} store depletion by thapsigargin, indicating activation of store-independent Ca^{2+} entry pathways by IR.

We further analyzed the function of Ca^{2+} signals in the cell cycle regulation of irradiated K562 cells. To blunt and to abrogate IR-mediated increases in $[Ca^{2+}]_i$, cells

Fig. 8. IR stimulates the activity of nuclear CaMKII and CaMKII-dependent accumulation in G_2 phase of the cell cycle as well as CaMKII-dependent clonogenic survival. A. Immunoblots showing the abundance of active (i.e., phosphorylated) CaMKII isoforms in the nuclei of control and irradiated (5 and 10 Gy) K562 cells 4 h p.i. (upper panel) and of the lamin A/C loading control (lower panel). The numbers indicate the ratio between the densitometrically semi-quantified phosphoCaMKII and lamin A/C protein amounts. B-C. Flow cytometry histograms (B) of propidium iodide (PI)-stained K562 cells and (C) mean percentage ($n = 11$) of control (0 Gy) and irradiated cells (10 Gy) residing in G_1 , S, G_2 , or sub G_1 population as indicated. Cells were pre-incubated (0.5 h), irradiated, and post-incubated (24 h) in the absence (vehicle, left in B and open bars in C) or presence (B, right and closed bars in C) of the CaMKII inhibitor KN93 (1 nM). D. Mean surviving fraction ($n = 12$) of control and irradiated (10 Gy) K562 cells pre- and post-incubated for 0.5 and 24 h, respectively, with 0 (vehicle, open bars) or 1 nM KN93 (closed bars) prior to diluting and plating the cells for the clonogenic survival assay. E. Mean specific surviving fraction of KN93 (1 nM) and vehicle-treated irradiated (10 Gy) K562 cells as calculated by normalizing the plating efficacies of KN93- and vehicle-treated irradiated cells to those of the KN93- and vehicle-treated non-irradiated controls, respectively (same data as in D).

were preincubated (0.5 h), irradiated (0 or 10 Gy) and post-incubated (24 h) with the membrane permeable Ca^{2+} chelator BAPTA-AM (10 μ M) and EGTA (3 mM) combined with the Ca^{2+} ionophore ionomycin (1 μ M), respectively, and cell cycle was analyzed by flow cytometry of Nicoletti stainings. Blunting of Ca^{2+} signals by intracellular Ca^{2+} buffering with BAPTA significantly attenuated the IR-induced decline in the G_1 and the S population as well as the increase in the G_2 population, while no effect was observed on the cell cycling of control cells (Fig. 6A and B). EGTA/ionomycin, which is thought to strongly deplete $[Ca^{2+}]_i$, almost abolished IR-induced decrease in G_1 and increase in G_2 (Fig. 6C) when compared with controls treated with ionomycin (1 μ M) alone. In contrast to BAPTA, EGTA/ionomycin conferred significant effects also on G_1 /S transition, S progression and G_2 accumulation of non-irradiated control cells (Fig. 6C).

In theory, the effect of Ca^{2+} buffering on cell cycle progression in irradiated cells could have been due to either a delayed accumulation in G_2 or to an accelerated G_2 /M checkpoint progression and entry into mitosis. To distinguish between these two possibilities, K562 cells were irradiated (0 or 10 Gy) and pulse-labeled with the thymidine analogue 5-bromo-2-deoxyuridine (BrdU) for 8 h. After 24 h p.i. BrdU incorporation and cell cycle was analyzed by flow cytometry using a combination of

anti-BrdU antibody and Nicoletti staining (Fig. 7). The data clearly indicate that Ca^{2+} buffering delayed the accumulation of the irradiated cells in G_2 . This is evident from the BAPTA-induced decline in the BrdU-positive G_2 population (G_2 high BrdU population in Fig. 7B, 4th diagram) which was not accompanied by an equivalent increase in the BrdU-positive G_1 population (G_1 high BrdU population in Fig. 7B, 5th diagram). The latter is expected in case the observed decline in G_2 was due to an accelerated G_2 /M progression. In fact, the BAPTA-induced decline in the BrdU-positive G_2 population of the irradiated K562 cells resulted from a delayed G_1 /S transition and S progression. This is evident from the BrdU-negative G_1 population (low BrdU population in Fig. 7B, 2nd diagram) that tended ($p = 0.12$) to be enlarged in the presence of BAPTA, as well as by the BAPTA-mediated increase in the total S population (Fig. 7B, 3rd diagram).

CaMKII isoforms are reportedly potential effector proteins of Ca^{2+} signals involved in the cell cycle control

of leukemia cells [6]. Therefore, we analyzed nuclear activation of CaMKII 4 h p.i. by Western blotting. As depicted in Fig. 8, IR (5 and 10 Gy) increased the nuclear abundance of Thr286-phosphorylated (i.e., active) CaMKII protein. The CaMKII inhibitor KN93 (1 nM) was employed to test for a CaMKII-mediated regulation of cell cycle and survival upon IR. K562 cells were pre-incubated (0.5 h), irradiated (0 or 10 Gy) and post-incubated (24 h) in the absence or presence of KN93 (1 nM). Thereafter, cells were either analyzed by flow cytometry using Nicoletti staining or diluted and plated for clonogenic survival. As shown in Fig. 8B-E, KN93 delayed accumulation in the G₂ phase of the cell cycle 24 h p.i. and impaired the clonogenic survival of irradiated but not of control cells. Taken together, these observations suggest an IR-specific function of CaMKII in cell cycle control and survival in K562 cells.

Discussion

In the present study, IR increased the activity of Ca²⁺ store-independent, Ca²⁺-permeable nonselective cation channels in the plasma membrane of K562 cells. Functional expression of cAMP-stimulated CFTR [16] and oxidation-induced ClC-3 anion channels [17], voltage-gated hERG1 [18] and Ca²⁺-activated SK4 (KCa_{3.1}) K⁺ channels [19], TRPC7 [20] and TRPV5 and -V6 nonselective cation channels [7] have been defined in K562 cells on the molecular level. In the present study, the Ca²⁺-permeable nonselective cation channels activated by IR exhibited a strongly inwardly rectifying current-voltage relationship highly reminiscent of Ca²⁺-selective TRPV5 and -V6 channels [21]. In K562 cells, TRPV5 and -V6 proteins may form functional heterotetrameric channels [7]. They are permeable for monovalent cations [22], and the monovalent cation currents are inhibited by micromolar concentrations of extracellular Ca²⁺ [7] suggestive of a competition of monovalent and Ca²⁺ cations within the channel pore. Accordingly, buffering of extracellular Ca²⁺ by EGTA activated in the present study a strongly inwardly rectifying cation current which was much more pronounced in the irradiated cells. Taken together, our data strongly suggest that the observed IR-stimulated Ca²⁺ entry into K562 cells occurred at least partially through TRPV5/6 channels. Similarly to the K562 cell line, IR stimulated a Ca²⁺-permeable cation conductance in primary chronic myeloid leukemia cells. This suggests that the observed phenomenon is not specific to the cell line K562 but might rather represent a

more general response of CML cells to genotoxic stress.

Sustained activity of these Ca²⁺-permeable channels might underlie the elevated [Ca²⁺]_i that was observed in K562 cells 48 h p.i. Reportedly, elevated [Ca²⁺]_i triggers the break-down of the phospholipid asymmetry in K562 cells [19, 20]. Accordingly, a higher percentage of irradiated K562 cells than control cells exhibited phospholipid scrambling 48 h p.i.. Notably, [Ca²⁺]_i of those cells rose above that of annexin V-binding, non-irradiated cells (see Fig. 1G), suggesting that the IR-stimulated increase in [Ca²⁺]_i even exceeded the threshold of phospholipid scrambling. This implies a pronounced perturbation of [Ca²⁺]_i in irradiated K562 cells which might contribute to the IR-induced death of these cells. On the other hand, in the minor fraction of cells which survived IR, the Ca²⁺-permeable channels might be involved in cell cycle control and DNA repair during genotoxic stress.

Mammalian cells responding to DNA damage initiate rapid but transient mechanisms promoting cell cycle arrest. In most cell types, these acute transient arrests are mediated through Ataxia-telangiectasia-mutated (ATM) / Ataxia-telangiectasia-and-Rad3-related (ATR) DNA-PK kinases-, Checkpoint kinases- and p53-dependent-pathways that target inhibition of Cdk1 and Cdk2 [23]. In hematopoietic cells, growth factor receptor-triggered phosphoinositide 3-kinase (PI3K) signaling has been proposed to contribute to both early DNA damage-induced activation of acute arrest checkpoints and subsequent release from G₁ and G₂ phase arrests [24]. It has been further reported that the surface expression of TRPV5 and -V6 channels is regulated by serum-and-glucocorticoid-inducible kinases (SGKs), known downstream targets of PI3K [25-27]. Moreover, the N-terminal amino acid sequence of TRPV6 suggests a putative pleckstrin-homology-domain which might hint to a direct regulation of the channel by 3'-phosphoinositides [28]. Thus, IR-stimulated PI3K signaling might underlie the TRPV5/6-mediated Ca²⁺ entry proposed by the present study.

Cell cycle progression in normal cells critically depends on Ca²⁺ signals including Ca²⁺ entry [3], and the cellular response to IR includes Ca²⁺ signaling [29-31]. Calmodulin (CaM) is a Ca²⁺-binding protein and a primary cellular sensor of [Ca²⁺]_i. Ca²⁺/CaM has been demonstrated to regulate the cell cycle [32], to interact with histone complexes [33], to enhance radioresistance by up-regulation of DNA repair pathways involving histone H2AX phosphorylation [34], to mediate IR-enhancement of double strand break-rejoining fidelity [35],

and to mediate IR-induced cell cycle arrest [36]. In seeming contradiction to these established functions of Ca^{2+} /CaM during genotoxic stress is the observation that IR down-regulates calmodulin mRNA levels within the first few hours following IR exposure [37]. The functional significance of this down-regulation for the Ca^{2+} /CaM signaling or the genotoxic stress response remains to be defined.

The Ca^{2+} signals are transduced into longer lasting biochemical events by CaMKII and other Ca^{2+} effector proteins [38]. CaMKII isoforms are encoded by 4 separate genes [39]. Multiple splice variants of these isoforms show differential expression and differ in their activity, their specificity for the different Ca^{2+} signals [40], and their ability to translocate to the nucleus [41]. Once activated by Ca^{2+} /calmodulin, auto-phosphorylation renders the kinases active independently of Ca^{2+} and calmodulin [39]. This autonomy differs between the splice variants [40] and may be prolonged by limited proteolytic cleavage [42]. By their autonomous activity, the CaMKII isoforms translate temporary Ca^{2+} signals into longer lasting protein phosphorylation events. Several functions of CaMKII in cell cycle control have been described as summarized in the following.

CaMKII has been demonstrated to activate MEK/ERK in colon adenocarcinoma cells, which in turn leads to phosphorylation and subsequent proteasomal degradation of p27 [43]. In osteosarcoma, CaMKII increases membrane-bound Tiam1 and GTP-bound Rac1 which decreases p21 gene expression [44]. Moreover, in breast cancer, CaMKII triggers the export of BRCA1 from the nucleus during the early part of the S phase [45]. This nuclear export decreases the BRCA1-dependent expression of p21. Furthermore, CaMKII stimulates cyclin D1 expression in breast cancer cells via nuclear factor kappaB [46]. Conversely, over-expression of the endogenous CaMKII inhibitory proteins hCaMKIINalpha and -beta in human colon adenocarcinoma [47] and ovarian cancer cells [48], respectively, stabilizes p27 or up-regulates p21 and down-regulates cyclin A, cyclin D1, cyclin E, and Cdk2. Thus, by up-regulating cyclin D1 and down-regulating p21 and p27, CaMKII activity leads to disinhibition of the cyclin-Cdk4 and cyclin-Cdk2 complexes, and thus promotes G_1 /S transition and S- G_2 /M cell cycle progression. Later on in cell cycle, CaMKII directly activates cdc25c triggering G_2 /M cell cycle transition [49, 50], is required for centrosome duplication [51], and regulates the microtubule depolymerase MCAK, which is essential for maintaining spindle bipolarity during mitosis [52]. Finally, CaMKII

regulates M phase exit [53].

In most primary CML cells and CML cell lines, cell proliferation specifically depends on activation of CaMKIIgamma that facilitates mitogen-activated protein (MAP) kinase, Janus-activated kinase/signal transducers and activators of transcription (Jak/Stat), as well as glycogen synthase kinase (GSK3beta)/beta-catenin pathways. CaMKIIgamma directly phosphorylates Stat3 and enhances its transcriptional activity. Accordingly, CML cells, induced to terminally differentiate or undergo growth arrest, display a marked reduction in the CaMKIIgamma autophosphorylation [6].

In addition to its cell cycle-promoting functions, CaMKII may also suppress cell cycle progression by inhibition of cdc25c [54] or by stabilization of p53 via CaMKII-dependent phosphorylation of Pirh2. The latter impairs the ubiquitination of p53 by Pirh2 in the G_1 phase of the cell cycle [55]. Furthermore, CaMKII has been demonstrated to impair nuclear translocation of nuclear factor of activated T cells (NFAT) by directly phosphorylating calcineurin [56], and to induce a G_2 /M cell cycle arrest when transiently expressed in a constitutively active form [57]. Moreover, CaMKII may inhibit DNA replication in the S phase of the cell cycle by phosphorylation of the PCNA binding domain of the large subunit of replication factor C [58]. Thus, CaMKII isoforms appear to interfere with cell cycle control in a complex manner at multiple pathways.

The present study suggests an additional function of CaMKII in the management of the cell cycle during genotoxic stress. IR has been demonstrated to increase the activity of CaMKII in normal fibroblasts where it contributes to the regulation of the S phase checkpoint [59]. In the present study, IR was demonstrated to stimulate the nuclear abundance of phosphorylated (i.e. active) CaMKII. Experimental suppression of intracellular Ca^{2+} signals by the membrane-permeable Ca^{2+} chelator BAPTA or by extracellular EGTA in combination with Ca^{2+} permeabilization of the plasma membrane delayed the IR-stimulated accumulation of K562 cells in G_2 phase. A similar, albeit less pronounced effect on G_2 accumulation was observed when CaMKII was inhibited by KN93, suggesting that the IR-induced Ca^{2+} signals confer their effect on cell cycle at least in part through activation of CaMKII. Notably, neither BAPTA nor KN93 affected cell cycle progression on non-irradiated control cells, indicating irradiation-specificity of the Ca^{2+} and CaMKII response. The additional effect of EGTA/ionomycin on the non-irradiated control cells might be explained by the complete abolishment of Ca^{2+} entry

and a (compared to BAPTA) stronger and complete depletion of cytosolic Ca^{2+} . Finally, KN93 decreased the clonogenic survival of the irradiated cells while having no effect on control cells, suggesting that control of the cell cycle by CaMKII fosters survival of the irradiated cells.

In summary, the present study demonstrates that IR-stimulated Ca^{2+} signaling contributes to G_1 -S progression and subsequent accumulation in the G_2 phase of the cell cycle in K562 leukemia cells. This Ca^{2+} signaling mechanism includes Ca^{2+} entry most probably through TRPV5/6 channels and activation of nuclear CaMKII.

Moreover, the present study suggests that genotoxic stress-induced activation of CaMKII contributes to the clonogenic survival of leukemia cells.

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