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Activation of deoxycytidine kinase by gamma-irradiation and inactivation by hyperosmotic shock in human lymphocytes

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Abstract

Deoxycytidine kinase (dCK) is a key enzyme in the intracellular metabolism of deoxynucleosides and their analogues, phosphorylating a wide range of drugs used in the chemotherapy of leukaemia and solid tumours. Previously, we found that activity of dCK can be enhanced by incubating primary cultures of lymphocytes with substrate analogues of the enzyme, as well as with various genotoxic agents. Here we present evidence that exposure of human lymphocytes to 0.5–2 Gy dosage of γ-radiation as well as incubation of cells with calyculin A, a potent inhibitor of protein phosphatase 1 and 2A, both elevate dCK activity without changing the level of dCK protein. When cells were gamma-irradiated in the presence of calyculin A, a more pronounced activation of dCK was observed. In contrast, both basal and stimulated dCK activities were reduced by hyperosmotic treatment of the cells. DNA repair determined by the Comet assay and by thymidine incorporation was induced by irradiation. Complete repair of γ-irradiated DNA was detected within 1 hr following the irradiation along with dCK activation, but the rate of repair was not accelerated by calyculin A. These data provide evidence for the activation of dCK upon DNA damage and repair that seems to be mediated by phosphorylation of the enzyme, suggesting the role of dCK in DNA repair processes.

Keywords: Deoxycytidine kinase; Nucleotide metabolism; γ-Irradiation; DNA repair; Enzyme activation; Calyculin A

1. Introduction

DNA replication and repair require a continuous supply of deoxynucleotide precursors provided either by the ''*de novo*'' or the ''salvage'' pathways. Some cell types, such as resting lymphocytes do not have any significant ''*de novo*'' synthesis, and precursors of DNA repair are formed via the ''salvage'' pathway. dCK (EC 2.7.1.74) is a key enzyme in this process, phosphorylating not only deoxycytidine but also purine deoxynucleosides, deoxyadenosine and deoxyguanosine [1], as a consequence of its broad substrate specificity. As phosphorylated deoxycytidine can be converted into thymine nucleotides, which is an important pathway in human lymphocytes [2], dCK is capable of supplying all four deoxynucleotide precursors for DNA repair during the resting state. dCK activity does not depend significantly on the cell cycle, in contrast to thymidine kinase 1 (TK1), which is characteristically an S-phase enzyme [3], suggesting that dCK has a specific importance in supplying DNA repair in resting lymphocytes. Moreover, this enzyme is responsible for the intracellular activation of many deoxyribonucleoside analogues, such as cladribine [4] (CdA) or gemcitabine [5] (dFdC), used in cancer and antiviral therapy [6].

Enhancement of dCK activity was previously reported as a result of intracellular metabolization of deoxynucleoside

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Abbreviations: dCK, deoxycytidine kinase; TK, thymidine kinase; ^{3}HdT, 2′-deoxy-[5-methyl-^{3}H]-thymidine; ^{3}HdC, 2′-deoxy-[5-^{3}H]-cytidine; dC, deoxycytidine; dT, deoxythymidine; PPase, protein phosphatase; CdA, 2-chlorodeoxyadenosine; dFdC, 2′,2′-difluoro-deoxycytidine; araC, arabinosylcytosine; DEAE, diethylaminoethyl; PBS, phosphate buffered saline; recdCK, recombinant dCK; LN, liponucleotide.

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analogues, such as cladribine [7], gemcitabine [8] and other purine and pyrimidine analogues [9]. Direct inhibition of DNA synthesis by aphidicolin [10] and by other genotoxic agents, such as etoposide [11] was also shown to elevate dCK activity. As dCK-activating agents encompass a relatively wide range, one could hypothesize that any disturbance of DNA synthesis and/or DNA damage might initiate significant dCK activation. To test this hypothesis, human resting lymphocytes were γ-irradiated and the effect on dCK activity was investigated. The results presented here showed that γ-irradiation of lymphocytes has a similar or even higher stimulation effect on dCK activity than the chemical agents investigated previously. dCK activity could be further potentiated by calyculin A, a known inhibitor of protein phosphatases 1 and 2A [12], suggesting that the mechanism of dCK activation involves a protein phosphorylation step. In addition, hyperosmotic stress considered to interfere with cellular phosphorylation signalling [20–22], profoundly reduced the extent of dCK activation elicited by either calyculin A or gamma-irradiation.

2. Materials and methods

2.1. Chemicals

^{3}HdT (925 GBq/mmol, 37 MBq/mL), ^{3}HdC (555 GBq/mmol, 37 MBq/mL), Hybond ECL nitrocellulose membranes and the Western blotting detection system were purchased from Amersham Life Sciences. Non-labelled nucleosides, okadaic acid, phenylmethylsulphonyl fluoride, dithiothreitol, Nonidet P-40, glycerol, Ponceau S and sorbitol were obtained from Sigma Chemical Co, calyculin A from Calbiochem, Kieselgel 60 F_{254} 25 DC sheets from Merck, Eagle's MEM from the National Institute of Public Health (Hungary). All other chemicals were of analytical grade and produced by Reanal (Hungary).

2.2. γ-Irradiation of human lymphocytes

Human lymphocytes were isolated from surgically removed tonsils of 3- to 6-year-old children [2] and irradiated with 0.5–4 Gy—as indicated—at room temperature in Eppendorf tubes with a ^{60}Co irradiation source (Siemens Gammatron-3; airdose rate was 0.4882 Gy/min; irradiation distance: 52.5 cm). The absorbed dose was adjusted by changing the irradiation time.

2.3. Alkaline single cell gel electrophoresis (Comet assay)

The Comet assay was performed as described by Singh *et al.* [13]. Briefly, 5×10^4 cells were resuspended in 85 μL low-melting point agarose (LMA, Gibco BRL), 0.5% in PBS, transferred to microscope slides pre-coated with 1% normal melting point agarose and covered with 85 μL LMA dropwise. Slides were immersed for 1 hr at 4° in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, pH 10, 1% Triton X-100 and 10% dimethylsulphoxide), laid into a horizontal electrophoresis apparatus filled with a highly alkalic running buffer containing 1 mM EDTA, 300 mM NaOH and were run for 20 min (1 V/cm, 300 mA). Electrophoretic separation of the damaged DNA was followed by a neutralization step (400 mM Tris–HCl, pH 7.5), and the specimens were stained with ethidium bromide (20 μg/mL), covered with coverslips and examined at 500× magnification through a fluorescent microscope. Twenty-five cells were randomly selected from each gel slab; comet "head" diameter and "tail" length were measured by means of a graduated grid, and the average of the tail/comet quotients was calculated, employing the Comet Single Cell Gel Electrophoresis Analysis Software Package (Kinetic Imaging Ltd.).

2.4. Metabolic labelling

Irradiated and control cell suspensions (5×10^6 cells in 500 mL Eagle's MEM) were pulse-labelled either with ^{3}HdC or with ^{3}HdT (1.0 μCi/mL; specific activities: 500–1000 cpm/pmol) for 30 min at 37°. Cells were washed three times with ice-cold PBS, followed by lysis with 70% ethanol at −20° overnight. Lysed cells were fractionated into ethanol soluble and insoluble parts by centrifugation. Labelling of the nucleotide pool (total uptake) and the liponucleotides was determined from the ethanol soluble supernatant [7,14]. Separation of phosphorylated nucleotides was based on their strong binding to DEAE-cellulose sheets. Phosphorylated ^{3}HdC partly participates in membrane phospholipid biosynthesis, forming liponucleotides such as dCDP-choline and dCDP-ethanolamine. The difference between the "total uptake" and the nucleotide fraction corresponded to the liponucleotide pool, which was also separated and determined directly by thin layer chromatography on Kieselgel silica sheets [7] and by high-performance liquid chromatography [14]. Incorporation into DNA was measured in the ethanol insoluble fraction after reprecipitation with 0.5 N perchloric acid and hydrolysis of nucleic acids at 90° for 1 hr, followed by a toluene-triton-based scintillation counting [2].

2.5. Preparation of cell extracts and assay of dCK and TK enzyme activities

Control and irradiated cell cultures containing 5×10^6 cells were extracted in 100 μL lysis buffer composed of 50 mM Tris–HCl, pH 7.6, 2 mM dithiothreitol, 0.5 mM phenylmethylsulphonyl fluoride, 20%, (v/v) glycerol and 0.5% Nonidet P-40 non-ionic detergent, by three consecutive freeze–thaw cycles (liquid nitrogen–ice). After centrifugation for 30 min at 14,000 *g* at 4°, the supernatants were applied for the determination of dCK and TK isoenzyme activities, using ^{3}HdC or ^{3}HdT as substrates (both 10 μM;

specific activities: 500–1000 cpm/pmol), respectively, in a kinase assay buffer containing 50 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM ATP, 2 mM dithiothreitol, 10 mM NaF and as much cell extract as to ensure the reaction to proceed in the linear kinetic range (5 μL). To stop the reaction, aliquots were spotted onto DEAE-cellulose sheets, which were subsequently washed, dried, eluted and radioactivity was counted as described [15]. Enzyme activity was expressed as pmol phosphorylated nucleoside pro 10^6 cells pro 1 hr. In order to determine the contribution of TK2 to overall ^{3}HdT phosphorylation, excess concentration of non-labelled dC (1 mM) was included in the TK reaction mixture [16]. The difference in ^{3}HdT phosphorylation capacity in the presence and in the absence of 1 mM dC is equal to the TK2 activity, corresponding to 2–3% of the total TK activity.

2.6. Immunoassay of dCK

Cell extracts were prepared as described for the enzyme assays. Five μL extracts (approximately 25 μg total protein) were resolved on 12% SDS–polyacrylamide gels, transferred to nitrocellulose membranes and stained with Ponceau S to check equal loading. Membranes were blocked overnight in a buffer containing 10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20 and 5% milk, subsequently washed and probed with a highly specific antibody raised against the C-terminal domain of the dCK protein [17] at 1:5000 dilution, followed by incubation with horseradish peroxidase conjugated anti-rabbit IgG (1:40,000). Immunocomplexes were visualized by the enhanced chemiluminescence reaction, according to the manufacturer's instructions. The bacterial expression plasmid pQEhdCK encoding the human dCK cDNA was kindly provided by Prof. S. Eriksson (the BMC, Uppsala, Sweden). 6× His-tagged recombinant dCK protein was produced and purified as described [18].

3. Results

3.1. Changes in the deoxynucleoside salvage pathways as a consequence of γ-irradiation

Primary cultures of human tonsillar lymphocytes were irradiated with gradually increasing doses, followed by pulse-labelling at 37° for 30 min in the presence of either ^{3}HdT or ^{3}HdC (see Fig. 1). After labelling, cells were precipitated with cold ethanol and radioactivity of intracellular metabolites was determined in each sample. In Fig. 1A, radioactivity found in the acid precipitable material (DNA) is indicated as the function of irradiation dose. As expected, increasing doses of irradiation induced a higher rate of incorporation of the deoxynucleotides into the DNA fraction, showing an enhanced rate of repair processes. Incorporation of exogenous deoxycytidine reached its maximum at 1 Gy dose of irradiation (Fig. 1A, ^{3}HdC), while thymidine incorporation slightly increased even at higher doses (Fig. 1A, ^{3}HdT). Fig. 1B displays the distribution of labelled intracellular metabolites among the different pools. The measure of dCK activity is the "total uptake", i.e. the total amount of deoxycytidine phosphorylated and either incorporated into the DNA or remained in the ethanol soluble pools as nucleotides (Nucl.) and liponucleotides (LN). The "total uptake" of deoxycytidine culminated at 1 Gy of irradiation, similarly to the profile of DNA labelling. It should be noted, however, that a significant portion—approximately 70%—of labelled deoxycytidine taken up by the lymphocytes is converted into thymine nucleotides resulting in loss of the radioactivity from ^{3}HdC [14]. The *ex vivo* activity of dCK is therefore higher than the sum of the radioactivity measured in the different cell fractions. Deoxynucleotide pool (Fig. 1B, ^{3}HdC Nucl.) labelling increased only marginally upon irradiation, while the production of liponucleotides (Fig. 1B, ^{3}HdC LN) peaked at 1 Gy dose. Only

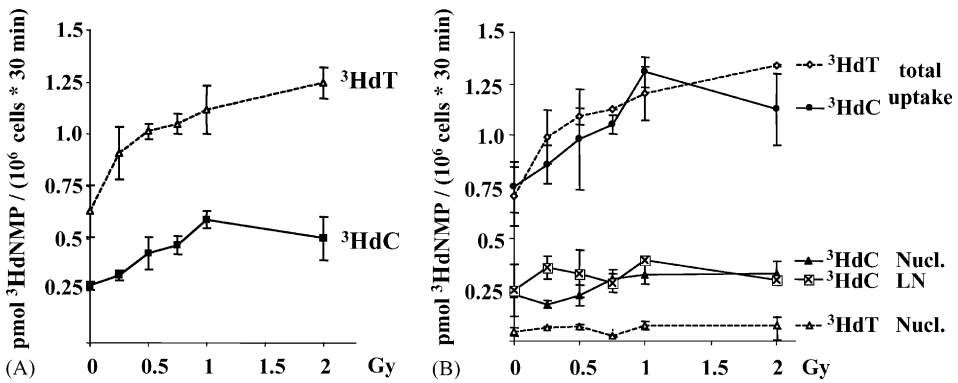

Fig. 1. Changes in deoxyribonucleotide salvage pathways as consequences of γ-irradiation in lymphocytes. Parallel cultures of tonsillar lymphocytes were exposed to different doses of γ-irradiation (0–2 Gy), then cells were pulse-labelled either with ^{3}HdT or with ^{3}HdC for 30 min at 37°. Radiolabelling of intracellular metabolites was measured after ethanol fractionation of cells. (A) Radioactivity incorporated into the insoluble macromolecular fraction (DNA). (B) Labelling of nucleotides ("Nucl."), liponucleotides ("LN"), and the total radioactivity taken up by the cells (the sum of labelling: Nucl. + LN + DNA; "total uptake"), respectively. Data are expressed in pmol of [^{3}H] nucleosides incorporated into the indicated fractions of 10^6 cells in 30 min.

about one-third of the labelled ^{3}HdC was found in the DNA fraction. On the contrary, the vast majority of ^{3}HdT was incorporated into DNA (about 95%; compare ^{3}HdT total uptake with ^{3}HdT in DNA in Fig. 1B and A, respectively), and only a small fraction remained in the ethanol soluble pools (Fig. 1B, ^{3}HdT Nucl.). These data show an overall elevation in the cellular uptake of deoxycytidine and thymidine due to the increased repair of DNA, as a consequence of irradiation.

3.2. Elevation of deoxycytidine kinase activity by gamma-irradiation and by calyculin A treatment in lymphocytes

Assay of the main pyrimidine salvage enzymes (dCK and TK isoenzymes) was performed in the crude extracts of control and irradiated cells (Fig. 2). Dose-dependent potentiation of the *in vitro* dCK activity followed a similar profile as it was shown in the *ex vivo* assay as the "total uptake" (Figs. 1B and 2A), however, the increase of *in vitro* dCK activity was significantly higher (about 3–4-fold). The irradiation of cells did not result in an immediate increase of dCK activity, a 15–30-min post-irradiation incubation at 37° was necessary to achieve the elevated activity (see Fig. 2B), while there was no change in dCK activity of control (non-irradiated) cells (Fig. 2B, $dCK_{control}$). In contrast to the significant activation of dCK, no differences were found in total TK enzyme activities between the control (Fig. 2, $TK_{control}$) and the irradiated (Fig. 2, TK_{γ}) cells. TK2 enzyme activity was selectively determined and found to be unchanged upon irradiation.

All samples whose deoxynucleoside kinase activities had been measured were also immunostained by a specific dCK antibody (Fig. 2C and D). Bands 5–10 in Fig. 2C and bands 1–12 in Fig. 2D correspond to the samples shown in Fig. 2A and B, respectively. Recombinant dCK was used as a positive control, having slightly slower mobility because of its N-terminal 6× His-tag [18]. In Fig. 2C, a clear-cut uniformity in the intensity of the bands from lanes 5 to 10 can be observed, showing no change in dCK protein content, despite the up to 4-fold increase in enzyme activity

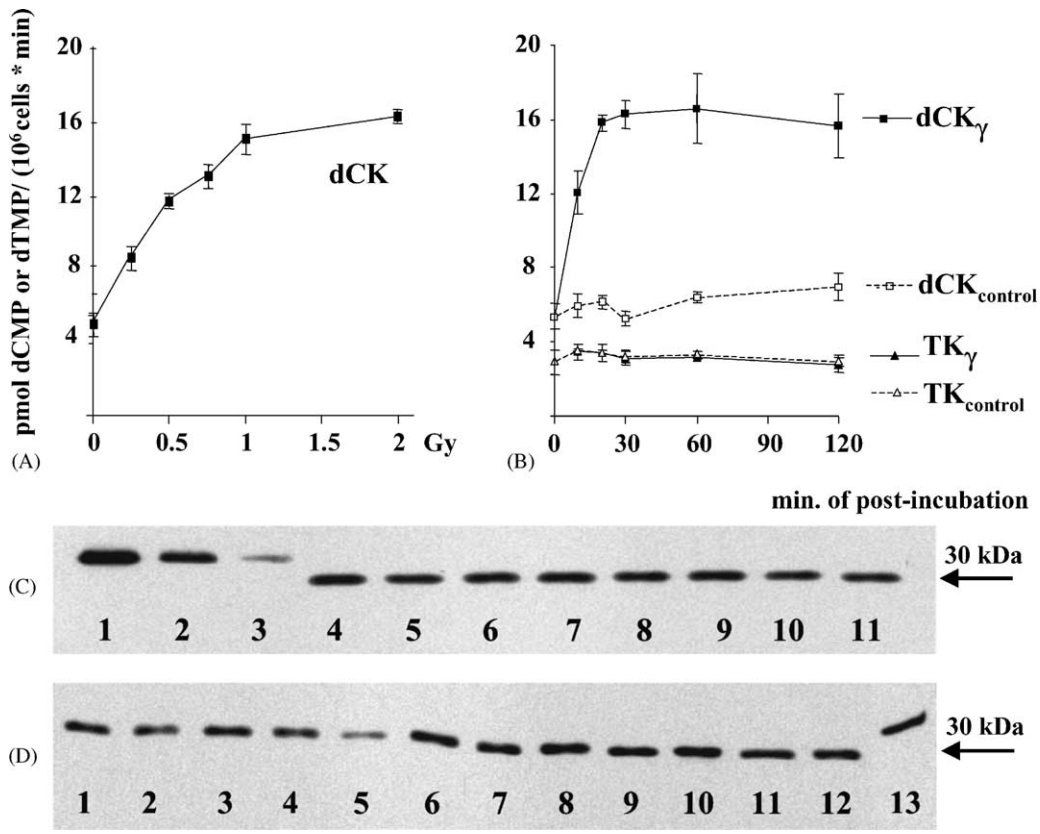

Fig. 2. Effect of γ-irradiation on deoxycytidine kinase and thymidine kinase activities. Human tonsillar lymphocytes were either exposed to increasing doses of γ-irradiation or used as control without being irradiated, followed by a post-incubation for different time periods at 37°. Crude cell extracts of control and irradiated cells were used for assay of deoxynucleoside kinase activities (A and B), as well as for determination of the amount of dCK protein by immunoassay (C and D). (A) dCK enzyme activities after different doses of γ-irradiation and a post-incubation period of 50 min. (B) Deoxynucleoside kinase (dCK and TK) enzyme activities, measured in crude extracts of control ($dCK_{control}$ and $TK_{control}$) and γ-irradiated (1 Gy) cells (dCK_{γ} and TK_{γ}), as the function of post-incubation time at 37°. (C) Immunostaining of dCK samples from the experiment shown in panel A. Labelling is as follows: 1: 20 ng recdCK, 2: 10 ng recdCK, 3: 5 ng recdCK; 4: control (0 Gy, no post-incubation); 5: control (0 Gy, 50-min post-incubation); 6: 0.25 Gy; 7: 0.5 Gy; 8: 0.75 Gy; 9: 1.0 Gy; 10: 2.0 Gy; (7–11: 50-min post-incubation); 11: treated with 3 μM 2-chloro-2′-deoxyadenosine for 50 min. (D) Immunostaining of dCK samples from the experiment shown in panel B. 1: control, 0 min; 2: 1.0 Gy, 0 min; 3: control, 10 min; 4: 1.0 Gy, 10 min; 5: control, 20 min; 6: 1.0 Gy, 20 min; 7: control, 30 min; 8: 1.0 Gy, 30 min; 9: control, 60 min; 10: 1.0 Gy, 60 min; 11: control, 120 min; 12: 1.0 Gy, 120 min; 13: 10 ng recdCK.

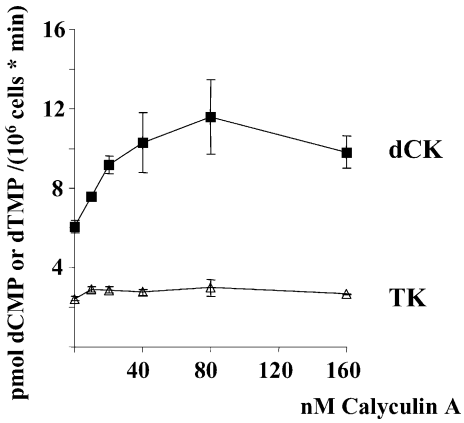

Fig. 3. Effect of calyculin A treatment on dCK and TK activities in lymphocytes. Human lymphocytes were incubated with the indicated concentrations of calyculin A at 37° for 60 min. Deoxycytidine kinase (dCK) and total thymidine kinase (TK) activities were measured in the crude extracts of treated cells as described in Section 2.5.

(Fig. 2A). Similar results are presented in panel D, where the samples from 2 to 13 are arranged so that control and treated specimens are in adjacent pairs for better comparison. The results show no tendentious alteration, except some inhomogeneity due to occasional differences in the blotting/staining procedure. Note that there is no elevation in the dCK protein level even upon treatment with its potent inducer 2-chlorodeoxyadenosine (Fig. 2C, lane 11), in accordance with our previous results [7], where secondary modification of the enzyme was suggested.

In order to clarify this issue, cell cultures were incubated with different concentrations of calyculin A, a protein phosphatase inhibitor, for 60 min at 37° and dCK and dTK activities were subsequently measured *in vitro* (Fig. 3). As shown in the figure, calyculin A enhanced dCK activity up to 200% of the untreated control in a dose-dependent manner, while it had no effects on TK activities. Okadaic acid, an other protein phosphatase inhibitor more specific for PP2A [19] was also tested under the same experimental conditions. In contrast to calyculin A, okadaic acid only slightly influenced dCK activity; the maximal stimulatory effect was 10% at 15 nM concentration, measured after a 60-min incubation (data not shown).

3.3. Comet assay in gamma-irradiated cells

We made attempts to prove whether the applied dosage of γ-irradiation induces DNA double strand breaks, visualized by the Comet assay, assuming that the extent of fragmentation is directly proportional to the length of the comet tail [13]. DNA fragmentation was quantitated by the ratio of the tail length/head diameter. This parameter was below 4 in non-irradiated cells and increased to 26 following the administration of 2 Gy irradiation, indicating a dramatic increase in DNA fragmentation (Table 1, γ-irradiation, 0 min). DNA strand breaks were completely repaired in 60 min after irradiation (the ratio gradually decreased from 26.26 to 3.75; see Table 1). Activity of dCK had a parallel increase during this short incubation period, suggesting that the signal for enzyme activation might originate from the damaged DNA (Fig. 2B). However, the DNA repair process could not be further stimulated in the presence of 80 nM calyculin A, arguing that enhanced dCK activity and a consequently better dNTP supply itself is not sufficient to drive higher repair rates. On the other hand, calyculin A might affect the repair apparatus more directly by interfering with multiple phosphorylation events—therefore data obtained in this experiment need further investigation.

Table 1
DNA repair as followed by the Comet assay

Treatment	Time (min)			
	0	15	30	60
Control	3.98	n.d.	n.d.	3.83
Calyculin A	3.51	n.d.	n.d.	3.06
γ-Irradiation	26.26	21.59	11.87	3.75
γ-Irradiation + calyculin A	15.60	10.73	9.88	5.60

Lymphocytes were incubated for 60 min at 37° in the presence or absence of 80 nM calyculin A (control and Cal A; upper part of the table) or irradiated with 1 Gy dose and subsequently incubated for 15, 30 and 60 min with or without 80 nM calyculin A (lower panel; γ-irradiation and γ-irradiation + Cal A, respectively). Average values of the tail length/head diameter quotients are displayed as described in Section 2.3; n.d.—no data.

3.4. Opposite effects of gamma-irradiation and hyperosmotic stress on dCK activity

To gain an insight into the mechanism of dCK activation/inactivation process, a series of dual treatments were applied and *in vitro* dCK and TK activities have been measured (Fig. 4, filled and blank columns, respectively). Samples 1–4 demonstrate the combined effect of calyculin A and 0.5 Gy of γ-irradiation, representing the additive effect of the stimulating agents.

Hyperosmotic stress has been shown to induce extensive changes in intracellular phosphorylation reactions, including the inactivation of S6 kinase (S6K) [20] and protein kinase B alpha [21], and potentiation of the MAPK-osmosensing pathways, for instance p38 and the Erk kinases [22]. Having obtained some hints pointing to the potential involvement of phosphorylation in the regulation of dCK activity, the effect of hyperosmolarity on dCK activity was investigated. The second series of samples (5–8) clearly illustrate the decrease in dCK activity as a consequence of hyperosmotic shock upon complementation with 400 mM sorbitol. Gamma-irradiation resulted in a 2–3-fold elevation in dCK activity either in the absence (sample 1 vs. sample 2), or in the presence (sample 5 vs. sample 6) of hyperosmotic shock except that the absolute values were lower after hyperosmotic shock. Similar type of interference was observed when the effect of calyculin A without (columns 1 vs. 3 and 4) or with 400 mM sorbitol (hyperosmotic

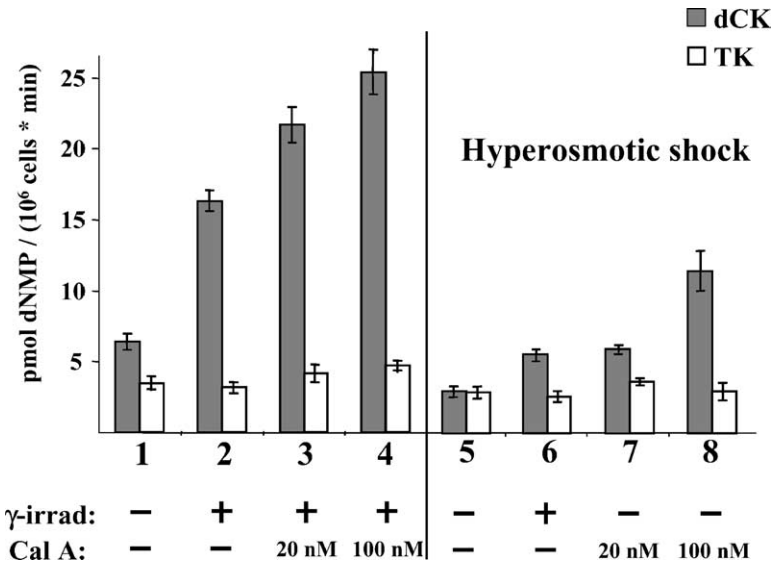

Fig. 4. Effects of γ-irradiation, hyperosmotic shock and calyculin A treatments on deoxynucleoside kinase activities. Parallel cultures of lymphocytes were pre-incubated with (+) or without (−) calyculin A (Cal A) for 10 min, then exposed (+) or not (−) to 1 Gy γ-irradiation (γ-irrad). Post-incubation was carried out at 37° for 40 min in the presence (1–4) or absence (5–8) of 400 mM sorbitol (hyperosmotic shock), followed by preparation of crude cell extracts and assay of dCK and TK enzyme activities, as described in Section 2.5.

shock; samples 5 vs. 7, 8) was investigated. The results of these combined treatments suggest independent and additional effects of the osmotic shock (inactivator), γ-irradiation (activator) and calyculin A (activator) on cells regarding the modulation of their dCK activities.

4. Discussion

Deoxynucleoside salvage pathways of human lymphocytes challenged with low doses (0.2–2 Gy) of ionizing radiation were investigated shortly (30–60 min) after the irradiation event. A dose-dependent increase in the uptake and incorporation of deoxycytidine and thymidine was revealed (Fig. 1). This was consistent with the dose-dependent increase in the amount of DNA strand breaks being almost completely repaired during the post-incubation period (Table 1). However, major differences were found in the metabolism of exogenous deoxycytidine and thymidine (see Fig. 1): while most of the radioactive thymine nucleotides were incorporated into the DNA fraction, thus less than 10% remained in the ethanol soluble nucleotides, only about half of the radioactivity derived from ^{3}HdC was used for DNA synthesis and the other half remained in the nucleotide and liponucleotide pools. These findings are in accordance with our previous data concerning human lymphocytes, and could be explained by the compartmentalization of dCTP pools [23], one for replicative DNA synthesis and the other one for DNA repair [24]. As about 90% of lymph node lymphocytes are in resting phase, as measured previously by flow cytometry [25], these cells require deoxynucleotides mainly for DNA repair. About one-third of the total dC uptake was used for liponucleotide synthesis (Fig. 1B, LN), previously identified as mainly dCDP-choline and dCDP-ethanolamine by HPLC [23,26] and this ratio was not influenced by irradiation under these conditions (Fig. 1B). While the total amount of both dC and dT phosphorylation showed a parallel increase with the irradiation dose, only dCK activity was found to be augmented in the cells (Fig. 2). Nevertheless, Kreder *et al*. have observed potentiation of the mitochondrial TK2 isoenzyme together with TK1 and dCK upon pulsed low-dose irradiation in SW-1573 cells, 6 hr after treatment [27]. TK2 induction might also have occurred under our experimental conditions, providing a plausible explanation for the dose-dependent enhancement in the utilisation of extracellular thymidine—in spite of constant TK levels. This possibility, however, was ruled out by selective determination of TK2 activity, showing no changes upon irradiation. It should be noted that TK2 was responsible for less than 2–3% of the overall thymidine phosphorylation capacity in tonsillar lymphocytes. The elevated thymidine incorporation after irradiation is due to the increased DNA repair as tested in the Comet assay (Table 1).

Boothman *et al*. performed a study on the radiation-induced activation of thymidine kinase [28]. They observed a dose-dependent transcriptional activation and enhanced levels of mRNA, protein and enzyme activity, which was more modest in normal fibroblasts than in their transformed counterparts. However, according to our data, low-dose irradiation does not affect TK activity at all in normal lymphnode lymphocytes. In their recent paper, Van Den Neste *et al*. proved that UV-C irradiation also elicits activation of DNA repair processes as measured by increased thymidine incorporation [29].

We have also shown that stimulation of dCK activity needs at least a 15–30-min post-incubation period of cell

cultures after irradiation (Fig. 2B), suggesting that irradiation is not the direct cause of dCK stimulation, but there seems to be several steps between DNA damage and the potentiation of dCK activity. This is consistent with our previous data concerning activation of dCK by nucleoside analogues and by aphidicolin, where the maximal activity of dCK was also achieved after a 1–2-hr incubation period [7,9,10].

Regarding the mechanism of activation of dCK as a consequence of γ-irradiation, Wei *et al*. found a 5-fold increase in the protein level and a 2.5-fold increase in the activity of dCK in cells upon administration of 7.5 Gy of irradiation dosage and up to 24 hr post-irradiational incubation time in the HT 29 colon cancer cell line [30]. They also demonstrated the activation of pyrimidine rather than purine salvage routes. Our results, however, suggest a different mechanism of dCK activation in normal quiescent lymphocytes, as no changes in the dCK protein level was found under our conditions (lower dose and shorter incubation; see Fig. 2C and D). In order to reconcile this apparent contradiction, we presume a two-step mechanism for the radiation-responsive induction of dCK activity. Our survey covered a short-term (1–2 hr) period; early dCK activation should be ascribed to post-translational modifications rather than changes in the protein content. Long-term follow-up (3–6 hr) indicated an adaptive transcriptional activation and subsequent elevation in dCK protein levels. Moreover, genetic rearrangements in transformed cells frequently influence the activity of signalling networks, potentially modulating dCK activity, and these alterations can account for the different extent of activation observed in normal vs. malignant cells.

The various mechanisms of dCK stimulation in normal and in malignant cells might be a striking difference between these cell types, and further comparative investigation might help to discover selective cytostatic agents.

The fact that the early event in dCK activation in normal lymphocytes is an increase in enzyme activity without any increase in the dCK protein level was also shown earlier, when the elevation of dCK activity was provoked by treating cells with deoxynucleoside analogues and aphidicolin [10]. Ionizing radiation, ultraviolet light and DNA-damaging drugs such as cisplatin and araC have been reported to induce the p21-activated protein kinase (γ-PAK) [31], suggesting that various DNA-attacking agents trigger the activation of this kinase through the sensor function of the p53 tumour suppressor gene. DNA-dependent protein kinase and ataxia telangiectasia mutated (ATM) kinase are also known to play central roles in the early response to agents inducing DNA strand breaks. As dCK is indispensable for the dNTP-supply for both homologous recombination and non-homologous end-joining mediated DNA double-strand breakage repair in lymphocytes [24], we speculate that its activation might be directly or indirectly connected to the action of these early stress kinases.

Regarding the mechanism of the post-translational activation of the dCK protein, a phophorylation process was suggested, based on evidence for *in vitro* phosphorylation and activation of dCK by protein kinase Cα [32]. Recombinant dCK, however, was efficiently phosphorylated by protein kinase A rather than by protein kinase C in an *in vitro* system, but phosphorylation did not alter the kinetic properties of the salvage kinase [33]. Recently, recombinant λ protein phosphatase treatment of dCK partially purified from human lymphocytes was shown to inactivate the enzyme, providing indirect evidence for the role of phosphorylation in dCK activation in an *in vitro* system [10].

Here we present new data suggesting the importance of reversible protein phosphorylation in dCK activation, using calyculin A, an equipotent protein phosphatase 1 and 2A inhibitor in human lymphocytes [12]. Our proposed model focusing on the transition between the active and inactive state of dCK is depicted in Fig. 5. Inactivation of the enzyme is accelerated by a calyculin A sensitive protein phosphatase. Notably, this phosphatase might not dephosphorylate dCK itself but could function at a more upstream level of a putative pathway by targeting either a kinase or an adaptor protein. On the other hand, the activation is stimulated by γ-irradiation. The calyculin A sensitive protein phosphatase 1 and 2A are serine/threonine-specific protein phosphatases, widely distributed in the cytoplasm of mammalian cells and have been reported to be involved in numerous signalling pathways [34]. Inhibition of protein phosphatase 2A induces serine/threonine phosphorylation, subcellular redistribution, and changes in the function of many key components of cell growth [35,36]. Xu *et al*. [24] demonstrated that salvaged deoxycytidine is preferentially utilised for DNA repair, suggesting a correlation between dCK activity, salvaged deoxycytidine pools and DNA repair. Combining these results, it can be concluded that these calyculin A sensitive protein phosphatases might play a remarkable role in the modulation of the salvage-dependent DNA repair system by affecting dCK activity.

In the present investigation, osmotic stress was shown to abrogate dCK activity. Osmotic stress has also been shown to inactivate the S6 kinase [20], as well as protein kinase Bα/Akt (PKBα) [21]. This inactivation was released by calyculin A, while other protein phosphatase inhibitors including okadaic acid had no effect [20]. In agreement with these, we were able to activate dCK by calyculin A, but other phosphatase inhibitors, such as okadaic acid—which is more specific for PP2A [19]—were much less effective (data not shown). This fact indicates the involvement of PP1 rather than PP2A in the activation process.

S6K and PKBα are members of the phosphatidylinositol-3-OH kinase signalling pathway, involved in the regulation of cell proliferation and growth [37]. They were also shown to take part in the cellular responses to stress and cell survival. Thus, both kinases were activated by low

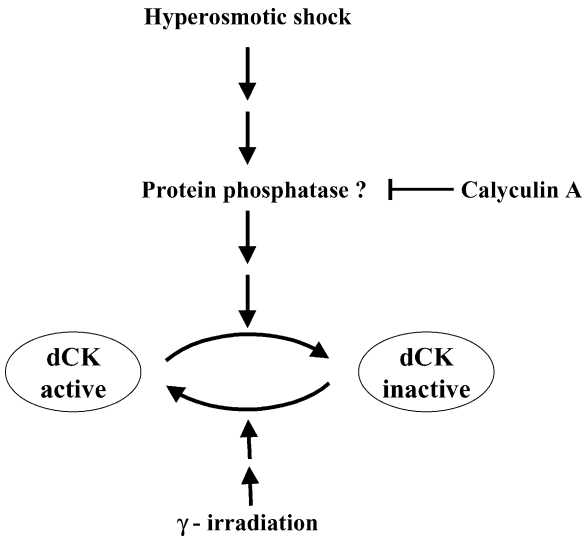

Fig. 5. A proposed model depicting regulatory effects influencing deoxycytidine kinase activity.

dose ionizing radiation in carcinoma cell lines [38], similar to our findings with dCK. Similarities in the regulation of these distinct kinases suggest that they are all involved in the same mechanism regarding cell survival.

Radiosensitivity of different cell types has been extensively investigated in a number of laboratories, as ionising radiation is a powerful tool to combat a cluster of human malignancies. Repair of DNA damage evoked by irradiation establishes a metabolic demand for enforced deoxynucleotide anabolism to supply DNA precursors. Changes in nucleotide metabolism after irradiation can be exploited in cancer therapy, especially when chemo- and radiotherapy are combined as previously suggested [28,39]. Higher dCK activity after exposure to ionizing radiation augments the effect of simultaneous chemotherapy with nucleoside analogues (CdA, dFdC, araC). This synergism provides the rationale for long-standing clinical therapeutic regimens implying that irradiation could render cancerous cells more sensitive to these drugs. However, calyculin A is probably not an eligible dCK sensitiser, as it has been reported to prevent apoptosis in γ-irradiated Burkitt's lymphoma cells [40]. This effect should be considered counter-productive since nucleoside analogues and irradiation kill malignant cells mainly via induction of apoptosis [29,41].

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