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Activation of deoxycytidine kinase by protein kinase inhibitors and okadaic acid in leukemic cells

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Abstract

Deoxycytidine kinase (dCK) is a key enzyme in the deoxynucleoside salvage pathway and in the activation of numerous nucleoside analogues used in cancer and antiviral chemotherapy. Recent studies indicate that dCK activity might be regulated through reversible phosphorylation. Here, we report the effects of a large panel of protein kinase inhibitors on dCK activity in the B-leukemia cell line EHEB, both in basal conditions and in the presence of the nucleoside analogue 2-chloro-2′-deoxyadenosine (CdA) which induces activation of dCK. Except staurosporine and H-7 that significantly reduced the activation of dCK by CdA, no specific protein kinase inhibitor diminished basal dCK activity or its activation by CdA. In contrast, genistein, a general protein tyrosine kinase inhibitor, and AG-490, an inhibitor of JAK2 and JAK3, increased basal dCK activity more than two-fold. Two specific inhibitors of the MAPK/ERK pathway, PD-98059 and U-0126, also enhanced dCK activity. These data suggest that the JAK/MAPK pathway could be involved in the regulation of dCK. Moreover, we show that the activity of dCK, raised by CdA, can return to its initial level by treatment with protein phosphatase-2A (PP2A). Accordingly, dCK activity in intact cells increased upon incubation with okadaic acid (OA) at concentrations that should inhibit PP2A, but not protein phosphatase-1. Activation of dCK by protein kinase inhibitors and OA was also observed in CCRF-CEM cells and in chronic lymphocytic leukemia B-lymphocytes, suggesting a general mechanism of post-translational regulation of dCK, which could be exploited to enhance the activation of antileukemic nucleoside analogues.

Keywords: B-CLL lymphocytes; 2-Chloro-2′-deoxyadenosine; Deoxycytidine kinase; Enzyme phosphorylation; Nucleoside analogue activation

1. Introduction

dCK (EC 2.7.1.74) catalyses the rate-limiting reaction in the salvage of deoxyribonucleosides, supplying cells with deoxyribonucleotides for DNA replicative and repair synthesis. The enzyme phosphorylates deoxycytidine, deoxyguanosine and deoxyadenosine, with ATP or UTP as phosphate donnor [1]. In addition to its natural substrates, dCK activates a number of clinically important antineoplastic nucleoside analogues, including CdA (cladribine) and F-Ara-A (9-β-D-arabinosyl-2-fluoroadenine), commonly used in the treatment of lymphoproliferative malignancies, particularly B-CLL [2,3]. Moreover, dCK phosphorylates the antiviral compounds 2′,3′-dideoxycytidine (zalcitabine) and 2′-deoxy-3′-thiacytidine (lamivudine) used for the treatment of HIV infection [4,5]. Expression of dCK is predominant in lymphoid cells [1], which explains the sensitivity of these cells to deoxyribonucleoside-induced cytotoxicity. Impaired dCK activity has been consistently shown to cause resistance to nucleoside analogues in various tumor cell lines [6–9]

Abbreviations: B-CLL, B-cell chronic lymphocytic leukemia; CdA, 2-chloro-2′-deoxyadenosine; dCK, deoxycytidine kinase; ERK, extracellular signal-regulated kinase; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; OA, okadaic acid; PI 3-kinase, phosphoinositide 3-kinase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PP1, protein phosphatase-1; PP2A, protein phosphatase-2A; λ-PP, protein phosphatase lambda; PTK, protein tyrosine kinase; VA, sodium orthovanadate

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and has also been correlated with clinical outcome [10–13], illustrating the key role of dCK in the action of these drugs. Studies of the mechanism(s) that control the activity of this enzyme are thus of particular interest.

In contrast to thymidine kinase 1, also a deoxynucleoside salvage enzyme, dCK is not cell-cycle regulated, although some variations in its activity have been observed during the progression of the cell cycle [14–16]. Physiologically, dCK activity could be down-regulated by dCTP. Indeed, the Ki value of dCTP for dCK is within the range of its intracellular concentration [17,18]. Interestingly, pharmacological reduction of the level of this regulator has been shown to enhance nucleoside analogue phosphorylation and cytotoxicity ([2] and references therein). Besides allosteric regulation by dCTP, compelling evidence exists that the activity of dCK might be regulated by post-translational modification(s) of the protein. Indeed, several agents, including nucleoside analogues such as CdA [19–21], inhibitors of DNA synthesis such as aphidicolin and etoposide [20,22], UV-C [23] and γ-irradiation [24] can produce a stable activation of dCK without any change in the dCK protein level. Moreover, it has been shown that dCK, partially purified from tonsillar lymphocytes [22] or overexpressed in HEK-293 cells [25], can be inactivated by treatment with λ-PP. These results strongly suggest that dCK activity can be regulated through reversible phosphorylation, a major mechanism for the regulation of many eucaryotic cellular processes.

The pathway leading to activation of dCK by phosphorylation is not known. Wang and Kucera [26] reported that PKCα can phosphorylate dCK purified from leukemic blasts and increase its activity. These results were, however, not reproduced with a human recombinant dCK [27]. To unravel the signal transduction pathway(s) involved in the regulation of dCK, we investigated the influence of general and selective pharmacological inhibitors of protein kinases in leukemic cells, either on basal dCK activity or on the increase of its activity induced by the nucleoside analogue CdA [19–21]. Preliminary results of this study have been presented at a symposium [28].

2. Materials and methods

2.1. Materials

CdA was synthesised and supplied by Prof. J. Marchand (Laboratory of Organic Chemistry, Université catholique de Louvain, Louvain-la-Neuve). [5-^{3}H]-deoxycytidine (24 Ci/mmol) was from Amersham International. Ficoll-plaque Plus (density: 1.077) was from Pharmacia Biotech. FCS and penicillin/streptomycin were purchased from BioWhittaker Europe. RPMI-1640 and all tissue culture reagents were from Gibco. Catalytic subunits of PP2A (specific activity: 10 U/mg protein), purified from beef heart according to Cohen et al. [29], was kindly provided by Prof. M. Rider (Hormone and Metabolism Research Unit, Université catholique de Louvain, Brussels, Belgium). U-0126 was obtained from Promega. AG-43, KN-62, H-89, Y-27632, daidzein, erbstatin analogue, rapamycin, calphostin C, bisindolylmaleimide I, PMA (phorbol-12-myristate-13-acetate), λ-PP, and protein tyrosine phosphatase IB were purchased from Calbiochem. Staurosporine, genistein, H-7, wortmannin, PDBu (phorbol-12,13-dibutyrate), dibutyryl-cAMP were purchased from Sigma Chemical Co. Herbimycin A, AG-490, PD-98059 and OA were from Biomol. Other chemicals, materials and reagents were from Acros, Merck, Sigma or Bio-Rad Laboratories.

2.2. Cell preparation and incubation

EHEB cells, a B-cell line established from B-CLL lymphocytes [30], and CCRF-CEM cells, a human T-acute lymphocytic leukemia cell line, were cultured in RPMI-1640 with Glutamax, supplemented with 10% heat-inactivated FCS at 37 °C in an atmosphere of 5% CO_2 in air. Both cell lines were routinely tested for *Mycoplasma* contamination. Before experiments, cells were counted and diluted to a concentration of 0.5×10^6 cells/ml. Freshly obtained peripheral blood from B-CLL patients was fractionated by Ficoll–Paque sedimentation. Mononuclear cells were washed with PBS and resuspended to a concentration of 5×10^6 cells/ml in RPMI-1640 supplemented with 10% FCS and 1% penicillin-streptomycin.

When used in combination with CdA, protein kinase effectors were added 30 min before the nucleoside analogue. Hydrophobic inhibitors were dissolved in DMSO. It was verified that DMSO, at a concentration of 0.5% (the maximal concentration used in the experiments) did not modify dCK activity.

2.3. Preparation of cell extracts and dCK assay

After incubation, cells were washed twice in cold PBS and resuspended in extraction buffer (buffer A) containing 50 mM Tris–HCl buffer, pH 7.6, 5 mM benzamidine, 0.5 mM *p*-toluenesulfonyl fluoride, 2 mM dithiothreitol, 20% glycerol, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, 1 mM β-glycerophosphate, 10 mM NaF, and freshly added 1 mM sodium orthovanadate (VA) to prevent an action of protein kinases and phosphatases during extraction. Cells were disrupted by three consecutive freeze-thawings. After centrifugation, the supernatants were used immediately, or frozen at −20 °C and stored for subsequent enzyme assays. It was verified that storage of extracts for a week at −20 °C did not modify enzyme activity compared to that of freshly prepared extracts.

Activity of dCK was measured as described by Arner et al. [31] with 10 μM [5-^{3}H]deoxycytidine (∼1000 cpm/pmol), in 100 μl of a reaction mixture containing 50 mM Tris–HC1, pH 7.6, 2 mM dithiothreitol, 5 mM $MgCl_2$,

5 mM ATP, 10 mM NaF and 0.05–0.07 mg of cellular protein. The enzyme assays were carried out at 37 °C and 10 μl aliquots were taken after several appropriate time intervals to yield a linear reaction rate. The protein content of cell extracts was measured by the method of Bradford [32], using bovine serum albumin as the standard.

2.4. Protein phosphatase treatment of cellular extracts

For dephosphorylation experiments, cell extraction was carried out in buffer A devoid of protein kinase and phosphatase inhibitors. For treatment with λ-PP, 120–150 μg of cell protein were incubated at 30 °C in the presence or absence of 1 mM VA with 333 units of λ-PP in a final volume of 100 μl, in a reaction mixture containing 50 mM Tris–HCl, pH 7.8, 2 mM $MnCl_2$ and 100 μg BSA, as described by Csapo et al. [22]. For treatment with PP2A purified from beef heart, 120–150 μg of cellular extract were incubated at 30 °C in the presence or in the absence of 1 μM OA with 8 units of PP2A in a final volume of 100 μl, in a reaction mixture containing 10 mM MOPS, pH 7, 0.5 mM EDTA, 0.1% 2-mercaptoethanol, and 50 mM $Mg(CH_3COO)_2$, as described by Deprez et al. [33]. For treatment with tyrosine phosphatase IB, 100 μg of cellular extract were incubated at 30 °C in the presence or in the absence of 1 mM VA with 0.15 unit of tyrosine phosphatase 1B in a final volume of 50 μl, in a reaction mixture containing 50 mM Hepes, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, as recommended by the manufacturer.

2.5. Statistical analysis

Differences in enzyme activity between cells incubated with or without protein kinase or phosphatase inhibitors were analysed for statistical significance by the two-tailed Student's *t*-test for paired samples, at a level of significance of $P = 0.05$. All means were calculated from at least three independent experiments.

3. Results

3.1. Effect of serine/threonine kinase inhibitors and activators on dCK activity

The effect of staurosporine and H-7, two broad-spectrum protein kinase inhibitors, was first investigated (Fig. 1). The inhibitors were added alone or 30 min before CdA, which induced a two- to three-fold increase of dCK activity in EHEB cells after 4 h of incubation, as described previously [19–21]. Both staurosporine (1 μM) and H-7 (100 μM) significantly reduced, by 48.1 ± 7.0% and 25.5 ± 5.3%, respectively, the increase of dCK activity provoked by CdA. No decrease was observed in basal conditions. Higher concentrations of staurosporine or H-7 were not more effective. Since staurosporine and H-7 are particularly effective against PKC, we studied the effects of calphostin C (up to 100 nM) and bisindolylmaleimide I (up to 5 μM), two more specific inhibitors of PKC, and of PMA (up to 1 μM) and PDBu (up to 20 nM), which activate PKC. None of these effectors modified dCK activity (data not shown), precluding a post-translational regulation of this enzyme by PKC in intact cells. Since staurosporine and H-7 might also inhibit PKA activity, we examined the effect of the highly specific PKA inhibitor, H-89 (up to 1 μM), and of the cell-permeable analogue of cAMP, dibutyryl cAMP (up to 100 μM). Both were without effect on dCK activity (not shown). We also tested other inhibitors of serine/threonine protein kinases susceptible to inhibition by staurosporine or H-7, namely rapamycin (100 nM), a specific inhibitor of ribosomal protein S6 kinase, KN-62 (5 μM), a specific inhibitor of Ca^{++}/calmodulin-dependent protein kinase II, Y-27635 (1 μM), an inhibitor of Rho-kinase, and finally, wortmannin (1 μM), an inhibitor of PI 3-kinase, but also of certain members of the PI 3-kinase superfamily of enzymes, such as the DNA-dependent protein kinase [34]. Although the concentrations used were shown to be efficient in intact cells, none of these inhibitors, tested in at least three separate experiments, did significantly modify basal or CdA-activated dCK (data not shown).

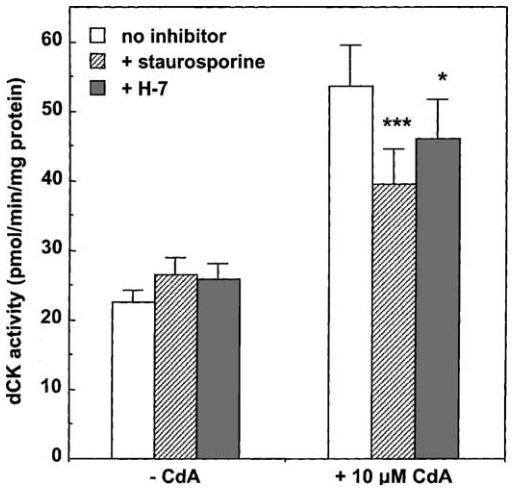

Fig. 1. Effect of staurosporine and H-7 on dCK activity. EHEB cells were pre-treated or not with 1 μM staurosporine or 100 μM H-7 for 30 min, and then incubated for 4 h in the absence or in the presence of 10 μM CdA. Results are means ± S.E.M. of five separate experiments. Significance relative to the absence of protein kinase inhibitors: $^{*}P < 0.05$; $^{***}P < 0.001$.

3.2. Effect of tyrosine kinase inhibitors on dCK activity

Because staurosporine can also inhibit PTK [35], we studied the effect of two widely used PTK inhibitors, genistein, a broad spectrum PTK inhibitor, and herbimycin A, more selective for cytosolic tyrosine kinases (Fig. 2). Genistein increased basal dCK activity, as also observed by Spasokoukotskaja et al. [36], and enhanced the activation of dCK by CdA. Unlike genistein, herbimycin A did not

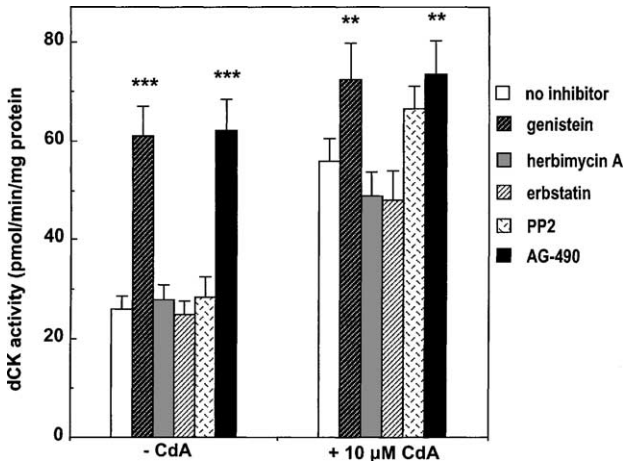

Fig. 2. Effect of tyrosine kinase inhibitors on dCK activity. EHEB cells were pre-treated or not for 30 min with various tyrosine kinase inhibitors (100 μM genistein, 1 μM herbimycin A, 100 μM erbstatin analogue, 10 μM PP2, 100 μM AG-490), and then incubated for 4 h in the absence or in the presence of 10 μM CdA. Results are means ± S.E.M. of six (control, genistein, herbimycin A and AG-490), four (erbstatin analogue) or three (PP2) separate experiments. Significance relative to the absence of protein kinase inhibitors: $^{**}P < 0.01$; $^{***}P < 0.001$.

influence dCK activity. To investigate the role of PTKs further, we used more selective tyrosine kinase inhibitors (Fig. 2). Erbstatin analogue, an inhibitor of EGFR-associated tyrosine kinase, and PP2, a potent and specific inhibitor of members of the Src tyrosine kinase family, did not change dCK activity. Nevertheless, AG-490, a tyrphostin which blocks JAK2 and JAK3, but not other cytosolic tyrosine kinases such as Lck, Lyn, Btk, Syk, Src, JAK1, or Tyk2 [37,38], increased the basal activity of dCK more than two-fold and also slightly enhanced the activation of dCK induced by CdA. To verify that the effects of genistein and AG-490 on dCK activity were actually related to the PTK inhibitory properties of these compounds, we investigated the effect of inactive analogues. AG-43, a negative control for AG-compounds [39], did not influence dCK activity (not shown). However, daidzein, an inactive analogue of genistein [40], produced an activation of dCK, lower than genistein, but significant. Dose- and time-effects of genistein, daidzein and AG-490 are compared in Fig. 3. AG-490 and genistein produced similar maximal level of activation of dCK after 4 h of incubation (Fig. 3A), but the rate of activation was clearly faster with genistein than with AG-490 (Fig. 3B).

3.3. Effect of ERK pathway inhibitors on dCK activity

Since AG-490 can inhibit the MAPK/ERK pathway due to its effect on JAK2 or JAK3 [41–44], we examined the effect of two specific and structurally distinct inhibitors of the MAPK/ERK signalling pathway, PD-98059 [45] and U-0126 [46]. As shown in Fig. 4, both inhibitors significantly increased dCK activity, whereas U-0124, an inactive analogue of U-0126 [46] was without significant effect. Like genistein and AG-490, PD-98059 and U-0126 slightly augmented (by approximately 20%) the activation of dCK by CdA (not shown).

3.4. Effect of various protein phosphatases on dCK activity

Since no specific protein kinase inhibitor could prevent activation of dCK by CdA, we investigated whether this

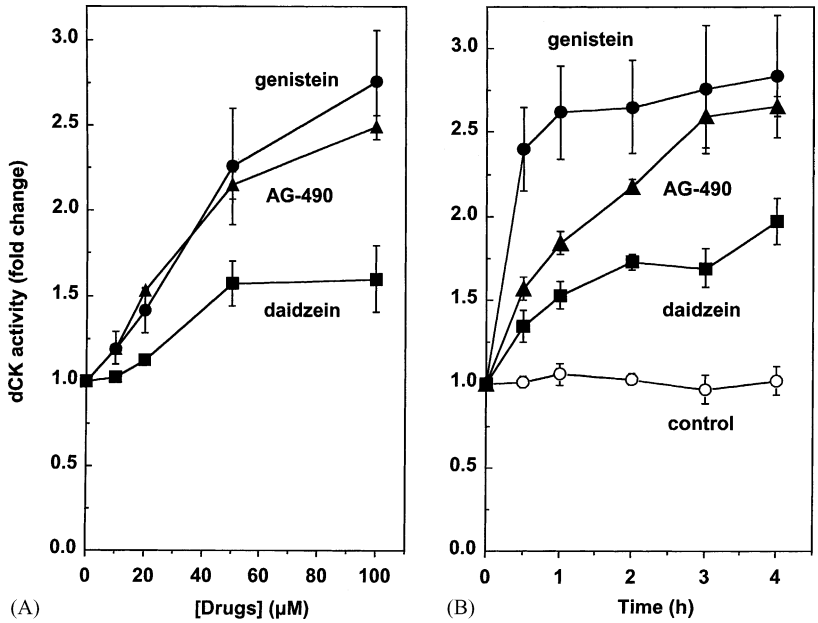

Fig. 3. Dose- and time-effects of genistein, daidzein and AG-490 on dCK activity. EHEB cells were incubated for 4 h with increasing concentrations of genistein, daidzein or AG-490 (A), or for various time periods in the presence of the same compounds at a concentration of 100 μM (B). Results are means ± S.E.M. of three separate experiments. Activity of dCK in absence of drugs (A) or at zero time (B) was 24.8 ± 4.6 pmol/min/mg protein and 20.8 ± 5.3 pmol/min/mg protein, respectively.

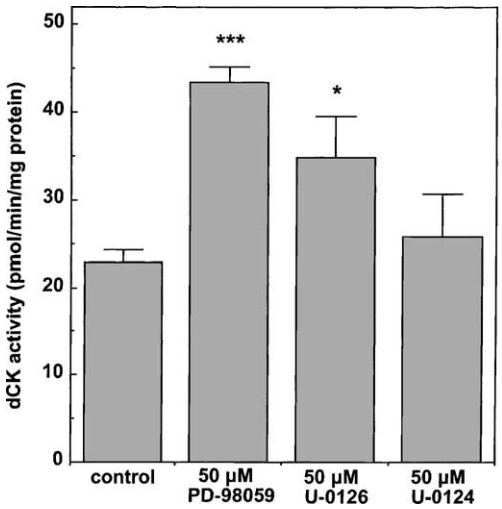

Fig. 4. Effect of inhibitors of the MAPK/ERK pathway on dCK activity. EHEB cells were incubated for 4 h with specific inhibitors of the ERK pathway, PD-98059 and U-0126, or with U-0124, an inactive analogue of U-0126. Results are means ± S.E.M. of four (PD-98059 and U-0126) or three (U-0124) separate experiments. Significance relative to the absence of protein kinase inhibitors: $^{*}P < 0.05$; $^{***}P < 0.001$.

process was really the result of an increased phosphorylation of the enzyme. Crude extracts of cells pre-incubated or not with 10 μM CdA, were treated for 30 min with λ-PP, according to the protocol described by Csapo et al. [22]. As illustrated in Fig. 5A, the aspecific λ-PP, which dephosphorylates Ser, Thr, and Tyr residues, strongly decreased dCK activity from control cells and from CdA-treated cells. Prevention of this effect by the phosphatase inhibitor VA established that the loss of dCK activity induced by λ-PP was indeed the result of a dephosphorylation of the enzyme. As depicted in Fig. 5B, purified PP2A [29], which dephosphorylates only Ser and Thr residues, also decreased dCK

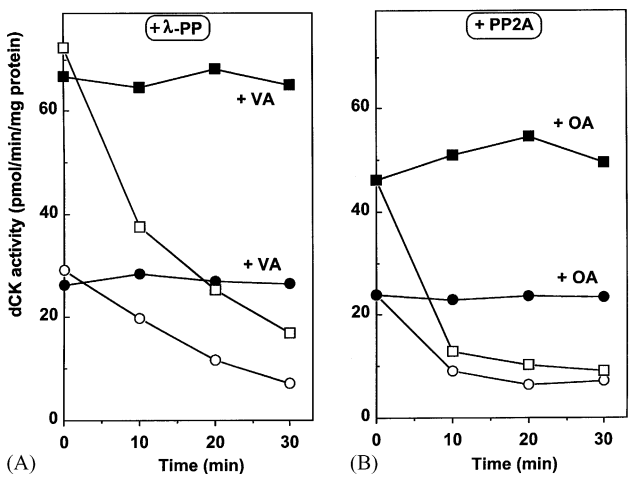

Fig. 5. Effect of protein phosphatase on dCK activity. Crude extracts of EHEB cells, pre-incubated for 4 h in the absence (○, ●) or in the presence (□, ■) of 10 μM CdA, were treated (A) with λ-PP in the presence (●, ■) or the absence (○, □) of 1 mM VA, or (B) with purified PP2A, in the presence (●, ■) or in the absence (○, □) of 1 μM OA, as described in the method section. Activity of dCK was determined at indicated times. Results are from representative experiments.

activity which reached a similar level, whether it had been previously increased by CdA or not. The effect of PP2A was prevented by OA, a potent inhibitor of this phosphatase. On the other hand, a recombinant purified protein tyrosine phosphatase IB was unable to decrease dCK activity (not shown). Experiments similar to those described in Fig. 5 were also performed on dCK activated by tyrosine kinase or ERK pathway inhibitors. In all these conditions, incubation with a protein phosphatase (λ-PP or PP2A) led to a decrease of dCK activity (data not shown).

3.5. Effect of okadaic acid on dCK activity

Recently, Csapo et al. [24] showed that dCK activity was increased approximately two-fold in human lymphocytes incubated with calyculin A, a potent inhibitor of both PP1 and PP2A, but very poorly by OA, an inhibitor more specific for PP2A. They concluded that dCK activity was regulated by PP1 rather than by PP2A, in contrast to the results presented in Fig. 5B. However, OA was used at the concentration of 15 nM in the study reported by Csapo et al. [24], a concentration reported to be insufficient to inhibit PP2A in intact cells. Several authors have indeed demonstrated that inhibition of PP2A inside cells may require up to 1 μM OA, a concentration at which PP1 activity is not yet affected [47,48]. Fig. 6A shows that OA can effectively increase dCK activity in EHEB cells, and that activation is nearly maximal at a concentration of 100 nM. Moreover, OA increased the activation of dCK by CdA, apparently in an additive way. As shown in Fig. 6B, the activation of dCK in the presence of 500 nM OA is gradual and a real plateau seemed not yet reached at the end of the incubation time studied. If we assume that dephosphorylation of dCK is completely inhibited by 500 nM OA, this rate of activation of dCK might reflect the activity of the protein kinase responsible for dCK phosphorylation in basal conditions.

3.6. Effect of protein kinase inhibitors and okadaic acid in CCRF-CEM and B-CLL cells

The effect of OA and of protein kinase inhibitors that were found to increase dCK activity in EHEB cells, was also investigated in CCRF-CEM cells and in B-CLL lymphocytes. As shown in Table 1, basal level of dCK activity was much higher in CCRF-CEM cells and in B-CLL lymphocytes than in EHEB cells. Still, genistein, daidzein, AG-490, PD-98059 and OA increased dCK activity in these two types of leukemic cells. The highest activation was obtained in the presence of 500 nM OA.

4. Discussion

As described in the introduction, there are several arguments in favour of a regulation of dCK through

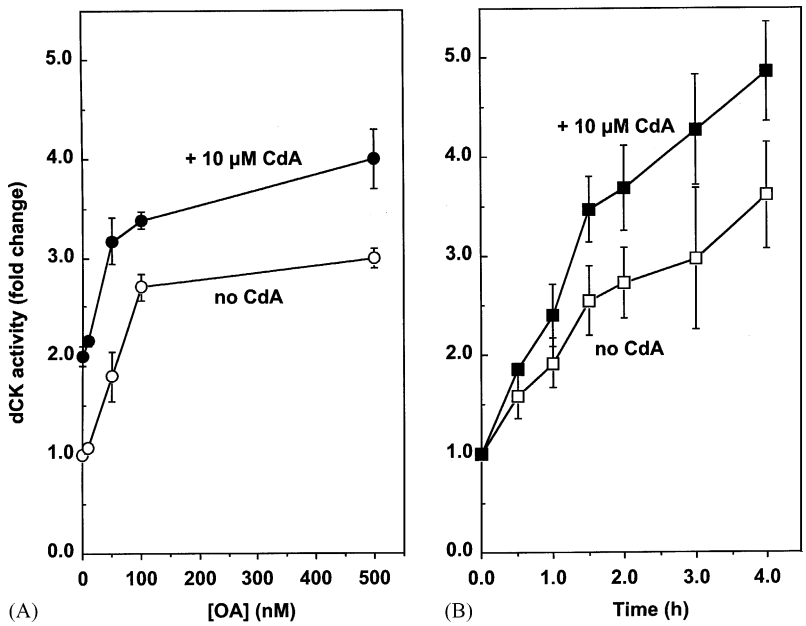

Fig. 6. Effect of okadaic acid on dCK activity. EHEB cells were incubated for 4 h with increasing concentrations of OA in the absence (○) or in the presence (●) of 10 μM CdA (A) or for various times in the presence of 500 nM OA in the absence (□) or in the presence (■) of 10 μM CdA (B). Results are means ± S.E.M. of three separate experiments. Activity of dCK in the absence of OA (A) or at zero time (B) was 27.4 ± 1.6 pmol/min/mg protein and 24.8 ± 1.3 pmol/min/mg protein, respectively.

reversible phosphorylation. This type of regulation implies the opposite actions of protein kinase(s) and protein phosphatase(s), whose identification represents a major interest for the understanding of the physiological regulation of the enzyme. To recognise protein kinases(s) involved in the regulation of dCK, we have used a large panel of cell-permeable protein kinase inhibitors. We have found no protein kinase inhibitors able to decrease dCK activity in basal conditions. On the contrary, we found that two PTK inhibitors, genistein and AG-490, and two inhibitors of the MAPK/ERK pathway, PD-98059 and U-0126, produced a significant activation of dCK.

Genistein is a general tyrosine kinase inhibitor which inhibits not only many tyrosine kinases, but also topoisomerase II [49]. Because inhibition of the latter enzyme by etoposide also increased dCK activity [20], we cannot exclude that activation of dCK by genistein is explained in part by an inhibitory effect on topoisomerase II. Our observation that daidzein, an analogue of genistein devoid of PTK inhibitory properties, also produced an activation of dCK, although less extensive than that of genistein (Fig. 3), confirms that the activation of dCK by genistein is not exclusively due to an inhibition of PTKs.

Unlike genistein, the tyrphostin AG-490 is considered a specific inhibitor that only targets the cytosolic tyrosine kinases JAK2 and JAK3, to the exclusion of other cytosolic tyrosine kinases [37,38]. Likewise, PD-98059 and U-0126, two structurally unrelated compounds, are reported to be specific inhibitors of the MAPK/ERK pathway [45,46]. Our observation that these specific inhibitors activated

Table 1
Deoxycytidine kinase activity in CCRF-CEM cells and B-CLL lymphocytes treated with protein kinase and phosphatase inhibitors

Drugs	CCRF-CEM cells		B-CLL lymphocytes	
	pmol/min/mg protein[a]	%[b]	pmol/min/mg protein[a]	%[b]
None	217 ± 17 (6)	*100*	91 ± 12 (3)	*100*
100 μM genistein	348 ± 29 (6)	*160****	220 ± 26 (3)	*242**
100 μM daidzein	378 ± 31 (3)	*174**	151 ± 28 (3)	*166*
100 μM AG-490	373 ± 38 (6)	*172***	186 ± 27 (3)	*205**
50 μM PD-98059	312 ± 33 (3)	*144* *	140 ± 22 (3)	*154**
500 nM okadaic acid	439 ± 23 (6)	*202****	271 ± 27 (3)	*298***

CCRF-CEM and B-CLL cells were incubated for 4 h before measurement of dCK activity. Activities are expressed in pmol/min/mg protein. Results are means ± S.E.M. of independent experiments. Significance between control and inhibitors are shown by the following *P*-values.

[a] Number in parentheses is the number of independent experiments.

[b] Activity is expressed in percentage of the control.

* $P \leq 0.05$.

** $P \leq 0.01$.

*** $P \leq 0.001$.

dCK, whereas AG-43, a negative control for AG-490, and U-0124, a negative control for U-0126, did not, suggest that dCK might be physiologically down-regulated by JAK2 and/or JAK3 and by the MAPK/ERK pathway. Moreover, since the MAPK/ERK signalling pathway may be a downstream substrate of JAK2 or JAK3 [41–44], regulation of dCK activity may involve the JAK2/MAPK or JAK3/MAPK pathways.

Activation of dCK by CdA, one of the most active drugs in the treatment of B-CLL, requires metabolisation of the nucleoside analogue [19,21] and occurs without new dCK protein synthesis or allosteric effect [19], which suggested a post-translational modification of the enzyme. Here, we show that CdA-activated dCK is highly susceptible to dephosphorylation by PP2A, which reduces its activity to that of basal dCK (Fig. 5). This result indicates that incubation of cells with CdA has raised the level of phosphorylation of dCK. However, apart from staurosporine and H-7, two general protein kinase inhibitors, which partially reduced the activation of dCK by CdA, we did not found specific protein kinase inhibitors able to prevent or reduce the activation of dCK by CdA (Section 3.1). Still, data obtained in these experiments allow to preclude a role of PKC, PKA, ribosomal protein S6 kinase, Ca^{++}/calmodulin-dependent protein kinase II, Rho-kinase and finally of PI3-kinases and of the DNA-dependent protein kinase, in the process of activation of dCK by CdA. This last stress kinase had previously been proposed to play a role in the activation of dCK by genotoxic agents, including nucleoside analogues [24], but it would not intervene in the activation of dCK by CdA, as demonstrated by the absence of effect of wortmannin on this process. The staurosporine- or H-7-sensitive protein kinase(s) involved in the activation of dCK by CdA, which could be different from the protein kinase(s) that control(s) dCK activity in basal conditions, remain(s) thus to be identified. Further studies are planned to investigate whether the activation of dCK by CdA could result from an inhibition of the JAK and/or of the MAPK/ERK pathways.

Experiments performed on cell lysates with PP2A (Fig. 5B) strongly indicate that the activity of dCK is modulated by the phosphorylation level of serine and/or threonine residue(s). The fact that OA, a specific inhibitor of phosphoserine/phosphothreonine phosphatase, increases dCK activity in intact cells (Fig. 6 and Table 1) accords with this hypothesis. OA inhibits PP1 and PP2A, but it has been shown that, up to 1 μM, it does not affect PP1 in intact cells [47,48]. Altogether, these results suggest that dCK might be an in vivo target of PP2A. Like PP1, PP2A is widely distributed in the cytoplasm of mammalian cells, and has been implicated in numerous signalling pathways and in the regulation of many cellular functions (reviewed in [50]). Time-course experiments show that the activation of dCK by OA is a rather slow process (Fig. 6B), suggesting that the kinase that counteracts the dephosphorylation of dCK in basal conditions is not very active. When CdA and OA are added together to cells, each at a concentration that produces a maximal effect, an up to five-fold activation of dCK can be observed, which is clearly higher than with each alone. This observation suggests that CdA and OA activate dCK by different pathways.

In summary, our study shows that agents known to modify the phosphorylation status of proteins can modulate the activity of dCK in several types of leukemic cells, including lymphocytes from patients with B-CLL. This strengthens the hypothesis of a phosphorylation/dephosphorylation process as a ubiquitous mechanism of dCK activity regulation, in which the JAK/MAPK pathway and PP2A could be particularly involved. Moreover, our observation that small molecules, and among them the soy isoflavones, genistein and daidzein, are able to enhance dCK activity, could be the starting point of new combination strategies aimed at enhancing the phosphorylation of nucleoside analogues and thereby their antileukemic efficacy.

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