Adenosine stimulates DNA fragmentation in human thymocytes by Ca^{2+} -mediated mechanisms

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Incubation of human thymocytes with an optimum concentration of adenosine and its receptor site agonist, 2-chloroadenosine, induced increases in intracellular cyclic AMP (cAMP) (from a resting 0.6 ± 0.1 to 4.1 ± 0.2 pmol/ 10^7 cells within 5 min) and Ca^{2+} (from the resting 85 ± 7 nM to a peak of 210 ± 25 nM) levels and resulted in internucleosomal DNA fragmentation and cell death (apoptosis). Other adenosine analogues were also effective at inducing DNA fragmentation, the order of potency being 2-*p*-(carboxyethylphenylethylamino)-5'-carboxyamido-adenosine < 5'-(*N*-ethylcarboxamide)adenosine $\leq$ cyclopentyladenosine < 2-chloroadenosine (2-CA). 2-CA treatment (with an optimum concentration of 40 μM) selectively depleted a thymocyte subpopulation (15–20% of the total cells) which expressed higher levels of the CD3 molecule and which was found mainly in the $\text{CD}4^+\text{CD}8^+$ double positive immature thymocyte population. DNA fragmentation was prevented by the addition of actinomycin D or cycloheximide to the thymocyte suspension, indicating that this process required both mRNA and protein synthesis.

Endonuclease activation and cell killing were dependent on an early, sustained increase in cytosolic Ca^{2+} concentration, most of which was of extracellular origin and was a result of an adenosine-induced inositol trisphosphate release. Other agents known to elevate intracellular cAMP levels by different mechanisms failed to induce similar DNA fragmentation, but enhanced the effect of adenosine. This suggested a supporting role for cAMP in adenosine-induced DNA fragmentation. Phorbol dibutyrate, a protein kinase C activator, previously shown to inhibit Ca^{2+} -dependent DNA fragmentation and cell killing in human thymocytes [McConkey, Hartzell, Jondal and Orrenius (1989) *J. Biol. Chem.* **264**, 13399–13402], at 60 ng/ml concentration also prevented adenosine-induced DNA fragmentation when added prior to adenosine. This suggested a complex cross-talk between the adenosine-triggered signal transduction cascade and the activation state of protein kinase C in regulating apoptosis of human thymocytes.

INTRODUCTION

Most cells have specific surface membrane receptors for adenosine, a naturally occurring metabolite, which are coupled to adenylate cyclase [1,2]. Lymphocytes from various species are shown to possess at least three types of adenosine receptors [3]. Extracellular A_1 receptors are stimulated by low (10^7 M) concentrations of adenosine and mediate decreases in cyclic AMP (cAMP) levels, extracellular A_2 receptors are stimulated by intermediate (10^{-5} M) concentrations of adenosine and mediate increases in cAMP levels, while intracellular P sites are stimulated by high (10^{-3} M) concentrations of adenosine and mediate decreases in cAMP levels. However, because of the low intracellular concentrations of adenosine, this latter effect may not have a physiological role [4].

Although adenosine and adenosine derivatives are able to modify cAMP levels in a wide variety of cell types [1,5], the actual relevance of cAMP as a second messenger mediating the physiological responses to adenosine has not been demonstrated in all tissues or cell systems [6–9]. Nonetheless, there is increasing evidence for the coupling of adenosine receptors to ion channels [10–12] and for adenosine-induced changes in the intracellular Ca^{2+} concentration in some cell types [6,13–15].

Adenosine has been reported to induce apoptosis in mouse thymocytes via membrane-bound extracellular, purinergic re-

ceptors [16]. This effect was accompanied by marked stimulation of membrane adenylate cyclase activity and cAMP accumulation, and was mimicked by other compounds known to elevate intracellular cAMP levels by different mechanisms. This suggested that the effect of adenosine on mouse thymocytes was mediated by cAMP-dependent mechanisms.

Apoptosis is a genetically programmed mechanism of cell death which is often biochemically characterized by internucleosomal DNA cleavage [17]. In thymus it is a critically important component of the intrathymic cell selection process which functions to prevent auto-immunity. Apoptotic deletion of T cell clones expressing auto-reactive or HLA-incompatible T-cell receptors occurs in the $\text{CD}4^+\text{CD}8^+$ 'double positive' immature differentiation stage. Cells expressing functionally acceptable T cell receptors become positively selected, escape the cell death pathway and differentiate into the mature $\text{CD}4^+$ or $\text{CD}8^+$ single positive thymocytes [18].

Despite the intensive biochemical research of the past few years still very little is known about the initiator and the effector elements of the various forms of programmed cell death [19]. The elusive nature of the killer gene products makes studying their regulation and control difficult. Nevertheless it seems well documented that in thymocytes elevation of the intracellular Ca^{2+} level induced by various agents can play a crucial role in triggering apoptosis. Agents reported to induce apoptosis by

Abbreviations used: cAMP, cyclic AMP; 2-CA, 2-chloroadenosine; $[\text{Ca}^{2+}]_i$, cytosolic Ca^{2+} concentration; CPA, cyclopentyladenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; CGS 21680, 2-*p*-(carboxyethylphenylethylamino)-5'-carboxyamido-adenosine; NECA, 5'-(*N*-ethylcarboxamide)-adenosine; IBMX, 3-isobutyl-1-methylxanthine; PdBu, phorbol dibutyrate; HA1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulphonamide; FCS, foetal calf serum; MPS, methylprednisolone; FITC, fluorescein isothiocyanate; PE, phycoerythrin; dbcAMP, dibutyryl-cAMP; IP_3 , inositol trisphosphate.

Ca²⁺-mediated mechanisms include antibody against the CD3–T cell receptor complex [20], ATP [21], tributyltin [22] and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin [23], and their effects are mimicked by treatment of the thymocytes with calcium ionophore [24]. Although the exact effector elements responding to the increases in intracellular Ca²⁺ are not well known yet, they may include enzymes in the cell death signalling pathway (e.g. protein kinase C and other protein kinases, phosphatases), killer genes (like *ced-4* in *Caenorhabditis elegans*, which codes a protein containing two sites similar to the Ca²⁺-binding EF-hand motif [25]) and enzymes related to the apoptotic programme. These last enzymes may include a Ca²⁺/Mg²⁺-dependent endonuclease which is perhaps equal to the DNase I [26], the tissue transglutaminase [27], and the calpain [28].

In this paper we report that human thymocytes exposed to adenosine and adenosine analogues which are known to activate adenylate cyclase and elevate intracellular cAMP levels via adenosine A₂ receptors in these cells [29] undergo programmed cell death. In contrast, other means of elevating intracellular concentrations of cAMP or mimicking its action on protein kinase A fail to induce DNA fragmentation and cell death. According to our data we suggest that in human thymocytes, unlike in mouse thymocytes, adenosine mediates its effect on DNA fragmentation primarily by increasing the intracellular Ca²⁺ concentration, but increases in the intracellular cAMP level can enhance the Ca²⁺-mediated response.

EXPERIMENTAL

All conjugated monoclonal antibody reagents were obtained from Dakoplatz AB (Glostrup, Denmark). Fura-2/AM (the acetoxymethyl ester), RNase A and T₁ were purchased from Boehringer (Mannheim, Germany); the cAMP radioimmunoassay kit and *myo*-[³H]inositol (18.2 Ci/mmol) were obtained from Amersham (Buckinghamshire, U.K.). Thapsigargin was obtained from GIBCO BRL (Gaithersburg, MD, U.S.A.). Cyclopentyladenosine (CPA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 2-*p*-(carboxyethylphenylethylamino)-5'-carboxy-amidoadenosine (CGS 21680) were from Research Biochemicals and were a kind gift from József Szentmiklóssy (Department of Pharmacology, University Medical School of Debrecen). 2-Chloroadenosine (2-CA), 5'-(*N*-ethylcarboxamide)adenosine (NECA), adenosine, forskolin, 1,9-dideoxyforskolin, dibutyl-*l*-cAMP, 3-isobutyl-1-methylxanthine (IBMX), cycloheximide, actinomycin D, phorbol dibutyrate (PdBu), H-7, *N*-(2-guanidinoethyl)-5-isoquinolinesulphonamide (HA 1004) and calcium ionophore A 23187 were from Sigma (St. Louis, MO, U.S.A.). Calphostin was a gift from Zoltán Szállási (N.I.H., U.S.A.) while the OKT3 antibody was a gift from Mikael Jondal (Department of Immunology, Karolinska Institute). All other reagents used were obtained from commercial sources and were of analytical grade.

Cell preparation and culture

Thymocyte suspensions were prepared from thymus glands from patients less than 2 years old undergoing corrective cardiac surgery by mincing the glands in RPMI 1640 media (GIBCO, Paisley, Scotland, U.K.) supplemented with 2% foetal calf serum (FCS; GIBCO), 2 mM glutamine and 100 i.u. of penicillin and 100 µg of streptomycin/ml. Thymocytes were washed three times and diluted to a final concentration of 10⁷ cells/ml before incubation at 37 °C in a humidified incubator under an atmosphere of 5% CO₂ and 95% air. Cell death was measured by trypan blue uptake. A total of 95–98% of cells routinely excluded trypan blue after the isolation procedures.

Quantification of DNA fragmentation

Thymocytes were incubated in 24-well plates with various agents. After 1 day 0.8 ml of cell suspensions were lysed by addition of 0.7 ml of ice-cold lysis buffer containing 0.5% (v/v) Triton X-100, 10 mM Tris, 20 mM EDTA, pH 8.0, before centrifugation for 15 min at 13000 *g* in a microcentrifuge. The DNA content of supernatant (DNA fragments) and pellets (intact chromatin) was precipitated with an equal amount of 10% trichloroacetic acid, resuspended in 5% trichloroacetic acid and quantified using the diphenylamine reagent [30]. Alternatively, the 13000 *g* supernatants were precipitated overnight in ethanol containing 0.15 mM NaCl. The pellets were redissolved in a buffer containing 10 mM Tris, 1 mM EDTA, pH 8.0, treated with RNase, sequentially extracted with equal volumes of phenol and chloroform/isoamyl alcohol (24:1) and precipitated in ethanol before electrophoresis for 3 h at 60 V in 1.8% agarose gel. DNA fragments were then visualized by u.v. light after staining the gel with ethidium bromide.

Characterization of thymocyte subpopulations

Thymocytes were incubated for 24 h in the absence (control) or the presence of either 40 µM 2-CA or 1 µM methylprednisolone (MPS). Viable cells were then recovered by Lymphoprep (Sigma) and centrifuged for 10 min at 200 *g* (4 °C) to separate dead from viable cells. Viable thymocytes did not show DNA fragmentation with propidium iodide staining. These thymocytes were washed twice and resuspended in ice-cold PBS containing 0.1% (w/v) sodium azide before staining either with fluorescein isothiocyanate (FITC)-labelled anti-CD4 and phycoerythrin (PE)-conjugated anti-CD8, or FITC-labelled anti-CD3. The cells were agitated, incubated for 30 min at 4 °C, washed twice with ice-cold PBS supplemented with 10% FCS and 0.1% sodium azide, and resuspended in PBS containing 0.1% sodium azide. Unstained thymocytes treated similarly served as auto-fluorescence controls, while thymocytes stained with non-reactive FITC-conjugated mouse IgG1 and PE-conjugated mouse IgG1 antibodies served as controls for non-specific staining. Dual fluorescence was analysed on a Becton Dickinson FACScan with excitation of the incident light at 488 nm. The log (integrated green fluorescence) (emission at 530 nm) and log (integrated red fluorescence) (emission at 585 nm) were collected after combined gating on forward angle light scatter and 90° light scatter. The overlap in green and red emission was corrected using an electronic compensation network.

Measurement of intracellular cAMP concentration

Thymocytes (5 × 10⁶/ml in RPMI solution supplemented with 10% FCS) were incubated at 37 °C in the presence of the indicated compounds for 5 min, then quickly washed with ice-cold PBS and precipitated with 96% ethanol. The pellets were sonicated, boiled for 2 min, then centrifuged at 20000 *g* for 5 min. The supernatants were dried under nitrogen and redissolved in 0.1 ml of buffer containing 10 mM Tris and 1 mM EDTA, pH 8.0. The cAMP content was then determined by using the Amersham radioimmunoassay kit.

Measurement of cytosolic Ca²⁺ concentration ([Ca²⁺]_i)

Thymocytes (10⁷ cells/ml) were incubated in the presence of 5 µM fura-2/AM in RPMI 1640 medium supplemented with 2% FCS for 30 min at room temperature, washed free from extracellular fura-2 with Krebs–Henseleit medium supplemented with 20 mM Hepes, 5.5 mM glucose, 1.3 mM CaCl₂, and 2% BSA,

pH 7.2, and incubated for another 15 min to allow the non-hydrolysed fura-2 to diffuse from the cells. 2-CA was added either to this medium and the changes in the cytosolic Ca^{2+} were determined by taking the samples at different time points, or it was added directly to the cuvette to determine the immediate $[\text{Ca}^{2+}]_i$ changes. Before measuring $[\text{Ca}^{2+}]_i$, cells were resuspended in the same medium supplemented with 0.2% BSA. Fluorescence intensity was determined on a Sigma ZFP 22 dual-wavelength spectrofluorimeter equipped with a thermostatically controlled cuvette holder to maintain the temperature at 30 °C to minimize dye leakage from the cells [31]. Changes in the fluorescence intensity were monitored by recording changes in the fluorescence ratio as previously described by Kass et al. [32].

Measurement of inositol phosphate production

myo-[^3H]inositol (2 $\mu\text{Ci}/\text{ml}$) overnight-prelabelled thymocytes were washed and incubated in Hanks solution supplemented with 25 mM Hepes, 15 mM Hepes, 15 mM glucose, 1.3 mM Ca^{2+} and 10 mM LiCl for 10 min at 37 °C (10^7 cells/ml). Aliquots of the cell suspension (2 ml) were then stimulated with the indicated agents for 10 min at 37 °C. Inositol phosphates were separated by anion-exchange chromatography on 1 ml Dowex AG 1-X8 (formate form; Bio-Rad Laboratories, Richmond, CA) columns, previously equilibrated with 0.1 M formic acid with subsequent washes using water, 60 mM sodium formate, and solutions containing increasing concentrations of ammonium formate in 0.1 M formic acid. The eluted radioactivity was quantified by liquid scintillation counting.

RESULTS

Adenosine is the only ribonucleoside which induces DNA fragmentation in human thymocytes

Adenosine which was previously reported to induce DNA fragmentation in mouse thymocytes by increasing intracellular cAMP levels via adenosine A_2 receptors [16] was added to human thymocytes at 1 mM concentration to compensate for a rapid decrease in adenosine levels due to metabolism. This treatment induced a significant increase in the DNA fragmentation (from 25.1 ± 1.7 to $38.7 \pm 0.8\%$) and loss of cell viability (from 89 ± 2 to $76 \pm 4\%$ determined after 24 h in culture). Other ribonucleosides had no effect. 2-CA, an adenosine analogue which is poorly transported into cells and is not deaminated, also produced a significant DNA fragmentation at micromolar concentrations (Figure 1). This suggested that adenosine mediated its effect on human thymocytes via adenosine receptors. Concentrations of 2-CA above 40 μM did not induce any further increase in DNA fragmentation but strongly decreased thymocyte viability. At the optimal 1 μM concentration, MPS, a well known apoptosis inducer of thymocytes [33], induced more extensive DNA fragmentation ($69.7 \pm 2.1\%$) than adenosine or 2-CA, suggesting that a larger fraction of cells were sensitive to treatment with the glucocorticoid.

Endonuclease activation in cells undergoing apoptosis results in the production of oligosome-length DNA fragments ('DNA ladder') which can be distinguished from the random cleavage observed in cells undergoing necrosis [33]. The chromatin cleavage pattern elicited by 2-CA was indistinguishable from that observed in glucocorticoid-treated thymocytes undergoing apoptosis (Figure 2), suggesting that a similar endonuclease activity was involved in both processes. This was further supported by the observation that Zn^{2+} ions, which were reported to inhibit DNA fragmentation induced by glucocorticoids [34], at 0.5 mM

Figure 1 Increase in the amount of DNA fragmented induced by various adenosine analogues in the absence or presence of DPCPX

Thymocytes (10^7 cells/ml) were incubated for 24 h in the presence of the indicated compounds. The amount of fragmented DNA was determined as described in the Experimental section. In the non-treated samples the amount of DNA fragmented was $24 \pm 3.2\%$. Data are means $\pm$ S.D. of three determinations. (a) CGS 21680 ($\blacktriangledown$), NECA ($\triangledown$), CPA ($\bullet$), 2-CA ($\circ$); (b) CPA ($\circ$) + 1 nM DPCPX ($\bullet$); (c) NECA ($\circ$) + 1 μM DPCPX ($\bullet$); (d) 2-CA ($\circ$) + 1 μM DPCPX ($\bullet$).

concentration also inhibited the DNA cleavage in 2-CA-treated thymocytes (results not shown).

Adenosine mediates its effects on DNA fragmentation via adenosine receptors

To investigate which of the adenosine receptors were involved in the induction of DNA cleavage by adenosine, we studied the effects of various adenosine receptor-site agonists. Adenosine analogues with substitution either in the N^6 position (CPA) or with substitution in the 5' position (NECA) induced DNA fragmentation in nanomolar concentrations (Figure 1). The most pronounced effect was observed with CGS 21680 which appeared to bind only to the A_{2a} sites in rat brain [35]. The A_1 -selective antagonist DPCPX inhibited the CPA-induced DNA fragmentation at 1 nM concentration while NECA and 2-CA induced DNA fragmentation at 1 μM concentration.

Adenosine depletes primarily a $\text{CD}8^+\text{CD}4^+$ thymocyte subpopulation expressing a higher level of the CD3 T cell receptor.

Previous work [36] has shown that treatment with antibody against the T cell receptor complex, glucocorticoid or calcium ionophore selectively depleted the immature, CD^4CD^8 double positive thymocyte cell population. Therefore, we examined whether the same population was sensitive to the 2-CA treatment.

Figure 2 Electrophoretic fractionation of DNA extracted from cultured human thymocytes

Thymocytes were cultured with or without 1 μ M MPS or 40 μ M 2-CA for 24 h. The DNA was extracted, electrophoresed on a 1.8% agarose gel as described in the Experimental section and visualized after staining with ethidium bromide. Lane 1, DNA from untreated thymocytes; lane 2, DNA from MPS-treated thymocytes; lane 3, DNA from 2-CA-treated thymocytes; lane 4, DNA from 2-CA-treated thymocytes after 30 min of 60 ng/ml PdBu pretreatment; and lane 5, DNA from 2-CA-treated thymocytes with PdBu added 30 min later.

Our observation that an optimal concentration of 2-CA affected a smaller population of cells than MPS suggested that a different or smaller population of thymocytes may be responsive to 2-CA. Phenotypic analysis of the viable cells after treatment with 1 μ M MPS or 40 μ M 2-CA showed indeed that, although in both cases the 'double positive' immature population was the most sensitive (Table 1), 2-CA depleted primarily the thymocytes expressing medium or high levels of CD3-T cell receptor molecule, while the glucocorticoid treatment affected cells expressing a lower level of CD3-T cell receptor (Table 2, Figure 3). The relative increase in the CD4⁺ population observed after both 2-CA and MPS treatment may have been due to the down-regulation of the CD8 molecule after initiation of apoptosis in the 'double positive' but still viable cells. This was observed previously also after ionomycin treatment in mouse thymocytes [37].

Table 2 Effects of MPS or 2-CA treatment on the survival of different subpopulations of thymocytes with respect to the expression of the CD3-T cell receptor complex

Thymocytes (40×10^6 cells) were incubated for 24 h in the presence or absence of 1 μ M MPS or 40 μ M 2-CA. The number of remaining viable cells in each subpopulations was determined as described in the Experimental section. Data in parentheses represent the percentage of control in each subpopulation. The most important changes are underlined.

Treatment	Total	Subpopulations ...	10 ⁻⁶ × Cell no.		
			CD3 ^{low/neg}	CD3 ^{med}	CD3 ^{high}
None	37.5		8.49 (100)	20.8 (100)	8.19 (100)
MPS	20.1		3.08 (36)	8.94 (41)	8.09 (99)
2-CA	30.5		8.48 (100)	15.5 (75)	6.52 (79)

Compounds that elevate intracellular cAMP do not increase the rate of DNA fragmentation in human thymocytes

Although it was previously demonstrated that human thymocytes possess adenosine A₂ receptors which are coupled to the adenylate cyclase system, other means of elevating intracellular concentrations of cAMP or mimicking its effect on protein kinase A failed to stimulate the amount of DNA fragmented (results not shown), although they increased the intracellular cAMP (Table 3). Among the compounds tested were: dibutyryl-cAMP (dbcAMP), a membrane-permeant analogue of cAMP; IBMX, a phosphodiesterase inhibitor; and cholera toxin, which is known to increase cAMP levels by ADP-ribosylation of the stimulatory subunit of adenylate cyclase [38]. Although forskolin, a direct activator of adenylate cyclase [39], induced DNA fragmentation in a dose-dependent manner, this effect appeared to be independent of cAMP formation, since 1,9-dideoxyforskolin, an analogue of forskolin which does not activate cyclase, exerted similar effects.

Adenosine mediates its effect on DNA fragmentation via increasing cytosolic Ca²⁺ concentration

It was previously shown that an increase in the cytosolic Ca²⁺ concentration preceded DNA fragmentation in thymocytes ex-

Table 1 Effects of MPS or 2-CA treatment on the survival of different subpopulations of thymocytes with respect to expression of the CD8 and CD4 surface molecules

Thymocytes (40×10^6 cells) were incubated for 24 h in the presence or absence of 1 μ M MPS or 40 μ M 2-CA. The number of remaining viable cells in each subpopulation was determined as described in the Experimental section. Data in parentheses represent the percentage of non-treated control in each subpopulation. The most important changes are underlined.

Treatment	Total	Subpopulations ...	10 ⁻⁶ × Cell no.			
			4 ⁻ 8 ⁺	4 ⁺ 8 ⁺	4 ⁻ 8 ⁻	4 ⁺ 8 ⁻
None	37.5		2.04 (100)	31.9 (100)	0.73 (100)	2.82 (100)
MPS	20.1		2.47 (121)	11.8 (37)	0.91 (123)	4.37 (154)
2-CA	30.5		1.71 (84)	23.3 (73)	1.02 (139)	4.49 (159)

Figure 3 Effects of MPS or 2-CA treatment on the survival of different subpopulations of thymocytes with respect to the expression of the CD3-T cell receptor complex

Thymocytes were incubated for 24 h in the presence or absence of 1 μ M MPS or 40 μ M 2-CA. The remaining viable cells were separated and stained with FITC-labelled anti-CD3 as described in the Experimental section.

posed to a number of different stimuli [20–24]. To determine whether a similar Ca^{2+} increase also preceded 2-CA-induced DNA fragmentation, thymocytes were loaded with the Ca^{2+} probe fura-2 and then incubated with 2-CA. As shown in Figure 4(a) in the presence of 1.3 mM extracellular Ca^{2+} 40 μ M 2-CA induced, within a few seconds, an increase of $[\text{Ca}^{2+}]_i$ from the resting level of 85 ± 7 nM to a peak of 210 ± 25 nM ($n = 5$). The increase in $[\text{Ca}^{2+}]_i$ reached the maximum value within 20 ± 4 s after 2-CA addition and then declined to a prolonged plateau level. This elevated calcium was maintained at least in the first

Table 3 Effect of 5 min treatment with various compounds on the cAMP content of human thymocytes

Compound	Concentration (μ M)	cAMP content (pmol/ 10^7 cells)*
None		0.6 ± 0.1
Adenosine	500	6.7 ± 0.3
2-CA	40	4.1 ± 0.2
Forskolin	5	9.5 ± 0.3
	25	20.4 ± 0.1
IBMX	250	3.0 ± 0.3
Cholera toxin	100 (ng/ml)	2.9 ± 0.1
2-CA	40	7.3 ± 0.2
+ IBMX	250	
Forskolin	5	30.0 ± 1.4
+ IBMX	250	
Forskolin	25	78.3 ± 2.7
+ IBMX	250	
PdBu	60 (ng/ml)	0.6 ± 0.1
2-CA	40	31.8 ± 0.4
+ PdBu	60 (ng/ml)	

* Data are means $\pm$ S.D. of determinations from three different thymuses.

Figure 4 (a) Effect of 2-CA on the cytoplasmic Ca^{2+} level of human thymocytes in the presence and absence of EGTA and (b) Inhibition of 2-CA-induced calcium response by PdBu and reversal of it by protein kinase C inhibitor H-7

Thymocytes (10^7 cells/ml) were loaded with fura-2/AM as described in the Experimental section. The cells were then washed and resuspended in a Krebs–Henseleit medium supplemented with 0.2% BSA. (a) At the indicated times the cells were stimulated with 40 μ M 2-CA in the presence and absence of 5 mM EGTA. (b) Fura 2-loaded thymocytes (10^7 ml cells in Krebs medium supplemented with 20 mM Hepes, 5.5 mM glucose and 2% BSA) were incubated in the presence of PdBu (60 ng/ml) for 10 min (left) or H7 (50 μ M) for 30 min, then PdBu (60 ng/ml) for another 10 min (right). Cells were washed and the increase in cytosolic Ca^{2+} was measured after addition of 40 μ M 2-CA. Changes in fura-2 fluorescence were detected by using a Sigma ZFP 22 dual-wavelength spectrofluorimeter. Each trace represents one of five experiments.

2 h (results not shown). Chelation of extracellular Ca^{2+} with EGTA did not inhibit the first part of the 2-CA-induced calcium response (Figure 4) suggesting that the first part was dependent on release of an intracellular calcium pool, while the second part of the response appeared to be dependent on extracellular calcium.

While elevated intracellular cAMP concentrations *per se* failed

Table 4 Effect of compounds that influence cytosolic Ca^{2+} concentration in thymocytes on DNA fragmentation and cell viability

Thymocytes (10^7 cells/ml) were incubated for 24 h in the presence of the indicated compounds. The amount of fragmented DNA and the cell viability were determined as described in the Experimental section. Data are means $\pm$ S.D. of determinations from three different thymuses.

Compound	Concentration (μM)	DNA fragmented (%)	Viability (%)
None		17.5 ± 1.1	93 ± 1
A23187	0.5	$31.4 \pm 0.7^*$	$81 \pm 2^*$
	1	$45.1 \pm 2.2^*$	$68 \pm 3^*$
	1.5	62.0 ± 3.1	$52 \pm 3^*$
	2	$36.1 \pm 0.4^*$	$42 \pm 1^*$
Quin-2	100	$10.2 \pm 0.3^*$	$96 \pm 1^*$
2-CA	40	$40.0 \pm 0.7^*$	$75 \pm 2^*$
2-CA	40	$14.5 \pm 2.1^\dagger$	$90 \pm 2^\dagger$
+ quin-2	100		
Calmidazolium	20	$19.7 \pm 0.9^\dagger$	$90 \pm 1^\dagger$
2-CA	40	$28.7 \pm 2.1^\dagger$	$83 \pm 2^\dagger$
+ calmidazolium	20		

* Significantly different from the non-treated control as determined by Student's paired t test, $P < 0.05$.

† Significantly different from the 2-CA (40 μM)-treated control as determined by Student's paired t test, $P < 0.05$.

to induce DNA fragmentation in human thymocytes, the calcium ionophore A23187 was as effective in human cells as previously found in rat and mouse thymocytes (Table 4). Furthermore chelation of intracellular Ca^{2+} using quin-2 blocked adenosine-stimulated DNA fragmentation, indicating that the process was calcium dependent. Calmidazolium also decreased the rate of adenosine-induced DNA fragmentation, suggesting that calmodulin-dependent protein kinase(s) may take part in the process.

The 2-CA-induced calcium response is independent of cAMP formation in human thymocytes

Since it was previously reported [40] that in human T lymphocytes cAMP may release an intracellular pool of calcium, there was a possibility that adenosine mediated its effects on the intracellular Ca^{2+} via cAMP. To test this possibility we determined whether the other compounds, which also increased the intracellular cAMP level in thymocytes (Table 3), exerted any effect on the $[\text{Ca}^{2+}]_i$ in human thymocytes.

None of these compounds had any effect on $[\text{Ca}^{2+}]_i$, except for forskolin, which induced a slight increase (results not shown). The effect of forskolin on $[\text{Ca}^{2+}]_i$, however, seemed to be independent of cAMP formation since the response was much smaller than the response to 2-CA, despite the fact that forskolin was the most effective at inducing increases in cAMP levels. IBMX, which potentiated the effect of forskolin on cAMP formation, did not potentiate its effect on $[\text{Ca}^{2+}]_i$, and dideoxy-forskolin, a forskolin analogue which does not activate adenylate cyclase, induced a similar increase. We do not know the mechanism responsible for this calcium increase. However it may be related to other effects associated with forskolin [41,42].

Adenosine-induced calcium release is dependent on inositol trisphosphate (IP_3) formation in human thymocytes

Since adenosine released calcium first from an intracellular pool, we tested whether the adenosine-sensitive pool was related to the

Table 5 Effect of 10 min 2-CA or OKT3 treatment on IP_3 production of human thymocytes preincubated with 10 mM LiCl for 10 min

$\text{myo-[}^3\text{H]inositol}$ -labelled thymocytes were washed and preincubated with 10 mM LiCl for 10 min as described in the Experimental section. 2-CA or OKT3 were added at the indicated concentrations, and the inositol phosphate production was stopped after 10 min of stimulation. In the case of 40 + P during the LiCl preincubation, 60 ng/ml PdBu was administered in addition to the 40 μM 2-CA, and the data are expressed as percentages of the 60 ng/ml PdBu-treated control. Inositol phosphates formed were separated by anion exchange chromatography and detected by liquid scintillation counting. The data are means $\pm$ S.E. of three separate experiments.

Treatment	IP_3 production (control %)
2-CA (μM)	
5	$156 \pm 2^*$
10	$238 \pm 23^*$
40	$306 \pm 15^*$
40 + P	100 ± 3
OKT3 ($\mu\text{g/ml}$)	
5	$397 \pm 11^*$
10	$679 \pm 23^*$

* Significantly different from the non-treated control as determined by Student's paired t test, $P < 0.05$.

IP_3 -sensitive intracellular pool. OKT3 antibody is a known activator of phospholipase C via the CD3 molecule of the T cell receptor [43]. In the absence of extracellular calcium, previous addition of 10 $\mu\text{g/ml}$ OKT3 antibody, which released the IP_3 -sensitive calcium pool within the CD3 receptor bearing thymocytes, prevented the increase in $[\text{Ca}^{2+}]_i$ induced by 40 μM 2-CA. Similarly, previous addition of 2-CA, in the absence of extracellular calcium, decreased the OKT3-induced calcium release (from 197 ± 20 nM to 117 ± 10 nM, whereas the initial concentration was 85 ± 10 nM, $n = 6$). These findings suggested that adenosine induced a calcium release from an IP_3 -sensitive intracellular pool which was within the OKT3-sensitive pool. This suggestion was further supported by the observation that previous addition of 1 μM thapsigargin, a microsomal Ca^{2+} translocase inhibitor, which has been shown to cause the release of the IP_3 -sensitive Ca^{2+} store in the absence of inositol phosphate production [44], completely inhibited the 2-CA-induced calcium response (results not shown).

Direct detection of inositol phosphate production after 2-CA addition in $\text{myo-[}^3\text{H]inositol}$ -labelled thymocytes revealed a dose-dependent release of IP_3 (Table 5). The observed IP_3 release was lower in the presence of 40 μM 2-CA than in the presence of 5 or 10 $\mu\text{g/ml}$ OKT3. This could be due to the fact that the functionally active adenosine receptor may appear later than the functionally active CD3 receptor during the maturation of the human thymocytes, since this is suggested by the observation that thymocytes expressing medium or high levels of CD3 receptor enter the apoptotic programme in the presence of 2-CA (Figure 3). So the observed, smaller IP_3 (and Ca^{2+}) release in the presence of adenosine could represent the response of a smaller, more mature subpopulation of thymocytes. These data provide evidence that in human thymocytes the adenosine receptor is coupled also to the phospholipase C signalling pathway.

Changes in the intracellular cAMP concentration induced by adenosine may contribute to its effects on DNA fragmentation

H-7, a non-specific inhibitor of the catalytic site, or calphostin, a highly specific inhibitor of the diacyl glycerol/PdBu binding site

Table 6 Effect of protein kinase inhibitors or compounds which elevate intracellular cAMP levels on adenosine-induced DNA fragmentation

Thymocytes (10^7 cells/ml) were incubated for 24 h in the presence of the indicated compounds. The amount of fragmented DNA was determined as described in the Experimental section. Data represent mean $\pm$ S.D. of determinations from three different thymuses.

Compound	Concentration (μ M)	DNA fragmentation (%)
None		20.4 ± 0.9
2-CA	40	40.4 ± 1.4
HA 1004	10	21.7 ± 1.1
2-CA + HA 1004	40	$32.7 \pm 2.1^*$
H-7	50	20.9 ± 0.3
2-CA + H-7	40	38.6 ± 1.2
Calphostin	50	21.7 ± 1.1
2-CA + calphostin	40	41.2 ± 1.3
2-CA	5	30.7 ± 2.1
Forskolin	5	22.4 ± 1.5
IBMX	250	22.7 ± 0.8
2-CA + forskolin	5	$43.7 \pm 2.1^\dagger$
2-CA + IBMX	5	$38.9 \pm 0.4^\dagger$

* Significantly different from the 2-CA (40 μ M)-treated control as determined by Student's paired *t* test, $P < 0.05$.

† Significantly different from the 2-CA (5 μ M)-treated control as determined by Student's paired *t* test, $P < 0.05$.

of protein kinase C had no effect on the adenosine-induced cell death (Table 6). This suggested that, while calcium was necessary, protein kinase C activation was not required for the adenosine-induced cell death pathway.

Although compounds which elevate cAMP failed to induce DNA fragmentation in human thymocytes, a partial prevention of the adenosine-induced DNA fragmentation was observed after addition of HA 1004, a potent inhibitor of protein kinase A (Table 6). It suggested that, although increases in the intracellular cAMP concentration alone may not be sufficient to induce DNA fragmentation, cAMP may still contribute to the effects of adenosine. To examine this possibility, 2-CA and IBMX or forskolin, at concentrations which alone did not induce a significant increase in DNA fragmentation, were added together to the cells. As shown in Table 6, both compounds potentiated the effects of 2-CA, suggesting a supporting role of cAMP.

cAMP does not potentiate the adenosine-induced calcium response

Since it was reported that in other cell types cAMP can mediate potentiation of influx of Ca^{2+} from extracellular sources [45,46] and can augment the IP_3 -mediated release of calcium from intracellular stores [47,48] it was investigated whether addition of cAMP or preincubation with IBMX, which can potentiate the adenosine-induced cAMP formation, or preincubation with HA 1004, which inhibits protein kinase A, could influence the adenosine-induced calcium release. However neither dbcAMP (100 μ M) and IBMX (250 μ M) nor HA 1004 (10 μ M) were found to exert an effect on the 2-CA-induced calcium response (results not shown). These data suggest that the supporting role of cAMP on the apoptosis induction of human thymocytes is not related to the control of calcium release.

DNA fragmentation induced by adenosine requires macromolecular synthesis

2-CA-induced DNA fragmentation was inhibited by actinomycin D at a concentration of 10 μ g/ml to below the control level, and by cycloheximide, at a concentration of 50 μ g/ml to near the control level (results not shown). Reduction of DNA fragmentation was accompanied by an increase in cell viability as judged by the trypan blue exclusion test. Both compounds also inhibited the spontaneous DNA fragmentation observed in the control samples, suggesting that ongoing transcription and protein synthesis were required for the induction of DNA cleavage.

Protein kinase C activation interferes with adenosine-induced cell death

Previous work has shown that activators of protein kinase C can inhibit the Ca^{2+} -stimulated pathway of endonuclease activation in thymocytes [49]. To test whether protein kinase C activation can interfere also with the adenosine-induced cell death, PdBu was added at various time points to the thymocyte culture. PdBu alone reduced the background cell death rate at concentrations higher than 10 ng/ml. It failed to inhibit 2-CA-induced DNA fragmentation if added simultaneously with or later than 2-CA. However, addition of phorbol ester 30 min before 2-CA caused complete protection from DNA fragmentation (Figure 2) and prevented the loss of cell viability measured by the trypan blue exclusion test. This effect of PdBu was dose dependent, starting at a concentration of 10 ng/ml and reaching a plateau level at 60 ng/ml (results not shown).

The fact that phorbol ester was effective only if it was added before 2-CA suggested that PdBu may interfere with an early step in the adenosine-triggered signalling pathway. To test this possibility it was investigated whether PdBu could influence the adenosine-induced calcium or cAMP response. As shown in Table 3, incubation of human thymocytes with 60 ng/ml PdBu prior to the addition of 2-CA potentiated the 2-CA-induced increase in intracellular cAMP, but impaired the 2-CA-induced increase in $[\text{Ca}]_i$ by nearly 100% (Figure 4b). PdBu did not alter the resting level of $[\text{Ca}^{2+}]_i$. Preincubation of thymocytes for 30 min with protein kinase C inhibitor H7 abolished the inhibitory effect of PdBu on the 2-CA-induced elevation in $[\text{Ca}^{2+}]_i$ (Figure 4b). In contrast, HA 1004 (10 μ M), another protein kinase inhibitor, which has little inhibitory action for protein kinase C, failed to restore the PdBu-induced inhibition of $[\text{Ca}^{2+}]_i$. These inhibitors alone did not alter the resting $[\text{Ca}^{2+}]_i$ or the 2-CA-induced increase in intracellular Ca^{2+} (results not shown). Our results indicated that protein kinase C is related to the regulation of the adenosine-induced Ca^{2+} mobilization in human thymocytes.

To determine the site at which protein kinase C activation inhibited calcium mobilization, the effect of PdBu on 2-CA-induced IP_3 release was studied. Preincubation of thymocytes with 60 ng/ml PdBu also inhibited the 2-CA-induced IP_3 formation by 100% (Table 5).

DISCUSSION

Timed induction of apoptosis appears to play an important role in normal cell turnover and development. Up to 90% of immature thymocytes die by apoptosis *in vivo*, and increasing evidence suggests that this phenomenon is a critical component of the intrathymic cell selection process that functions to prevent autoimmunity and to remove thymocytes expressing functionally inactive T cell receptors.

The present study has shown that human thymocytes exposed to adenosine undergo programmed cell death. The cell death induced by adenosine was dependent on active protein and RNA synthesis and involved activation of an endonuclease which resulted in DNA fragmentation similar to that observed in the glucocorticoid-induced process. Zn^{2+} ions, which were previously reported to inhibit endonuclease activation induced by glucocorticosteroids in thymocytes [34], also inhibited the adenosine-induced DNA fragmentation in this study.

It should be noted that, generally, human thymocytes showed a lower background level of DNA fragmentation with more pronounced differences between individual cultures than normally observed in rat thymocytes. This did not correlate with the age or the sex of the donors and may have reflected the stress situation induced by hospitalization and the operations performed on the children. Despite the differences in background level, the percentage increase in the amount of DNA fragmented after various treatments was similar in each individual.

Lymphocytes from various species have been reported to possess three types of adenosine receptors, all of which are linked to adenylate cyclase [3]. In the present study, the involvement of adenosine receptors in initiating programmed cell death was shown by using various adenosine receptor site agonists. 2-CA, CPA, NECA and CGS 21680 all induced DNA fragmentation, the order of potency being $CGS\ 21680 < NECA \leq CPA < 2-CA$. The observed response did not fall clearly into one of the two expected patterns for A_1 or A_2 receptors, as NECA and CPA were almost equipotent. The results with DPCPX showed that whereas the effect of CPA was inhibited by an A_1 -selective concentration of DPCPX (1 nM) [50,51], much higher concentrations (1 μ M) were required to inhibit 2-CA or NECA. This suggested that human thymocytes contain a mixture of A_1 and A_2 receptors, with NECA and 2-CA acting on the A_2 population and being inhibited by DPCPX with a dissociation constant in the micromolar range, whereas CPA acts on the A_1 receptors and is inhibited by DPCPX with a dissociation constant in the nanomolar range. The log concentration-response curve to CPA was somewhat steeper in the presence of 1 nM DPCPX, suggesting that at higher concentrations CPA may have been acting on the A_2 receptors as well. The finding that CGS 21680 was also effective at inducing DNA fragmentation showed that, of the A_2 receptors, at least the A_{2a} is present on the surface of human thymocytes.

Although adenosine has been reported to induce cell death by cAMP-mediated mechanisms in mouse thymocytes [16] other means of elevating intracellular concentrations of cAMP did not cause apoptosis in human thymocytes. This finding suggested that adenosine may trigger cell death by different mechanisms in human and mouse thymocytes. Since it has been previously demonstrated that a sustained increase in cytosolic Ca^{2+} concentration is required for the induction of the cell death programme by various stimuli in thymocytes, a series of experiments were carried out to investigate a possible role for Ca^{2+} in the adenosine-induced apoptosis. Our results indicated that the adenosine-induced cell death process is dependent on an early and sustained increase in cytosolic Ca^{2+} concentration, and that is further stimulated by the elevated intracellular cAMP level induced by adenosine.

Although it has been suggested that in human T lymphocytes cAMP-elevating agents can release Ca^{2+} from an intracellular pool [40], in human thymocytes the adenosine-induced calcium release was dependent on IP_3 formation and cAMP had no effect on it.

Although calcium ionophore or glucocorticoid treatments were as effective at inducing cell death in human thymocytes as

previously reported for rodent cells [20], it appears that 2-CA treatment affected a relatively smaller population of thymocytes. Thus, population analysis after 2-CA treatment revealed that, although 2-CA depleted mainly the $CD4^+CD8^+$ 'double positive' thymocyte population, the number of viable thymocytes in the $CD4^-CD8^+$ population also decreased. A more striking difference was found concerning the number of cells expressing different levels of the CD3 molecule. While MPS treatment depleted cells expressing a lower level of the CD3 molecule, 2-CA killed thymocytes expressing relatively higher CD3 levels. These results suggested that a more mature population of thymocytes is sensitive to 2-CA as compared with the cells that are killed by glucocorticoid treatment. Although there are no data available for possible differences in adenosine receptor expression during T cell development, our findings suggest that the expression of a functionally active adenosine receptor molecule may coincide with that of the functionally active CD3 T cell receptor. This suggestion is further supported by the findings which demonstrate that previous addition of OKT3 antibody prevents the adenosine-induced calcium release.

A previous paper [52] suggests that adenosine (and cAMP) mediate their effect on mouse thymocytes via modification of the glucocorticoid receptor activity. Our finding that adenosine and MPS affect different populations excludes this possibility in human thymocytes.

In thymocytes a complex signalling system determines whether a cell enters the cell death programme, or is positively selected and differentiates to a functionally mature T cell. Although the mechanism of positive selection is not fully understood and the extracellular signal(s) involved are not known yet, there is increasing evidence that the activation of protein kinase C may be involved positively in this process [49]. Our results provide further evidence for this hypothesis by showing inhibition of the adenosine-induced cell death process by PdBu, a known activator of protein kinase C. From this point of view it is worth noting that adenosine depletes a thymocyte subpopulation which already expresses high levels of the T cell receptor. The presence of the functionally acceptable T cell receptor is required for the T cell receptor-mediated positive selection [53]. During this positive selection process protein kinase C may be activated and can protect thymocytes against the adenosine-induced cell death. Although the sites at which protein kinase C activation interferes with the signalling process in apoptosis induced by various stimuli may be very different, the data presented in this paper suggest that in the adenosine-triggered signalling pathway protein kinase C activation inhibits an early step, namely the immediate release of IP_3 and the consequent increase in cytosolic Ca^{2+} concentration.

Our previous work [54–56] has demonstrated that adenosine may have a role in controlling the proliferation of mitogen-stimulated mature peripheral lymphocytes. The data presented in this paper suggest a specific role for adenosine in also controlling the selection of human thymocytes expressing functionally active T cell receptors in the later phases of T cell development. The effect of adenosine may be additional to that of the glucocorticoid hormones, affecting a more mature population of thymocytes. There are no data available on the extracellular adenosine concentrations within the thymus, but because of the very high rate of cell death adenosine may be constantly released from the dying cells. This could provide a high extracellular adenosine concentration in the deeper cortical layers of the thymus, sufficient to induce apoptosis of the more mature thymocytes.

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