Journal of Cellular Biochemistry 96:1296–1310 (2005)

Differential Effect of Insulin and Elevated Glucose Level on Adenosine Handling in Rat T Lymphocytes

Tadeusz Pawelczyk,[1]* Monika Sakowicz-Burkiewicz,[1] Katarzyna Kocbuch,[1] and Andrzej Szutowicz[2]

[1]Department of Molecular Medicine, Medical University of Gdansk, 80-211 Gdansk, Poland
[2]Departemnt of Laboratory Medicine, Medical University of Gdansk, 80-211 Gdansk, Poland

Abstract Reduced proliferation potential is among other T cell functional defects long known feature of diabetes. However, the mechanism responsible for this impairment is still unknown. Our study was undertaken to investigate the effect of changes in glucose and insulin concentrations on adenosine metabolism, transport and receptor-mediated action in rat T lymphocytes. Presented results indicate that vulnerability of T cells to metabolic stress is determined by insulin but not by glucose concentration. However, glucose and insulin differentially affected the activities of adenosine metabolizing enzymes in resting and proliferating T cells. The Con A-induced proliferation of cultured T lymphocytes did not depended on expression level and functional state of nucleoside transporters. Inhibition of adenosine kinase (AK) with 5-iodotubercidin lowers the proliferation potential of T cells to the level observed for insulin-deprived cells. Moreover, insulin-deprived T lymphocytes but not cells cultured in the presence of insulin released significant quantities of adenosine. Under resting conditions, the cAMP level was fivefold higher in cells deprived of insulin comparing to cells cultured in the presence of insulin. Exposition of insulin-deprived T lymphocytes to specific antagonist (ZM241385) of A2a receptor but not to specific antagonist (Alloxazine) of A2b receptor suppressed cAMP elevation and completely restored the proliferation potential of T cells. Concluding, adenosine released by insulin-deprived T cells due to suppressed AK activity by acting on A2a receptors leads to increases in cAMP level and suppression of T cell proliferation. We assume that this mechanism may significantly contribute to immune impairment observed in diabetes. J. Cell. Biochem. 96: 1296–1310, 2005.

Key words: glucose; insulin; adenosine metabolizing enzymes; nucleoside transporters; adenosine receptors; cAMP; T cell proliferation

INTRODUCTION

Several aspects of immunity are altered in diabetic patients. Clinical observations indicate that diabetic patients are more vulnerable to some specific infections (mostly caused by bacteria and fungi) and ultimate complications [Joshi et al., 1999]. Since, cell-mediated immunity is mediated by T lymphocytes (beside macrophages) its prolonged suppression could account for impaired host defense against intracellular pathogens such bacteria and fungi. Studies on human peripheral blood mononuclear cells from diabetic patients demonstrated decreased basal production of cytokines [Kaye et al., 1986; Pickup et al., 2000]. Moreover, diabetic patients display suppressed proliferative response of T cells to the primary protein antigens [Schloot et al., 1997; Eibl et al., 2002]. It was reported that IL-2 is unable to restore the decreased proliferative response of diabetic lymphocytes to phytohemagglutinin (PHA) [Chang and Shaio, 1995]. Authors of that report suggested that the impaired proliferation of lymphocytes in diabetic patients might be the result of reduced percentage of interleukin-2-receptor-bearing cells together with decreased expression of complement receptor CR-3. Nevertheless, the mechanisms responsible for impaired lymphocyte proliferation in diabetic patients are largely unclear.

Grant sponsor: State Committee for Scientific Research (KBN) (to TP); Grant number: 2 P05A 081 29.

*Correspondence to: Tadeusz Pawelczyk, Department of Molecular Medicine, Medical University of Gdansk, ul. Debinki 7, paw. 29, 80-211 Gdansk, Poland.
E-mail: tkpaw@amg.gda.pl

Received 2 June 2005; Accepted 9 August 2005
DOI 10.1002/jcb.20642

Cell proliferation is associated with activation of multiple biochemical activities necessary for membrane lipid biosynthesis, protein glycosylation, RNA, and DNA synthesis. These biochemical processes require appropriate fuel supply in order to proceeds. Recent evidence has shown that the rate of glucose and glutamine oxidation was significantly decreased in diabetic rat lymphocytes [Otton et al., 2002]. This may lead to impairment in energy provision especially under conditions of increased energy consumption and elevation of nucleotides catabolites level including adenosine.

The importance of adenosine for the function of the immune system has been documented by many experimental and clinical observations [Birch and Polmar, 1982; Hershfield, 2005]. Adenosine can affect T-lymphocyte activation, proliferation, cytokine production, and cell mediated cytolysis [Wolberg et al., 1975; Dos-Reis et al., 1986; Antonysamy et al., 1995; Apasov et al., 1995]. Under normal conditions adenosine metabolism and transport processes maintain its local concentration relatively constant. Previously we reported that the expression levels of nucleoside transporters in T lymphocytes isolated from diabetic rat were significantly altered concomitant with impaired adenosine transport [Sakowicz et al., 2004]. Moreover, the expression level of adenosine kinase (AK) was greatly reduced in diabetic T cells [Pawelczyk et al., 2003]. In this report we demonstrate that rat T lymphocytes cultured under conditions of insulin deprivation are more vulnerable to metabolic stress than normal cells and release under metabolic load increased quantities of adenosine, which suppress Con A-induced proliferation of T cells.

MATERIALS AND METHODS

Reagents

Histopaque-1077, insulin, penicillin, streptomycin, glucose, nitrobenzyltioinosine, adenosine, Con A, RPMI-1640 medium, nonselective A2 receptor antagonist 3,7-dimethyl-1-propargylxanthine (DPMX), and the selective A2b antagonist Alloxazine were obtained from Sigma-Aldrich Sp. z o.o. (Poznan, Poland). The selective A2a antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4-a][1,3,5]triazin-5-ylamino]ethyl)-phenol (ZM 241385) was purchased from Tocris Bioscence (Nortpoint, UK). 5-Iodotubercidin was from ICN Pharmaceuticals (Irvine, CA). [^{3}H] thymidine was from Amersham (Buckinghamshire, England). [8-^{14}C] adenine was from Moravek Biochemicals, Inc., (Brea, CA). TLC sheets DC Alufolien Kieselgel 60 F_{254} were from Merck Sp. z o.o. (Warsaw, Poland). Fluorescein conjugate mouse anti rat CD2[LFA-2] clone OX-34 was from Chemicon International (Hofheim, Germany). Fluorescein conjugate mouse anti rat CD3 clone G4.18 was from BD Bioscences (Heidelberg, Germany).

Cells and Culture Conditions

T cells were isolated from male Wistar rat spleen as described previously [Sakowicz et al., 2004]. Briefly, mononuclear cells were isolated by centrifugation of the cell suspension through Histopaque-1077 at 700*g* for 30 min at room temperature. The cells were then separated into adhesive and nonadhesive by the panning method [Severson et al., 1987] relying on incubation (1 h at 37°C) of cell suspension in the presence of 3% BSA in plastic bottles with surface for adhesive cells (Sarstedt AG & Co., Numbrecht, Germany). Following incubation, the nonadhesive cells were collected by centrifugation and suspended in RPMI-1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum. The cells were analyzed for surface phenotype by flow cytometry. The nonadherent fraction (T cells) contained 92%–97% CD2 (OX-34) and 86%–90% CD3 (G4.18) positive cells. The number of viable cells was determined by Trypan Blue dye exclusion. Only cell preparations with a 95% viability or greater were used. Cells were cultured in flat-bottomed culture bottles in humidified atmosphere containing 5% CO_2 at 37°C at a density of $0.5–1 \times 10^6$ cells/ml in a total volume of 6 ml RPMI-1640 medium supplemented with antibiotics (at concentrations as stated above) and 10% fetal bovine serum, and containing glucose and insulin at concentration and for the time detailed in the figure legends.

Ex vivo Proliferation Assay

For proliferation assays the purified cells were cultured at concentration of $4–8 \times 10^5$ cells/ml for 48 h in RPMI-1640 medium at glucose and insulin concentrations as indicated. The inhibitors and adenosine receptors antagonists were added at concentrations and for time duration as indicated in figure legends. On the third day T cells were stimulated with Con A at

the indicated concentrations for 48 h and then counted. The number of viable cells for each condition was measured by trypan blue exclusion with a hemocytometer. In order to analyze the effect of Con A on DNA synthesis, the medium was replaced (after 48 h) by the same medium containing 1 μCi/ml [H^3]-thymidine and the cells were incubated for 8 h, and then harvested by centrifugation. The cells were washed three times with PBS and the radioactivity was measured using Wallac 1409 liquid-scintillation counter.

Measurement of Purine Nucleotides Level in the Cells

The level of ATP, ADP, and AMP in the cells was determined by HPLC using PerkinElmer Instrument consisting of the Series 200 LC pump, the 2001c Autosampler, the series 200 Diode Array Detector II set for 254 nm, and the TotalChrom Client/Server. The nucleotides were separated on Supelcosil LC-18-T 5 μm column (25 cm × 0.45 cm). The column was washed with 50 mM sodium phosphate buffer, pH 6.0, containing 4 mM tetrabutylammonium hydrogen sulfate and eluted with linear gradient from 0% to 30% methanol over 3 ml (flow rate 1.2 ml/min).

Measurement of ATP Catabolism and Adenosine Release

In order to evaluate the adenosine release during ATP catabolism cells were first incubated for 1 h with 10 μCi of [8-^{14}C]adenine to label intracellular ATP. After 1 h the cells were washed with fresh growth medium and resuspended in glucose-free growth medium (10^6 cells/0.1 ml) and incubated in the presence of 5 mM 2-deoxyglucose. At appropriate (indicated on Figures) time points 200 μl of cell suspension was withdrawn, centrifuged and resulting supernatant (culture medium) and pellet (cells) were extracted with 0.4 M perchloric acid. Obtained perchloric acid extracts were neutralized and purine compounds were separated on silica gel (with fluorescent indicator) aluminum plate. In order to localize the purine compounds on the same place together with analyzed sample, purine nucleotides (ATP, ADP, AMP) or adenosine, inosine, and hypoxanthine standards were spotted. For nucleotides separation the plate was developed in 1,4 dioxane/25% ammonia/water (6:1:3.8 v/v/v/v). For separation of purine nucleosides and bases the plate was developed in butan-1-ol/methanol/ethyl acetate/ammonia (7:3:4:4, v/v/v/v). Purine compounds were located under UV, the spots were cut out and the radioactivity was counted.

Measurement of Enzyme Activities

The cells (40×10^6) were suspended in 0.5 ml of 50 mM Tris-HCl buffer, pH 7.2, containing 0.2 mM Pefabloc SC, and 5 μM leupeptin, and sonicated (2 × 10 s). Resulting cell extract was centrifuged at 50,000*g* for 45 min, and obtained supernatant was stored at −20°C as the cytosolic fraction. The sediment from 50,000*g* centrifugation was washed twice by suspension in homogenization buffer. The pellet was finally suspended in homogenization buffer containing 0.2% Triton X-100 and homogenized. The resulting homogenate was used as membrane fraction. The enzymes activities were assayed as described previously [Pawelczyk et al., 1992]. The activities of 5′-nucleotidase (5′-NT), adenosine deaminase (ADA), and AMP deaminase were measured spectrophotometrically with 100 μM AMP as substrate. The activity of AK was assayed by the radiochemical method with 1 μM adenosine as substrate. All enzyme assays were done under conditions where the product formation was linear with time and with the amount of added protein during consumption of up to 20% of the substrate. The enzyme activities were measured at 25°C.

Real-time PCR Analysis

The levels of nucleoside transporters transcripts were analyzed by real-time PCR performed in a Light Cycler 2.0 (Roche, Mannheim, Germany) using the Light Cycler DNA SYBR Green I Kit. The reaction mixture contained 2 μl Master Mix, 1 pmol of each primer, and 1 μl of cDNA. For β-actin cDNA amplification GAAATCGTGCGT GACATTAAG (forward) and CTAGAAGCATTTGCGGTGGA (reverse) primers were used. This pair of primers based on cDNA sequences from GenBank; accession number NM031144. For rENT1 cDNA amplification CCCTGGTAAGGTGGAGA TGG (forward) and GCCACTGAGGAAGAGGGTGCTG (reverse) primers were used. For rENT2 cDNA amplification CTTCTTCATTACCGCCATCCCG (forward) and GCCACTGAGGAAGAGGGTGCTG (reverse) primers were used. For rCNT2 cDNA amplification GGAGCTCATGGAAG TCGGAAC (forward) and CCATGA ACACCCTCTTAAGCCA (reverse) primers were used. The

rENT1, rENT2, and rCNT2 primers were based on cDNA sequences from GenBank; accession number AF015304, AF015305, and U66723, respectively. As negative controls water and yeast tRNA was run with every PCR. In order to control the PCR product melting curve analysis was performed. The ratio of β-actin and AR was calculated for each sample. Analysis of the data was done using Light Cycler software 4.0.

Statistical Analysis

The statistical analysis was carried out using the STATISTICA 5PL statistical package (StatSoft). Statistical significance was determined using the *t*-test. *P* values below 0.05 were considered as significant.

RESULTS

ATP Catabolism and Release of Purine Nucleosides

In T lymphocytes ATP seems to be the major source for adenosine formation, therefore, we determined the level of adenine nucleotides in cells cultured at different glucose and insulin concentrations. The insulin concentration in the culture medium supplemented with 10% fetal bovine serum was in the range of 1–3×10^{-11} M. Our experiments indicated (not shown) that at such a low concentration insulin has no effect on any measured parameters, therefore, hereafter cells cultured in medium containing 10% fetal bovine serum are considered as cells cultured in the absence of insulin. The levels of ATP, ADP, and AMP in T cells cultured in the absence of insulin were slightly lower than levels of these nucleotides determined in cells cultured in the presence of insulin irrespectively of glucose concentration, but these differences were not significant statistically (Table I).

In order to examine the influence of glucose and insulin on the rate of ATP degradation induced by metabolic stress the cells were labeled with [8-^{14}C]adenine. After 1 h incubation, the radioactivity was almost exclusively (more than 90%) incorporated into ATP (not shown). ADP and AMP contained less than 8% of radioactivity. There were no significant differences in the levels of radiolabeled ATP in cells cultured at low and high glucose irrespectively of insulin presence (Fig. 1). To study the effect of metabolic stress on ATP catabolism, the cells were transferred to appropriate (with or without insulin) glucose-deficient RPMI medium and after 1 h incubation ATP degradation was induced by addition of 2-deoxyglucose (5 mM). During the first 10 min from addition of 2-deoxyglucose, the decline rate of cellular ATP level was significantly faster in cells cultured originally in the absence of insulin irrespective of glucose concentration (Fig. 1). In 10 min after addition of 2-deoxyglucose about 60% and 80% of ATP was degraded in cells cultured in the presence and absence of 10 nM insulin, respectively. During incubation of T cells under noninduced conditions (first hour) there was no detectable adenosine, and very small level of inosine, and hypoxanthine in incubation medium containing 10 nM insulin, whereas, significant quantities of adenosine were detected in medium containing no insulin (Fig. 2). Accelerated ATP catabolism induced by 2-oxoglucose was accompanied by appearance of adenosine, inosine, and hypoxanthine in cell culture medium. There were marked differences between the cell cultures in the amount and ratio of these nucleosides and base generation. The levels of adenosine, inosine, and hypoxanthine measured in incubation medium of cells cultured in the absence of insulin rose significantly faster and to the higher levels then

TABLE I. Purine Nucleotides Levels in Cultured Rat T Lymphocytes Under Resting Conditions

	ATP	ADP	AMP
Culture conditions	(nmol/10^6 cells)		
5 mM glucose, 10 nM insulin	1.51 ± 0.12	0.17 ± 0.05	0.08 ± 0.02
5 mM glucose, no insulin	1.42 ± 0.11	0.16 ± 0.03	0.07 ± 0.02
20 mM glucose, 10 nM insulin	1.38 ± 0.10	0.14 ± 0.04	0.06 ± 0.01
20 mM glucose, no insulin	1.33 ± 0.10	0.13 ± 0.02	0.06 ± 0.02

T lymphocytes were cultured for 2 days in medium containing glucose and insulin at concentrations as indicated. No insulin refers to concentration lower than 2×10^{-11} M. On third day cells (60×10^6) were harvested and nucleotides were determined by HPLC as described in "Materials and Methods." The data represent the mean ± SD from three independent experiments.

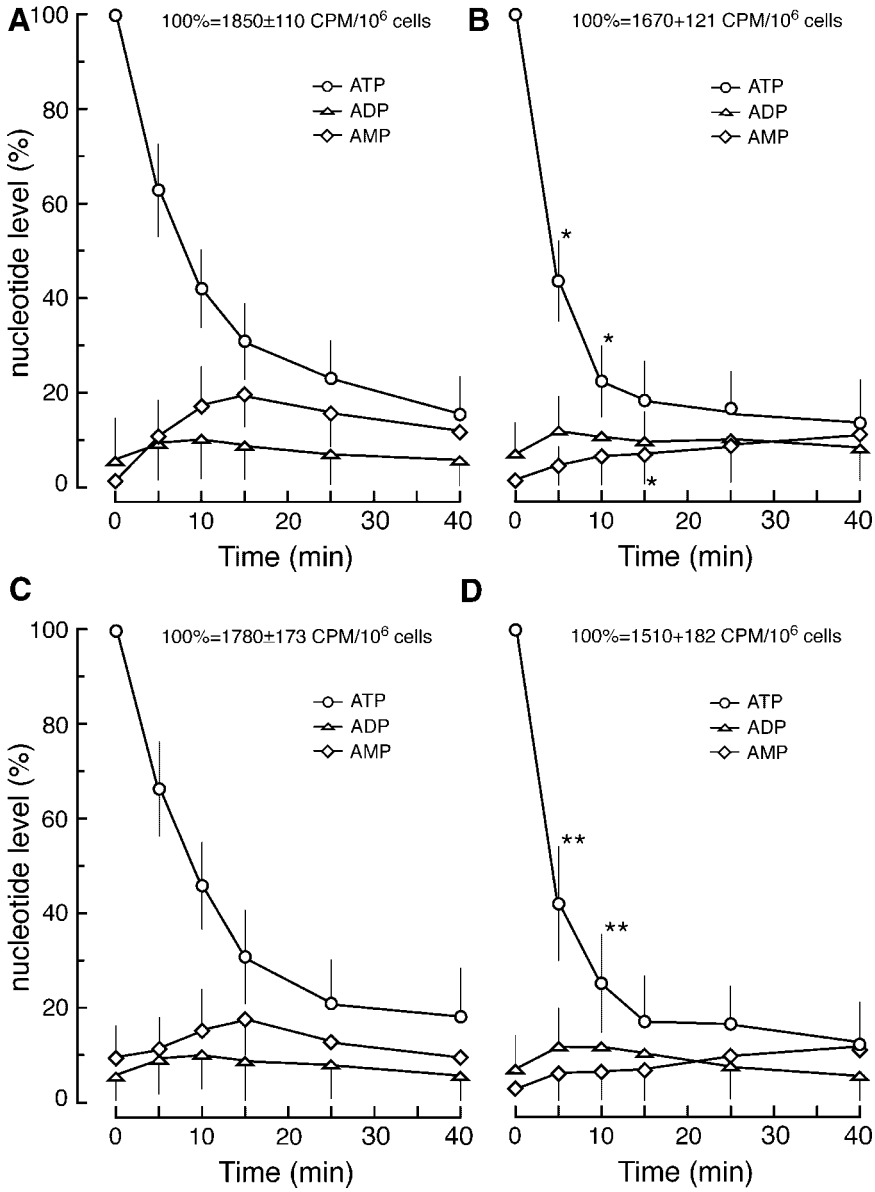

Fig. 1. 2-deoxyglucose-induced ATP degradation in rat T lymphocytes. T lymphocytes were cultured for 2 days in medium containing 5 mM glucose and 10 mM insulin (**A**), or in medium containing 5 mM glucose and no insulin (**B**), or in medium containing 20 mM glucose and 10 nM insulin (**C**), or in medium containing 20 mM glucose and no insulin (**D**). No insulin refers to concentration less than 2×10^{-11} M. On third day cells were labeled with [8-^{14}C]adenine as described in ''Materials and Methods.'' After washing out unincorporated radioactivity the cells were transferred to the glucose-free culture medium, with (A, C) or without (B, D) 10 nM insulin and incubated for 1 h. After that time 2-deoxyglucose was added to culture medium to reach 5 mM concentration. At indicated times, 2×10^6 cells were withdrawn and the level of radioactivity in purine nucleotides was analyzed by TLC as described in ''Materials and Methods.'' The data represent the mean ± SD from three independent experiments. (*) $P < 0.05$ versus (5 mM gluc + Ins); (**) $P < 0.05$ versus (20 mM gluc + Ins).

levels of these compounds in medium of cells cultured in the presence of 10 nM insulin irrespectively of glucose concentration (Fig. 2). These results indicated that vulnerability of T cells to metabolic stress evaluated by the rate of ATP level decline and adenosine outflow from the cell is determined by insulin presence but not by glucose concentration.

Glucose and Insulin Differentially Affect the Activities of Adenosine Metabolizing Enzymes in Resting and Proliferating T Lymphocytes

Under resting conditions total (measured in whole cell lysate) activity of adenosine deaminase (ADA) increased in cells cultured at high glucose concentration irrespectively of insulin

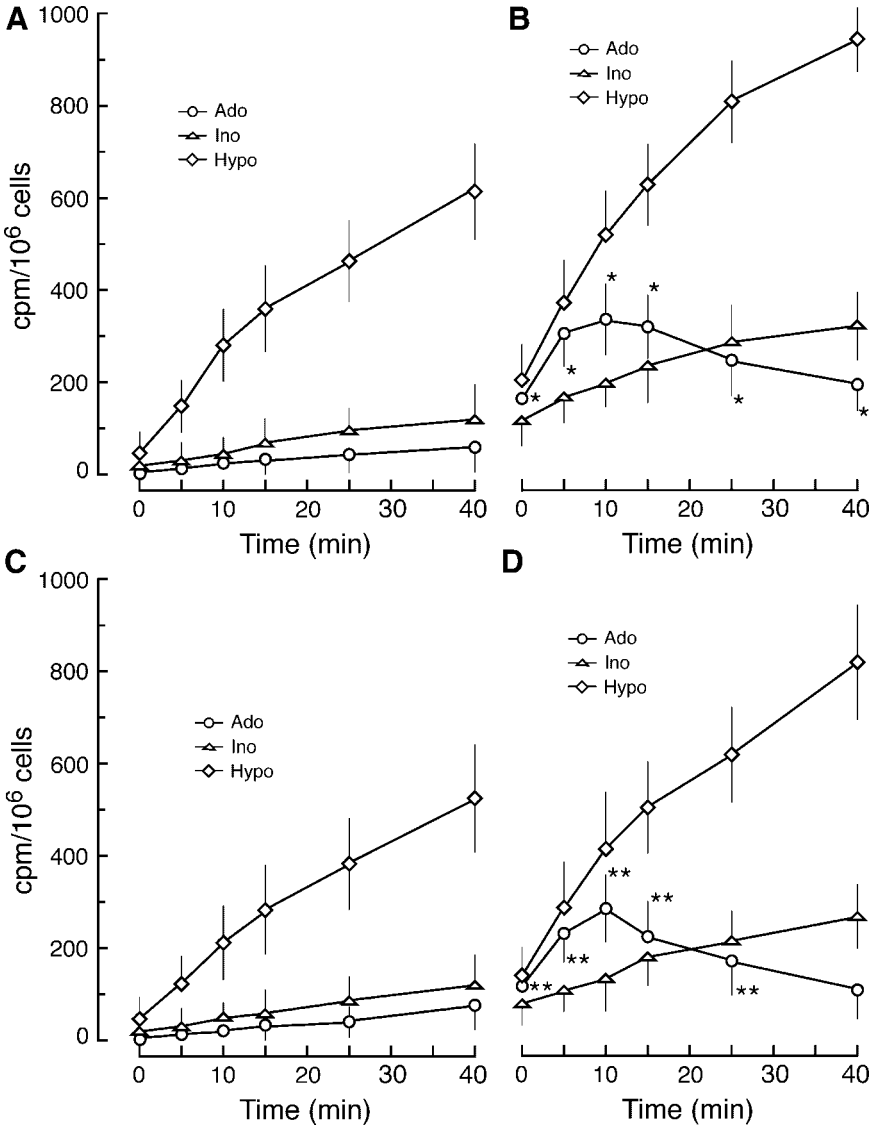

Fig. 2. Outflow of purine nucleosides and bases from T lymphocytes during 2-deoxyglucose-induced ATP degradation. T lymphocytes were cultured for 2 days in medium containing: 5 mM glucose and 10 nM insulin (**A**); 5 mM glucose and no insulin (**B**); 20 mM glucose and 10 nM insulin (**C**); 20 mM glucose and no insulin (**D**). No insulin refers to concentration less than 2×10^{-11} M. On third day cells were labeled with [8-^{14}C]adenine as described in ''Materials and Methods.'' After washing out unincorporated radioactivity the cells were transferred to the glucose-free culture medium (12×10^6 cells/ml), with (A, C) or without (B, D) 10 nM insulin and incubated for 1 h. After that time 2-deoxyglucose was added to reach 5 mM concentration. At indicated times, 200 µl of the culture medium was withdrawn and the level of radioactivity in adenosine, inosine, and hypoxanthine was analyzed by TLC as described in ''Materials and Methods.'' The data represent the mean ± SD from four (A, B) and three (C, D) independent experiments. (*) $P < 0.05$ versus (5 mM gluc + Ins); (**) $P < 0.05$ versus (20 mM gluc + Ins).

presence (Table II). However, the activity of ADA in cytosolic fraction of T cells cultured at high glucose concentration (20 mM) was not changed and the observed increases in ADA activity originated from membrane fraction where ADA activity raised twofold. Similar increase (twofold) in activity of ecto 5″-nucleotidase were observed in membranes of T cells cultured at high glucose concentration but this was not associated with statistically significant increases in total activity of 5′-nucleotidase (5′-NT) (Table II). Activity of AK was the only enzymatic activity dependent on insulin presence. T lymphocytes cultured in medium without insulin displayed 70%–75% reduction in AK activity compared to cells incubated in the presence of 10 nM insulin irrespective of glucose concentration (Table II).

It was reported previously that stimulation of human T lymphocytes with PHA leads to

TABLE II. Differential Effect of Insulin and Glucose on Adenosine Metabolizing Enzymes in Cultured Rat T Lymphocytes

Enzyme	Enzyme activity (nmol/min/mg)[a]			
	5 mM glucose, 10 nM insulin	5 mM glucose, no insulin	20 mM glucose, 10 nM insulin	20 mM glucose, no insulin
5′-nucleotidase				
Total	2.61 ± 0.40	2.70 ± 0.36	3.12 ± 0.44	3.20 ± 0.48
Cytoplasm	2.46 ± 0.37	2.31 ± 0.41	2.43 ± 0.34	2.53 ± 0.36
Membranes	$0.24 \pm 0.05^{b,c}$	$0.29 \pm 0.08^{b,c}$	$0.66 \pm 0.18^{d,e}$	$0.57 \pm 0.15^{d,e}$
AMP deaminase				
Total	9.13 ± 1.34	10.25 ± 2.04	11.08 ± 2.73	12.19 ± 2.61
Cytoplasm	8.27 ± 1.29	9.11 ± 1.46	8.92 ± 2.04	11.41 ± 2.48
Membranes	ND	ND	ND	ND
Adenosine deaminase				
Total	$90.41 \pm 6.08^{b,c}$	$93.76 \pm 5.82^{b,c}$	$108.31 \pm 5.10^{d,e}$	$113.73 \pm 5.71^{d,e}$
Cytoplasm	80.23 ± 5.31	79.41 ± 4.22	83.25 ± 4.94	85.05 ± 4.80
Membranes	$9.07 \pm 1.16^{b,c}$	$11.32 \pm 1.29^{b,c}$	$18.50 \pm 1.86^{d,e}$	$21.21 \pm 2.53^{d,e}$
Adenosine kinase				
Total	$29.37 \pm 2.14^{c,e}$	$8.44 \pm 0.73^{b,d}$	$30.17 \pm 1.97^{c,e}$	$7.86 \pm 0.54^{b,d}$
Cytoplasm	$27.91 \pm 1.90^{c,e}$	$7.61 \pm 0.52^{b,d}$	$29.93 \pm 1.18^{c,e}$	$7.32 \pm 0.49^{b,d}$
Membranes	ND	ND	ND	ND

T lymphocytes were cultured for 2 days in medium containing glucose and insulin at concentrations as indicated. No insulin refers to concentration lower than 2×10^{-11} M. On third day cells (50×10^6) were harvested and enzymes activities in whole homogenates (total) and cytosolic and membrane fractions were determined as described in "Materials and Methods." The data represent the mean ± SD from five independent experiments.
[a]Enzymes activities are presented in nmol/min/mg of total protein ($\sim 23 \times 10^6$ cells).
[b]$P < 0.05$ versus (20 mM glucose, 10 nM insulin).
[c]$P < 0.05$ versus (20 mM glucose, no insulin).
[d]$P < 0.05$ versus (5 mM glucose, 10 nM insulin).
[e]$P < 0.05$ versus (5 mM glucose, no insulin).

alteration of several pathways of nucleotide metabolism and to changes in activities of adenosine metabolizing enzymes [Snyder et al., 1976; Barankiewicz and Cohen, 1987]. Therefore, in our experiments we examined the impact of various glucose and insulin concentrations on activities of enzymes metabolizing adenosine in proliferating T lymphocytes. In Con A-induced T cells changes in the activities of investigated enzymes depended on glucose and insulin concentrations in the culture medium. The cytosolic activity of 5′-NT decreased significantly in cells proliferating in the presence of 5 mM glucose and 10 nM insulin, whereas in membrane fraction its activity raised (Table II and III). On the other hand, no changes in 5′-NT activity were observed in cells proliferating in the absence of insulin regardless of glucose concentration. The activity of AMP deaminase increased in proliferating T cells regardless insulin and glucose concentrations. Comparing to nonproliferating cells (Table II) there was no change in activity of ADA in cells proliferating in the presence of insulin irrespective of glucose concentration (Table III). In the absence of insulin cytosolic activity of ADA decreased in proliferating T cells. On the other hand, ADA activity in the membrane fraction of cells proliferating in the absence of insulin depended on glucose concentration. During T cells proliferation activity of AK did not changed regardless insulin and glucose concentrations (Table III).

Proliferation Potential of T Lymphocytes Depends on Insulin Presence but not on Glucose Concentration

In order to asses the impact of changes in glucose and insulin concentration on proliferation potential of T cells we stimulated cultured cells with various concentrations of Con A. Performed experiments showed that T cells cultured in the absence of insulin in response to stimulation by Con A proliferate less than cells cultured in the presence of 10 nM insulin irrespective of glucose concentration (Fig. 3A). Although, in the absence of insulin we observed slightly lower proliferation rate (~10%) of cells cultured at 20 mM glucose comparing to cells cultured at 5 mM glucose but this difference did not reach the statistical significance. The maximal effect of insulin on Con A-induced T cells proliferation was observed at 10 nM concentration, and only slight increase in proliferation rate was seen with 100 nM insulin (Fig. 3B).

TABLE III. Con A-induced Changes in Activities of Adenosine Metabolizing Enzymes in Cultured Rat T Lymphocytes

	Enzyme activity (nmol/min/mg)[a]			
Enzyme	5 mM glucose, 10 nM insulin	5 mM glucose, no insulin	20 mM glucose, 10 nM insulin	20 mM glucose, no insulin
5′-nucleotidase				
Total	$1.61 \pm 0.36^{c,e,f}$	$2.79 \pm 0.43^{b,d}$	$1.82 \pm 0.44^{c,e,f}$	$3.11 \pm 0.48^{b,d}$
Cytoplasm	$0.76 \pm 0.28^{c,e,f}$	2.45 ± 0.47^{d}	$0.97 \pm 0.34^{c,e,f}$	$2.38 \pm 0.32^{b,d}$
Membranes	$0.64 \pm 0.13^{c,f}$	0.31 ± 0.09^{b}	0.69 ± 0.15^{c}	0.54 ± 0.14^{c}
AMP deaminase				
Total	$12.66 \pm 1.72^{c,e,f}$	$15.16 \pm 1.81^{b,f}$	16.02 ± 1.64^{f}	$17.69 \pm 2.20^{b,f}$
Cytoplasm	$11.51 \pm 1.34^{c,e,f}$	$14.11 \pm 1.52^{b,f}$	$15.82 \pm 1.73^{b,f}$	$16.53 \pm 1.84^{b,f}$
Membranes	ND	ND	ND	ND
Adenosine deaminase				
Total	$87.31 \pm 5.10^{c,e}$	$68.21 \pm 4.61^{b,d,f}$	$102.04 \pm 5.73^{b,c,e}$	$71.24 \pm 5.28^{b,d,f}$
Cytoplasm	$78.23 \pm 4.74^{c,e}$	$53.15 \pm 4.17^{b,d,f}$	$79.28 \pm 4.61^{c,e}$	$61.11 \pm 4.17^{b,d,f}$
Membranes	9.50 ± 1.32	10.79 ± 1.29	$19.62 \pm 1.72^{b,c,e}$	8.42 ± 1.21^{f}
Adenosine kinase				
Total	$30.62 \pm 2.71^{c,e}$	$9.18 \pm 0.73^{b,d}$	$32.17 \pm 2.82^{c,e}$	$8.65 \pm 0.43^{b,d}$
Cytoplasm	$28.80 \pm 2.12^{c,e}$	$8.64 \pm 0.64^{b,d}$	$30.33 \pm 2.36^{c,e}$	$8.13 \pm 0.51^{b,d}$
Membranes	ND	ND	ND	ND

T lymphocytes were cultured for two days in medium containing glucose and insulin at concentrations as indicated. No insulin refers to concentration lower than 2×10^{-11} M. On the third day T cells were stimulated with Con A (2.5 μg/ml) and after 48 h the cells ($\sim 50 \times 10^6$) were harvested and enzymes activities in whole homogenates (total) and cytosolic and membrane fractions were determined as described in "Materials and Methods." The data represent the mean ± SD from four independent experiments.
[a]Enzymes activities are presented in nmol/min/mg of total protein ($\sim 23 \times 10^6$ cells).
[b]$P < 0.05$ versus (5 mM glucose, 10 nM insulin).
[c]$P < 0.05$ versus (5 mM glucose, no insulin).
[d]$P < 0.05$ versus (20 mM glucose, 10 nM insulin).
[e]$P < 0.05$ versus (20 mM glucose, no insulin).
[f]$P < 0.05$ versus cells not induced with Con A.

Proliferation of T Lymphocytes Does Not Depend on Nucleoside Transporers

Previously we have reported that the expression level of nucleoside transporters (NT) is altered in diabetic rat T lymphocytes leading to decreased adenosine uptake by these cells [Sakowicz et al., 2004]. In the present study we examined the impact of alterations in NT expression level on proliferation rate of T cells. Rat T lymphocytes express ENT1, ENT2, and CNT2 transporter. Expression of ENT1 is inhibited by high glucose concentration but is not influenced by insulin, which suppresses expression of CNT2 and stimulates expression of ENT2 transporter (Fig. 4A). During T cell proliferation the expression level of ENT2 did not change regardless of insulin and glucose concentration. Expression of CNT2 decreased by ~50% in cells proliferating in medium with no insulin irrespective of glucose concentration, whereas 50% decrease of ENT1 expression was observed in T cells proliferating in high glucose medium regardless of insulin concentration (Fig. 4B). Comparison of T cells proliferation rate with changes in the expression level of NT showed that there was no relation between these two processes. T lymphocytes cultured in the presence of insulin (10 nM) and low (5 mM) or high (20 mM) glucose displayed the same proliferation potential (Fig. 5) despite significant changes in NT expression level (Fig. 4). Previously we have demonstrated that overall adenosine uptake by T cells cultured at 5 mM glucose and 10 nM insulin was twofold higher than adenosine uptake by cells cultured at 20 mM glucose and 10 nM insulin [Sakowicz et al., 2004]. On the other hand, adenosine uptake by T cells cultured at 20 mM glucose in the presence or absence of insulin was the same, whereas here we demonstrate that the proliferation rate of cells cultured at 20 mM glucose depended on insulin (Fig. 5). Moreover, the presence of 1 μM NBTI (an inhibitor of ENT1) in the incubation medium have no effect on T cells proliferation rate (Fig. 5) despite significant decreases in adenosine uptake [Sakowicz et al., 2004]. These results suggested that other factors than altered NT function are responsible for impaired proliferation of T lymphocytes deprived of insulin.

T Cell Proliferation Depends on Adenosine Kinase Activity

Our experiments showed that Con A-induced T cells proliferation depends on insulin but not

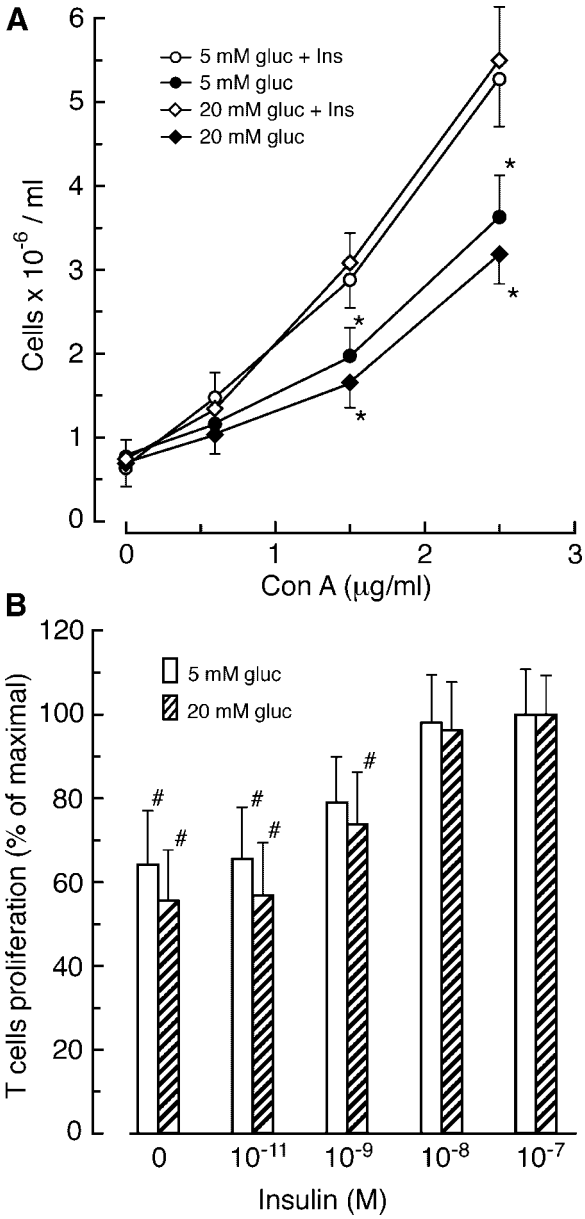

Fig. 3. The insulin and glucose effect on ConA-induced proliferation of rat T lymphocytes. T cells isolated from normal rat were cultured as described under ''Materials and Methods'' for 2 days in the medium containing: (**A**) 5 mM glucose and 10 nM insulin (5 mM gluc + Ins); 5 mM glucose and no insulin (5 mM gluc); 20 mM glucose and 10 nM insulin (20 mM gluc + Ins); 20 mM glucose and no insulin (20 mM gluc); (**B**) no serum and at glucose and insulin concentrations as indicated. Concentration (10^{-11} M) of insulin determined in RPMI medium supplemented with 10% fetal bovine serum. On the third day T cells were stimulated with Con A at concentrations as indicated (A) or 2.5 μg/ml (B) and after 48 h the number of viable cells was determined as described in ''Materials and Methods.'' The data represent the mean ± SD from five (A) or three (B) independent experiments. (*) $P < 0.05$ versus (5 mM gluc + Ins); (#) $P < 0.05$ versus (10^{-7} M insulin).

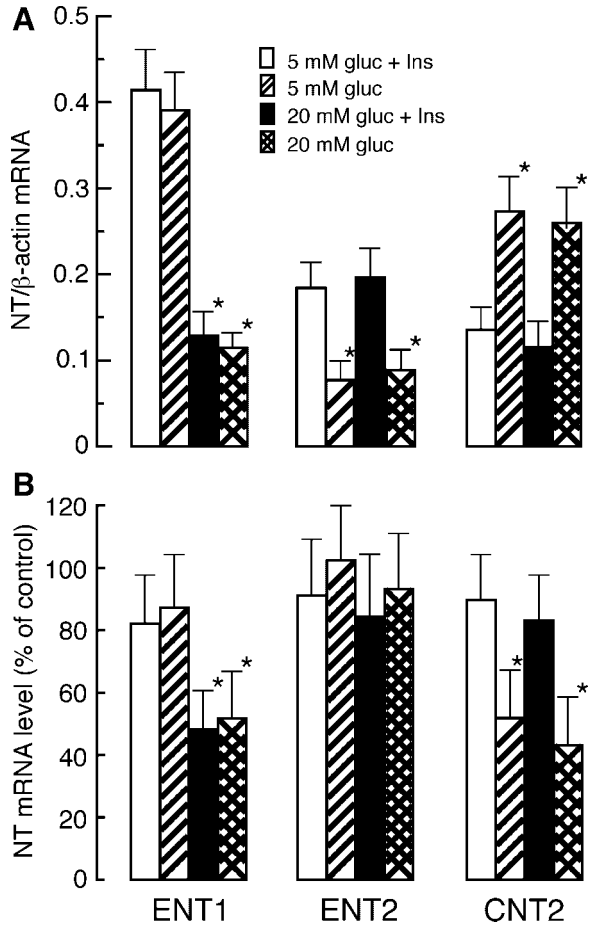

Fig. 4. Differential effect of insulin and glucose on expression of nucleoside transporters in rat T lymphocytes. T cells isolated from normal rat were cultured as described under ''Materials and Methods'' for 2 days in the medium containing: 5 mM glucose and 10 nM insulin (5 mM gluc + Ins); 5 mM glucose and no insulin (5 mM gluc); 20 mM glucose and 10 nM insulin (20 mM gluc + Ins); 20 mM glucose and no insulin (20 mM gluc). No insulin refers to concentration lower than 3×10^{-11} M. On third day: cells were harvested and levels of NT mRNAs were determined by real-time PCR as described in Materials and Methods (**A**); or 2.5 μg/ml Con A was added and cells were cultured for 24 h and then harvested and NT mRNAs levels were determined (**B**). Each NT mRNA level in part B is presented as % of mRNA level under resting conditions (showed in part A). The data represent the mean ± SD from six (A) or three (B) independent experiments. (*) $P < 0.05$ versus (5 mM gluc + Ins).

glucose concentration (Fig. 3). On the other hand among investigated enzymes AK is the only enzyme in T cells, which activity depended on insulin but not on glucose concentration (Table II). Therefore we examined the impact of changes in AK activity on T cells proliferation. In our experiments we used 5-iodotubercidin (IT) a specific inhibitor of AK [Wiesner et al., 1999]. Including of 0.5 μM IT in the culture

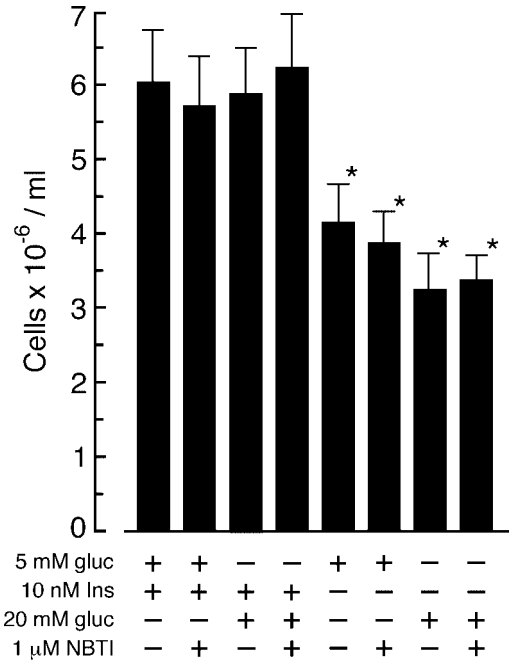

Fig. 5. The effect of ENT1 inhibition on Con A-induced proliferation of rat T lymphocytes. T cells isolated from normal rat were cultured as described under ''Materials and Methods'' for 2 days in the medium containing glucose and insulin at indicated concentrations. Where 10 nM insulin was not added its concentration was lower than 2×10^{-11} M. On third day 2.5 μg/ml Con A was added and after 48 h the number of viable cells was determined as described in ''Materials and Methods.'' The NBTI was added 1 h before Con A. The data represent the mean ± SD from four independent experiments. (*) $P < 0.05$ versus (5 mM gluc + Ins).

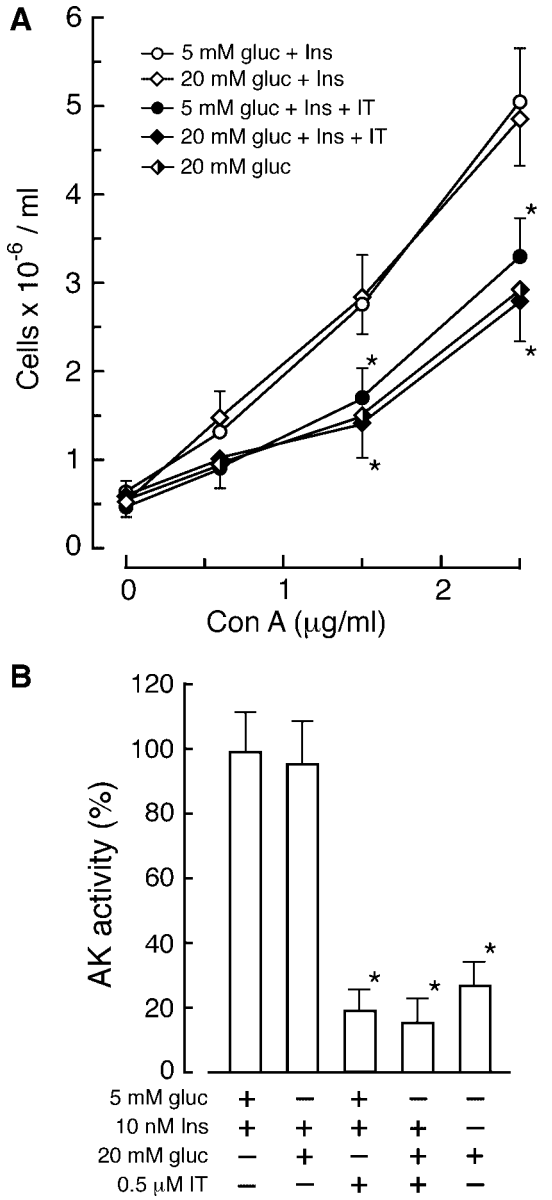

Fig. 6. Impact of adenosine kinase inhibition on rate of ConA-induced T lymphocytes proliferation. T cells isolated from normal rat were cultured as described under ''Materials and Methods'' for 2 days in the medium containing: 5 mM glucose and 10 nM insulin (5 mM gluc + Ins); 5 mM glucose, 10 nM insulin and 0.5 μM 5-iodotubercidin (5 mM gluc + Ins + IT); 20 mM glucose, 10 nM insulin and 0.5 μM 5-iodotubercidin (5 mM gluc + Ins + IT); 20 mM glucose and 10 nM insulin (20 mM gluc + Ins); 20 mM glucose and no insulin (20 mM gluc). On the third day T cells were stimulated with Con A at concentrations as indicated (**A**) and after 48 h the number of viable cells was determined as described in ''Materials and Methods.'' **B**: Activity of AK was determined in cells cultured for 2 days at insulin, glucose and IT at concentrations as indicated. The insulin concentration determined in RPMI medium not supplemented with 10 nM insulin was 2×10^{-11} M. The data represent the mean ± SD from five (A) or six (B) independent experiments. (*) $P < 0.05$ versus (5 mM gluc + Ins).

medium reduced (within 1 h) the activity of AK by 95% in cells cultured at 5 mM glucose and the presence of 10 nM insulin (not shown). The inhibitory effect of single addition of IT to the culture medium sustained at least over 60 h however, during this time AK activity slowly increased and at 24 h reached 20% of the activity measured in cells cultured without IT (Fig. 6). Conducted experiment showed that inhibition of AK activity in cells cultured in the presence of 10 nM insulin lowered the proliferation potential of these cells to the same level as cells cultured without added insulin regardless of glucose concentration (Fig. 6).

Adenosine Receptors and Proliferation of T Cells

Results of conducted experiments suggested that observed impaired T cells proliferation could be the receptor-mediated effect of adenosine. In order to examine the involvement of

adenosine receptors (AR) in alteration of T cells proliferation we used specific AR antagonists. Exposition of cells proliferating in medium containing 20 mM glucose and no insulin to DPMX (1 μM), a nonselective A2 receptor antagonist slightly increased the proliferation rate of T cells, but this increase was insignificant statistically (Fig. 7). Alloxazine (1 μM), which is relative specific antagonist of A2b receptor, had no effect on T cells proliferation rate. On the other hand, including into culture medium ZM241385 (1 μM) a selective antagonist of A2a receptor completely restored the proliferation potential of T cells (Fig. 7). Moreover, the presence of 1 μM ZM241385 but not Alloxazine or DPMX prevented the increases in cAMP level seen in cells cultured in the absence of insulin (Fig. 8). Under resting conditions, the cAMP level was fivefold higher in cells cultured at 20 mM glucose and the absence of insulin (concentration of insulin $<3 \times 10^{-11}$ M) comparing to cells cultured at 20 mM glucose and the presence of 10 nM insulin. During Con A-induced proliferation the level of cAMP raised threefold in T cells proliferating in the absence of insulin but not in cells cultured in the presence of 10 nM insulin (Fig. 8).

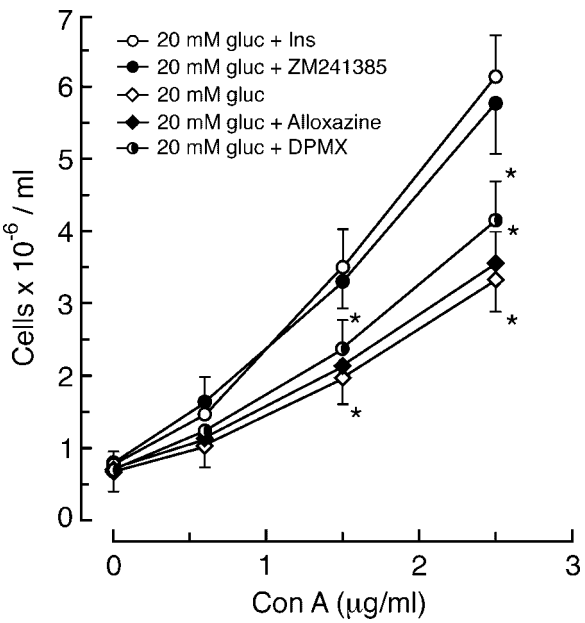

Fig. 7. Identification of A2a receptor as the adenosine receptor responsible for suppression of T cell proliferation. Cells were cultured for 2 days at 20 mM glucose and 10 nM insulin (20 mM gluc + Ins) or 20 mM glucose and no insulin (20 mM gluc). No insulin refers to concentration lower than 3×10^{-11} M. On the third day cells were induced to proliferation by addition of Con A and after 48 h the number of viable cells was determined as described in ''Materials and Methods.'' One hour before addition of Con A cells were exposed to 1 μM ZM241385 (specific A2a receptor antagonist), or 1 μM Alloxazine (specific A2b receptor antagonist), or 1 μM DPMX (nonspecific A2 receptor antagonist). The data represent the mean ± SD from four independent experiments. (*) $P < 0.005$ versus (20 mM gluc + 10 nM Ins).

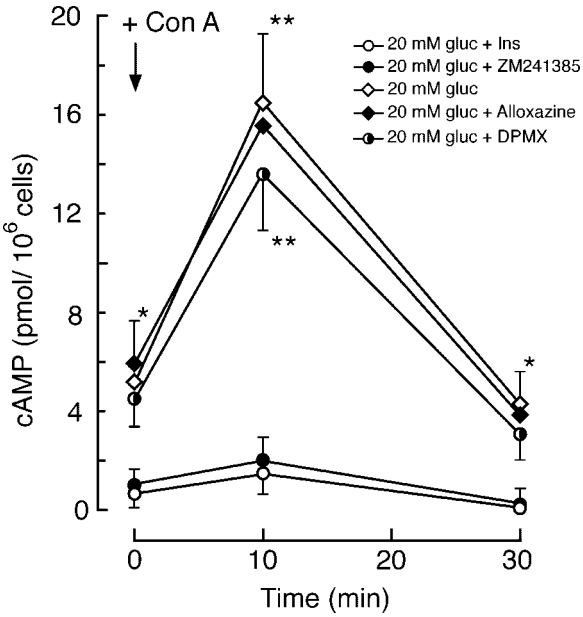

Fig. 8. Changes of cAMP level in Con A-induced T lymphocytes. T Cells were cultured as described under ''Materials and Methods'' for 2 days in the medium containing 20 mM glucose and 10 nM insulin (20 mM gluc + Ins) or 20 mM glucose and no insulin (20 mM gluc). No insulin refers to concentration lower than 3×10^{-11} M. On the third day cells cultured in the medium containing 20 mM glucose and no insulin were exposed to 1 μM ZM241385 (20 mM gluc + ZM241385) or 1 μM Alloxazine (20 mM gluc + Alloxazine), or 1 μM DPMX (20 mM gluc + DPMX) and after 1 h incubation were induced to proliferation by addition 2.5 μg/ml Con A. At indicated time points cells were separated from the culture medium and cellular cAMP was determined as described in ''Materials and Methods.'' The data represent the mean ± SD from four independent experiments. (*) $P < 0.05$ versus (20 mM gluc + Ins); (**) $P < 0.05$ versus (time 0).

In summary, results of our experiments showed that impaired proliferation of rat T lymphocytes cultured under conditions of insulin deprivation result from suppressed expression of AK what in turn leads to increased release of adenosine from the cell. Released adenosine by acting on A2a receptor induces cAMP production and suppresses proliferation of T cells (Fig. 9).

DISCUSSION

The proliferative response of T cell to pathogens is a crucial step in cell-mediated immunity. Proliferation of activated T cells is associated

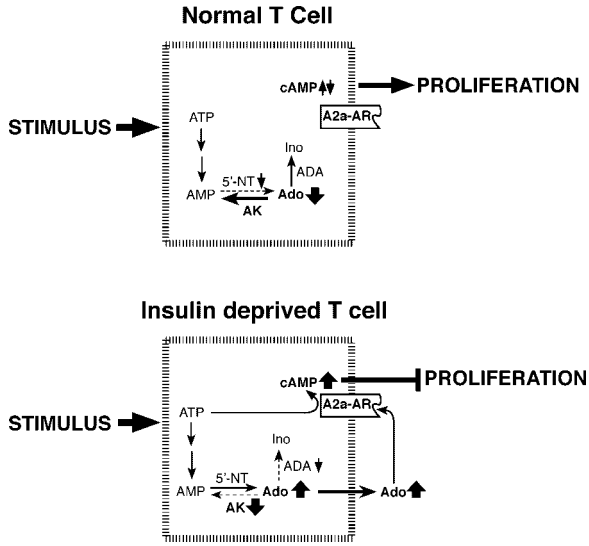

Fig. 9. The postulated mechanism leading to suppression of T lymphocyte proliferation under condition of insulin deprivation. A2a-AR, adenosine receptor type A2a; 5′-NT, 5′-nucleotidase; AK, adenosine kinase; Ado, adenosine; ADA, adenosine deaminase; Ino, inosine.

with DNA and RNA synthesis, and stimulation of de novo purine and pyrimidine synthesis pathways [Shambaugh and Blumenschein, 1974; Fairbanks et al., 1995]. Although, resting human lymphocytes display very low de novo nucleotide biosynthesis, this process is activated 30–500 times during proliferation [Marijnen et al., 1989]. Studies with the use of specific inhibitors of purine and pyrimidine nucleotide synthesis demonstrated that depletion of nucleotide pool affect cell-cycle progression, clonal expansion, differentiation, and survival of T lymphocytes [Turka et al., 1991; Laliberte et al., 1998; Quemeneur et al., 2004]. Previous reports showed that inhibition of pyrimidine nucleotide synthesis abrogated progression of cell cycle from early to late S phase [Quemeneur et al., 2003]. On the other hand, depletion of purine nucleotides resulted in arresting of activated T cells in the G_1 phase of the cell cycle [Turka et al., 1991; Laliberte et al., 1998]. The level of nucleotide pool in the cell depends on the rate of nucleotide utilization and synthesis. Our results presented in this report indicate that the level of purine nucleotides did not differ significantly in cells deprived of insulin and cultured at high glucose comparing to cells cultured at low glucose concentration and the presence of insulin (Table I). This indicates that under resting conditions the nucleotide conserving system is able to maintain equally well constant nucleotide pool in both types of T cell cultures. However, the 2-deoxyglucose-induced fall in ATP level proceeded faster in T cells cultured at high glucose and the absence of insulin indicating some impairment in the nucleotide preserving system.

Cell proliferation is associated with increases in energy consumption designated for synthesis of nucleic acid precursors and other biochemical activities therefore proliferating cell may be considered as being under metabolic stress conditions. During metabolic stress the efficiency of nucleotide synthesis depends not only on de novo synthetic routes but also on salvage reactions, which require far less energy than does de novo synthesis. The phosphorylation of adenosine to AMP catalyzed by AK would be considered as such a reaction. Lowered AK activity resulted from decreased expression of *AK* gene was observed in lymphocytes isolated from diabetic rat [Pawelczyk et al., 2003]. Decreased activity of AK in diabetic T lymphocytes may result in lowering the AMP-adenosine turnover and lead to increase of adenosine level in the cell. Despite the low rate of AMP-adenosine substrate cycle in T lymphocytes [Szondy and Newsholme, 1989] pharmacological blockade of AK activity was demonstrated to induce adenosine release from T cells [Barankiewicz et al., 1990]. Our results presented in this report indicate that under metabolic stress conditions T cells cultured at high glucose and the absence of insulin release higher quantities of adenosine comparing to cells cultured at low glucose and the presence of insulin.

The way of adenosine outflow from T cells cultured at high glucose and the absence of insulin is unclear. The activity of equilibrative transport system by which the nucleoside can be transported out of the cell is lowered by 60% in T lymphocytes cultured at high glucose and the absence of insulin [Sakowicz et al., 2004]. However, it is possible that this residual activity of nucleoside transport system is still sufficient to move adenosine out of the cell. It is also possible that there are other routes by which adenosine can leave the cell. Cells possess various efflux transporters including multidrug resistant (MDR) channels. Among several proteins able to bind and transport drugs at the expense of ATP hydrolysis the P-glycoprotein (P-gp) is able to transport a vide range of

neutral compounds [Borst et al., 2000]. Other efflux proteins like organic anion transporters (OAT) are also capable to mediate either the exchange or the facilitated transport of several nucleoside analogues [Pastor-Anglada et al., 2005]. It was reported that rat OAT1 is capable to mediate high-affinity transport (K_m in the range 50–250 μM) of several pyrimidine and purine nucleoside analogs that possess no typical anionic moiety [Wada et al., 2000]. Nevertheless, data presented in this report indicate that rat T lymphocytes cultured under diabetic-like conditions are more vulnerable to metabolic stress than normal cells and release under metabolic load increased quantities of adenosine.

It was reported previously that activation and proliferation of macrophages was highly dependent on nucleoside transporters [Soler et al., 2001]. Our experiments demonstrated that proliferation potential of T lymphocytes cultured at low glucose was impaired to the same extend as that of T cells cultured at high glucose despite significant changes in nucleoside transporters expression level. Moreover, inhibiting of nucleoside transport with NBTI did not affect the proliferation rate of T lymphocytes indicating that T cells proliferation is highly independent on nucleoside transporters functional state.

Decreased activity of AK was common feature of T cells cultured in the absence of insulin regardless of glucose concentration. Moreover, we demonstrated that by changing the activity of AK we could manipulate the proliferation rate of T lymphocytes independently on the cell growing conditions. This indicates that lowered activity of AK is the crucial factor responsible for decreased proliferation potential of insulin deprived T lymphocytes. However, it should be noted that during proliferation of insulin deprived T cells the cytosolic activity of ADA decreased (Table III), what might additionally contribute to increases in adenosine level in the cell. The results described here indicate that adenosine released by activated diabetic T lymphocytes inhibits cell proliferation by acting on A2a receptors what in turn leads to increases in cAMP level. To date in T lymphocytes expression of A1, A2a, A2b, and A3 have been demonstrated [Koshiba et al., 1999; Mirabet et al., 1999; Gessi et al., 2004; Zhang et al., 2004]. However, the expression level of particular adenosine receptor depends on subtype of T lymphocyte and state of the cell. In human both CD4+ and CD8+ cells express the A3 receptor, but during proliferation its expression level raises only in the CD4+ cells [Gessi et al., 2004]. The A2b receptor was shown to be equally expressed in the CD4+ and CD8+ human cells [Mirabet et al., 1999]. It was reported that in human lymphocytes the expression level of A2a adenosine receptor is much higher in T cells than in B cells [Koshiba et al., 1999]. Moreover, not all T cells possess this type of adenosine receptor. Only 22% of human CD4+ cells and 12% of CD8+ cells expressed A2a receptor. There is no data on distribution of A2a receptor in rat lymphocytes, but it might be assumed that not all subtypes of rat T cells express this type of adenosine receptor. Therefore, the percentage of rat T cells bearing A2a receptor could be mirrored by observed in our experiments 30%–40% suppression of T cell proliferation.

Reduced proliferation potential is among other T cell functional defects long known feature of experimental and human diabetes [Puxty and Fox, 1984]. However, the mechanism responsible for suppression of diabetic T cell proliferation is still unknown. Our results described in this report demonstrate that adenosine released by insulin deprived T cells inhibits cell proliferation by acting on A2a receptors what in turn leads to increases in cAMP level and suppression of T cell proliferation. We assume that this mechanism may significantly contribute to immune impairment observed in diabetes.

REFERENCES

Antonysamy MA, Moticka EJ, Ramkumar V. 1995. Adenosine acts as an endogenous modulator of IL-2-dependent proliferation of cytotoxic T lymphocytes. J Immunol 155: 2813–2821.

Apasov S, Koshiba M, Redegeld F, Sitkovsky MV. 1995. Role of extracellular ATP and P_1 and P_2 classes of purinergic receptors in T-cell development and cytotoxic T lymphocyte effector functions. Immunol Rev 146:5–19.

Barankiewicz J, Cohen A. 1987. Purine nucleotide metabolism in phytohemagglutinin-induced human T lymphocytes. Arch Biochem Biophys 258:167–175.

Barankiewicz J, Ronlov G, Jimenez R, Gruber HE. 1990. Selective adenosine release from human B but not T lymphoid cell line. J Biol Chem 265:15738–15743.

Birch RE, Polmar SH. 1982. Pharmacological modification of immunoregulatory T lymphocytes. I. Effect of adenosine, H1 and H2 histamine agonists upon T lymphocyte regulation of B lymphocyte differentiation in vitro. Clin Exp Immunol 48:218–230.

Borst P, Evers R, Kool M, Wijnholds J. 2000. A family of drug transporters: The multidrug resistance-associated proteins. J Natl Cancer Inst 92:1295–1302.

Chang FY, Shaio MF. 1995. Decreased cell-mediated immunity in patients with non-insulin-dependent diabetes mellitus. Diab Res Clin Pract 28:137–146.

DosReis GA, Nobrega AF, de Carvalho RP. 1986. Purinergic modulation of T-lymphocyte activation: Differential susceptibility of distinct activation steps and correlation with intracellular 3′,5′-cyclic adenosine monophosphate accumulation. Cell Immunol 101:213–231.

Eibl N, Spatz M, Fischer GF, Mayr WR, Samstag A, Wolf HM, Schernthaner G, Eibl MM. 2002. Impaired primary immune response in type-1 diabetes: Results from a controlled vaccination study. Clin Immunol 103:249–259.

Fairbanks LD, Bofill M, Ruckemann K, Simmonds HA. 1995. Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. J Biol Chem 270:29682–29689.

Gessi S, Varani K, Merighi S, Cattabriga E, Avitabile A, Gavioli R, Fortini C, Leung E, Mac Lennan S, Borea PA. 2004. Expression of A3 adenosine receptors in human lymphocytes: Up-regulation in T cell activation. Mol Pharmacol 65:711–719.

Hershfield MS. 2005. New insights into adenosine-receptor-mediated immunosuppression and the role of adenosine in causing the immunodeficiency associated with adenosine deaminase deficiency. Eur J Immunol 35: 25–30.

Joshi N, Caputo GM, Weitekamp MR, Karchmer AW. 1999. Infections in patients with diabetes mellitus. N Engl J Med 341:1906–1912.

Kaye WA, Adri MN, Soeldner JS, Rabinowe SL, Kaldany A, Kahn CR, Bistrian B, Srikanta S, Ganda OP, Eisenbarth GS. 1986. Acquired defect in interleukin-2 production in patients with type 1 diabetes mellitus. N Engl J Med 315: 920–924.

Koshiba M, Rosin DL, Hayashi N, Linden J, Sitkovsky MV. 1999. Patterns of A_{2A} extracellular adenosine receptor expression in different functional subsets of human peripheral T cells. Flow cytometry studies with anti-A_{2A} receptor monoclonal antibodies. Mol Pharmacol 55: 614–624.

Laliberte J, Yee A, Xiong Y, Mitchell BS. 1998. Effects of guanine nucleotide depletion on cell cycle progression in human T lymphocytes. Blood 91:2896–2904.

Marijnen YM, de Korte D, Haverkort WA, den Breejen EJ, van Gennip AH, Roos D. 1989. Studies on the incorporation of precursors into purine and pyrimidine nucleotides via 'de novo' and 'salvage' pathways in normal lymphocytes and lymphoblastic cell-line cells. Biochim Biophys Acta 1012:148–155.

Mirabet M, Herrera C, Cordero OJ, Mallol J, Lluis C, Franco R. 1999. Expression of A_{2B} adenosine receptors in human lymphocytes: Their role in T cell activation. J Cell Sci 112:491–502.

Otton R, Mendonca JR, Curi R. 2002. Diabetes causes marked changes in lymphocyte metabolism. J Endocrinol 174:55–61.

Pastor-Anglada M, Cano-Soldado P, Molina-Arcas M, Lostao MP, Larrayoz I, Martinez-Picado J, Casado FJ. 2005. Cell entry and export of nucleoside analogues. Virus Res 107:151–164.

Pawelczyk T, Bizon D, Angielski S. 1992. The distribution of enzymes involved in purine metabolism in rat kidney. Biochem Biophys Acta 1116:309–314.

Pawelczyk T, Sakowicz M, Podgorska M, Szczepanska-Konkel M. 2003. Insulin induces expression of adenosine kinase gene in rat lymphocytes by signaling through the mitogen-activated protein kinase pathway. Exp Cell Res 286:152–163.

Pickup JC, Chusney GD, Thomas SM, Burt D. 2000. Plasma interleukin-6, tumor necrosis factor alpha and blood cytokine production in type 2 diabetes. Life Sci 67:291–300.

Puxty JHA, Fox RA. 1984. Diabetes and infection. In: Fox RA, editor. Immunology and infection in the eldery. Edinburgh: Churchill Livingstone. pp 79–201.

Quemeneur L, Gerland LM, Flacher M, Ffrench M, Revillard JP, Genestier L. 2003. Differential control of cell cycle, proliferation, and survival of primary T lymphocytes by purine and pyrimidine nucleotides. J Immunol 170:4986–4995.

Quemeneur L, Beloeil L, Michallet MC, Angelov G, Tomkowiak M, Revillard JP, Marvel J. 2004. Restriction of de novo nucleotide biosynthesis interferes with clonal expansion and differentiation into effector and memory CD8 T cells. J Immunol 173:4945–4952.

Sakowicz M, Szutowicz A, Pawelczyk T. 2004. Insulin and glucose induced changes in expression level of nucleoside transporters and adenosine transport in rat T lymphocytes. Biochem Pharmacol 68:1309–1320.

Schloot NC, Roep BO, Wegmann DR, Yu L, Wang TB, Eisenbarth GS. 1997. T-cell reactivity to GAD65 peptide sequences shared with coxsackie virus protein in recentonest IDDM, post-onset IDDM patients and controls subjects. Diabetologia 40:332–338.

Severson CD, Burg DL, Lafrenz DE, Feldbush TL. 1987. An alternative method of panning for rat B lymphocytes. Immunol Lett 15:291–295.

Shambaugh GE III, Blumenschein GR. 1974. Combined effects of phytohemagglutinin and staphylococcal enterotoxin B on deoxyribonucleic acid synthesis during blast transformation in human lymphocytes. Infect Immunol 9:384–390.

Snyder FF, Mendelsohn J, Seegmiller JE. 1976. Adenosine metabolism in phytohemagglutinin-stimulated human lymphocytes. J Clin Invest 58:654–666.

Soler C, Garcia-Manteiga J, Valdes R, Xaus J, Comalada M, Casado FJ, Pastor-Anglada M, Celada A, Felipe A. 2001. Macrophages require different nucleoside transport systems for proliferation and activation. FASEB J 15: 1979–1988.

Szondy Z, Newsholme EA. 1989. The rate of the AMP/adenosine substrate cycle in concanavalin-A-stimulated rat lymphocytes. Biochem J 261:739–742.

Turka LA, Dayton J, Sinclair G, Thompson CB, Mitchell BS. 1991. Guanine ribonucleotide depletion inhibits T cell activation. Mechanism of action of the immunosuppressive drug mizoribine. J Clin Invest 87:940–948.

Wada S, Tsuda M, Sekine T, Cha SH, Kimura M, Kanai Y, Endou H. 2000. Rat multispecific organic anion transporter 1 (rOAT1) transports zidovudine, acyclovir, and other antiviral nucleoside analogs. J Pharmacol Exp Ther 249:844–849.

Wiesner JB, Ugarkar BG, Castellino AJ, Barankiewicz J, Dumas DP, Gruber HE, Foster AC, Erion MD. 1999. Adenosine kinase inhibitors as a novel approach to anticonvulsant therapy. J Pharmacol Exp Ther 289: 1669–1677.

Wolberg G, Zimmerman TP, Hiemstra K, Winston M, Chu LC. 1975. Adenosine inhibition of lymphocyte-mediated cytolysis: Possible role of cyclic adenosine monophosphate. Science 187:957–959.

Zhang H, Conrad DM, Butler JJ, Zhao C, Blay J, Hoskin DW. 2004. Adenosine acts through A_2 receptors to inhibit IL-2-induced tyrosine phosphorylation of STAT5 in T lymphocytes: Role of cyclic adenosine 3′,5′-monophosphate and phosphatases. J Immunol 173:932–944.