JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2010, 61, 4, 491-499
www.jpp.krakow.pl

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ADENOSINE 5'-TRIPHOSPHATE IS THE PREDOMINANT SOURCE OF PERIPHERAL ADENOSINE IN HUMAN B LYMPHOBLASTS

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Adenosine 5'-triphosphate (ATP) and adenosine are the crucial endogenous signaling molecules in immunity and inflammation. In this study we identified the source of extracellular adenosine in human B lymphoblasts, and evaluate the ATP release and metabolism. We observed that the B cells continuously released substantial quantities of ATP (35 pmol/10^6 cells) when subjected to slow motion in the incubation medium. The adenosine level in the B cell incubation medium was very low, and increased (5-fold) upon inhibition of adenosine deaminase activity with 10 μM of 2-deoxycoformycin (DCF). Inclusion of an inhibitor of equilibrate nucleoside transport (nitrobenzylthioinosine) in the incubation medium in the presence of DCF resulted in the elevation of adenosine level by 9-fold. Inhibition of ecto-ATPase activity with 100 μM of ARL67156 was associated with a 2-fold increase of the extracellular ATP level and a 3-fold decrease of adenosine concentration in the cell culture media. Inclusion of α,β-methyleneadenosine 5'-diphosphate, a selective inhibitor of ecto-5'-nucleotidase in the incubation medium resulted in a significant decrease (7-fold) the adenosine concentration. In conclusion, our results indicate that ATP released from the B cell is the primary source of peripheral adenosine, and that the activities of ecto enzymes and the efficiency of Ado uptake through the nucleoside transporters determine the Ado level on the B cell surface.

Key words: *adenosine, adenosine 5'-triphosphate release, B lymphocyte, ecto-ATPase, ecto-5'-nucleotidase, adenosine deaminase*

INTRODUCTION

The function of immune cell is subjected to regulation by various signaling molecules released by immune and non-immune cells. These molecules are necessary for initiating the primary immune response and for controlling the course of action. Following release, some compounds may act on specific receptors, be converted into a bioactive agent, or both. Extracellular ATP may function as endogenous signaling molecule, or/and be hydrolyzed into adenosine (1, 2). Both of these compounds acting on specific purinergic receptors function as endogenous signaling molecules that control the immune response (3). Almost all cells contain cell-surface P1 and P2 receptors for adenosine and ATP, respectively. There are four adenosine receptors (A_1, A_{2A}, A_{2B}, A_3), which bind adenosine with different affinities (4). The P2 receptors are subdivided into two subfamilies namely P2X and P2Y (5). These receptors operate in both auto- and paracrine fashion contributing to the engineering of inflammation and immune response (3). The other component of purinergic signaling system comprises a variety of ecto-nucleotidases hydrolyzing nucleotides to generate nucleosides that interact with adenosine receptors (6). The third, and final, component of the purinergic signaling network is the nucleoside transport system in the cells. Adenosine and other nucleosides and nucleobases are transported across the plasma membrane by specific transport proteins, which can be divided into two categories based on their transport mechanism. Nucleoside transport by equilibrate transporters (ENT) is bidirectional and is driven by the concentration gradient, whereas the function of concentrative transporters (CNT) is based on the electrochemical ion gradient and is coupled to sodium ions (7). Collectively, the bioavailability of adenosine at the receptor site is determined by its generation and catabolism rates, and by transport across the plasma membrane. Despite substantial progress in the understanding of the involvement of purines in regulation of immune cell function, the current knowledge about the pathway that determines the adenosine level on periphery of human lymphocytes, especially the B cells, is rather limited. B cells are the core constituent of immunological system, and when stimulated by an antigen proliferate and differentiate into cells that produce and secrete antibodies. Therefore, the mechanisms that affect the function of B lymphocytes have an important impact on the humoral immune response. Previously it had been demonstrated that non-stimulated human leukemic T cells and B cell lymphoblasts maintain a sufficient amount of ATP on their surface to reach the micromolar concentration in the immediate vicinity of the cell (8). Our research was undertaken to investigate the extracellular source of adenosine in unstimulated B lymphocytes. We evaluated the ATP release and its metabolism in human B cell line SKW6.4.

MATERIALS AND METHODS

Reagents

Penicillin, streptomycin, nitrobenzylthioinosine (NBTI), α, β-methyleneadenosine 5'-diphosphate (AOPCP), 6-N,N-Diethyl-D-β-γ-dibromomethylene adenosine triphosphate (ARL 67156), erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA), ATP, ADP, AMP, adenosine, inosine, hypoxanthine, RPMI-1640 medium, were obtained from Sigma-Aldrich Sp. z o.o. (Poznan, Poland). 2-deoxycoformycin (DCF) was from Park Davies (Ann Arbor, MI, USA). [8-^{14}C]adenine was from Moravek Biochemicals Inc. (Brea, CA, USA). Thin-layer chromatography (TLC) sheets DC Alufolien Kieselgel 60 F_{254} were from Merck Sp. z o.o. (Warsaw, Poland).

Cells and culture conditions

The SKW6.4 cell line was kindly provided by dr. Peter H Krammer from the German Cancer Research Centre in Heidelberg. This cell line is a human IL-6-dependent, IgM secreting B-cell line. Cells were maintained under standard conditions (5% CO_2-95% air, 98% humidity and 37°C) in RPMI-1640 medium, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum (Gibco). Cells were cultured in flat-bottomed culture bottles (Sarsted) and when necessary were split to maintain a density of ~10^6 cells/ml. For the experiments the cells were harvested and suspended in RPMI-1640 medium to obtain the density of 10^7 cells/ml and incubated with a smooth continuous orbital motion (100 RPM) in cell dishes placed on the compact slow-speed orbital shaker installed inside the cell culture chamber. The number of viable cells was determined by Trypan Blue dye exclusion, and by measurement of lactate dehydrogenase activity in the incubation media. Only cell cultures with a 95% viability or greater were used. Total lactate dehydrogenase activity did not change in the course of incubation.

Radiolabeling of cellular ATP

To investigate the ATP release and catabolism, cells were first incubated for 3 hours with 10 µCi [8-^{14}C]adenine (45 mCi mmol^{-1}) to label intracellular ATP. After incubation for 3 hours, the cells were washed with RPMI-1640 medium. Measuring the radioactivity in liquid-scintillation counter monitored the efficiency of consecutive washes. Examination of radiolabeled cell extracts by TLC showed that ~95% of the cellular acid-soluble radioactivity was incorporated in ATP, ADP, and AMP of which ATP accounted ~85% (not shown).

Measurement of ATP level and ATP catabolism

The cells were suspended in the incubation medium right before a time course of ATP released assay. ATP content in the cells and incubation media was measured using a luciferase-based bioluminescent ATP assay kit (Sigma) as described previously (9). The ATP level was expressed in nmol or pmol/10^6 cells. The catabolism of ATP was determined by examination of radiolabeled compounds. The fast separation of cells from incubation medium was achieved by centrifugation (100 µl of cell suspension) trough a silicone oil as described previously (10). Resulting aqueous layer (culture media) and the pellet (cells) were collected and extracted with 0.4 M perchloric acid, neutralize and concentrated on speed vac (Sorval) when necessary. The radioactive purine compounds were separated on silica gel (with fluorescent indicator) aluminum plate as described previously (11). For nucleotides separation the plate was developed in 1,4 dioxane/25% ammonia/water (6:1:3.8, 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.

Statistical analysis

The statistical calculation was performed with ANOVA and Dunnett's test for comparison to control group. Paired Student's t-test was performed when two groups were analyzed. *P* values below 0.05 were considered as significant.

RESULTS

The release of ATP from B lymphoblasts

To investigate the release of ATP from the SKW6.4 cells we labeled the intracellular pool of ATP with [^{14}C]adenine. Examination of the B lymphoblasts culture media indicated that the level of radiolabeled ATP changed over the time. Relatively high levels of ATP were detected immediately after the change of medium. However, during cell incubation the extracellular ATP level decreased steadily, reaching the base line value within an hour (*Fig. 1A*). During the first hour of cell incubation, the decrease of ATP level in the culture media was accompanied by increases in AMP and ADP levels. We have observed that single pipetting of the cell suspension up and down resulted in a marked outflow of ATP from the cells (not shown). This observation is consistent with reports demonstrating that ATP is released from a

Time (h)	Radioactivity incorporated		
	ATP	ADP	AMP
	(cpm/10^6 cells)		
0	235 832 ± 21 167	36 418 ± 4 102	10 535 ± 1 229
1	229 740 ± 20 096	34 632 ± 3 948	9 287 ± 1 053
2	222 510 ± 20 684	32 868 ± 3 385	9 494 ± 929
3	216 877 ± 19 541	30 658 ± 3 251	8 333 ± 985*
4	210 359 ± 18 637	28 822 ± 3 240*	7 971 ± 669*
5	196 166 ± 16 939*	26 827 ± 3 056*	7 401 ± 625*
6	189 495 ± 10 215*	24 211 ± 2 531*	6 879 ± 667*

Table 1. The level of radioactivity incorporated into cellular adenine nucleotides during incubation of B lymphoblasts. The SKW6.4 cells were labeled with [8-^{14}C]adenine as described under "Materials and Methods". At time indicated the cells were separated, extracted with perchloric acid, and acid-soluble radiolabeled purine nucleotides were separated by thin-layer chromatography, located under UV, and the radioactivity was counted. The data represent the mean±S.D. from four independent experiments. *, P<0.05 *vs*. 0 time.

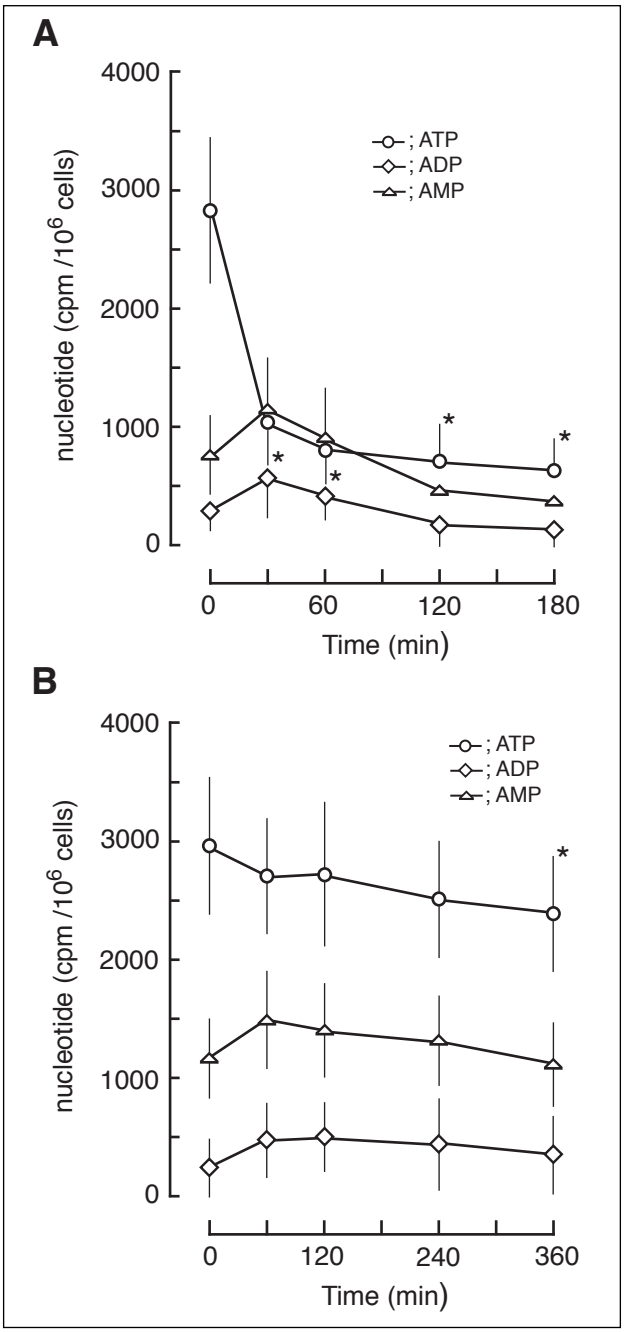

Fig. 1. The levels of adenine nucleotides in the culture medium of B lymphoblasts during incubation. The cells were labeled with [8-^{14}C]adenine as described under "Materials and Methods", washed and incubated in RPMI-1640 medium without mixing (**A**), or with orbital mixing at 100 RPM (**B**). At time indicated 100 µl of cell suspension was withdrawn, placed on the top of oil, and immediately centrifuged. The top layer was separated, extracted with perchloric acid, and acid-soluble radiolabeled purine nucleotides were separated by thin-layer chromatography as described under "Materials and Methods". The data represent the mean±S.D. from at least three independent experiments. *, P≤0.05 *vs*. 0 time.

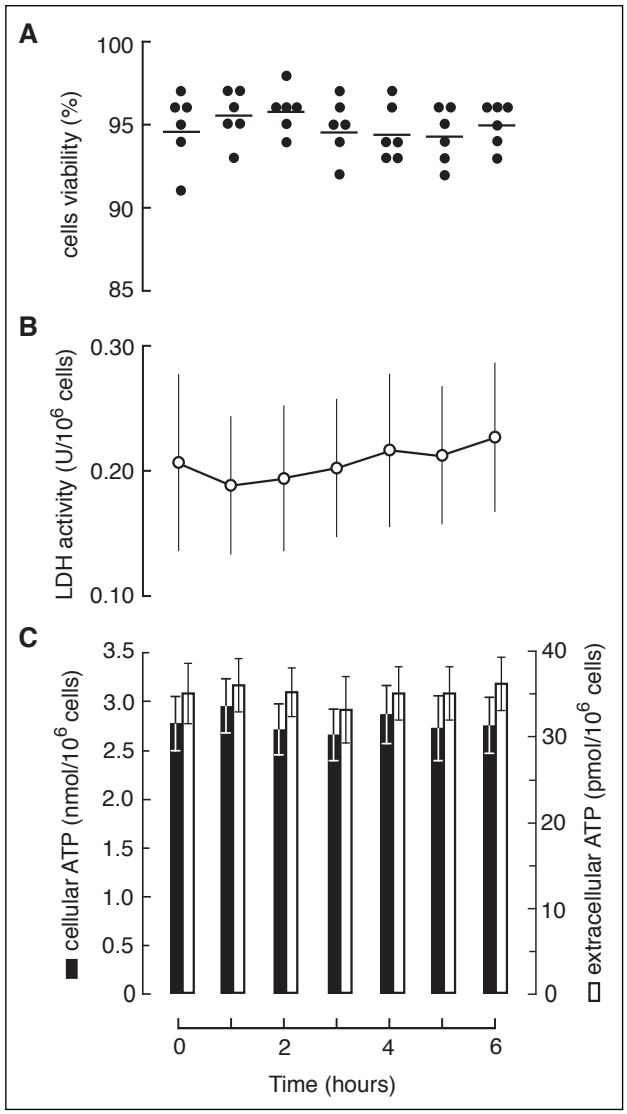

Fig. 2. Cell viability during incubation with mixing. The SKW6.4 cells were incubated with orbital mixing (100 RPM) in RPMI-1640 medium, and at time indicated the cell viability was determined by: Trypan Blue dye exclusion (**A**), determination of lactate dehydrogenase activity in incubation medium (**B**), measurement of cellular and extracellular ATP level using a luciferase-based bioluminescent assay (**C**). The presented results are the means±S.D. from six independent experiments.

variety of cells in response to perturbation of the cell surface by altered flow of the aqueous medium (12-16). In order to standardize the experimental conditions in terms of stable ATP release (over time) we incubated the cells in culture dishes using gentle orbital mixing (100 RPM). Under these conditions the ATP level in the cell culture medium remained relatively stable. Measurements performed showed that the radiolabeled ATP present in the incubation medium represented up to 1.3±0.1% of radioactivity incorporated into cellular ATP (*Fig. 1B* and *Table 1*). Over the six hours of incubation the level of radiolabeled ATP in the incubation medium decreased ~20% (*Fig. 1B*). A similar decline rate of radioactivity incorporated in the cellular adenine nucleotides was observed (*Table 1*). The possible cell lysis that may have occurred during the cells mixing was evaluated based on assessment of cell viability, measurements of cellular pool of ATP, and by monitoring the activity of the lactate dehydrogenase in the incubation medium. As can be seen in *Fig. 2*, the cells viability was high (~95%) and remained unchanged over the six hours of incubation and mixing. The incubation conditions influenced neither the cellular ATP content nor the lactate

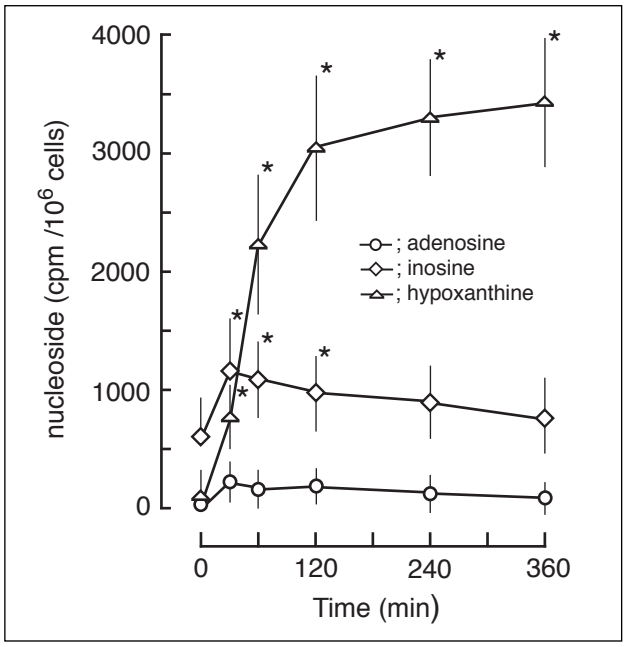

Fig. 3. The level of adenosine, inosine and hypoxanthine in culture media of B lymphoblast. The cells were labeled with [8-^{14}C]adenine as described under "Materials and Methods", washed and incubated with orbital mixing (100 RPM) in RPMI-1640 medium. At time indicated 100 µl of cell suspension was withdrawn, placed on the top of oil, and immediately centrifuged. The top layer was separated, extracted with perchloric acid, and acid-soluble radiolabeled purine nucleosides and bases were separated by thin-layer chromatography as described under "Materials and Methods". The data represent the mean±S.D. from four independent experiments. *, P≤0.05 *vs*. 0 time.

leakage of cellular ATP into the medium. To resolve this question, we determined the viability of the cells incubated with and without the mixing. Measurements performed showed that under these two conditions the cell viability was the same over the six hours of incubation (not shown). Whereas, the ATP content measured in medium after 1 hour of incubation with and without mixing reach the level of ~10 pmol/10^6 cells and 36 pmol/10^6 cells, respectively (not shown). This indicated that the ATP release was specifically induced by medium displacement, but not the nonspecific leakage.

B lymphoblast peripheral adenosine is generated on the cell surface and is not released from the cell

Examination of the purine nucleosides and bases levels in the culture media showed very low level of adenosine (*Fig. 3*). We observed that inosine and hypoxanthine were the major products of extracellular adenine nucleotides catabolism. To evaluate the impact of adenosine deaminase (ADA) activity on the extracellular adenosine level, we used 2-deoxycoformycin (DCF), a potent and specific inhibitor of this enzyme (17). It was reported that DCF at 20 µM concentration does not affect AMP deaminase activity in human B lymphoblasts (18). Inclusion in the incubation medium DCF (10 µM) resulted in a substantial elevation of adenosine level (up to 5-fold) and a decrease of inosine and hypoxanthine concentration (*Fig. 4*). Similar changes in purine nucleosides and bases levels were observed in the incubation media of cells treated with EHNA (1 µM), another selective and potent inhibitor of ADA (not shown). This observation would indicate that significant quantities of adenosine are formed on the periphery of human B lymphoblasts or that adenosine is effectively released from the cells. Studies performed on human B cell lines and murine bone macrophages

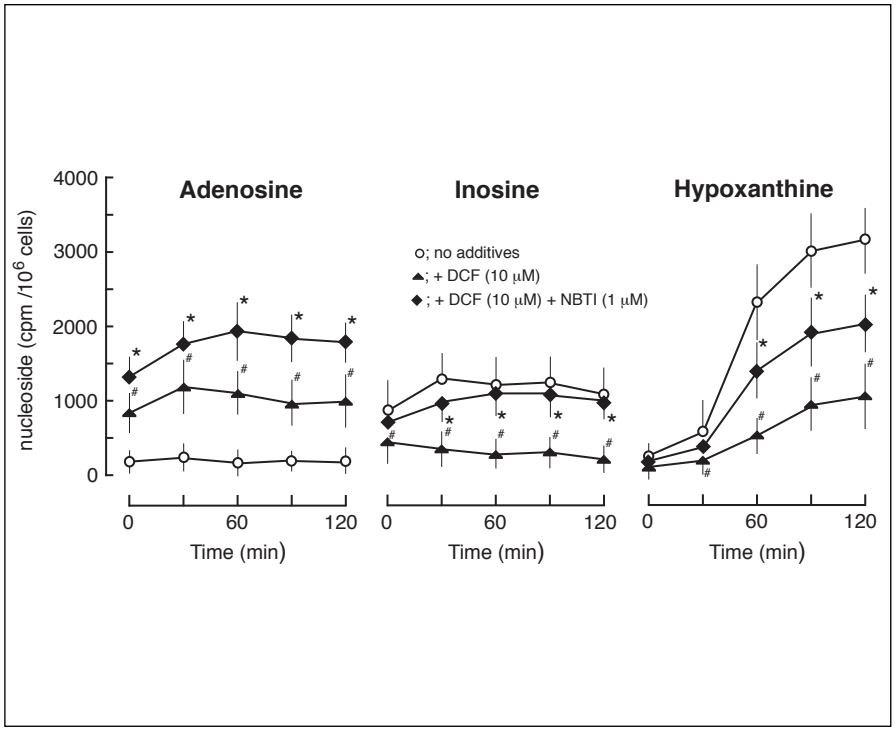

Fig. 4. The impact of adenosine deaminase and nucleoside transporters inhibition on the adenosine, inosine and hypoxanthine levels in the culture media of B lymphoblast. The cells were labeled with [8-^{14}C]adenine as described under "Materials and Methods", washed and incubated with orbital mixing (100 RPM) in RPMI-1640 medium. The incubation medium contained 10 µM 2-deoxycoformycin (DCF) (closed triangle), or 10 µM DCF and 1 µM nitrobenzylthioinosine (NBTI) (closed square). Control cells were incubated without additives (open symbols). At time indicated 100 µl of cell suspension was withdrawn, placed on the top of oil, and immediately centrifuged. The top layer was separated, extracted with perchloric acid, and acid-soluble radiolabeled purine nucleosides and bases were separated by thin-layer chromatography as described under "Materials and Methods". The data represent the mean±S.D. from four independent experiments. *, P≤0.05 *vs*. DCF; #, P≤0.05 *vs*. no additives.

dehydrogenase release rate (*Fig. 2*). However, due to high ATP concentration gradient across the plasma membrane, relatively low damage to the cells could induce sufficient nonspecific

showed that these cells express both the equilibtrative and concentrative nucleoside transport systems (19, 20). Previously, we have shown that nitrobenzylthioinosine (NBTI) sensitive

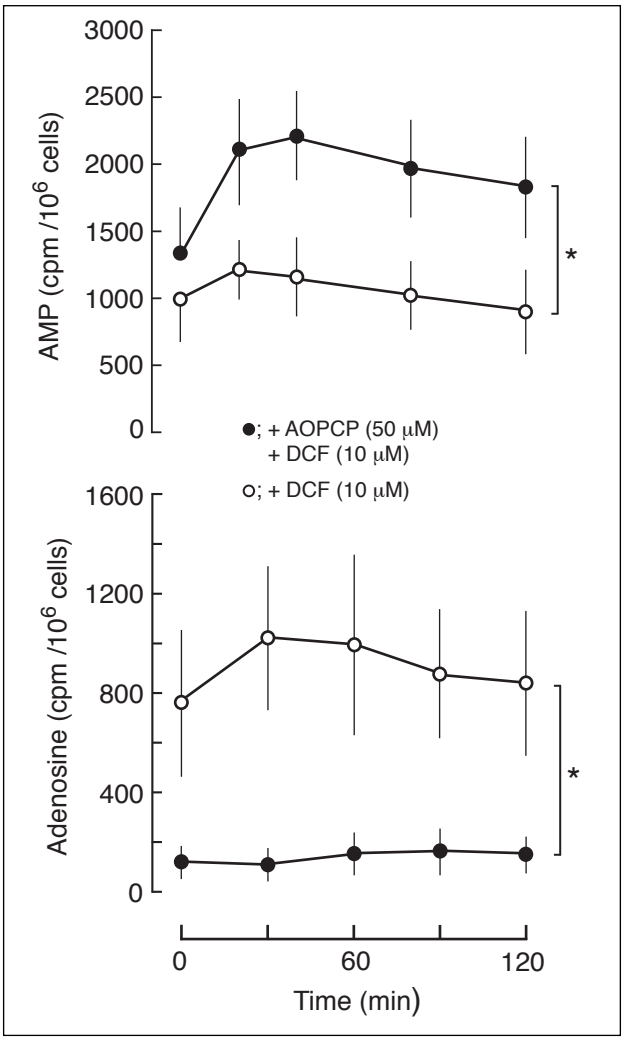

Fig. 5. The effect of ecto-5'-nucleotidase inhibition with α,β-methyleneadenosine 5'-diphosphate (AOPCP) on AMP and adenosine level in B lymphoblast culture media. The cells were labeled with [8-^{14}C]adenine as described under "Materials and Methods", washed and incubated with orbital mixing (100 RPM) in RPMI-1640 medium containing 10 μM 2-deoxycoformycin (DCF), or 10 μM DCF and 50 μM AOPCP. At time indicated 100 μl of cell suspension was withdrawn, placed on the top of oil, and immediately centrifuged. The top layer was separated, extracted with perchloric acid, and acid-soluble radiolabeled purine compounds were separated by thin-layer chromatography as described under "Materials and Methods". The data represent the mean±S.D. from four independent experiments. *, $P<0.05$.

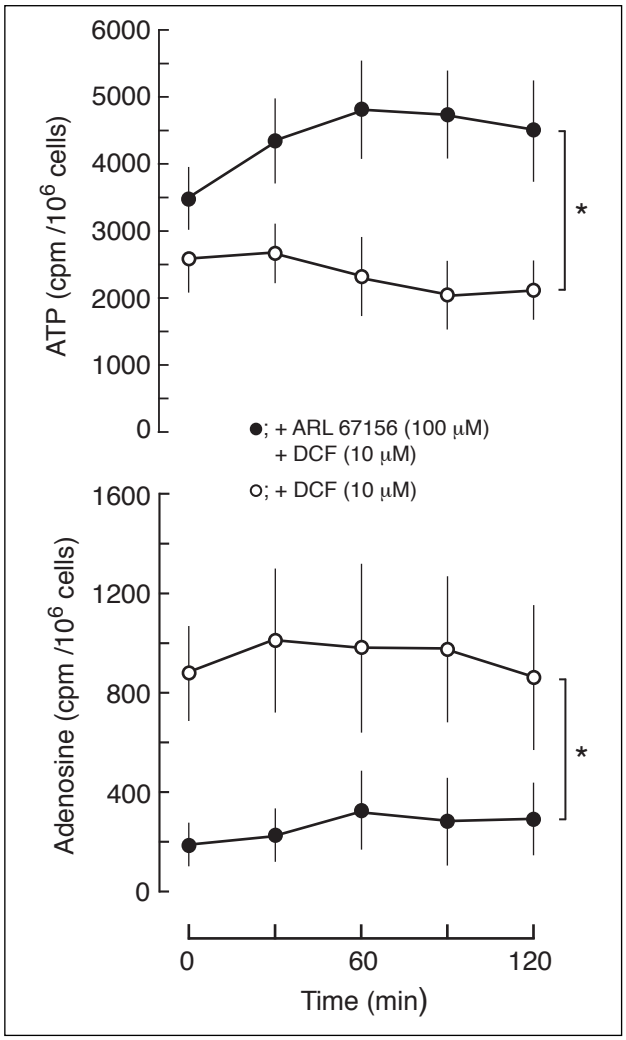

Fig. 6. The effect of ecto-nucleoside triphosphate diphosphohydrolase inhibition with ARL 67156 on ATP and adenosine level in B lymphoblast culture media. The cells were labeled with [8-^{14}C]adenine as described under "Materials and Methods", washed and incubated with orbital mixing (100 RPM) in RPMI-1640 medium containing 10 μM 2-deoxycoformycin (DCF), or in medium containing 10 μM DCF and 100 μM ARL 67156. At time indicated 100 μl of cell suspension was withdrawn, placed on the top of oil, and immediately centrifuged. The top layer was separated, extracted with perchloric acid, and acid-soluble radiolabeled purine compounds were separated by thin-layer chromatography as described under "Materials and Methods". The data represent the mean±S.D. from five independent experiments. *, $P<0.05$.

transport accounts for ~70% of overall equilibtrative adenosine transport in rat B lymphocytes (21). Inclusion in the incubation medium of NBTI (1 μM) in the presence of DCF (10 μM) resulted in the elevation of extracellular adenosine, inosine and hypoxanthine levels (*Fig. 4*). This indicates that most of the extracellular adenosine was generated on the B cell surface and was not released by the cell.

Catabolism of ATP released from the B lymphoblast is the predominant source of adenosine in the extracellular milieu

Extracellular nucleotides are hydrolyzed to the respective nucleosides by a network of ecto-enzymes located on the cell surface. Ecto-5'-nucleotidase (ecto-5'-NT) is among various ectonucleotidases the most specific enzyme responsible for the hydrolysis of AMP and the production of adenosine. It has been reported that some lymphoid cells do not express ecto-5'-NT (22), however our previous work showed that the SKW 6.4 cells possess ecto-5'-NT activity (23). To investigate the contribution of ecto-5'-NT activity to the peripheral B lymphoblast adenosine level, we used α,β-methyleneadenosine 5'-diphosphate (AOPCP), a selective and powerful inhibitor of ecto-5'NT (24). In the experiments carried out in the presence of 50 μM AOPCP and 10 μM DCF, we observed a modest increase of AMP level and a significant decrease of adenosine content in the incubation medium. At 1 hour of incubation, the AMP level increased 2-fold

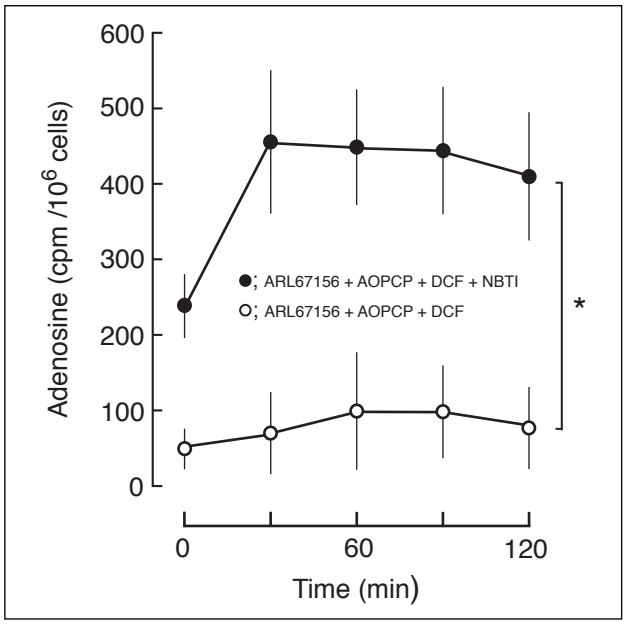

Fig. 7. The impact of nucleoside transporters inhibition on adenosine level in culture media of B lymphoblasts exposed to adenosine deaminase, ecto-5'nucleotidase, and ecto-nucleoside triphosphate diphosphohydrolase inhibitors. The cells were labeled with [8-^{14}C]adenine as described under "Materials and Methods", washed and incubated with orbital mixing (100 RPM) in RPMI-1640 medium containing 10 μM DCF, 50 μM AOPCP, 100 μM ARL 67156, and 1 μM NBTI (closed symbols). Control cells were incubated without NBTI (open symbols). At time indicated 100 μl of cell suspension was withdrawn, placed on the top of oil, and immediately centrifuged. The top layer was separated, extracted with perchloric acid, and acid-soluble radiolabeled purine compounds were separated by thin-layer chromatography as described under "Materials and Methods". The data represent the mean±S.D. from five independent experiments. *, P<0.05

and adenosine decreased ~7-fold (*Fig. 5*). These observations indicated that the activity of ecto-5'-NT was responsible for generation of adenosine on B lymphoblasts periphery. The ecto-nucleoside triphosphate diphosphohydrolases (E-NTDPases) initiate catabolism of extracellular ATP. Among the blood cells, NTPDase1/CD39 has been identified as a major nucleotide-hydrolyzing enzyme on blood cells including B-cells (25, 26). ARL 67156 is the commercially available inhibitor for ecto-ATPases. This compound inhibits NTPDases in a competitive manner; therefore its inhibition efficiency depends on the inhibitor/substrate concentration ratio. It was reported that at ARL/ATP ratio of 100:10 up to 80% inhibition of human NTPDase1 and NTPDase3 occurred (27). In our experiments (10^6 cells/0.1 ml) the concentration of ATP in the incubation media ranged from 0.3 to 0.4 μM (*Fig. 2*); therefore we assumed that 100 μM ARL 67156 (ARL/ATP ratio ~300) should significantly inhibit most of the NTPDases on SKW6.4 cells. Accordingly, incubation of the cells in the presence of ARL 67156 (100 μM) and DCF (10 μM) resulted (at 1 hour of incubation) in a ~2-fold increase of ATP level and a ~3-fold decrease of the adenosine concentration in the medium (*Fig. 6*). The radiolabeled adenosine in the incubation medium containing DCF (10 μM) reached the level of ~1000 cpm/10^6 cells (*Fig. 6*), but simultaneous inhibition of ecto-5'-NT (50 μM AOPCP) and NTPDases (100 μM ARL67156) resulted in a lowering of the adenosine level by a factor of 10 (*Fig. 7*). Introduction of NBTI (10 μM) into the incubation medium under these conditions resulted (at 1 hour of incubation) in the elevation of the adenosine level by 4.5-fold (*Fig. 7*). These observations indicate that the adenosine found on the B lymphoblast periphery originated mostly from the sequential catabolism of extracellular purine nucleotides (possibly ATP), and its concentration depended on the generation/cellular uptake ratio (*Fig. 8*).

DISCUSSION

Our data presented in this report indicate that adenosine is continuously produced on the B-cell surface, however under normal conditions its level is very low owing to efficient uptake by the nucleoside transport system and deamination to inosine by the ecto adenosine deaminase. We also documented that the adenosine appearing in the incubation media result exclusively from sequential breakdown of ATP released from the cell.

The nucleotides released have been observed in excitatory and non-excitatory cells in response to various stimuli (3, 6). Modest mechanical forces of hemodynamic origin and forces associated with increased perfusion flow rates have been shown to induce ATP release from vascular smooth muscle cells, endothelial cells, and from T lymphocytes (3, 6, 12-16). We observed that B lymphoblasts release the measurable quantity of ATP, however when put in motion by gentle swirling, the amount of ATP released increased 3-fold reaching ~35 pmol/10^6 cells. Since the blood cells are subjected to motion physiologically by flow of blood in the body's circulatory system, we assumed that examination of the ATP and adenosine level would be more appropriate in cells subjected to the movement in the incubation medium. Under physiological conditions nucleotides may leave the cells by conductive transport through membrane channels, facilitated diffusion by nucleotide-specific transporters or by means of exocytosis. However, to date the precise mechanism of ATP release from the lymphocytes has not been fully resolved. Recently, it was reported that T cells release ATP through pannexin-1 hemichannels after TCR activation. The patch clamp studies performed on the membrane of *Xenopus oocytes* expressing human pannexin-1 indicated that the channel formed by this protein could be opened by mechanical perturbation and is permeable for ATP (28, 29). However, whether pannexin-1 channel operate in lymphocytes to release ATP in response to the mechanical stress remains to be elucidated. It should be stressed that ATP outside the cell may also be generated during diadenosine polyphosphate (Ap_nA) metabolism (30). These compounds are released by various cells and affect the target cell *via* purinoceptors (31). Recently the regulatory role of Ap_nAs has been observed in several tissues, especially the renal vasculature system (32-34). Regarding B lymphocytes there is no available data on nucleotide release in either conditions, therefore our report provides the first data on ATP release from this cell type.

The effect of ATP and adenosine on immune cells depends on the purinergic receptors. Most cells express both P1 and P2 receptors subtypes, which bind their purine ligands with different affinities (3). In B lymphocyte, the presence of both P1 and P2 receptors have been reported (3, 35). Therefore, the relative density of the particular receptor type and the concentration of signaling purine determine the outcome of purinergic signaling. The ATP concentrations measured in the blood or interstitial space usually reach nanomolar concentrations (36, 37), which are far below the EC_{50} values of most P2 receptors (3). However, accumulation of ATP especially in the nearest vicinity of the cell surface might result in generation of relatively high local ATP concentration. Recently, a micromolar level of ATP was detected in the periphery of lymphoid cells (8). Such a mass of ATP might constitute a

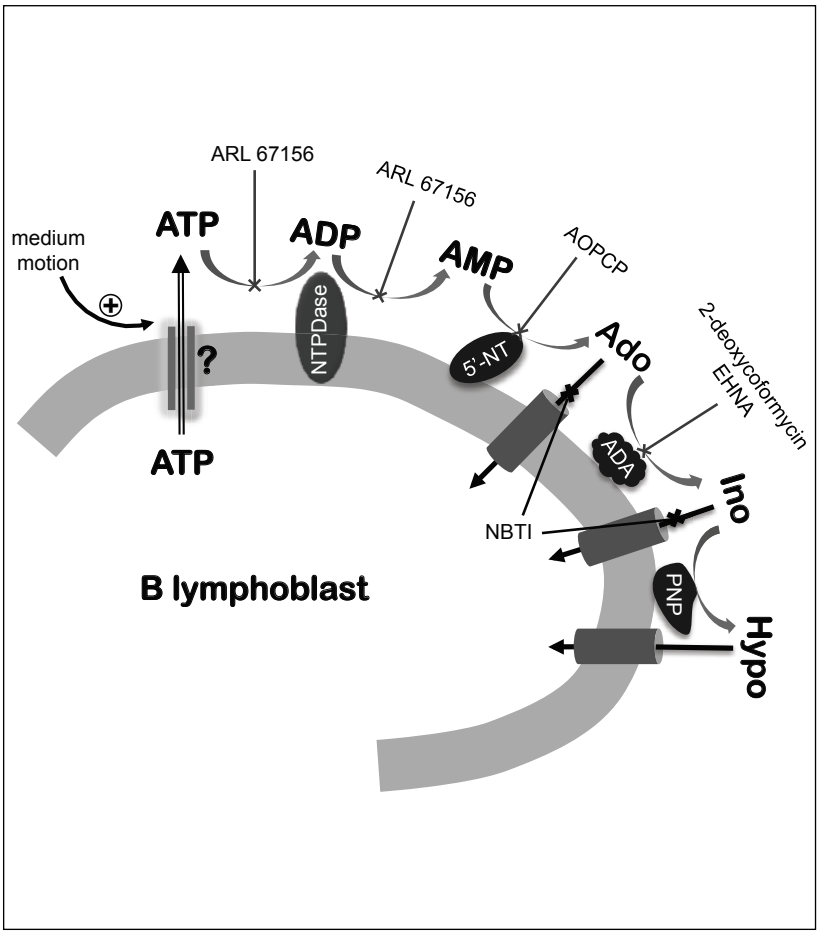

Fig. 8. The proposed route of ATP catabolism and adenosine handling on the surface of B lymphocyte. Our data indicate that ATP is continuously released from B cells by unidentified mechanosensitive channel (possibly trough pannexin channel). The ATP released is hydrolyzed to AMP by ecto-nucleotide triphosphate diphosphohydrolase (NTPDase). It was documented that single NTPDase can dephosphorylate both ATP and ADP nucleotide (44, 45). Four members of the NTPDase family (NTPDase1, 2, 3, and 8) are expressed as cell surface-located enzymes (6). NTPDase1 was identified as a CD39 antigen and major nucleotide-metabolizing enzyme on T and B lymphocytes (26, 46). Adenosine resulting from ecto-5'-nucleotidase (5'-NT) action on AMP can be transported into the cell by nucleoside transporters or be subjected to deamination to inosine by the ecto adenosine deaminase (ADA). Inosine can be transported into the cell or be converted to hypoxanthine by the purine nucleoside phosphorylase (PNP) located on the cell surface (6). The target for each pharmacological inhibitor used in our study is indicated. Adenosine and inosine are substrates of equilibtrative (ENT1, ENT2) and concentrative (CNT2) nucleoside transporters. Nitrobenzylthioinosine (NBTI) sensitive transport accounts for ~65% of overall adenosine transport in B lymphocytes (21). ENT2 transporter, which is not sensitive to inhibition by NBTI transports the hypoxanthine (7).

substantial source of adenosine owing to the network of nucleotide- and nucleoside-converting ectoenzymes located on the cell surface (2, 6). Accordingly, we have observed that inhibition of particular steps of ATP catabolism resulted in a significant lowering of the adenosine level. On the other hand, inhibition of adenosine transport with NBTI resulted in a substantial increase of the adenosine level in the incubation media. This indicates that the majority of extracellular adenosine was generated on the cell surface and was not released from the cells. Therefore, the conditions leading to altered function of nucleoside transporters might result in an elevation of the adenosine level on cell surface. We have previously documented that adenosine transport in B and T cells is significantly impaired in diabetes (10, 11, 21). The other crucial elements determining the extracellular nucleoside/nucleotide ratio are the activities of the ecto-enzymes on the cell surface. We have observed that inhibition of the ecto-nucleoside triphosphate diphosphohydrolases (E-NTDPases) with ARL67156 resulted in a substantial increase of extracellular ATP level and a decrease of adenosine concentration in the incubation medium. Inhibition of ecto-5'-nuleotidase was associated with a decreased extracellular adenosine level. These observations are in accordance with studies performed on ecto-enzyme knock-out models (38, 39). However, we have noted some disproportionality between the magnitude of changes in AMP and adenosine level induced by inhibition of ecto-5'-NT. As can be seen in *Fig. 5*, the 2-fold increase of AMP level was accompanied by a decrease as high as 7-fold in the adenosine level. In our present study we did not investigate in detail the extracellular metabolism of nucleotides, but it has been reported that outside of the cell, AMP may be phosphorylated back into ADP in a reaction catalyzed by ecto-adenylate kinase (22). The activity of ecto-adenylate kinase has been identified on a variety of cells including human vascular endothelial cells (40), lymphocytes (22), hepatocytes (41), keratinocytes (43), and airway epithelial cells (44). It is assumed that under normal physiological conditions the ecto-adenylate kinase do not compete with ecto-nucleotidases for nucleotides, but during increases in extracellular nucleotide level the ecto-adenylate kinase might significantly contribute to AMP consumption.

In conclusion, the available data indicate that the ATP and adenosine actions on immune cells are extremely complex and interdependent. In general ATP molecule is considered as a proinflammatory and immunostimulatory compound, whereas adenosine suppresses the response of immune cells. Since, both molecules may act on the same cell simultaneously the net effect depends on the balance between Ado and ATP action. Our study's data indicate that ATP released from the B cell is the primary source of peripheral adenosine, and that the activities of ecto enzymes and efficiency of Ado uptake trough the nucleoside transporters determine the Ado level in the immediate vicinity of the cell surface. Thus, with an unchanged ATP release, any conditions altering the ecto-enzymes activities or/and the nucleoside transporters function may lead to a change in the ATP/Ado ratio on the cell surface.

Acknowledgements: This work was supported by the Polish Ministry of Science and Higher Education grant no. 2 P05A 081 29 to TP. Special thanks to dr. Inna N. Lavrik for technical advise concerning the cell culture maintenance.

Conflict of interests: None declared.

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Received: March 11, 2010
Accepted: June 15, 2010

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