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PROTEIN KINASE C MEDIATED HIGH GLUCOSE EFFECT ON ADENOSINE RECEPTORS EXPRESSION IN RAT B LYMPHOCYTES

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Hyperglycemia-induced alterations of adenosine receptors (ARs) expression are implicated in the pathomechanism leading to impaired function of the lymphocytes in diabetes. However, the signaling pathways utilized by glucose to regulate ARs expression are unknown. This work was undertaken to investigate the impact of high glucose level on the ARs expression in rat B lymphocytes. The results presented in this report demonstrate that rat B lymphocytes express all four types of ARs at the mRNA and protein level. Exposing B cells to high glucose (25 mM) suppressed the expression of A_1-AR, A_{2B}-AR, and A_3-AR, but had no effect on the expression of A_{2A}-AR. A selective inhibitor of Ca^{2+}-dependent protein kinase C (PKC) isoforms suppressed the high glucose effect on A_3-AR expression. Inhibition of PKC-δ with rottlerin blocked the high glucose effect on A_1-AR mRNA level. An inhibitor of Raf-1 kinase completely blocked the high glucose effect on A_{2B}-AR expression. The suppression of A_1-AR and A_{2B}-AR mRNA expression induced by high glucose was blocked by an inhibitor (PD98059) of MAPK kinase (MEK). In conclusion, high glucose utilizes a signaling pathway involving some elements of the MAPK pathway and different PKC isoforms to suppress the expression of A_1-AR, A_{2B}-AR, and A_3-AR in rat B lymphocytes.

Key words: *glucose, B lymphocyte, adenosine receptors, protein kinase C, MAP kinases*

INTRODUCTION

B-cells are the core constituent of the immunological system. When stimulated by an antigen, a mature B-cell proliferates and differentiates to cell producing and secreting antibodies (Ab). Therefore, the mechanisms that affect the function of B-lymphocytes have a significant impact on the humoral immune response. Impaired lymphocyte function and enhanced susceptibility to infections is a common feature of human diabetes (1). The reason for this increased susceptibility in diabetic patients is not fully understood. Some indications point to adenosine as a factor of the pathomechanism leading to the altered functioning of the lymphocytes in diabetes (2, 3).

Adenosine is an endogenous compound exerting a potent action on the immune system. Clinical observations of patients with severe combined immunodeficiency have documented the importance of adenosine to the development and function of the immune cells (4). The experimental data gathered to date indicate the ability of adenosine to affect events such as lymphocyte activation, proliferation, cytokines production, and lymphocyte-mediated cytolysis (5). Adenosine is formed both in the extra- and intracellular space (6, 7) and exerts its biological effect by coupling with cell-surface receptors in a paracrine or autocrine fashion. To date, four adenosine receptors (ARs) have been identified, namely A_1, A_{2A}, A_{2B}, and A_3 (8). Development of diabetes results in an altered expression of ARs in many types of cells (9, 10). Moreover, it appears that diabetes-induced changes in ARs expression are tissue and cell type specific (11). Experiments on cells in culture have indicated that these alterations are largely induced by changes in glucose concentrations (2). Little is known about the ARs status in B lymphocytes during the development of diabetes. There is also a lack of data concerning the consequences for ARs expression in B lymphocytes arising from changes in glucose concentration. Therefore, the objective of our study was to investigate the expression level of adenosine receptors in B lymphocytes cultured at different glucose concentrations. We evaluated the signaling pathways utilized by glucose to regulate the ARs expression level in rat B lymphocytes.

MATERIALS AND METHODS

Reagents

Histopaque-1077, insulin, penicillin, streptomycin, 2'-amino-3'-methoxyflavone (PD 98059), 3-(3,5-dibromo-4-hydroxybenzyliden)-5-iodo-1,3-dihydroindol-2-one (GW 5074), glucose, wortmannin, leupeptin, thiobutabarbital sodium (Inactin), and RPMI-1640 medium, were obtained from Sigma-Aldrich Sp. z o.o. (Poznan, Poland). Bisindolylmaleimide I (Bis I), 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-imidazole (SB 203580), rottlerin, calphostin C, [12-(2-cyanoethyl)]Q1-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole (Go 6976), {2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-3(1H-indol-3-

yl)maleimide (Go 6983), and cell permeable PKC-ζ pseudosubstrate sequence (Myr-SIYRRGARRWRKL-OH) were obtained from Calbiochem-Merck Sp. z o.o. (Warsaw, Poland). All primers used were from Integrated DNA Technologies, Inc. (Coralville, IA, USA). Total RNA Prep Plus Kit was from A&A Biotechnology (Gdansk, Poland). Primary rabbit polyclonal antibodies to A_1-AR (A-268), and β-actin, were from Sigma-Aldrich Sp. z o.o. (Poznan, Poland). Rabbit polyclonal antibody to A_{2B}-AR (AB1589P) was from Chemicon International (Temecula, CA, USA). Goat polyclonal antibodies to A_{2A}-AR (R-18), and A_3-AR (C-17), were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Fluorescein conjugate mouse anti rat CD2[LFA-2] (clone OX-34) was from Chemicon International (Hofheim, Germany). Fluorescein conjugate Armenian hamster anti rat CD40 (clone HM40-3), and mouse anti rat CD19 (clone 1D3) were from BD Biosciences (Heidelberg, Germany).

Animals

Male Wistar rats (200-240 g) fed on Altromin C 1000 diet (Altromin GmbH, Lage, Germany) were used for all experiments. All animals had access to food and water *ad libitum*.

Cells and culture conditions

Single cell suspension of splenocytes was prepared by pressing spleens through sterilized 20 mm pore size nylon mesh gauze in the presence of sterile saline. Mononuclear cells were isolated by centrifugation of the cell suspension through Histopaque-1077 at 400 x g for 30 minutes at room temperature. Cells found at the saline/Histopaque interface were washed and suspended in RPMI-1640 medium supplemented with 3% BSA. The cells were then separated into adhesive and nonadhesive by the panning method as described previously (12). The purity of isolated cell fractions was examined by flow cytometry. The adherent fraction (B cells) contained 95-97% CD2 (OX-34) negative cells, 89-93% CD40 (HM40-3) and 85-90% CD19 (1D3) 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 for 48 hours in flat-bottomed culture bottles in humidified atmosphere containing 5% CO_2 at 37°C at a density of 2-4 x 10^6 cells/ml in RPMI-1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 mg/ml), and 10% fetal bovine serum and containing glucose at concentration detailed in the figure legends. The compounds examined (glucose, and inhibitors) were added to lymphocyte culture in the concentration and the order and for the time detailed in the figure legends. Inhibitors were dissolved in a small volume (less than 0.2% of the total volume of culture medium) of DMSO. The osmolarity of the culture medium was maintained constant by the addition of appropriate amount of D-mannitol.

Real-time PCR analysis

The levels of ARs transcripts were analyzed by real-time PCR performed in a Light Cycler 2.0 (Roche Diagnostics GmbH) using the Light Cycler DNA SYBR Green I Kit. The reaction mixture contained 1 ml Master Mix, 5 pmol of each primer and 2 µl of cDNA. The primers for A_1-AR, A_{2A}-AR, A_{2B}-AR, A_3-AR, and β-actin cDNA amplification were as described previously (11). As a negative controls water was run with every PCR. The specificity of product was controlled by melting curve analysis, and by agarose gel electrophoresis. The ratio of AR/ β-actin was calculated for each sample. Analysis of the data was done using Light Cycler software 4.0.

Western blot analysis

The extract of B cells was obtained by sonication (3 x 15 s) of cell suspension in 20 mM Tris-HCl, pH 7.2, 1 mM dithiothreitol, 0.2 mM Pefabloc SC, and 5 mM leupeptin. The proteins from obtained extract were separated by 12% SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred to Immobilon poly(vinylidene difluoride) transfer membrane. The membrane was incubated at 4°C (overnight) with 3% BSA in Tris-buffered saline (TBS). The membrane was then cut horizontally at appropriate position (based on positions of prestained molecular mass markers), and incubated with appropriate primary antibodies. Next the membrane strips were incubated with alkaline phosphatase-conjugated secondary antibodies. Membrane bound antibodies were visualized with 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium. For A_1-AR and A_{2B}-AR the β-actin was used as a reference protein. The p-14-3-3 protein was a reference protein for A_{2A}-AR and A_3-AR.

Statistical analysis

The statistical calculation was performed with ANOVA or 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 glucose effect on adenosine receptors proteins level in rat B lymphocytes

To investigate the glucose effect on adenosine receptors (ARs) expression in rat B lymphocytes, we first determined which AR types are present in these cells. The AR proteins were analyzed by Western blot with type-specific polyclonal antibodies. Our investigation revealed the presence of all four ARs proteins in the rat B cell extract, although the protein bands of A_{2A}-AR and A_3AR were weak (*Fig. 1*). The levels of ARs proteins were similar in isolated cells before and after 3 days of culture in an RPMI medium containing 5 mM glucose (not shown). This indicates that

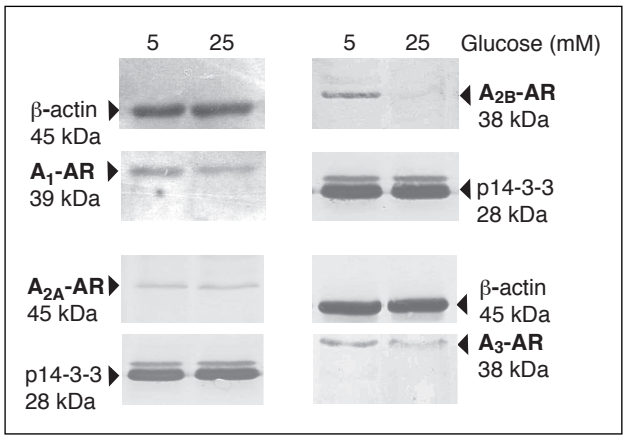

Fig. 1. The protein level of adenosine receptors in rat B lymphocytes cultured in various glucose concentrations. B lymphocytes isolated from rat spleen were cultured for 48 hours at the indicated glucose concentrations and the protein extracts were prepared as described under "Materials and Methods". The proteins were separated on 12% SDS-PAGE and immunoblotted with appropriate antibodies. The presented blots are representative of those obtained in at least three independent experiments.

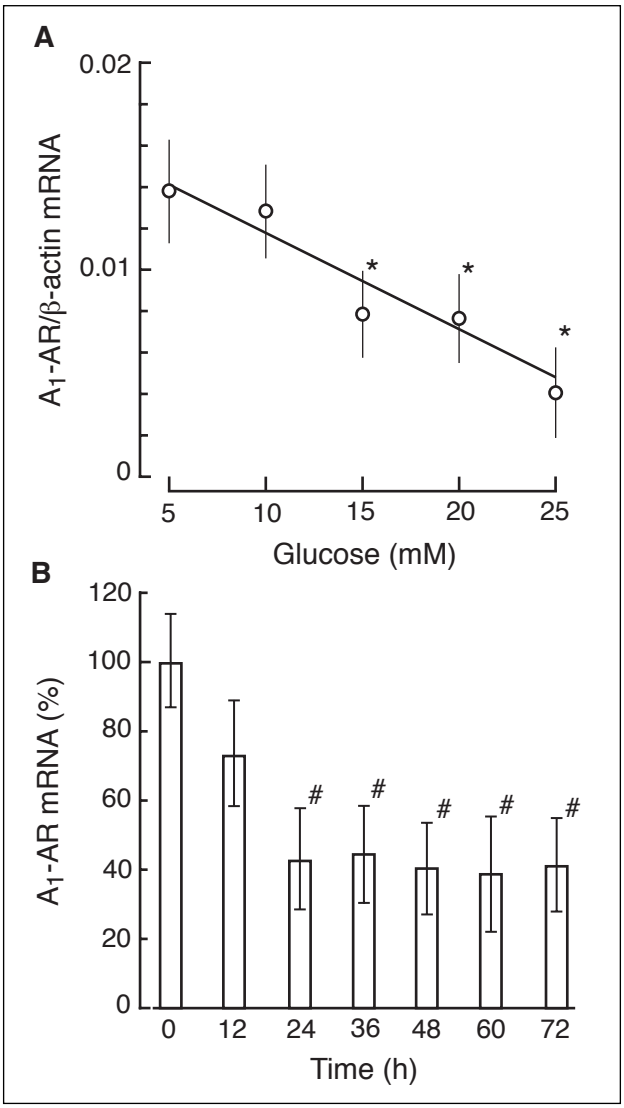

Fig. 2. The glucose effect on the abundance of A_1-AR mRNA in rat B lymphocytes. (**A**) Dose-dependent course of glucose action on the A_1-AR mRNA level. Cells were cultured for 48 hours in the presence of glucose at the concentrations indicated. Next, cells were harvested, total RNA was extracted, and the A_1-AR mRNA level was determined by Real-time PCR as described under "Materials and Methods". The data represent the mean ±SD from three experiments. (*) $P<0.05$ versus 5 mM glucose. (**B**) Time course of glucose action on the A_1-AR mRNA level. Cells were cultured in the presence of 5 mM glucose for 20 hours. On the second day (time 0) cells were transferred to the culture medium containing 25 mM glucose. At time indicated, cells were harvested and the A_1-AR mRNA level was determined as described above. The data represent the mean ±SD from three experiments. (*) $P<0.05$ versus 0 time.

the expression level of ARs did not change significantly during the first days of cell culture (up to 3 days). Exposition of B cells to 25 mM glucose for 48 h resulted in a decrease of the AR proteins (A_{2B}-AR and A_3-AR, to undetectable levels) with the exception of A_{2A}-AR, which did not change significantly (*Fig. 1*).

The high glucose effect on A_1-AR expression in rat B lymphocytes

The level of ARs proteins was very low in the B cell extracts; the Western blot analysis had therefore given us the qualitative data but was of no use in terms of quantitative analysis. To examine the expression of ARs we determined the levels of mRNAs by real-time PCR. *Fig. 2* depicts the concentration and time-dependent effect of glucose on the expression of A_1-AR. Glucose at a concentration of up to 10 mM did not significantly affect the A_1-AR mRNA level; at higher concentration, however, it suppressed the expression of this receptor (*Fig. 2A*). The maximal effect of 25 mM glucose on the A_1-AR mRNA level was observed at the 24th hour (*Fig. 2B*).

The activation of the mitogen-activated protein kinase (MAPK) pathway and protein kinase C (PKC) is the mechanistic basis of high glucose-induced changes in the cell (*Fig. 3*). To define the key steps of the glucose signaling involved in suppression of A_1-AR expression we used specific inhibitors. The exact target of a particular inhibitor is indicated on the scheme presented in *Fig. 3*. Optimal concentrations of the inhibitors were determined by pilot studies (not shown) and are in accordance with previous reports (13-17). Wortmannin, an inhibitor of phosphatidyl 3-kinase (PI3-K) did not affect the glucose action on the A_1-AR transcript level (*Fig. 4*). Pretreatment of B cells with PD 98059 (10 μM), an MAPK kinase (MEK) inhibitor, abolished the high glucose effect on A_1-AR transcript level. To further elucidate the glucose-signaling pathway in rat B lymphocytes we examined the high glucose effect on the A_1-AR mRNA level under a condition of Raf kinase inhibition. Raf-1 kinase is an upstream effector of MEK. The inclusion of a selective and potent Raf-1 inhibitor (GW 5074) in the incubation medium had no effect on high glucose-induced suppression of A_1-AR expression. The inhibition of p38 MAP kinase with SB 203580 (1 μM) did not affect the action of glucose on the A_1-AR mRNA level. To evaluate the role of PKC in high glucose-induced suppression of A1-AR mRNA we used different PKC inhibitors. Exposing B cells to 1 μM bisindolylmaleimide I (Bis I), an isozyme non-selective PKC inhibitor, prevented the glucose-induced suppression of A_1-AR (*Fig. 4*). A similar result was observed with Calphostin C, which is a highly specific PKC inhibitor that competes at the diacylglicerol binding site (*Fig. 5*). This implies the involvement of conventional and/or novel PKC isozymes. Treatment of B cells with a selective inhibitor of Ca^{2+}-dependent PKC isozymes (Go 6976) had no effect on the A_1-AR mRNA level, suggesting the involvement of novel PKCs. According to previous reports, even at a micromolar concentration, Go 6976 has no effect on the Ca^{2+}-independent PKC isozymes (13). Exposing B cells to Go 6983, an inhibitor of several PKC isozymes (with the exception of PKC-μ), effectively blocked the high glucose effect on A_1-AR expression. The glucose effect on A_1-AR expression was also abolished by rottlerin (10 μM), a selective inhibitor of PKC-δ and PKC-Θ (*Fig. 5*).

The high glucose effect on A_{2B}-AR expression in rat B lymphocytes

Exposing rat B lymphocytes to an increased concentration of glucose resulted in a decrease of the A_{2B}-AR transcript level. The maximal effect of 25 mM glucose (a 60% decrease) was seen at the 48th hour incubation of the cells (*Fig. 6*). Exposing the B cells to high glucose in the presence of wortmannin (0.1 μM) did not affect the glucose action on the A_{2B}-AR transcript level, implying that PI3-K is not involved. Pretreatment of the cells with GW 5074 (10 μM), or PD 98059 (10 μM) resulted in suppression of the glucose effect on A_{2B}-AR mRNA level (*Fig. 7*). This indicates that high glucose-induced A_{2B}-AR downregulation is mediated *via* activation of the Raf-1/MAPK/ERK pathway. Incubation of the cells with a p38 MAPK inhibitor (SB 203580) for 1 hour before the exposure to 25 mM glucose had no effect on the glucose action on the A_{2B}-

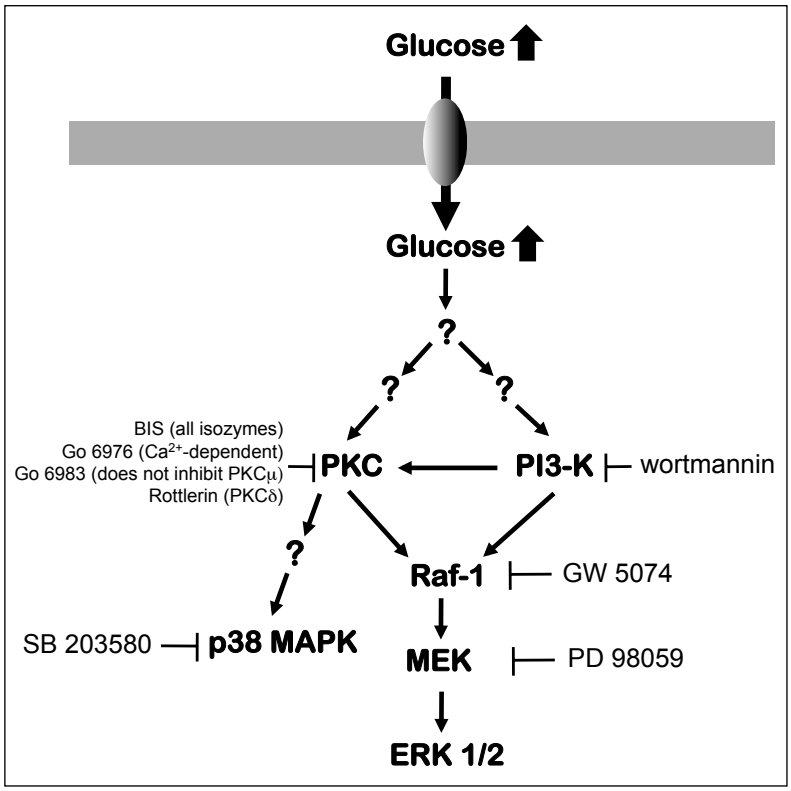

Fig. 3. Schematic sketch depicting potential signaling cascade transmitting the high glucose signal. The exact mechanism by which high glucose activates PKC and PI3-K remains undefined. The target for each pharmacological inhibitor is indicated. Abbreviations are described in the text.

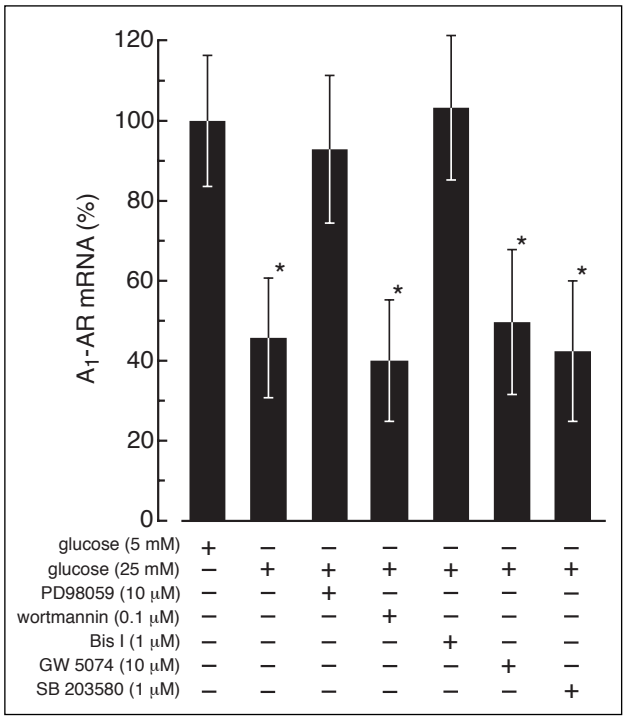

Fig. 4. The effect of PI3-K, Raf-1, MEK, p38 MAPK, and PKC inhibitors on high glucose-induced suppression of the A_1-AR mRNA level in rat B lymphocytes. The cells were cultured in RPMI medium containing 5 mM glucose for 20 hours and then for 24 hours in medium containing 25 mM glucose. One hour before raising the glucose concentration to the culture medium examined inhibitors were added at indicated concentrations. Next the cells were harvested and the A_1-AR mRNA was quantified as described under "Materials and Methods". To the control cells appropriate volume of solvent (DMSO) was added. The data represent the mean ±SD from four experiments. (*) $P<0.05$ versus 5 mM glucose.

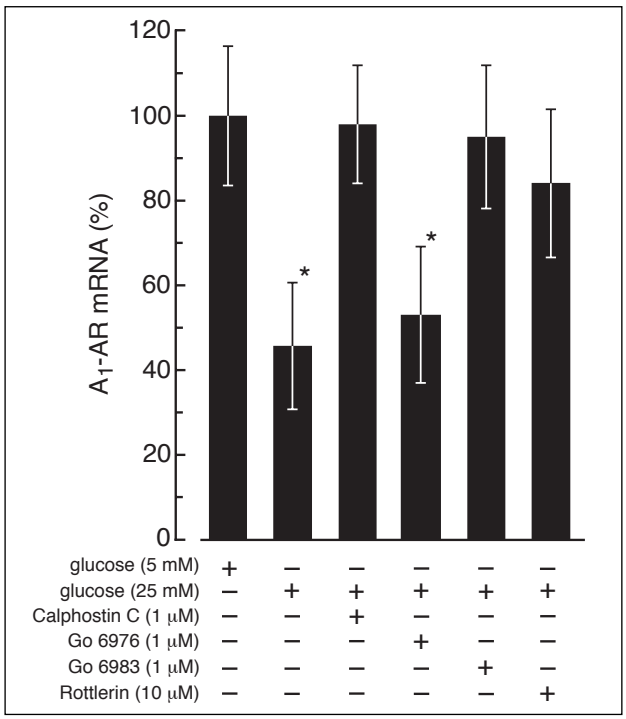

Fig. 5. The effect of PKC inhibitors on high glucose-induced suppression of the A_1-AR mRNA level in rat B lymphocytes. The cells were cultured in RPMI medium containing 5 mM glucose for 20 hours and then for 24 hours in medium containing 25 mM glucose. One hour before raising the glucose concentration to the culture medium examined inhibitors were added at indicated concentrations. Next the cells were harvested and the A_1-AR mRNA was quantified as described under "Materials and Methods". To the control cells appropriate volume of solvent (DMSO) was added. The data represent the mean ±SD from four experiments. (*) $P<0.05$ versus 5 mM glucose.

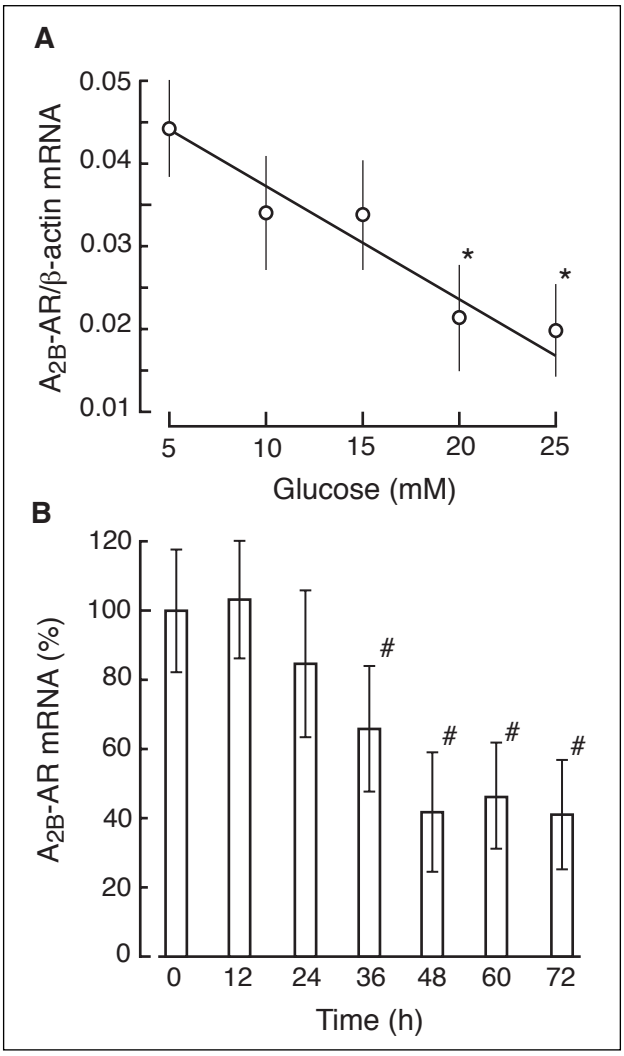

Fig. 6. The glucose effect on the abundance of A_{2B}-AR mRNA in rat B lymphocytes. (**A**) Dose-dependent course of glucose action on the A_{2B}-AR mRNA level. Cells were cultured for 48 hours in the presence of glucose at the concentrations indicated. Next, cells were harvested, total RNA was extracted, and the A_{2B}-AR mRNA level was determined as described under "Materials and Methods". The data represent the mean ±SD from three experiments. (*) $P<0.05$ versus 5 mM glucose. (**B**) Time course of glucose action on the A_{2B}-AR mRNA level. Cells were cultured in the presence of 5 mM glucose for 20 hours. On the second day (time 0) cells were transferred to the culture medium containing 25 mM glucose. At time indicated, cells were harvested and the A_{2B}-AR mRNA level was determined as described above. The data represent the mean ±SD from three experiments. (*) $P<0.05$ versus 0 time.

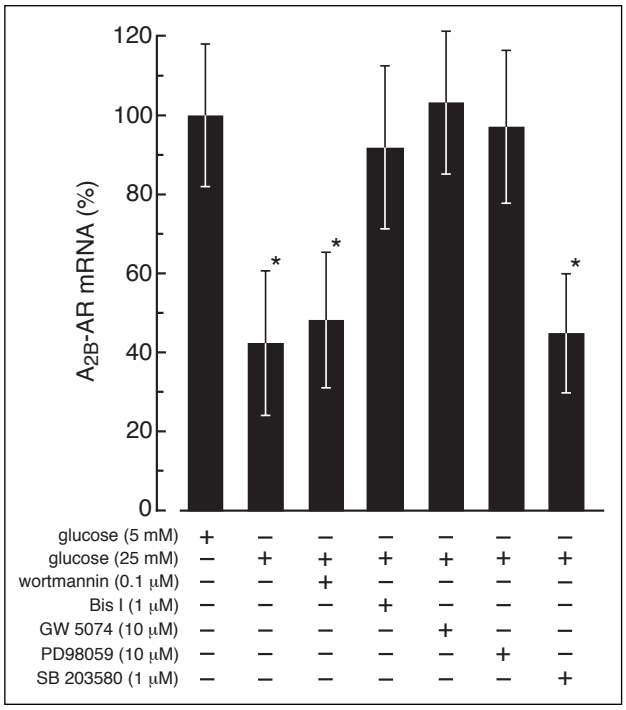

Fig. 7. The effect of PI3-K, Raf-1, MEK, p38 MAPK, and PKC inhibitors on high glucose-induced suppression of the A_{2B}-AR mRNA level in rat B lymphocytes. The cells were cultured in RPMI medium containing 5 mM glucose for 20 hours and then for 48 hours in medium containing 25 mM glucose. One hour before raising the glucose concentration to the culture medium examined inhibitors were added at indicated concentrations. Next the cells were harvested and the A_{2B}-AR mRNA was quantified as described under "Materials and Methods". To the control cells appropriate volume of solvent (DMSO) was added. The data represent the mean ±SD from four experiments. (*) $P<0.05$ versus 5 mM glucose.

AR mRNA level. Inclusion of 1 μM Bis I in the incubation medium completely prevented the glucose-induced suppression of A_{2B}-AR expression (*Fig. 7*). To determine the role of PKC isoforms in the glucose signaling cascade, the association of a particular PKC isoform with the downregulation of A_{2B}-AR was assessed by the use of several specific inhibitors. As shown in *Fig. 8*, pretreatment of the cells with a selective inhibitor of Ca^{2+}-dependent PKCs (Go 6976) did not affect the glucose action on the A_{2B}-AR transcript level. The presence of another PKC inhibitor (Go 6983), which is not effective in inhibiting PKC-μ isoform, prevented the glucose action on A_{2B}-AR mRNA level. The suppression of A_{2B}-AR expression evoked by high glucose was also affected by neither the PKC-δ and PKC-Θ inhibitor (Rottlerin) nor the PKC-ζ inhibitor (myristoylated pseudosubstrate peptide).

The high glucose effect on A_3-AR expression in rat B lymphocytes

Rat B lymphocytes incubated at an increased concentration of glucose displayed decreased level of A_3-AR mRNA. Examination of the glucose dose response effect of A_3-AR expression revealed that the maximal effect could be observed at ~20 mM glucose (*Fig. 9A*). The maximal effect of 25 mM glucose (~50% decrease) was observed at the 48th hour of the cells' incubation (*Fig. 9B*). The glucose effect on A_3-AR expression was blocked by neither the PI3-K inhibitor (wortmannin) nor the MAPK inhibitors (GW 5074, PD98059, SB 203580). However, pretreatment of the cells with 1 μM Bis I resulted in suppression of the high glucose effect on A_3-AR expression, implying the involvement of PKC (*Fig. 10*). To further examine the hypothesis that A_3-AR expression in rat B lymphocytes is affected by high glucose in a PKC-dependent manner we used other inhibitors that selectively inhibit PKC isoforms. Inhibition of PKC-δ and PKC-Θ with Rottlerin or PKC-ζ with myristoylated pseudosubstrate peptide, had no effect on high glucose-induced suppression of A_3-AR expression. However, the addition of a selective inhibitor of

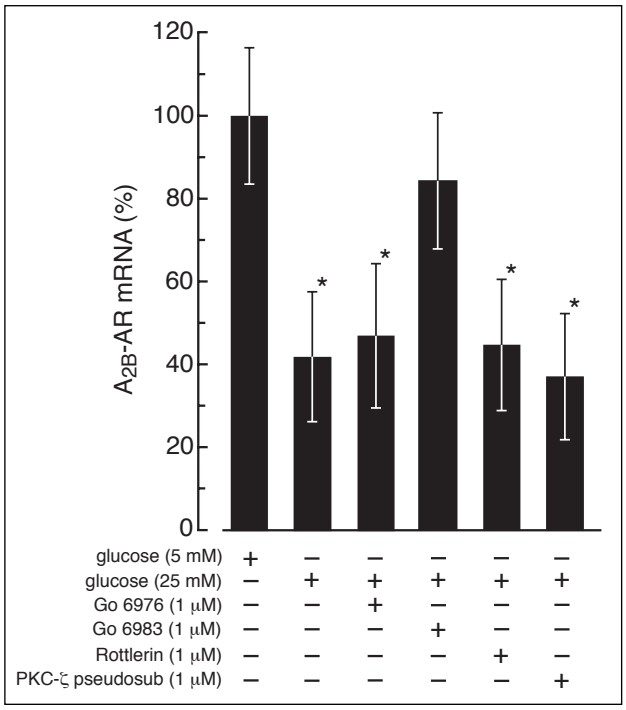

Fig. 8. The effect of PKC inhibitors on high glucose-induced suppression of the A_{2B}-AR mRNA level in rat B lymphocytes. The cells were cultured in RPMI medium containing 5 mM glucose for 20 hours and then for 48 hours in medium containing 25 mM glucose. One hour before raising the glucose concentration to the culture medium examined inhibitors were added at indicated concentrations. Next the cells were harvested and the A_{2B}-AR mRNA was quantified as described under "Materials and Methods". Where it was necessary to the control cells appropriate volume of solvent (DMSO) was added. The data represent the mean ±SD from four experiments. (*) $P<0.05$ versus 5 mM glucose.

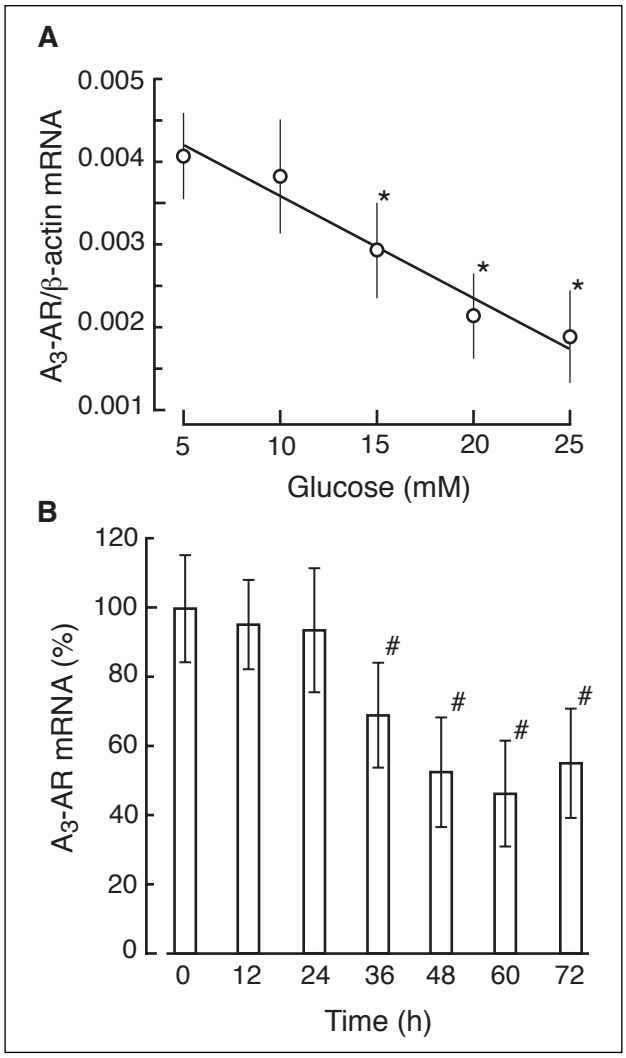

Fig. 9. The glucose effect on the abundance of A_3-AR mRNA in rat B lymphocytes. (**A**) Dose-dependent course of glucose action on the A_3-AR mRNA level. Cells were cultured for 48 hours in the presence of glucose at the concentrations indicated. Next, cells were harvested, total RNA was extracted, and the A_3-AR mRNA level was determined as described under "Materials and Methods". The data represent the mean ±SD from three experiments. (*) $P<0.05$ versus 5 mM glucose. (**B**) Time course of glucose action on the A_3-AR mRNA level. Cells were cultured in the presence of 5 mM glucose for 20 hours. On the second day (time 0) cells were transferred to the culture medium containing 25 mM glucose. At time indicated, cells were harvested and the A_3-AR mRNA level was determined as described above. The data represent the mean ±SD from three experiments. (*) $P<0.05$ versus 0 time.

Ca^{2+}-dependent PKC isoforms (Go 6976) to the incubation medium prevented the high glucose effect on A_3-AR expression (*Fig. 11*).

DISCUSSION

The presence of all four adenosine receptor (AR) types on immune cells has been reported previously (5), although information about the ARs expression pattern on particular lymphocyte subtype is incomplete. In most cases, the expression of a particular AR type was inferred from studies using specific pharmacological ligands. Investigation performed on human peripheral lymphocytes has documented the presence of A_{2A}-AR protein in functional subsets of peripheral T cells, but failed to detect this protein in B cells (18). Expression of mRNA for all four ARs has been reported in mice B lymphocytes, but the ARs proteins were not investigated in that study (19). Our work shows that rat B lymphocytes express all four types of adenosine receptors at the mRNA and protein level, although the A_{2A}-AR and A_3-AR proteins were barely detectable.

Previously reported data indicate that the development of diabetes results in an altered expression of adenosine receptors in a variety of cell types, including rat T lymphocytes (2, 9-11). However, regulation of adenosine receptors in B cells had not yet been studied in relation to changes in glucose concentration. Our study, performed on cardiac fibroblasts, documented that changes in the glucose level have a significant impact on ARs expression (20); however, the signaling pathways utilized by glucose to regulate ARs expression was not investigated. In the present study, we demonstrate that in rat B cells high glucose suppresses the expression of A_1AR, A_{2B}-AR, and A_3-AR in a PKC-dependent manner, but does not affect the expression level of A_{2A}-AR.

The high glucose effects on PKC expression and activity have been studied using the animal model of diabetes and in cultured cells (21, 22). Many investigators have documented increased activity of different PKC isozymes in cells exposed to

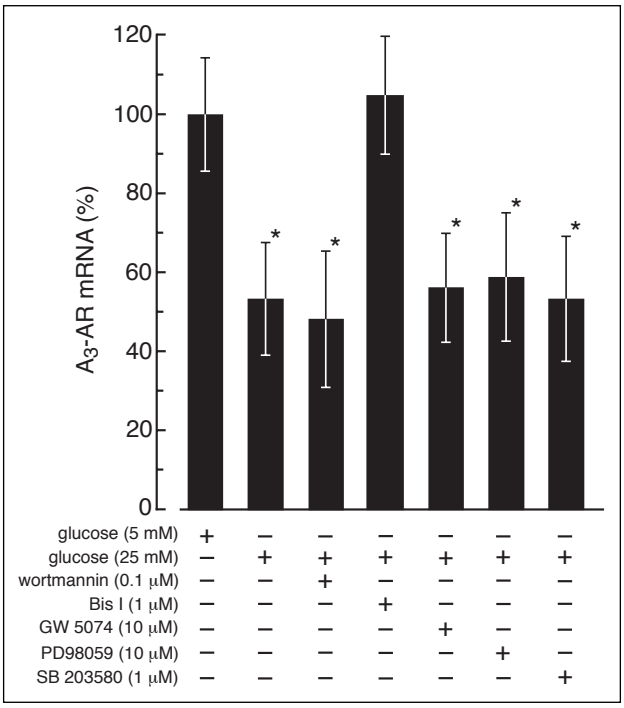

Fig. 10. The effect of PI3-K, Raf-1, MEK, p38 MAPK, and PKC inhibitors on high glucose-induced suppression of the A_3-AR mRNA level in rat B lymphocytes. The cells were cultured in RPMI medium containing 5 mM glucose for 20 hours and then for 48 hours in medium containing 25 mM glucose. One hour before raising the glucose concentration to the culture medium examined inhibitors were added at indicated concentrations. Next the cells were harvested and the A_3-AR mRNA was quantified as described under "Materials and Methods". To the control cells appropriate volume of solvent (DMSO) was added. The data represent the mean ±SD from four experiments. (*) $P<0.05$ versus 5 mM glucose.

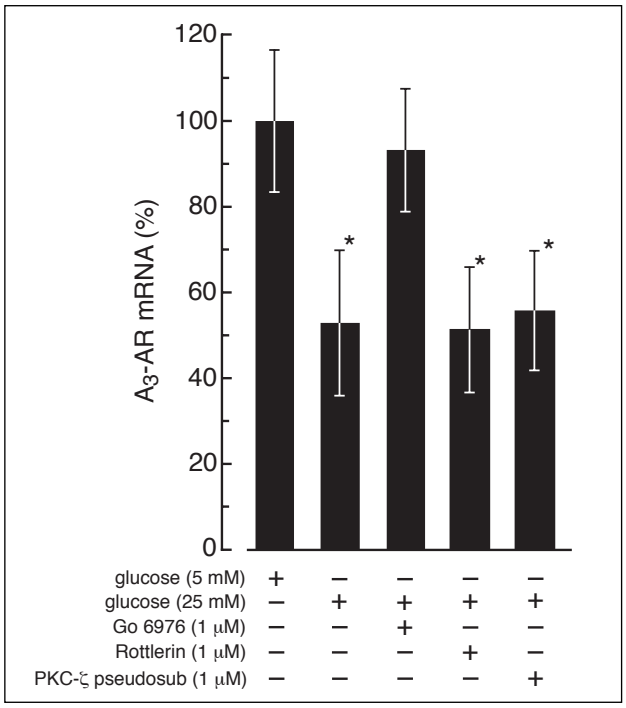

Fig. 11. The effect of PKC inhibitors on high glucose-induced suppression of the A_3-AR mRNA level in rat B lymphocytes. The cells were cultured in RPMI medium containing 5 mM glucose for 20 hours and then for 48 hours in medium containing 25 mM glucose. One hour before raising the glucose concentration to the culture medium examined inhibitors were added at indicated concentrations. Next the cells were harvested and the A_3-AR mRNA was quantified as described under "Materials and Methods". Where it was necessary to the control cells appropriate volume of solvent (DMSO) was added. The data represent the mean ±SD from four experiments. (*) $P<0.05$ versus 5 mM glucose.

high glucose (23-26). The involvement of PKC in the regulation of A_1-AR expression in hamster ductus deferens tumor (DDT_1MF-2) cells has been reported recently (27). It has been demonstrated that the PKC-induced expression of A_1-AR gene is mediated by NF-κB. The PKC-depended NF-κB activation required an increase in cellular Ca^{2+} concentration, implying the involvement of conventional PKC isozymes. This assumption supports the observation that B cells isolated from the PKC-β-deficient mice show a defect in NF-κB activation (28). In our study, pretreatment of B cells with a Ca^{2+}-dependent PKC inhibitor (Go6976) suppressed the high glucose effect on A_3-AR mRNA expression, but had no effect on high glucose-induced suppression of A_1-AR and A_{2B}-AR mRNA expression. This suggests the involvement of conventional PKC isozymes in mediating the high glucose effect on A_3-AR expression. Go6983, a potent inhibitor of several PKC isozymes (though not PKC-μ), completely blocked the high glucose effect on A_1AR and A_{2B}AR expression. Pretreatment of B lymphocytes with rottlerin, reported to be a selective inhibitor of PKC-δ and PKC-Θ (14, 29), abolished the high glucose effect on the expression of A_1-AR mRNA, but has no effect on A_{2B}-AR expression. Since B cells do not express the PKC-Θ (30, 31), it might be suggested that PKC-δ is the principal PKC isozyme mediating the high glucose effect on A_1-AR mRNA expression in rat B cells. The observation that the high glucose effect on A_{2B}-AR expression was blocked by a nonselective PKC inhibitor but was not affected by inhibitors of the Ca^{2+}-dependent PKC isoforms, PKC-ζ and PKC-δ might suggest that in rat B lymphocytes high glucose-induced suppression of A_{2B}-AR expression requires the activity of some novel PKC isoforms i.e. PKC-ε or/and PKC-η.

It has been observed that exposure of primary cell cultures to high glucose results in the elevation of the total mass of PKC-α, –δ, –βII, and -ε (32-34). A significant increase of PKC-δ was observed within 24 h of the cells exposure to high glucose, whereas PKC-α, -βII, and -ε had increased by 48 h (34, 35). The timing of maximal suppressions of ARs expression observed in our experiments correlates well with the induction of the appropriate PKCs observed in the above mentioned studies. The exact mechanism whereby rat B cell PKCs are activated by high glucose remains to be evaluated, although it is possible that activation of the polyol pathway under high glucose concentration leads to a *de novo* synthesis of DAG, which in turn stimulates PKCs (35). There is also a growing body of evidence regarding the activation of PKC by high glucose-induced oxidative stress (36, 37).

The activation of the MAPK signaling pathways is perhaps the most common event to be involved in a high glucose-mediated induction of PKC (33, 38, 39). PKC is often found to be associated with this pathway at the level of Raf-1 kinase. We have observed that inhibition of the Raf/MEK/ERK1/2 signaling cascade, at the level of Raf-1, completely blocked the high glucose effect on A_{2B}-AR expression, but had no effect on the expression of A_1-AR, and A_3-AR. The suppression of A_{2B}-AR evoked by high glucose was also abolished by an MAPK kinase (MEK) inhibitor which is an

upstream effector of ERK1/2. It has been reported that the same PKC-δ signaling pathways are associated with the activation of extracellular signal-regulated kinases 1/2 (ERK1/2), and p38 mitogen-activated protein kinase (p38 MAPK) (40-42). We observed that inhibition of A_1-AR expression by high glucose was not prevented by the pretreatment of rat B lymphocytes with an inhibitor of p38 MAPK; however, the MEK inhibitor suppressed the high glucose effect on A_1-AR expression. This indicates that high glucose suppresses expression of A_1-AR in rat B cells by activating the PKC-δ and MEK/ERK1/2 pathways. The involvement of PKC-α in activation of the Raf-1/MEK/ERK1/2 pathway has been reported (25, 43, 44). However, in our study pretreatment, the B lymphocytes with an Raf-1, MEK or p38 MAPK inhibitor (GW5074, PD98059, SB203580, respectively) has no effect on high glucose-induced and PKC-α/β dependent suppression of A_3-AR expression.

In summary, our study has documented that in rat B lymphocytes, high glucose suppresses the expression of A_1, A_{2B} and A_3 adenosine receptors utilizing a different signaling pathway involving various protein kinase C isozymes, whereas the expression level of A_{2A}-AR is not affected by changes in glucose concentration. Therefore, it is possible that, in diabetes the A_{2A}-AR may become the predominant adenosine receptor on B cells. Previously, we have reported that T lymphocytes exposed to high glucose release the increased amount of adenosine (2). Thus, under high glucose conditions B cells might be more sensitive to suppression by the adenosine released from interacting T lymphocytes. These outcomes may be related to the impaired lymphocyte function observed in diabetes.

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