



The cGMP Synthesis and PKG1 Expression in Murine Lymphoid Organs

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Abstract. Numerous reports indicate that cyclic 3', 5' guanosine monophosphate (cGMP) is involved in the regulation of immune processes. However, the mechanisms responsible for the synthesis of this nucleotide and its signaling pathways in immune cells are still not well recognized. The aim of our studies was to establish: 1) which form of guanylyl cyclase (GC) synthesizes cGMP in murine lymphoid organs and 2) whether the same organs express the isoforms PKG1 α and/or PKG1 β of protein kinase G, known as possible target for synthesized cGMP. Cells isolated from thymus, lymph nodes, and spleen were treated with activators (SNP, ANP, CNP, STa) of soluble or particulate cyclases. Sodium nitroprusside (SNP) elevated intracellular cGMP 2-fold in thymic and lymph node cells and about 10-fold in spleen cells. Atrial natriuretic peptide (ANP) caused modest but statistically significant increases of cGMP in cells of all three organs. Additionally, spleen cells elevated their cGMP content about 2-fold in response to C-type natriuretic protein (CNP). In cellular homogenates of the all analyzed organs, the antibody anti-PKG1 β stained the 78 kDa band corresponding to the molecular mass of PKG1. Only homogenates of spleen cells were stained by the antibody recognizing PKG1 α . Our results indicate that in the investigated organs cGMP may be synthesized mainly by soluble GC in response to nitric oxide. The modest increase of cGMP upon stimulation by ANP suggests that in all these organs either exists only a small subpopulation of cells that express particulate cyclase GC-A or GC-A is expressed at very low level. In spleen cells, however, cyclase GC-B appears to be the more active enzyme. Elevated cGMP concentration may in turn activate PKG1 β in thymus, lymph node, and spleen cells and also PKG1 α in spleen cells.

Key words: cyclic nucleotides; guanylyl cyclases; protein kinases; signal transduction; lymphoid organs.

Introduction

Cyclic nucleotides (cAMP and cGMP) are involved in many cellular processes, including gene expression, differentiation, and maturation. The participation of

cAMP in the functions of the immune system cells is well documented^{9, 14} but, although it is well known that cGMP is also present in most of them^{1, 3, 10, 21, 25, 26}, its role there is still poorly understood. Cyclic GMP is suggested to be involved in the proliferation of lympho-

Abbreviations used: ANP – atrial natriuretic peptide, CNP – C-type natriuretic peptide, CREB – cAMP-response element binding protein, GC – guanylyl cyclase, IBMX – 3-isobutyl-1-methylxanthine, NO – nitric oxide, PKG – cGMP-regulated protein kinase, SNP – sodium nitroprusside, STa – heat-stable enterotoxin from *E. coli*, VASP – vasodilator-stimulated phosphoprotein.

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cytes^{5, 20}, the chemotaxis and adhesion of granulocytes²² and macrophages^{15, 33}, gene expression for iNOS and TNF- α in macrophages^{10, 17} and dendritic cells²⁷. Intracellular level of cGMP depends on the activity of guanylyl cyclases (GC) which synthesize the nucleotide from GTP and phosphodiesterases which hydrolyze it to GMP. Guanylyl cyclases exist as cytosolic (soluble, sGC) or membrane-bound (particulate, pGC) enzymes¹⁸. Both forms differ in structure, mechanisms of activation, subcellular localization, and supposedly have distinct intracellular functions^{12, 18}. Soluble cyclases are activated by nitric oxide (NO), a known mediator of immune reactions^{1, 2, 8, 20, 27, 29, 35}. Three isoforms (GC-A, GC-B, and GC-C) of particulate cyclases are receptors for peptide hormones and are activated either by natriuretic peptides (GC-A, GC-B) or by guanylin, uroguanylin, lymphoguanylin, and bacterial enterotoxin STa (GC-C)¹⁸. The mRNA for natriuretic peptide A (ANP) has been identified in lymphoid organs of the mouse, pig, and chicken³⁴. The mRNAs for guanylin, uroguanylin⁴, and lymphoguanylin⁶ have been detected in thymus, lymph nodes, and spleen of the opossum. Soluble, particulate, or both forms of GC were reported to be present in the T cells^{11, 21, 25}, monocytes, macrophages^{17, 26} and neutrophils^{1, 35} of various species. Once synthesized, cGMP may activate multiple effector proteins, including phosphodiesterases³⁰ and protein kinases²³. The latter proteins are of special interest since they have been documented to be responsible for cGMP signaling in several cell types^{5, 15, 16, 22, 28}. Recently, we reported that murine T cells during maturation display different patterns of response to activators of soluble and particulate cyclases and that protein kinase G (PKG1) is a possible target for cGMP in thymocytes¹⁹.

The aim of the present study was to determine which form of GC might be responsible for cGMP synthesis in cells isolated from murine lymphoid organs and which isoform of PKG is expressed in these cells.

Materials and Methods

Mice. C57BL/6 mice were obtained from the colony at the Institute of Immunology and Experimental Therapy, Wrocław. All mice were used at 8 week of age.

Cells. Thymocytes, splenocytes, and lymph node cells were isolated as previously described²⁴. Briefly, thymus, spleen and lymph nodes were removed aseptically from mice according to procedures approved by The Local Ethics Committee For Animal Experimentation in Wrocław. Cell suspensions were prepared by

gentle pressing the organs through a 60-mesh nylon screen into RPMI 1640 without supplements. After washing twice, the cells were resuspended in phosphate-buffered saline (PBS) containing 2.5% fetal calf serum (FCS). The isolated cells were always >95% viable as determined by trypan blue exclusion.

Staining of cells and flow cytometry. Cells were stained and analyzed in a flow cytometer as described elsewhere³¹. Briefly, separated cells were stained using PE-labeled anti-mouse CD4 and/or FITC-labeled anti-mouse CD8 antibodies (Sigma, Schnellendorf Germany). After 15 min incubation on ice, the cells were washed and subjected to analysis in a FACScalibur (Becton-Dickinson Biosciences, Heidelberg, Germany) flow cytometer. In each sample, 1×10^4 cells were counted and analyzed using CellQuest and WinMDI software, respectively.

Induction and measurement of intracellular cGMP. All cell suspensions (3×10^6 cells/ml) were allowed to rest for 10 min at 37°C before the experiments. Then IBMX (Sigma, Schnellendorf, Germany), the inhibitor of phosphodiesterases, was added to a final concentration of 0.5 mM and the cells were transferred to the 24-well microplate. Each well contained 3×10^6 cells in a final volume of 1 ml. After 10 subsequent minutes of incubation at 37°C, the cells were supplemented with activators of guanylyl cyclases: SNP (Sigma, Schnellendorf, Germany), ANP (Calbiochem-Novabiochem, San Diego, USA), CNP (Calbiochem-Novabiochem, San Diego, USA), or STa (Sigma, Schnellendorf, Germany) at concentrations as indicated. All samples were prepared in quadruplicates. After 30 min incubation, the reaction was terminated by boiling, the cells were disintegrated, and intracellular cGMP was determined using the competitive ELISA method based on rabbit anti-cGMP antibodies obtained in our laboratory. The amount of cGMP in the samples was determined from the reference curve performed for each set of experiments and is expressed as femtomol of cGMP per 1 mg of protein.

Western blot analysis. The cells were homogenized in a glass/glass homogenizer at 4°C in 10 mM HEPES, pH 7.5, containing 1 mM benzamidine, 1 mM PMSF, 1 μ g/ml pepstatin, and 1 μ g/ml aprotinin (Sigma, Steinheim, Germany). Homogenates were then centrifuged for 30 min at 21,500 $\times$ g to separate soluble and insoluble proteins. Both fractions (insoluble and soluble), each containing 50 μ g of protein as determined by the method of Lowry, were resolved under reducing conditions on SDS-PAGE using 10% acrylamide gels and electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The mem-

branes were then blocked with 5% (w/v) non-fat dry milk and incubated for 1.5 h with rabbit antibodies anti-PKG1 α or anti-PKG1 β (StressGen Biotechnologies, Victoria, Canada). After washing with TBST (50 mM Tris pH 7.5, 150 mM NaCl and 0.05% Tween20) and then three times with TBS, the bound primary antibodies were incubated with anti-rabbit IgG goat antibodies conjugated to alkaline phosphatase (Promega, Madison, USA) and visualized using NBT/BCIP-based substrate (Promega, Madison, USA).

Statistics. The results obtained from at least 8 independent measurements are presented as mean $\pm$ SD. Statistical differences between the means of two populations were evaluated using Student's *t*-test and were regarded as significant at $p < 0.05$.

Results

The cells isolated from thymus contained 90% double-positive (CD4 $^{+}$ CD8 $^{+}$) and 9% single-positive (CD4 $^{+}$ or CD8 $^{+}$) cells as determined by means of flow cytometry. Lymph node cells consisted of CD4 $^{-}$ CD8 $^{-}$ (47%), CD4 $^{+}$ (35%), and CD8 $^{+}$ (17%) single-positive cells, while spleen cells contained 73% CD4 $^{-}$ CD8 $^{-}$, 18% CD4 $^{+}$, and 9% CD8 $^{+}$ cells (Fig. 1).

To establish whether lymphoid organs are able to synthesize cGMP in response to activators of soluble and/or particulate cyclases, the isolated cells were treated either with SNP (donor of NO, activator of sGC) or with ANP, CNP, STa (activators of GC-A, GC-B and GC-C, respectively). Treatment of thymic cells with SNP caused about 2-fold increase in intracellular cGMP content in comparison with untreated cells.

Among the activators of particulate cyclases only ANP caused a slight but statistically significant ($p < 0.01$) increase in intracellular cGMP. Similar GC activities were observed in lymph node cells, where SNP caused about 2-fold increase in cGMP content, ANP elevated the intracellular level of the nucleotide by factor of 1.4 ($p < 0.05$), and the other activators of pGCs had no effect. In spleen cells, the amount of cGMP was again only slightly elevated by ANP, but about 2-fold by CNP and markedly (about 10-fold) by SNP (Fig. 2).

The main enzyme regulated by cGMP in most types of cells is PKG1, which may be expressed as two splice variants, PKG1 α or PKG1 β ^{7, 23}. Using antibodies specific to each isoform, we tested expression of PKG1 α and PKG1 β in the studied lymphoid organs. The results of immunostaining of blots containing electrotransferred cellular homogenates are shown in Fig. 3. In the homogenates of thymic and lymph node cells, only antibody specific to PKG1 β stained the band of ≈ 78 kDa that corresponds to the enzyme's molecular mass. There was a lack of reactivity in these homogenates with antibodies against PKG1 α . In the homogenates of spleen cells, both antibodies recognized the bands corresponding to the isoforms of PKG1. The appearance of a lower-molecular-weight band (about 75 kDa) reflects a shift in the electromobility of both PKG1 isoforms, which may result from the autophosphorylation or cGMP-bound states that change the overall charge of the enzymes⁷. In the preliminary experiments we observed the strongest immunoreactivity of antibodies against PKG1 α and PKG1 β with extracts of murine lung and liver, respectively. Therefore, extracts of both organs were used as references for the detection of the PKG1 isoforms.

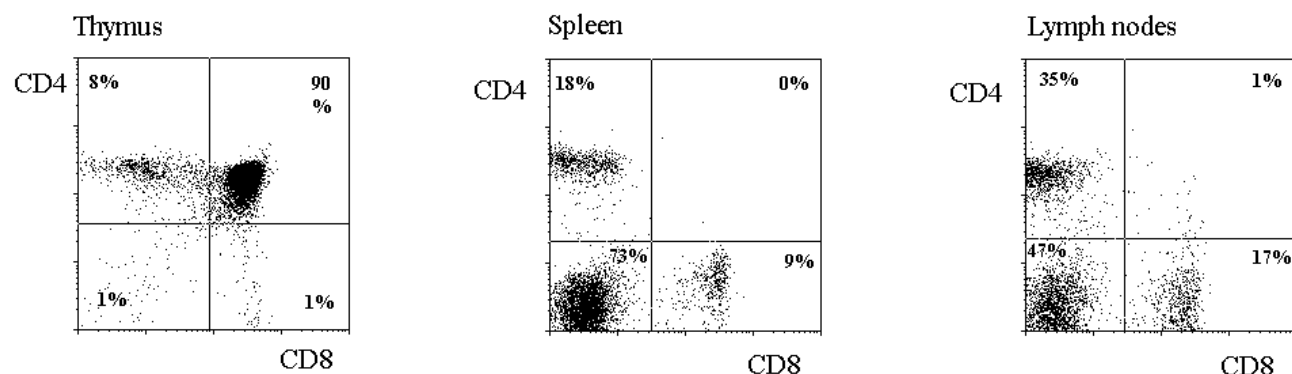


Fig. 1. Cytofluorimetric analysis of cells from murine lymphoid organs. Cells isolated from thymus, lymph nodes, and spleen were stained with antibodies anti-CD4 and anti-CD8 and analyzed in flow cytometer as described in Materials and Methods. (The results of one representative experiment are shown)

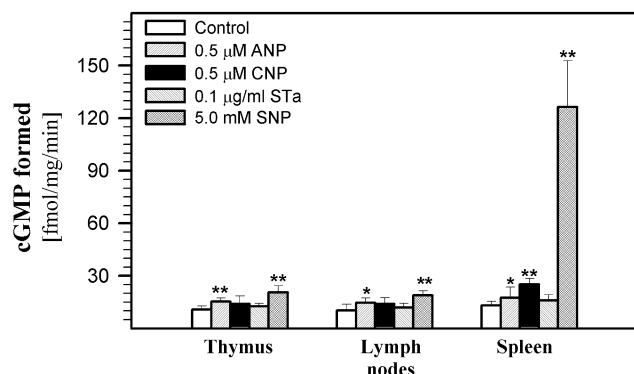


Fig. 2. Synthesis of cGMP by cells of mouse lymphoid organs in response to activators of GC. Cells were incubated without (control) or in the presence of SNP, ANP, CNP or STa and the intracellular cGMP levels were determined as described in Materials and Methods. Results are normalized and are expressed as femtomoles of cGMP formed per minute in the samples containing 1 mg of total protein. * $p < 0.05$; ** $p < 0.01$

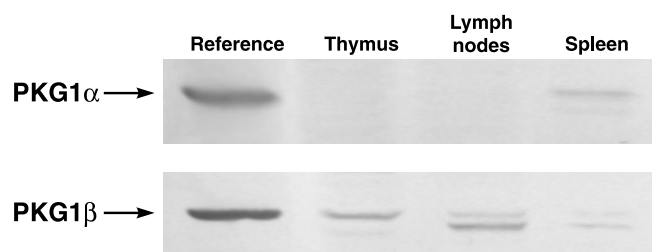


Fig. 3. Western blot detection of PKG1 α and PKG1 β in mouse lymphoid organs. The soluble protein extracts (50 μ g) from cells of lymphatic organs were subjected to SDS-PAGE and Western blotting analysis as described in Materials and Methods by using the primary antibodies anti-PKG1 α (diluted 1:1000) or anti-PKG1 β (diluted 1:500). The position of the PKG1 isoforms are indicated by arrows. The extracts containing 20 μ g of soluble proteins from mouse lung and liver were used as reference standards for PKG1 α and PKG1 β , respectively

Discussion

Using known activators of pGCs and sGC, we have demonstrated that, although both forms of GCs contribute to cGMP synthesis in murine lymphoid organs, the activity of soluble GC is markedly higher in all of them. The difference between the activities of soluble and particulate cyclases was especially pronounced in the case of spleen cells. This may be related to the presence in the spleen of a certain population of cells (e.g. spleen-specific T lymphocytes, spleen-specific macrophages or other spleen-specific cells), which can respond more efficiently to SNP. Identification of these interesting cells needs more study. In all investigated organs the level of cGMP increases in response to ANP indicating that synthesis of nucleotide is mediated also

by GC-A. This observation is consistent with earlier reports that ANP is expressed in thymus, lymph nodes, and spleen³⁴ and possibly exerts its effect through the activation of GC-A^{21, 32}. Moreover, it was also shown that expression of GC-A in human T lymphocytes isolated from peripheral blood depends on environmental conditions and may be induced by yet unrecognized factors¹¹. The very weak response to ANP observed by us suggests therefore that in tested organs either GC-A is expressed at extremely low level or there exists only a small subpopulation of cells bearing this enzyme. In spleen cells the GC-B appears to be more active particulate cyclase than GC-A. In none of studied cells the activity of GC-C was observed. An increased level of intracellular cGMP may, in turn, activate PKG1. Our observation that PKG1 β is present in all analyzed organs is supported by the recent finding of FISHER et al.⁵ that this isoform of PKG1 is expressed in human peripheral T cells, where it plays an important role in cGMP-mediated inhibition of proliferation. Among the potential proteins phosphorylated by PKG1 are vimentin, VASP, and CREB^{22, 23}. It is also suggested that PKG1 may regulate activation of the transcription factor NF- κ B¹⁵.

In summary, our results indicate that signaling via the cGMP pathway in lymphoid tissues may be mediated by the PKG1 and is possible in response to different extracellular signals which generate NO. Synthesis of cGMP in response to natriuretic peptides additionally suggests that in lymphoid organs exist cells which express particulate cyclases. This observation is very important in light of recent reports that ANP acting through activation of cyclase GC-A may influence the activity of transcription factors AP-1 and NF- κ B^{13, 17}.

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