Activation of cGMP-dependent Protein Kinase Iβ Inhibits Interleukin 2 Release and Proliferation of T Cell Receptor-stimulated Human Peripheral T Cells*

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Thomas A. Fischer†‡, Alois Palmetshofer†‡, Stepan Gambaryan†, Elke Butt†, Christian Jassoy‡‡, Ulrich Walter‡, Sieghart Sopper†‡**, and Suzanne M. Lohmann¶
From the †Department of Medicine II, University of Mainz, D-55101 Mainz, Germany, the ‡Institute of Clinical Biochemistry and Pathobiology, and the **Institute of Virology and Immunobiology, University Medical Clinic, D-97080 Würzburg, Germany

Several major functions of type I cGMP-dependent protein kinase (cGK I) have been established in smooth muscle cells, platelets, endothelial cells, and cardiac myocytes. Here we demonstrate that cGK Iβ is endogenously expressed in freshly purified human peripheral blood T lymphocytes and inhibits their proliferation and interleukin 2 release. Incubation of human T cells with the NO donor, sodium nitroprusside, or the membrane-permeant cGMP analogs PET-cGMP and 8-pCPT-cGMP, activated cGK I and produced (i) a distinct pattern of phosphorylation of vasodilator-stimulated phosphoprotein, (ii) stimulation of the mitogen-activated protein kinase, (iii) inhibition of interleukin 2 release and (iv) inhibition of cell proliferation. cGK I was lost during in vitro culturing of primary T cells and was not detectable in transformed T cell lines. The proliferation of these cGK I-deficient cells was not inhibited by even high cGMP concentrations indicating that cGK I, but not cGMP-regulated phosphodiesterases or channels, cAMP-dependent protein kinase, or other potential cGMP mediators, was responsible for inhibition of T cell proliferation. Consistent with this, overexpression of cGK Iβ, but not an inactive cGK Iβ mutant, restored cGMP-dependent inhibition of cell proliferation of Jurkat cells. Thus, the NO/cGMP/cGK signaling system is a negative regulator of T cell activation and proliferation and of potential significance for countering inflammatory or lymphoproliferative processes.

Effective T cell activation can be elicited by a combination of (i) engagement of the T cell antigen receptor (TCR)1-CD3 complex with foreign antigen presented on major histocompatibility complex molecules and (ii) ligation of the CD28 T cell antigen by B7 molecules (CD80 and CD86) on the surface of antigen-presenting cells, resulting in transcription factor activation, interleukin 2 (IL-2) production, and cell proliferation (1-4). Activation and subsequent proliferation of resting T lymphocytes is an essential process in the T cell-mediated immune response. In vitro, T cells respond to stimulation of the TCR-CD3 complex (without costimulation) with IL-2-dependent induction of cell proliferation (2, 5). A costimulatory signal from the CD28 pathway synergizes with the signal from the TCR-CD3 complex to further increase cell proliferation and IL-2 production, and T cell proliferation becomes largely independent of IL-2 (6). Intracellular signaling pathways, which may mediate T cell activation, include phosphorylation cascades that stimulate MAP kinases including ERK (3, 7, 8) and p38 kinase (9, 10), as well as phosphatidylinositol 3-kinase (11, 12) and its target Akt/protein kinase B (13).

Reports of cyclic nucleotide effects on T cell activation indicate that cAMP is inhibitory, whereas effects of cGMP have not been conclusively elucidated. In fact, lowering of cAMP levels by induction of phosphodiesterase-7 was reported to be required for T cell activation (14). Nitric oxide, which activates soluble guanylate cyclase and increases intracellular cGMP, has inhibitory effects on T cell proliferation (15) and cytokine secretion (16), stimulates e-Jun N-terminal kinase, ERK, and p38 activities in Jurkat T cells (17), and decreases tyrosine phosphorylation of Jak3/STAT5 and proliferation in cultured T cells (18). However, NO can elicit effects via both cGMP-dependent and -independent pathways, and increasing intracellular cGMP may affect not only cGMP-dependent protein kinase but also cGMP-regulated phosphodiesterases or channels and cAMP-dependent protein kinase (19, 20).

The present study focuses on the role of cGK in mediating cGMP effects on human peripheral blood T cells. In general, there exist two mammalian isomers of cGMP-dependent protein kinase, cGK I and cGK II (19-24), as well as a splice variants of cGK I (cGK Iα and Iβ) (21, 22) with differing N-terminal ends. cGK I is expressed in platelets, vascular smooth muscle cells, fibroblasts, certain endothelial cells,
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lung, cerebellum, and heart, whereas cGK II is expressed in intestinal epithelium and many regions of brain and kidney (19, 20, 23–26). In human T cells, effects of the NO donor sodium nitroprusside (SNP) were compared with those of membrane-permeant, selective activators of cGK such as PET-cGMP and 8-pCPT-cGMP (27). Our results demonstrate that primary human T cells contain endogenous cGK Iβ, which mediates inhibitory effects of cGMP on IL-2 production and proliferation. In contrast to the bulk of published studies, only freshly prepared cells were used for examining cGK I effects on T cells because our studies demonstrated that cGMP effects were absent in cGK I-deficient cultured cells; however, cGMP effects could be restored by transfection of these cells with cGK I.

**EXPERIMENTAL PROCEDURES**

**Materials**—Anti-CD3, CD4, or CD8-conjugated Dynabeads used for affinity purification of T cells were purchased from Dynal (Hamburg, Germany). Stimulatory anti-CD3 antibodies were either 12F6, a kind gift from Johnson T. Wang (Massachusetts General Hospital, Boston, MA), or produced from the OKT3 hybridoma obtained from the American Tissue Culture Collection. Fluorochrome-conjugated antibodies against CD2, CD3, CD14, and CD19 were obtained from Immunotech (Hamburg, Germany), against CD8 from Pharmingen (Hamburg, Germany), against CD4 from Exalux (Boston, MA), and against CD41a from Southern Biotechnology Associates (Birmingham, AL). Fluorochrome-conjugated isotype control antibodies were from Dako (Hamburg, Germany).

SNP, 3-(5'-hydroxymethyl-2-furyl)-1-benzylindazol (YC-1), and forskolin were from Calbiochem (Bad Soden, Germany). cGMP analogs PET-cGMP and 8-pCPT-cGMP, as well as the cAMP analog Sp-5,6-dichlorobenzimidazole ribose-3',5'- cyclic monophosphorothioate (cBIMPS) were from BioLog (Bremen, Germany).

Jurkat-E6 cells were obtained from American Tissue Culture Collection and A0.01 human T lymphoma cells from the Center for Disease Control (Atlanta, GA); fetal calf serum and cell culture media were from Life Technologies, Inc., nitrocellulose membranes were from Schleicher & Schuell, and polyvinyl difluoride membranes from Millipore (Eschborn, Germany). Standard chemicals were obtained from Sigma.

Purified recombinant cGK I protein, used for preabsorbing anti-cGK I antibody, has been described (28). Purified dephospho-VASP was kindly provided by Christiane Baust (Institute of Molecular Pathology, University of Heidelberg, Germany). Anti-cGK I antibody (29) and monoclonal anti-phosphoSer239/VASP 16C2 anti-body (30) were produced as described. Antibodies against dual phosphorylated, activated MAP kinasas, ERK1/2, and p38, as well as antibodies for total ERK1/2 and p38 were obtained from New England Biolabs (Schwalbach, Germany).

**Isolation of Human Peripheral Blood Mononuclear Cells, Purified T Lymphocytes, and Platelets**—PBMC were isolated from freshly drawn heparinized human blood using Ficoll gradient centrifugation (31) and then resuspended in RPMI 1640 medium. T cells were purified from PBMC using anti-CD3-conjugated Dynabeads, or CD4+ and CD8+ T cell subsets were obtained by incubating PBMC with anti-CD4+ or anti-CD8-conjugated Dynabeads at 4 °C for 1 h and then following the supplier's protocol. Affinity purification of T cells was performed using either fluorochrome-conjugated antibodies against T cell antigens together with fluorescence-activated cell sorting (see below) or using T cell immunoaffinity columns and a protocol provided by Cedar Lane (Hornby, Canada) for negative-selection of T cells (removal of B cells and monocytes). Purified human platelets were prepared as described elsewhere (32).

For experiments, PBMC or purified T cells were incubated at 37 °C in RPMI 1640 medium containing 5% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, or in the case of long term culturing (less than 2 weeks), in the same buffer except with 10% fetal calf serum plus 100 units/ml IL-2, and an initial addition of 100 ng/ml anti-CD3 antibody.

Fluorescence-activated Cell Sorting of Human T Lymphocytes—PBMC were resuspended in phosphate-buffered saline containing 0.1% bovine serum albumin and then incubated with fluorochrome-conjugated anti-CD3 and anti-CD41a antibodies at 4 °C for 1 h. After washing to remove excess antibodies, cells were sorted to obtain pure T cells devoid of platelets (CD3+/CD41a-) separate from a T cell fraction with adherent platelets (CD3+/CD41a+). T cell purity was determined by distinguishing T cells from other cells, using fluorochrome-conjugated antibodies directed against cell-specific antigens such as CD2, CD4, CD8 (T cells), CD19 (B cells), CD14 (monocytes), and CD41a (platelets).

Briefly, affinity-purified cells were incubated with a combination of up to three differently labeled antibodies at 4 °C for 1 h and then washed with phosphate-buffered saline containing 0.1% bovine serum albumin, followed by a final wash using a FACS buffer consisting of 1% bovine serum albumin, 0.05% Tween 20, and 0.15% NaCl (1:5 diluted Tris-Cl, pH 7.5, 100 μM NaCl, 0.1% Tween 20) to block nonspecific binding sites. For FACS calibration purposes, fluorescein isothiocyanate- and phycoerythrin-isotype-matched control antibodies were used.

Reversion Transcription-PCR—Total RNA was isolated from immune-affinity column-purified T cells using Trizol (Life Technologies, Inc.) and reverse transcription was performed with random hexamers using a first strand cDNA synthesis kit from MBI Fermentas (St. Leon-Rot, Germany) according to the supplier's protocol. PCR amplification of cGK was performed using oligonucleotide primer pairs specific for each cGK splice variant (cGK Iα-83 sense: 5'-GTCGAGAGGAGGAAGAATT-3' and cGK Iβ-954 antisense: 5'-CAATCAACAGGGCGATTACAG-3', or cGK Iδ-69 sense: 5'-ATGGGACCTTGCGGATTATTAA-3' and cGK Iβ-1269 antisense: 5'-TTGGATTCTTGTGACTTTCCA-3', resulting in PCR products of 872 nucleotides (cGK Iα) and 1201 nucleotides (cGK Iβ), respectively. PCR amplification was performed using 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. Products were analyzed by agarose gel electrophoresis.

Analysis of cGK Activation by Detection of VASP Phosphorylation in Human PBMC, Purified T Cells, and Platelets Either in Lysates or Intact Cells—In vitro cGK I activity was determined by analyzing VASP phosphorylation in cell lysates of affinity-purified T lymphocytes as well as platelets, following a previously published protocol (33).

Purified T cells were lysed in 10 mM Hepes, pH 7.4, 5 mM MgCl2, 0.2 mM EDTA, and a protease inhibitor mix (Complete™, Roche Molecular Biochemicals), and then lysates were passed through 25 gauge needles to shear chromosomal DNA. In vitro kinase reactions with cell lysates were carried out in buffer (20 mM Tris-Cl, pH 7.4, 10 mM MgCl2, 5 μM β-mercaptoethanol, 0.1% bovine serum albumin, and 50 μM ATP), in the absence or presence of 0.5 μM PET-cGMP and 0.1 μM of purified recombinant dephospho-VASP, at 30 °C for 30 min. Subsequently, samples were processed for SDS-PAGE and Western blot analysis of VASP phosphorylation as described below.

In intact cell experiments, PBMC (preincubated at 37 °C for 3 h to overcome potential preactivation arising from handling during preparation) or platelets were resuspended in serum-free RPMI medium and stimulated with the absence of agents such as TPA or Poly(I:C) or cGMP or cAMP levels (see details under “Results” and in the figure legends) and briefly centrifuged to obtain cell pellets which were processed for SDS-PAGE and Western blot analysis of VASP phosphorylation as described below.

Analysis of cGMP-dependent Activation of the MAP Kinases ERK 1/2 and p38, in Human PBMC and Immunoaffinity Column-Purified T Lymphocytes and Platelets—PBMC or purified T lymphocytes or platelets were resuspended in serum-free RPMI medium for 3 h at 37 °C and then stimulated in the absence or presence of agents which elevate cGMP levels and briefly centrifuged to obtain cell pellets that were processed for SDS-PAGE and Western blot analysis of phosphorylation as described below.

Western Blot Analysis of Protein Kinases (cGK I, ERK1/2, and p38) and VASP Phosphorylation—Intact cells or cell lysates were added to SDS gel loading buffer and heated at 95 °C for 10 min, then analyzed by SDS-PAGE (8% gels, for cGK I and VASP) and Western blotting. Western blot nitrocellulose membranes were blocked with 1% hemoglobin in phosphate-buffered saline and divided horizontally to stain the high molecular weight range of proteins with polyclonal anti-cGK I anti-serum (diluted 1:3000) and the lower range with monoclonal anti-phosphoSer239/VASP 16C2 antibodies (1 μg/ml), followed by either horseradish peroxidase-coupled goat anti-rabbit or goat anti-mouse secondary antibodies (1:5000; Bio-Rad), respectively. Signals were visualized using the ECL system (Amersham Pharmacia Biotech).

For determining ERK and p38 activation, stimulated cells were analyzed by SDS-PAGE (using 10% gels) and blotted to poly(vinylidene difluoride) membranes. Phospho-ERK1/2 antibodies (1 μg/ml), followed by either horseradish peroxidase-coupled goat anti-rabbit or goat anti-mouse secondary antibodies (1:5000; Bio-Rad), respectively. Signals were visualized using the ECL system (Amersham Pharmacia Biotech).

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ethanol, and 2% SDS and then blocked in 5% nonfat dry milk (Bio-Rad) and incubated with anti-ERK1/2 or anti-p38 phosphorylation-independent antibodies, followed by ECL detection (data not shown).

**Determination of T Cell Proliferation**—PBMC (2 x 10^5/well of 24-well plates) were incubated with or without agents that elevate cAMP or cGMP levels, at 37 °C for 1 h under serum-free conditions prior to adding anti-CD3 antibody (12F6 or OKT3, diluted 1:2500), alone or together with anti-CD28 antibody (0.1 μg/ml), and incubating in 10% serum-containing medium for analysis of proliferation after 5 days. Subsequently, PBMC were counted using a Cell Counter (Coulter, Krefeld, Germany), and the inhibition of anti-CD3 antibody-induced cell proliferation caused by cAMP or cGMP analogs or SNP was determined.

**Proliferation of cGK Iβ-expressing Jurkat Cells**—Jurkat E6.1 cells (5 x 10^5/ml) were cotransfected with pCMV-cGK Iβ (34) or with pCMV-cGK Iβ-K405A mutant (35), and pCMV-EGFP (kindly provided by S. Schneider-Rasp) at a ratio of 3:2, using Fugene-6 reagent (Roche Molecular Biochemicals). After 16 h of transfection, cells were seeded at a density of 200,000 cells/well and subsequently incubated for 72 h in the presence of various concentrations of 8-pCPT-cGMP. Using flow cytometry, the impact of cGK I expression and activation with 8-pCPT-cGMP on Jurkat cell proliferation was determined. In the presence of 8-pCPT-cGMP, the ratio of green fluorescent cells (e.g. containing cGK I β and EGFP) to nonfluorescent cells was calculated and divided by the same ratio for cells exposed to medium only (non-8-pCPT-cGMP treated cells). This gave a Jurkat proliferation ratio which was converted to percent.

**Enzyme-linked Immunosorbent Assay of IL-2**—PBMC cultured in triplicate at 3 x 10^5/ml (24-well plate) in RPMI 1640/10% fetal calf serum were stimulated with anti-CD3 (1:2500) in the presence of various concentrations of 8-pCPT-cGMP. After 48 h of stimulation, cell supernatants were collected, and IL-2 concentrations were determined using the OPTEIA IL-2 set according to the supplier’s protocol (BD/ Pharmingen, Heidelberg, Germany).

**RESULTS**

**Highly Purified Primary Human T Lymphocytes, but Neither Cultured T Cells nor T Cell Lines, Display Endogenous cGK I Expression and Activity**—A rich source of cGK I in human blood is platelets that can easily contaminate crude lymphocyte preparations. Therefore, for examining the expression of cGK I in T lymphocytes, it was crucial to use highly purified cells (Fig. 1A). T cells were prepared from freshly isolated PBMC using anti-CD3-conjugated Dynabeads or were isolated from PBMC by labeling with fluorochrome-conjugated anti-CD3 and anti-CD41a (platelet-specific marker) and using FACS to separate pure T cells devoid of platelets (CD3^+/CD41a^-) from T cells with adherent platelets (CD3^+/CD41a^+). Western blot analysis using polyclonal anti-cGK I antibody (Fig. 1A) detected cGK I in unsorted as well as both of the FACS-separated T cell fractions, indicating that T cells devoid of platelets (CD41α^-) indeed contain endogenous cGK I. To estimate the abundance of cGK I expression in T cells, signal intensities from FACS-sorted T cells were compared with those observed in increasing amounts of platelet extracts with a known content of cGK I (38.1 pmol cGK I/10^9 platelets, equivalent to 3 ng of cGK I protein/10^6 cells (32)). T cell-specific cGK I expression was estimated at ~5 ng/10^6 T cells (Fig. 1A).

Anti-CD8^- and anti-CD4^-conjugated Dynabeads used to purify T cell subsets (with less than 5% contaminating platelets; data not shown) demonstrated endogenous cGK I expression in both CD8^- and CD4^- T cells (Fig. 1B). However, transformed T cell lines (Jurkat-E6 or A3.01) contained no detectable cGK I, and that observed in primary T cell cultures was lost after 48 h of culture (Fig. 1, B and C, respectively). None of the T cells investigated (freshly isolated, cultured, or transformed) expressed detectable cGK II by either Western or PCR analysis (data not shown). The cGK I isoform expressed in T cells was identified by reverse transcriptase-PCR amplification of RNA derived from immunoaffinity-column-purified T cells using cGK Iα and cGK Iβ specific primer pairs (see “Experimental Procedures”). As shown in Fig. 1D, only the cGK Iβ splice variant was detected in human T cells.

Evidence that the band identified by Western blot analysis as cGK I in fresh T cells indeed demonstrated that cGK activity was obtained by studying cGK I phosphorylation of the endog-
Fig. 2. Demonstration of cGK activity, i.e. cGMP-dependent phosphorylation of VASP, in lysates of freshly isolated, affinity-purified human T cells (A) but not in cultured T lymphocytes (B). Purified T cells either freshly isolated (A) or cultured for 10 days in vitro (B) were compared with purified platelets (C) with respect to cGK I activity in vitro assays. T cells purified from PBMC using anti-CD3-conjugated Dynabeads or purified platelets were lysed as described under “Experimental Procedures” and then incubated in the absence (lanes 1 and 5) or presence (lanes 2, 3, and 6) of 0.5 μM PET-cGMP for 30 min, and in some cases with 0.1 μM exogenously added, purified recombinant dephospho-VASP (rVASP, lanes 2, 5, and 6) to ensure saturating concentrations of substrate. The phosphorylation of VASP, a well-established substrate of cGK, was subsequently determined using monoclonal anti-phospho(Ser239)-VASP antibody 16C2 on Western blots. Shown for T cell and platelet lysates (30 μg of protein/lane) is the phosphorylation of VASP on Ser157, either without (46-kDa VASP), or with (50-kDa VASP) concomitant phosphorylation of Ser157. Phospho-VASP in samples was identified using a standard (Std) of phosphorylated VASP derived from extracts made from 8-pCPT-cGMP-stimulated platelets (lane 1, A–C). The experiments shown were performed five times with cell preparations from five different donors.

Fig. 3. Analysis of VASP phosphorylation demonstrating distinct characteristics of cGK I activation in freshly isolated human PBMC versus platelets and loss of cGMP- but not cAMP-dependent VASP phosphorylation in cultured PBMC. PBMC either freshly isolated or cultured for 10 days in vitro or isolated platelets were incubated in the absence (control) or the presence of 100 μM SNP, 50 μM YC-1, 200 μM PET-cGMP, or 500 μM cBIMPS, for 25 min (A) or the times indicated (B). Cells immediately harvested in SDS gel loading buffer were subsequently analyzed (30 μg of protein/lane) for VASP phosphorylation using the monoclonal anti-phospho(Ser239)-VASP antibody 16C2. Observed is the phosphorylation of VASP on Ser157, either without (46-kDa VASP) or with (50-kDa VASP) concomitant phosphorylation of Ser157. Phospho-VASP was identified in lymphocyte samples using a standard (Std) of phosphorylated VASP derived from extracts made from 8-pCPT-cGMP-stimulated platelets. A, VASP phosphorylation in response to elevation of cAMP was observed in both fresh and cultured PBMC; however, in response to elevation of cGMP only in fresh PBMC (cGK I is lost from cultured T cells, as shown in Fig. 1C). Note the absence of SNP-stimulated VASP phosphorylation at the 25-min time point for fresh PBMC in A, a time at which stimulation by SNP has already returned to base line in PBMC but not platelets (see SNP time course in the description of B below). Shown are data representative of those obtained with PBMC from three different donors and platelets from two different donors. B, time course of SNP- and YC-1-induced VASP phosphorylation in freshly isolated PBMC and platelets. The data shown are representative of three independent experiments with cells obtained from different donors.

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markedly less than VASP phosphorylation in platelets (Fig. 3, A and B, respectively, showing analysis of equal amounts of protein), as had also been observed in cell lysates above. Examination of a detailed time course of SNP stimulation revealed that peak VASP phosphorylation occurred transiently after 5 min SNP and then rapidly declined (Fig. 3B), unlike the SNP stimulation in platelets that persisted throughout 2 h. YC-1 (50 μM), a nitric oxide-independent activator of guanylate cyclase and an inhibitor of several phosphodiesterases, caused strong and long lasting stimulation of VASP phosphorylation in PBMC (Fig. 3). SNP plus YC-1 treatment produced somewhat more VASP phosphorylation than YC-1 alone. cBIMPS, a selective activator of cAK, also strongly stimulated VASP phosphorylation in PBMC and platelets. Whereas the cBIMPS stimulatory effect was preserved in cultured PBMC, none of the effects of cGMP-elevating agents were preserved (Fig. 3A), indicating that their effects were lost along with cGK I and could not be mediated by cAK.
Selected cellular targets of NO and cGMP: The proliferative effects of NO and cGMP (Figs. 1–3) were not mediated by cGK I, but were mediated by cAK, as assessed through experiments in transfectants (data not shown). The proliferation of T cells costimulated with anti-CD3 plus anti-CD28 (Fig. 5E) was inhibited by 8-pCPT-cGMP (40 and 30% inhibition, respectively).

cGMP/cGK Inhibition of T Cell Proliferation Partially Results from Reduced IL-2 Expression—IL-2 is an important regulator of T cell proliferation and is released by activated T cells. Anti-CD3-stimulated IL-2 release was reduced by 8-pCPT-cGMP to 42% of control (from 64 to 37 pg of IL-2/10^7 cells; Fig. 6). To test whether the observed cGMP-dependent inhibition of T cell proliferation was due to reduction of IL-2 levels, recombinant human IL-2 was added to anti-CD3-stimulated PBMC cultures in the presence or absence of 8-pCPT-cGMP. IL-2 (500 units/ml) effectively diminished 8-pCPT-cGMP inhibition of anti-CD3-stimulated proliferation (Fig. 7). Costimulation with anti-CD3 plus anti-CD28 elicits IL-2 independent proliferation, which is also inhibited by 8-pCPT-cGMP (Fig. 5F), but this was, as expected, not reversed by addition of exogenous IL-2 (data not shown).

Reconstitution of cGK Expression in Jurkat-E6 Cells Restores cGK-mediated Inhibition of Cell Proliferation—Jurkat-E6 cells were found to be deficient in cGK I (Fig. 1B), and their proliferation was resistant to inhibition by 8-pCPT-cGMP (Fig. 5D). To provide clear evidence that cGMP-dependent inhibition of T cell proliferation was indeed mediated by cGK, cytokomalgivirus-promoter-driven cDNA constructs encoding either cGK Iβ or an inactive cGK Iβ-mutant (cGK Iβ K405A) were transfected into Jurkat cells, and, subsequently, the number of cGK expressing cells were determined in the presence of increasing concentrations of 8-pCPT-cGMP. As observed previously for cGK I-deficient Jurkat cells in Fig. 5D, the proliferation of cGK Iβ-K405A-transfected, 8-pCPT-cGMP treated Jurkat cells was not inhibited relative to that of similarly transfected, non-8-pCPT-cGMP-treated cells (Fig. 8). In contrast, the proliferation of cGK Iβ (wild type)-transfected, 8-pCPT-cGMP treated Jurkat cells was inhibited relative to that of the corresponding non-8-pCPT-cGMP-treated cells, indicating that cGMP-dependent inhibition of T cell proliferation can indeed be reconstituted by cGK Iβ transfection.

DISCUSSION

Human T cells devoid of platelets were shown to express endogenous cGK I, which could be stimulated by NO and cGMP analogs to inhibit T cell proliferation. Both CD4+ and CD8+ T cells expressed endogenous cGK Iβ; however, neither cell lines such as Jurkat and A3.01 nor cultured T cells retained cGK I. Thus, NO or cGMP effects reported in such cells are unlikely to be mediated by cGK I. In our experiments employing freshly isolated T cells, effects of NO and cGMP analogs were clearly mediated by cGK I and not cAK because these agents were unable to phosphorylate VASP in cultured PBMC that had lost cGK I, but contained cAK that did phosphorylate VASP in response to the cAMP analog cBIMPS. The pattern of VASP phosphorylation by cGK I in response to SNP was particularly different in PBMC compared with platelets. Whereas SNP-stimulated VASP phosphorylation persisted for over 2 h in platelets, it lasted only about 5 min in lymphocytes, suggesting the presence of a highly efficient cGMP phosphodiesterase in lymphocytes. Experiments using other types of agonists gave results in agreement with this. PET-cGMP (a hydrolysis-resistant analog), YC-1 (an activator of soluble guanylate cyclase) but also an inhibitor of several phosphodiesterases including ones that hydrolyze cGMP (38)), as well as SNP plus YC-1, had much longer lasting effects than SNP alone.

In our experiments, stimulation of cGK I activated ERK and p38 MAP kinases, and in contrast to cAMP (37), cGMP did not inhibit anti-CD3-induced MAP kinase activation (data not shown). NO, cGMP, or cGK I stimulation or inhibition of MAP

Fig. 4. cGMP-dependent activation (dual phosphorylation) of ERK and p38 in immunoaffinity column-purified human T lymphocytes. A, purified T cells (10⁶/lane) were incubated in serum-free medium in the absence (ctrl) or presence of 200 μM PET-cGMP or 8-pCPT-cGMP or 100 μM SNP for the times indicated and then harvested immediately in SDS gel loading buffer for Western blot analysis using polyclonal antibodies which recognize dual phosphorylated, activated ERK, or p38 MAP kinases. The Western blots shown are representative of three independent experiments. B, purified T cells (10⁶ cells/lane) were incubated in serum-free medium in the absence or presence of the indicated concentrations of 8-pCPT-cGMP for 15 min, followed by Western blot analysis as described for A. Data are representative of three independent determinations with cells from three different donors.

SNP and cGMP Analog Activation of cGK Leads to Dose-dependent Transient Activation of ERK and p38 in Immunoaffinity Column-Purified T Cells—Stimulation of purified T cells with 200 μM PET-cGMP or 8-pCPT-cGMP or with 100 μM SNP caused dual phosphorylation/activation of both ERK and p38 over a time period of 5–60 min (Western blot; Fig. 4A), with peak activation between 10 and 15 min. Induction of both ERK- and p38-phosphorylation by 8-pCPT-cGMP for 15 min was dose-dependent (Fig. 4B).

SNP and cGMP or cAMP Analog Activation of cGK I Inhibits TCR-CD3-induced Proliferation in PBMC—Proliferation of smooth muscle cells and other cGK I positive cells is inhibited by cGMP analogs (see “Discussion”). Because cAMP is a known inhibitor of T cell proliferation, stimulators of cGK/cGK were compared with the cAMP analog cBIMPS with regard to their effects on proliferation of TCR-CD3-activated PBMC (Fig. 5; note that cell number increase is shown in A, percentage of control proliferation in the absence of inhibitor in B–D). cBIMPS (200 μM) abolished CD3-activated T cell proliferation, whereas 200 μM PET-cGMP (or 8-pCPT-cGMP) and 100 μM SNP inhibited proliferation about 50, 40, and 70%, respectively, in freshly prepared PBMC (Fig. 5, A and B) shown to contain cGK I (Figs. 1–3). In contrast, cBIMPS (200 μM) abolished CD3-activated PBMC (Fig. 5; note that cell number increase is shown in A, percentage of control proliferation in the absence of inhibitor in B–D). cBIMPS (200 μM) abolished CD3-activated T cell proliferation, whereas 200 μM PET-cGMP (or 8-pCPT-cGMP) and 100 μM SNP inhibited proliferation about 50, 40, and 70%, respectively, in freshly prepared PBMC (Fig. 5, A and B) shown to contain cGK I (Figs. 1–3). In cultures cultured for 3 days prior to the proliferation assay) that have lost cGK I, the inhibitory effect of cBIMPS was highly preserved; however, no inhibition by 8-pCPT-cGMP was observed, and inhibition by SNP was reduced from 70% in fresh PBMC to 50% (Fig. 5, B and C). Jurkat-E6 cells (Fig. 5D) containing no detectable cGK I (Fig. 1B) also did not respond to 8-pCPT-cGMP, but Jurkat proliferation was inhibited by cBIMPS (60%) and by SNP (40%). Not only the proliferation of T cells stimulated with anti-CD3 antibody alone (Fig. 5B) but also that of T cells costimulated with anti-CD3 plus anti-CD28 (Fig. 5E) was inhibited by 8-pCPT-cGMP (40 and 30% inhibition, respectively).
kinases has been demonstrated in other cell types including cGK I-transfected baby hamster kidney cells (38), 293T fibroblasts (39), CHO cells (40), Jurkat T cells (17), macrophages (41), mesangial cells (42), and also low passage rat aorta vascular smooth muscle cells (43). Responses to NO, cGMP, or cGK not only varied in different cell types with respect to activation or inhibition of MAP kinases but also proliferation. In many cases, a definitive relationship between cGK and effects on MAP kinases and proliferation is not possible because certain cells may not contain cGK (e.g., Jurkat cells), and this was not always investigated. Also some criteria that have been used to define cGK effects in cells, such as inhibition by agents such as KT5823, may be unreliable because this agent has been shown to actually enhance cGMP effects in certain cell types such as platelets and mesangial cells (44). Our own results in T cells indicate that short term cGK I activation of MAP kinases does not activate T cells, instead cGK I inhibits proliferation of activated T cells. This is in distinct contrast to cAMP-dependent inhibition of T cell proliferation reported to be related to MAP kinase inhibition (37) and thus represents a major difference in cAMP and cGMP effects on T cells.

Selective activation of cGK I by cGMP analogs, as well as by SNP, inhibited proliferation of human T cells, although these were only 40–70% as effective as the cAMP analog cBIMPS, which caused complete inhibition. In Jurkat T cells and PBMC cultured for 3 days prior to the proliferation assay, i.e. cells that contain no detectable cGK I, the inhibitory effect of 8-pCPT-cGMP was lost, whereas that of cBIMPS was unchanged and that of SNP was only somewhat reduced. These results indicated that cGMP effects are not mediated by cAK and that SNP has additional inhibitory effects on T cell proliferation that are clearly cGK-independent. cGMP inhibition of cell proliferation could be reconstituted in Jurkat cells overexpressing wild type cGK I but not the inactive mutant cGK Iβ–K405A, further demonstrating that the observed inhibition of proliferation was indeed cGK-dependent and not due to cross-activation of cAK. cGK-mediated inhibition of cellular proliferation has also been described for other cell types such as vascular smooth muscle cells (45, 46) and cardiac fibroblasts (47). Furthermore, vector-mediated overexpression of eNOS or cGK I has been reported to decrease neointima proliferation in balloon catheter-damaged carotid arteries (48, 49).

In our studies, the effect of cGMP analogs on proliferation appeared specific because the effect is absent in cGK I-deficient cells (whereas that of cAMP analogs is intact) but can be reconstituted by transfection of cGK I. This agrees with other...
stimulated human PBMC. PBMC (3 × 10^6/well) stimulated with anti-CD3 (1:2500) were incubated with or without 8-pCPT-cGMP, and subsequently IL-2 concentrations were determined in supernatants using enzyme-linked immunosorbent assay. The data represent the mean values ± S.E. of five independent experiments with triplicate determinations performed on blood from different donors. Data significantly differing from control values are indicated with asterisks (p < 0.05, Student’s paired t test).

Inhibition of T cell activation by the NO/cGMP/cGK signal-pathway may be of potential significance for counteracting inflammatory or lymphoproliferative processes. Studies have shown that NO (53, 54), as well as cGMP (18), is involved in inhibition of T cell activation and inflammation. NO has also been reported to inhibit the proliferation of human leukemia cells (55); however, this requires study in greater detail. Because there are also cGMP-independent effects of NO and cGMP can affect other mediators than cGK, the role of cGMP and cGK in inflammation and lymphoproliferative disorders needs to be directly investigated in the future.

In summary, our data show that cGK Iβ is present only in freshly purified T cells, that cGK I-dependent inhibition of T cell proliferation is clearly distinct from that which is cAMP mediated, that cGK I-dependent inhibition of T cell proliferation results in part from inhibition of IL-2 release, and that not all NO inhibitory effects on T cell proliferation are controlled by cGK I. cGK I suppression of T cell proliferation may be an important independent adjunct to the inhibition mediated by cAMP and have implications for diverse aspects of immune function.

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Activation of cGMP-dependent Protein Kinase Iβ Inhibits Interleukin 2 Release and Proliferation of T Cell Receptor-stimulated Human Peripheral T Cells

Thomas A. Fischer, Alois Palmetshofer, Stepan Gambaryan, Elke Butt, Christian Jassoy, Ulrich Walter, Sieghart Sopper and Suzanne M. Lohmann

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