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Inhibition of Antigen-Specific T Cell Proliferation and Cytokine Production by Protein Kinase A Type I

Einar Martin Aandahl,† Walter J. Moretto,‡ Patrick A. Haslett,§ Torkel Vang,† Tone Bryn,† Kjetil Tasken,§ and Douglas F. Nixon*  

CAMP inhibits biochemical events leading to T cell activation by triggering of an inhibitory protein kinase A (PKA)-C-terminal Src kinase pathway assembled in lipid rafts. In this study, we demonstrate that activation of PKA type I by Sp-8-bromo-cAMPS (a CAMP agonist) has profound inhibitory effects on Ag-specific immune responses in peripheral effector T cells. Activation of PKA type I inhibits both cytokine production and proliferative responses in both CD4+ and CD8+ T cells in a concentration-dependent manner. The observed effects of CAMP appeared to occur endogenously in T cells and were not dependent on APC. The inhibition of responses was not due to apoptosis of specific T cells and was reversible by a PKA type I-selective cAMP antagonist. This supports the notion of PKA type I as a key enzyme in the negative regulation of immune responses and a potential target for inhibiting autoreactive T cells. The Journal of Immunology, 2002, 169: 802–808.

Adequate activation of T cells is crucial in the defense against infectious pathogens and neoplasms, whereas exaggerated immune activation or immune attack against self-Ags may lead to disease. The processes that regulate the threshold for and the precise level of immune activation are still poorly understood. Engagement of the TCR elicits a series of complex biochemical events involving activation of the Src family tyrosine kinases Lck and Fyn (1). These kinases mediate the initial tyrosine phosphorylation of immune receptor tyrosine-based activation motifs in the TCR-CD3 complex and subsequent recruitment of the tyrosine kinase Zap-70 to the CD3 and TCR ζ-chain (2, 3). Zap-70 tyrosine phosphorylates lipid raft-associated adaptor molecules such as the linker for activation of T cells (4, 5), which further recruit several downstream Src homology 2 domain-containing signaling molecules (6–8). The assembly of TCR signaling complexes occurs in specialized membrane microdomains with high cholesterol and glycolipid contents called glycosphingolipid-enriched micromembranes or lipid rafts (reviewed in Refs. 9 and 10). Rafts also serve as a scaffold for assembly of a recently unraveled inhibitory pathway which involves protein kinase A (PKA)3 type I that in response to cAMP activates the C-terminal Src kinase (Csk) by phosphorylation of residue S364 (11). Activated Csk subsequently phosphorylates the C-terminal inhibitory tyrosine residue in Lck and thereby acts as a negative regulator of TCR signaling (12–14). This may be an important mechanism whereby CAMP-inducing ligands such as PGE and β-adrenergic stimuli inhibit mitogenic signaling pathways and regulate set-point for activation of T cells. However, CAMP negatively regulates signaling events at multiple levels (15). In addition to ligand-receptor interaction, production of cAMP may also be induced during T cell activation or by intracellular events not involving engagement of transmembrane receptors and contribute to termination of the activating signal, but such mechanisms remain to be elucidated.

The role of the cAMP-PKA type I pathway as a potent inhibitory regulator has not been clearly defined functionally in the different immune responses in CD4 and CD8 T cells, nor has it been defined in the context of Ag-specific immune functions. In this study, we have investigated the effect of PKA type I on production of IFN-γ, TNF-α, IL-2, and IL-4 as well as proliferation of T cells with various Ags. We report that PKA type I acts as a consistent and universal inhibitor of T cell function, reducing the population of cytokine-expressing cells as well as inhibiting proliferation in a concentration-dependent manner.

Materials and Methods

Study subjects and samples

PBMC from healthy blood donors (n = 13) were isolated from heparinized whole blood by Ficoll-Paque PLUS density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden), and washed twice in RPMI 1640 (Life Technologies, Grand Island, NY) with 15% fetal calf serum. When used, peripheral human T cells were purified by negative selection (16). All experiments were performed with freshly isolated PBMC or T cells.

Reagents

Sp-8-bromo-cAMP-phosphorothioate (Sp-8-Br-cAMPS) and Rp-8-bromo-cAMP-phosphorothioate (Rp-8-Br-cAMPS; BioLog Life Sciences, San Diego, CA) were dissolved to stock concentrations of 10 mM in PBS (Life Technologies), PGE2, 3-isobutyl-1-methylxanthine (IBMX), and rolipram were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved to stock concentrations in DMSO.

Ags and Abs

Staphylococcal enterotoxin B (SEB; Sigma-Aldrich) was dissolved to a stock concentration of 100 μg/ml in PBS. A final concentration of 10 μg/ml was used in all intracellular cytokine flow cytometry assays, while...
a final concentration of 100 ng/ml was used in the CFSE dilution proliferation assays. CMV lysates and matched control Ag (BioWhittaker, Walkersville, MD) were used at a 1:12.5 dilution. For CD8 T cell responses, PBMC were stimulated with a HLA-A*0201 epitope of CMVpp65 (residues 495–503) at a final concentration of 5 μg/ml. Tuberculin purified protein derivative (PPD; Statens Seruminstitut, Copenhagen, Denmark) was used at a final concentration of 10 μg/ml. For cell surface and intracellular cytokine staining, different combinations of mouse anti-human mAbs were used: CD3 FITC, PerCP and allophycocyanin, CD4 PerCP and allophycocyanin, CD8 PerCP and allophycocyanin, CD69 FITC, IFN-γ PE, TNF-α PE, IL-2 PE, and IL-10 PE (BD Biosciences, Mountain View, CA).

**Intracellular cytokine staining assay**

Freshly isolated PBMC were preincubated with cAMP analogs or cAMP-inducing pharmacological compound 1 h before stimulation with Ag. The cells were then incubated for 1 h if not otherwise noted before addition of brefeldin A (Sigma-Aldrich) at a final concentration of 10 μg/ml and incubation for another 5 h. The cells were washed in PBS, 0.02% EDTA, and 1% BSA, fixed, and permeabilized in FACS permeabilizing buffer (BD Biosciences) for 10 min. After washing, staining was performed with a mixture of FITC-, PE-, PerCP-, and allophycocyanin-conjugated Abs for 30 min at 4°C in the dark before subsequent washing, fixing, and flow cytometry analysis using a BD Biosciences FACSCalibur instrument.

**ELISPOT assay**

Each well of a sterile multiscreen 96-well filtration plate (Millipore MAHAS4510; Millipore, Bedford, MA) was coated with 50 μl anti-IFN-γ mAb (Mabtech, Stockholm, Sweden) at a concentration of 5 μg/ml in 1 M sodium bicarbonate buffer (pH 9.5). After an overnight incubation at 4°C, each well was washed four times with PBS (Cellgro, Herndon, VA) and blocked with 50 μl 5% pooled human serum in RPMI 1640 for 1 h at 37°C. A total of 2.0 × 10^6 PBMC was added to each well, and SEB was added at a concentration of 1.0 ng/ml. After incubation overnight (14 h) at 37°C, plates were washed four times using PBS with 0.05% Tween 20 (Fisher-Biotech, Fair Lawn, NJ). Biotinylated anti-IFN-γ mAb 7-B6-1 (MabTech) was added at 1 μg/ml in 50 μl PBS and the plate was incubated for 2 h at 37°C. Next, plates were washed four times using 0.1% Tween 20 in PBS and then treated with avidin-conjugated HRP H (Vector Laboratories, Burlingame, CA). After 1 h, plates were washed four times with 0.1% Tween 20 in PBS. Fifty microliters of stable diaminobenzidine tetrahydrochloride substrate (Research Genetics, Huntsville, AL) was added to each well for 5 min and then washed away with water. IFN-γ spot-forming cells were visualized and counted using an AID ELISPOT reader system (Autoimmun Diagnostika, Strassburg, Germany). Raw counts were standardized to express the frequency of spot-forming cells per million PBMC.

**CFSE proliferation assay**

Fresh PBMC were resuspended at a concentration of 10^6 cells/ml in RPMI 1640 and labeled with CFSE (Molecular Probes, Eugene, OR) by incubation for 15 min in 37°C CO₂ at a final concentration of 2 μM. Labeling was quenched with RPMI 1640 supplemented with 15% FCS and the cells were washed twice before culturing in flat-bottom 96-well plates. FACS analysis was performed after 96 h of incubation. In the assays where CFSE labeling was combined with staining for intracellular cytokines, brefeldin A was added for the last 6 h, the cells were then transferred to V-bottom 96-well plates, and the procedure for intracellular cytokine staining was conducted before FACS analysis.

**Assessment of Csk activity**

Purified peripheral T cells (40 × 10^6 cells/ml in RPMI 1640) were incubated at 37°C for 30 min with or without the cAMP analog Sp-8-Br-cAMPS at indicated concentrations. Thereafter, cells were disrupted in lysis buffer (50 mM HEPES (pH 7.4), 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, 50 mM α-m-1-glucoside, 10 mM NaPO₄, 1 mM NaVO₄, 50 mM NaF, and 1 mM PMSF) and subjected to immunoprecipitation with anti-Csk Ab (catalog no. Sc-286; Santa Cruz Biotechnology, Santa Cruz, CA). After overnight incubation at 4°C, protein A-Sepharose was added and the incubation was continued for 1 h. Immune complexes were washed three times in lysis buffer and three times in CSK kinase assay buffer (50 mM HEPES and 5 mM MgCl₂, pH 7.4), followed by Csk kinase assays and Western blot analysis. The tyrosine kinase activity of human Csk was measured as incorporation of [32P]phosphate into the synthetic polypeptide acid poly(Glu,Tyr) 4:1 (Sigma-Aldrich). A standard protocol was followed with reaction volumes of 50 μl containing HEPES buffer (pH 7.4), 5 mM MgCl₂, 200 μM [γ-32P]ATP (0.15 Ci/mmol), 200 μg/ml poly(Glu,Tyr), and immunoprecipitated Csk. The incubation temperature was 30°C, and the incubation time was 12 min.

**Results**

**cAMP and PKA type I inhibits SEB-induced T cell activation and expression of IFN-γ**

To explore possible effects of cAMP and PKA type I on early functional events in immune activation, we measured the expression of the activation marker CD69 and production of IFN-γ after activation of PBMC with the superantigen SEB for 6 h. The expression of CD69 and IFN-γ were measured by flow cytometry in the presence and absence of the PKA agonist Sp-8-Br-cAMPS alone or in combination with the PKA type I selective antagonist Rp-8-Br-cAMPS. The cells were preincubated with cAMP analogs for 1 h to allow diffusion of the compounds, followed by activation with SEB. Brefeldin A was then added 1 h after activation with SEB, and the cultures were incubated for 5 h for cytokine accumulation.

The expression of both CD69 and IFN-γ were markedly reduced by preincubation with 250 μM Sp-8-Br-cAMPS (agonist) in CD3 T cells compared with control (Fig. 1, a and b). To clarify whether this inhibited cytokine production is mediated through PKA type I, associated with the TCR/CD3 and lipid rafts, or PKA type II, whose localization is mainly confined to the perinuclear and Golgi centrosomal regions in lymphocytes (17), we preincubated the cells with 250 μM Sp-8-Br-cAMPS in combination with 1000 μM of the PKA type I selective antagonist Rp-8-Br-cAMPS (Fig. 1c). The antagonist reversed almost completely the inhibitory effect of the agonist, indicating that the inhibitory effect of CAMP in the early T cell activation is mainly due to activation of PKA type I. The antagonist had no effect on CD69 and IFN-γ expression alone (Fig. 1d).

![FIGURE 1. cAMP inhibits IFN-γ and CD69 expression. PBMC were stimulated for 6 h with SEB in the absence of cAMP analog (a), in the presence of Sp-8-Br-cAMPS alone (b) or in combination with Rp-8-Br-cAMPS (c), or in the presence of Rp-8-Br-cAMPS alone (d). Cells were preincubated with cAMP analogs 1 h before stimulation with SEB. Brefeldin A was added for the last 5 h to promote cytokine accumulation. The cells were then fixed, permeabilized, and stained for CD3, CD69, and intracellular IFN-γ flow cytometry analysis. The lymphocyte population was gated for CD3+ cells and analyzed for CD69 vs IFN-γ expression. Data are representative of N = 8 observations.](http://www.jimmunol.org/Downloadedfrom)
The inhibitory effect of cAMP and PKA type I is not due to apoptosis

To investigate whether the inhibitory effect of cAMP and PKA type I was due to induction of apoptosis, we evaluated the morphology of unstimulated and SEB-stimulated PBMC with and without preincubation with 1000 μM Sp-8-Br-cAMPS and with and without incubation with brefeldin A for the last 5 h of the incubation time by flow cytometry on forward and side scatter. The cultures were incubated for various periods of time ranging from 6 to 48 h and did not demonstrate the morphological changes characteristic of apoptosis, with decrease in forward scatter and increased side scatter, in the presence of the cAMP analog (data not shown). Furthermore, apoptosis was also assessed by Annexin V

Increase in endogenous cAMP by physiological agonists and pharmacological agents also inhibits cytokine production

The effect of PGE2 on SEB-induced IFN-γ expression was assessed by intracellular flow cytometry in the presence and absence of 1000 μM Rp-8-Br-cAMPS. PGE2 increased endogenous cAMP production through E2 and E4 receptors on T cells and inhibited cytokine production as shown in Fig. 4. In addition, IBMX and rolipram, which are nonselective and type 4-specific phosphodiesterase inhibitors, respectively, that inhibit degradation of endogenous cAMP, also inhibited IFN-γ release. The PKA type I selective antagonist fully or partially reversed the inhibitory effect of the various compounds (Fig. 4).

cAMP and PKA type I inhibits both CD4 and CD8 T cell activation independently of APC

To further address the question of which T cell compartment is affected by cAMP in the inhibition of immune activation, we investigated the effects of cAMP and PKA type I on CD4 and CD8 T cells. As shown in Fig. 5, a and b, the expression of both IFN-γ and TNF-α was inhibited in a concentration-dependent manner. However, this experiment was performed with SEB stimulation of PBMC containing APC. To eliminate the possibility that the effects observed could be due to suppressive effects of cAMP on the function of APC, we isolated APC by adherence and loaded the cells with SEB followed by paraformaldehyde fixation. We then set up an autologous coculture with fixed, Ag-loaded APC, and an APC-depleted PBMC population at a 1:10 ratio. The coculture was incubated for 6 h before addition of brefeldin A. Cytokine accumulation was allowed to proceed for 14 h. The expression of IFN-γ was assessed by intracellular flow cytometry in CD4 and CD8 T cells and a concentration-dependent inhibition of cytokine expression was observed with increasing concentrations of Sp-8-Br-cAMPS showing that inhibition is independent of APC (Fig. 5c).

Coupled inhibition of cytokine expression and proliferation

To further determine the extent of the cAMP-mediated inhibition of immune functions, we investigated the effects on proliferation of CD3, CD4, and CD8 T cells by the CFSE dilution assay. As Fig. 6a shows, a concentration-dependent decrease in SEB-induced proliferation with increasing concentrations of Sp-8-Br-cAMPS is observed in all three cell populations. The inhibition was reversible with the PKA type I selective antagonist Rp-8-Br-cAMPS (data not shown).

By combining the CFSE dilution assay with intracellular flow cytometry, we measured the effect of cAMP on both immune functions simultaneously. As shown in Fig. 6b, there is a sequential
reduction of CFSE fluorescence intensity for each cell division, with the highest fraction of IFN-γ-expressing T cells being in the later daughter cell generations. With increasing concentrations of Sp-8-Br-cAMPS, there was a profound reduction in IFN-γ expression and a marked suppression of proliferation shown by disappearance of the last two cell divisions with 1000 μM Sp-8-Br-cAMPS.

cAMP and PKA type I inhibits Ag-specific immune responses

We next examined the effect of cAMP and PKA type I on Ag-specific immune responses (Fig. 7a). As seen with SEB-induced immune responses, we observed a concentration-dependent inhibition of CMV-induced IFN-γ expression by increasing concentrations of Sp-8-Br-cAMPS in both the CD4 and CD8 T cell populations. To induce optimal stimulation, the CD4 T cell population was stimulated by CMV-infected cell extracts, while the CD8 T cell population was stimulated by CMVpp65. The range of inhibition was from 60 to 80% in both cell populations.

We also examined the effect of PKA type I activation on tuberculin PPD-induced T cell proliferation and IFN-γ production by combining the CFSE dilution assay with intracellular flow cytometry (Fig. 7b). As observed with the SEB- and CMV-induced immune responses, the T cell proliferation as well as IFN-γ expression induced by tuberculin PPD declined in a concentration-dependent manner.
Data are representative for 

with Sp-8-Br-cAMPS. Furthermore, both T cell proliferation and IFN-γ expression were fully reversible with the cAMP antagonist Rp-8-Br-cAMPS (data not shown).

cAMP activates Csk
To assess the effect the cAMP agonist used in this study on the previously mapped mechanism for cAMP-PKA inhibition of T cell functions (11), we examined whether Sp-8-Br-cAMPS lead to an increase in Csk activity. Csk phosphotransferase activity was assessed in vitro after treatment with increasing concentrations with the cAMP analog resulting in a concentration-dependent increase in Csk activity with a >2-fold increase in the activity with the highest concentration used (Fig. 8).

Discussion
We and others have shown that cAMP and PKA type I act as an acute inhibitor of TCR/CD3 signaling on the molecular level. In this study, we present data showing that cAMP through activation of PKA type I leads to an activation of Csk activity in the T cells. We have previously shown that cAMP through activation of PKA type I is a universal inhibitor of T cell function. Preincubation with Sp-8-Br-cAMPS inhibited Ag-induced expression of IFN-γ, TNF-α, IL-2, and IL-4 in a dose-dependent manner. The inhibition involved both CD4 and CD8 T cells, and the effect was independent of APC and did not induce apoptosis. Furthermore, the cAMP analog inhibited both SEB-induced proliferation and Ag-specific immune responses to CMV and tuberculosis PPD. Co-incubation with Rp-8-Br-cAMPS, which is a selective inhibitor of PKA type I, completely reversed the inhibitory effect of the PKA agonist Sp-8-Br-cAMPS. This indicates that the effects observed are due to activation of PKA type I and that inhibition of T cell function following cAMP incubation can be reversed in a timely manner with a cAMP antagonist.

In our experiments, the sensitivity to cAMP varied among the cytokines. This may be due to different thresholds for inhibition or different kinetics. However, different accumulation periods revealed a consistent inhibition, indicating that the inhibitory function is not a transient effect which is overcome or bypassed later in the activation process. Thus, the different sensitivities observed may rather be due to distinct pathways being involved in the transcriptional activation of the various cytokines downstream of the initial activation process.

We also investigated the effects of cAMP and PKA type I on Ag-driven T cell proliferation. Clonal expansion and proliferation are the basis for establishment of an adequate and efficient immune response. Recent studies have shown that effector functions of Ag-specific T lymphocytes can be segregated and may be independently regulated (18). In hepatitis B infection, immunological clearance appears to predominantly mediated by cytokine release rather than direct cell lysis of hepatitis B virus-infected cells (19).

In this study, we find that the proliferation and cytokine production of effector T cells can be segregated based upon the dosage of Sp-8-Br-cAMPS used. At the highest concentration of compound (1000 μM), both proliferation and cytokine production were suppressed whereas at a concentration of 250 μM, T cells proliferated but did not produce cytokine.

Finally, we investigated whether cAMP agonist treatment leads to an activation of Csk activity in the T cells. We have previously shown that cAMP through activation of PKA type I leads to an activation of Csk in the lipid raft fraction of the cell membrane (11). Csk inhibits T cell activation by phosphorylating Lck on residue Y505, leading to a conformational change and inactivation of the enzyme. We report here that the cAMP compound used in this study (Sp-8-Br-cAMPS) leads to a dose-dependent increase in Csk activity. This points to Csk as an early target for cAMP-induced inhibition of T cell activation specifically acting through PKA type I, although additionally other targets downstream for the initial activation events may be involved.

The effect of increasing concentrations of Sp-8-Br-cAMPS on IFN-γ expression in CD4 and CD8 T cells in coculture with paraformaldehyde-fixed APC cells loaded with SEB.
Specific manipulation of aberrant T cell activity in autoimmune disease, transplantation, or delayed-type hypersensitivity is a long sought-after clinical goal. Current strategies for immunosuppression can be divided into separate categories depending upon mode of action of the therapeutic agent. First, lymphocyte cell division can be interrupted with drugs such as azathioprine, cyclophosphamide, or mycophenolic acid. Second, lymphocyte depletion can be achieved through drugs which produce lysis and/or inactivation (monoclonal OKT3 antilymphocyte Abs or thymoglobulin). Third, interruption of lymphocyte maturational events may be obtained by the calcineurin-inhibiting agents cyclosporine or tacrolimus (FK506) or with rapamycin. Fourth, the accessory signals generated by APC can be interrupted by distinct agents, including the receptor conjugate CTLA4-Ig and anti-B7 or anti-CD40 ligand mAbs. Finally, there are strategies which specifically target Ag-reactive T cells such as by blocking CD8 binding using α3 domain mutants of MHC class I-peptide complexes (20). In organ transplantation, cyclosporine and tacrolimus (FK506) have been used in clinical practice. These agents are potent, but nonselective and long-term sequelae of their use include serious side effects as posttransplant lymphoproliferative disease associated with broad-spectrum immunosuppression (21). Other agents under evaluation such as FTY720 induce apoptosis of T cells, leading to protracted loss of lymphocytes (22). The need for selective and reversible inhibitors of T lymphocyte function remains. Further investigation of the immunosuppressive properties of PKA type I by cAMP analogs is therefore warranted.

FIGURE 6. Inhibition of proliferation of CD3, CD4, and CD8 T cells. PBMC were labeled with CFSE and stimulated by SEB for 96 h in the absence and presence of increasing concentrations of Sp-8-Br-cAMPS. Cells were preincubated with cAMP analog for 1 h before stimulation with SEB. The cells were then fixed, permeabilized, and stained for CD3, CD4, and CD8 before analysis by flow cytometry (a). The lymphocyte population was gated on CD3⁺, CD3⁺CD4⁻, and CD3⁺CD8⁺ T cells and analyzed for CFSE fluorescent intensity. Representative data (n = 3) are shown. b, Brefeldin A was added for the last 6 h of the incubation period before fixation, permeabilization, and staining for CD3 and intracellular IFN-γ. The lymphocyte population was gated for CD3⁺ cells and analyzed for CFSE vs IFN-γ expression. Representative data (n = 4) are shown.

FIGURE 7. Inhibition of CMV- and tuberculin PPD-induced immune responses. PBMC were stimulated for 6 h with CMV lysate (CD4 T cell responses) or CMVpp65 (CD8 T cell responses) in the absence and presence of increasing concentrations of Sp-8-Br-cAMPS (a). Cell cultures were treated and prepared as described previously and stained for CD3, CD4, CD8, and intracellular IFN-γ before analysis by flow cytometry. b, PBMC were labeled with CFSE and stimulated by tuberculin PPD for 96 h in the absence and presence of increasing concentrations of Sp-8-Br-cAMPS. Brefeldin A was added for the last 6 h of the incubation period before fixation, permeabilization, and staining for CD3, CD4, and intracellular IFN-γ. The lymphocyte population was gated for CD3⁺, CD4⁺, and CD8⁺ T cells and analyzed for CFSE vs IFN-γ expression.
FIGURE 8. Effect of Sp-8-Br-cAMPS on Csk activity in T cells. Purified peripheral T cells were incubated at 37°C for 30 min with or without the cAMP analog Sp-8-Br-cAMPS at the indicated concentrations. Thereafter, cells were lysed and phosphotransferase activity of immunoprecipitated Csk was assessed as described in Materials and Methods. Average ± half-range of duplicate measurements are shown and data are representative of two independent experiments. Equal amounts of immunoprecipitated Csk in each reaction mixture was verified by immunoblotting.

References