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Co-regulation of Antigen-specific T Lymphocyte Responses by Type I and Type II Cyclic AMP-Dependent Protein Kinases (cAK)

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ABSTRACT. While a differential sensitivity to cyclic AMP (cAMP)-mediated signaling between Th1 and Th2 cells has been hypothesized, differential activity of downstream signaling through cAMP-dependent protein kinase (cAK) isoforms remains unexplored. We herein report the effects of type 1- and type 2-specific cAK agonists and antagonists on proliferative responses and cytokine generation from ragweed-driven peripheral blood mononuclear cells (PBMCs) and Amb a 1-specific Th1 and Th2 clones. Rp-8-Cl- and Rp-8-CPT-cAMP were utilized as single agent antagonists of cAKI and cAKII, respectively; 8-AHA-cAMP, with and without 8-PIP-cAMP, and 8-CPT-cAMP, with and without 6-Bnz-cAMP, were used as synergistic agonist pairs specific for the cAKI and cAKII, respectively. Activation of either cAKI or cAKII individually was ineffective in down-regulating proliferative responses of PBMCs or T cell clones; concentration–response curves for the Th1 and Th2 clones were identical. Moreover, inhibition of either cAKI or cAKII individually was ineffective in overcoming the down-regulatory effects of phosphodiesterase inhibition. Activation of either cAKI or cAKII individually was ineffective in down-regulating proinflammatory cytokine generation from T cell clones (interleukin-4 from Th2; interferon-γ from Th1). However, concurrent activation of both cAKI and cAKII produced down-regulatory effects equivalent to those of the phosphodiesterase inhibitor on both proliferation and cytokine generation. These data suggest a critical role for concurrent activation of cAKI and cAKII in the functional efficacy of antigen-driven downstream signaling due to elevations of intracellular cAMP and argue against differential regulation of Th1 and Th2 responses by cAK subtypes. BIOCHEM PHARMACOL **56**;7:871–879, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. human; T lymphocytes; cAMP-dependent protein kinase; phosphodiesterase; interleukin-4; interferon-γ

Elevations of intracellular cAMP† have been shown to down-regulate antigen- and mitogen-driven proliferation and cytokine generation from human PBMCs, purified peripheral blood T cells, and subset-specific T cell clones [1–5]. While elevations of intracellular cAMP may be achieved through the activation of adenylyl cyclase, these elevations are short-lived, are accompanied by elevations of cyclic nucleotide PDE activity, and do not affect T cell responses significantly [6]. We have shown recently that elevations of intracellular cAMP may be achieved through the selective inhibition of either type 3 or type 4 PDE isozymes; however, down-regulation of T cell responses was associated exclusively with inhibition of PDE4, implicating selectivity most likely on the basis of subcellular compartmentalization [5]. To date, the potential selectivity of downstream signaling pathways of cAMP in antigen-driven human T lymphocytes remains largely unexplored.

The primary downstream signaling effector for elevations of intracellular cAMP is the family of cAKs, comprised of two main subtypes (cAKI and cAKII). Each subtype consists of multiple, distinct isoforms of regulatory and catalytic subunits [7]. Each isozyme exists as a tetramer composed of a homo- or heterodimer of regulatory subunits and a homo- or heterodimer of catalytic subunits. The two regulatory subunits may cooperatively bind four cAMP molecules, resulting in dissociation from and activation of the two catalytic subunits [8, 9]. These serine-threonine kinases affect multiple downstream targets, including the families of CREBs and the activation of transcription factors (ATFs) [10]. Compartmentalization of cAKs is suggested by the finding that cAKI is predominantly membrane bound and may be associated with the CD3 complex, while cAKII

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† *Abbreviations:* cAK, cAMP-dependent protein kinase; cAMP, adenosine 3′,5′-cyclic monophosphate; APC, antigen-presenting cells; CREB, cyclic nucleotide regulatory element binding protein; DTT, dithiothreitol; IFN, interferon; IL, interleukin; PBMCs, peripheral blood mononuclear cells; PDE, phosphodiesterase; PMSF, phenylmethylsulfonyl fluoride; RT–PCR, reverse transcription–polymerase chain reaction; and RW, ragweed.

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is predominantly cytosolic [11, 12]. Previous studies have provided evidence for selectivity of cAK subtype activation and function during mitogen-driven proliferation and cytokine generation from human T cell lines [12–14]. To date, studies demonstrating selectivity of cAK subtype activation and function in antigen-driven human T cells have not been performed.

We herein present data showing the requirement for activation of both cAKI and cAKII for the transduction of down-regulatory signals in human, antigen-driven T lymphocytes. Furthermore, using phenotypically specific Th1 and Th2 clones, we demonstrate a lack of differential regulation of proliferation and cytokine generation at the level of cAKs.

MATERIALS AND METHODS

Selection of Subjects

Subjects for these studies were allergic volunteers from the Johns Hopkins Asthma and Allergy Center who showed positive epicutaneous skin tests to a 100,000 AU stock of Short Ragweed Extract (ALK Laboratories Inc.). All subjects were symptomatic by history with upper and/or lower airway allergic disease upon RW exposure. Subjects had received tetanus booster immunizations within the preceding 3 years and were off all medications for 1 month preceding the study; none of the subjects had undergone immunotherapy.

Selection and Use of cAK Agonists and Antagonists

A panel of cyclic nucleotide analogues (see Table 1) for use as selective cAK subtype agonists or antagonists was purchased from BioLog Life Sciences Institute; the efficacy and specificity of these analogues has been reported previously [8, 12, 15, 16]. Briefly, subtype-specific agonists were used in pairs. Each analogue showed specificity for either the A or B binding site of the R subunit of the specific cAK subtype; selective activation of that cAK subtype, requiring simultaneous binding at both the A and B binding sites, was reflected in a left shift of the concentration–response curve of the partial agonist upon addition of a single concentration of the synergistic agonist. The antagonists were selective, single agent, competitive inhibitors. The selective type 4 phosphodiesterase (PDE4) inhibitor rolipram was used alone or in combination with these agents in many of the experiments.

PBMC Isolation and Derivation of Antigen-specific T Lymphocyte Clones

PBMCs were isolated by gradient centrifugation on Ficoll-Paque (Pharmacia Inc.) and resuspended in RPMI (BioWhittaker) supplemented with penicillin (50 U/mL)/streptomycin (50 μg/mL) and 5% human AB serum (all from Life Technologies), as previously described [1]. These cells either were used immediately for PBMC studies or were irradiated for use as APCs in conjunction with the Th1 and Th2 clones. Platelet contamination of these preparations was $< 1\%$; viability by trypan blue exclusion was uniformly $\geq 99\%$.

Antigen-specific T cell clones were derived and characterized as previously described [17]. Briefly, PBMCs from an asthmatic, rhinitic subject with epicutaneous skin test reactivity to short ragweed (*Ambrosia artemisiifolia*) and a short ragweed-specific IgE = 1761 ng/mL by radioallergosorbant test (RAST) were cultured in the presence of short ragweed antigen at 10 μg/mL. This primary culture underwent two successive biweekly restimulations of the antigen-specific T cells with a major short ragweed antigen, Amb a 1, in the presence of autologous APCs. The resulting antigen-specific T cell line was cloned and subcloned using the limiting dilution technique. All T cell clones were $CD3^+CD4^+CD8^-$ by flow cytometry. Proliferative responses restricted to Amb a 1 confirmed antigen specificity. Of thirty clones obtained by this procedure, eight were selected for further analysis based on similar proliferation characteristics. Cytokine profiles were determined by both RT–PCR and ELISA for secreted protein; based on these data, phenotypic assignment to Th0, Th1, or Th2 was made.

Preparation of Cell Lysates and In Vitro *Kinase Assays*

Cell extracts were prepared by the method of Bauman *et al.* [14]. Briefly, cells were incubated with agonists or antagonists (30 min) or rolipram (2 hr), washed once in cold PBS, resuspended in 1 mL of lysing buffer [10 mM Tris (pH 7.4) with 1 mM DTT, 1 mM EDTA, and 300 μM PMSF], and sonicated for a total of 20 s. Supernatants were centrifuged free of cellular debris and either placed on ice for immediate use or stored at −70°. Cyclic AK-specific kinase activity was determined with the Protein Kinase A Assay System (Life Technologies, Inc.) according to the manufacturer's instructions.

Proliferation Assays

Proliferation assays were performed as previously described using conditions optimized for cell number, clone/APC ratio, antigen concentration, and kinetics [1, 17, 18]. Briefly, either 2×10^5 PBMCs or 2×10^4 clonal T cells with 1.5×10^5 APCs were cultured in 96-well flat-bottom plates in the absence or presence of antigen and/or various concentrations of specific cyclic nucleotide analogues. Antigen concentrations were 10 μg/mL for RW and 0.1 lF/mL for tetanus toxoid. Cells were preincubated with the cyclic nucleotide analogues for 1 hr prior to the addition of antigen, although neither longer preincubations nor simultaneous addition of cyclic nucleotide analogue and antigen significantly altered these results (data not shown). Negative (medium alone) and positive (antigen only) controls were performed with each culture. No exogenous cytokine was used in these assays. All conditions were performed in

triplicate and incubated for 72 hr at 37° with 5% CO_2. Each culture was pulsed with 1 μCi of tritiated thymidine, incubated for an additional 20 hr, harvested onto glass fiber filters in a multichannel cell harvester (Cambridge Technologies Inc.) and counted for 1 min in an automated beta counter (Beckman Instruments Inc.). Cell viability prior to harvest was uniformly >97% by trypan blue exclusion.

Cytokine Gene Expression Assays

Cytokine gene expression assays were performed as previously described using conditions optimized for cell number, clone/APC ratio, antigen concentration, and kinetics [2, 17, 18]. Briefly, either 5×10^6 PBMCs or 2×10^5 clonal T cells with 3×10^6 APCs were cultured in the absence or presence of antigen (RW 10 μg/mL or tetanus toxoid 0.1 lF/mL) and/or various concentrations of specific cyclic nucleotide analogues. Cells were preincubated with the cyclic nucleotide analogues for 1 hr prior to the addition of antigen; no exogenous cytokine was used. After a 12-hr incubation with antigen in slanted polypropylene tubes, the cells were pelleted, washed, and subjected to RNA isolation by the RNAzol[B] technique, according to the manufacturer's instructions (Tel-Test Inc.). Diethylpyrocarbonate-treated water without SDS was used for the final resuspension step. RNA was stored at −80° until studied. Normalization of RNA to approximately 100 ng/μL was achieved with a combination of spectrophotometry, ethidium bromide-stained gel electrophoresis, and RT–PCR for a constitutive marker gene, β-actin. $A_{260/280}$ values >1.7 were uniformly obtained. Semi-quantitative RT–PCR was performed with 5 mM magnesium and oligo dT priming using standard reagents (Perkin Elmer Cetus) and cytokine-specific primer pairs designed in our laboratory and made at the DNA Core Facility of The Johns Hopkins University [18]. Strict RNase-free conditions were maintained throughout these experiments. All PCR products were visualized by ethidium bromide-stained gel electrophoresis and photographed.

Cytokine Protein Secretion Assays

Cytokine protein secretion was assessed by ELISA, using Cytoscreen Immunoassay kits (Biosource, Inc.) according to the manufacturer's instructions. Quantitation was achieved using the WHO standards provided by the company. Briefly, culture supernatants were collected after 12 hr, centrifuged free of debris, and stored at −20° until assayed. Dilutions of samples were performed in culture medium. All standards and samples were tested in duplicate. Most samples were analyzed at two different dilutions and compared for internal consistency.

Statistical Analysis

Mean and standard error values, as well as *t*-test comparisons, were derived using StatView (BrainPower, Inc.) on a Macintosh Classic computer. *P* values are paired, two-tail. Percent inhibition for each condition was calculated based on inhibition relative to stimulated, drug-free mean counts, corrected in each case for background counts with medium alone. The IC_{50} values represent the concentration of drug at 50% inhibition; EC_{50} values represent the concentration of drug at 50% efficacy.

RESULTS

In Vitro *Kinase Activity from PBMCs*

Figure 1 depicts the percent activated cAK from PBMCs in the presence and absence of subtype-specific cAK agonists and antagonists. The concentrations of agonists and antagonists used are shown in Table 1; both 8-AHA and 8-CPT were used at 10 μM. Values for each culture condition are expressed as percent activated cAK relative to that induced by 10 μM cAMP, corrected in each case for kinase activity in the presence of a 1 μM concentration of the cAK inhibitor, PKI. While resting cells showed 8% activity, treatment with the selective type 4 phosphodiesterase (PDE4) inhibitor, rolipram, significantly increased this activity to 22% ($P < 0.05$). Each of the agonist pairs increased cAK activity to a higher level than did rolipram (48 and 63%, respectively, for cAKI and cAKII agonist pairs, $P < 0.01$), and the combination of cAKI and cAKII agonists increased percent cAK activity to 92% ($P < 0.001$ vs medium alone; $P < 0.05$ vs cAKI or cAKII agonist pairs). Neither the cAKI nor the cAKII antagonist was efficacious as a single agent inhibitor of the rolipram effect. Finally, the cAKII antagonist, but not the cAKI antagonist, showed significant partial agonist activity when evaluated in the absence of rolipram (16%; $P < 0.05$ vs medium alone). These data show the ability of these agents to affect cAK activity in intact cells, while also suggesting limitations to their use.

Antigen-driven Proliferation of PBMCs

Figure 2 depicts the modulation of RW-driven proliferation of PBMCs in the presence of synergistic agonist pairs specific for cAKI and cAKII (upper and lower panels, respectively). In both instances, the partial agonists produced a clear, concentration-dependent inhibition of RW-driven proliferation; a significant left shift of the concentration–response curve upon the addition of a single, low concentration of the synergistic agonist (8-PIP, 90 μM; 6-Bnz, 3 μM) would imply specificity of signaling for that cAK subtype. However, neither concentration–response curve showed a significant left shift; IC_{50} and EC_{50} values were unchanged with the addition of the synergistic agonist ($IC_{50} = 5 \times 10^{-5}$ M and 3×10^{-6} M; $EC_{50} = 3 \times 10^{-5}$ M and 3×10^{-6} M, respectively, for cAKI and cAKII). Varying the concentration of each synergistic agonist from 100 to 0.1 μM was ineffective in shifting the partial agonist concentration–response curve (data not shown). These data suggest an inability of activation of either cAK subtype

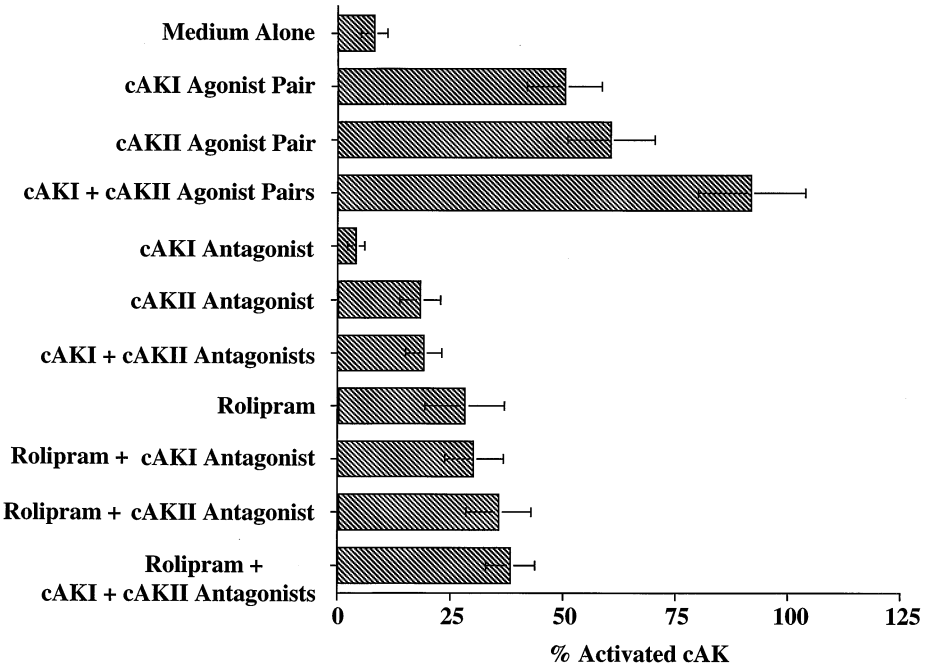

FIG. 1. Modulation of cAK activity in PBMCs by cAKI- and cAKII-specific agonists and antagonists. Data are presented as the percent activated cAK relative to total cAK activity induced by 10 μM cAMP (56,000 ± 2,200 cpm), corrected for background in the presence of 1 μM PKI (9,800 ± 460 cpm). The effect of 10 μM rolipram is shown for comparison. All values are means ± SEM; N = 3 individual experiments on three different subjects.

individually to selectively modulate antigen-driven proliferative responses in human PBMCs.

Antigen-driven Proliferation of Th1 and Th2 Clones

Figures 3 and 4 depict the modulation of RW-driven proliferation of Th1 and Th2 clones, respectively, in the presence of the same synergistic agonist pairs used in Fig. 2. Once again, the partial agonists specific for cAKI and cAKII each produced clear, concentration-dependent inhibition of RW-driven proliferation. Moreover, no significant differences between Th1 and Th2 clones were evident. Finally, no significant left-shifts of the concentration–response curves for either of the cAK partial agonists in either of the T cell phenotypes were evident upon the addition of a single, low concentration of the specific synergistic agonist. These data suggest an inability of activation of either cAK subtype individually to selectively or differentially modulate antigen-driven proliferative responses in human Th1 and Th2 clones.

Figure 5 depicts the modulation of RW-driven proliferation of Th1 and Th2 clones in the presence of both agonist pairs simultaneously. The simultaneous use of the partial agonists for cAKI and cAKII, each at the abscissa-specified concentration, produced a clear, concentration-dependent inhibition of RW-driven proliferation, which was slightly more pronounced than that achieved with either partial agonist alone (Figs. 3 and 4). Upon the simultaneous addition of single, low concentrations of both specific synergistic agonists (as specified in Table 1), a significant left shift of the concentration–response curves was obtained ($P < 0.05$ at 10^{-7} M, $P < 0.01$ at 10^{-6} M

TABLE 1. cAK agonists and antagonists

Chemical name	Abbreviation	Activity	Site specificity	Optimum concentration
cAK Type I (cAKI)				
8-(4-Aminohexyl) aminoadenosine-3′,5′-cyclic monophosphate	8-AHA	Partial agonist	RIB	0.01–100 μM
8-Piperidinoadenosine-3′,5′-cyclic monophosphate	8-PIP	Synergistic agonist	RIA/RIIB	90 μM
8-Chloroadenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer	Rp-8-Cl	Antagonist		10 μM
cAK Type II (cAKII)				
8-(4-Chlorophenylthio) adenosine-3′,5′-cyclic monophosphate	8-CPT	Partial agonist	RIIB	0.01–100 μM
N^6-Benzoyladenosine-3′,5′-cyclic monophosphate	6-Bnz	Synergistic agonist	RIA/RIIA	3 μM
8-(4-Chlorophenylthio) adenosine-3′,5′-cyclic monophosphorothioate, Rp-isomer	Rp-8-CPT	Antagonist		10 μM

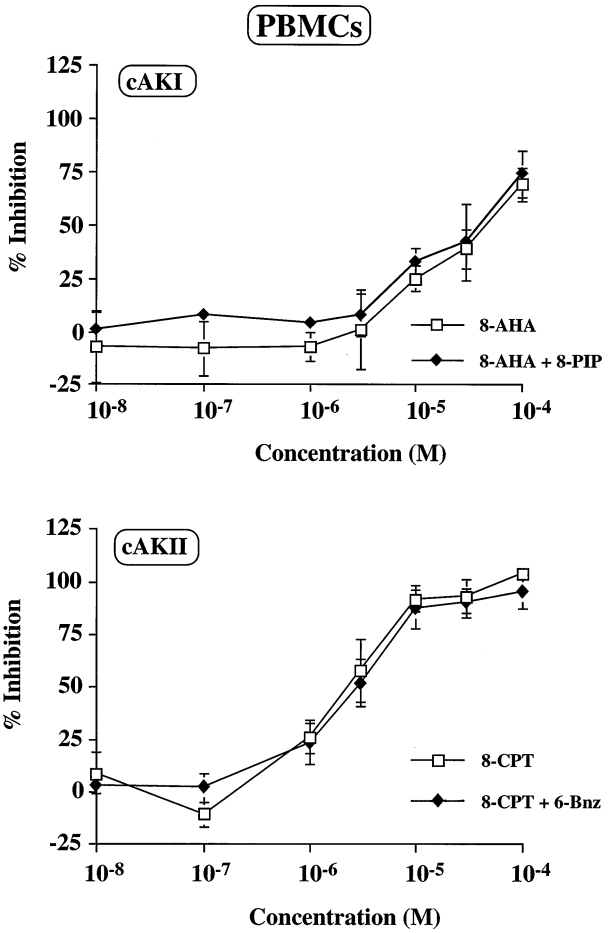

FIG. 2. Modulation of RW-driven PBMC proliferative responses by cAKI (top) and cAKII (bottom) agonists. Data are presented as percent inhibition ± SEM relative to stimulated, drug-free cultures, corrected for background with medium alone (4,300 ± 400 and 710 ± 60 cpm, respectively). N = 5 individual experiments on five different subjects.

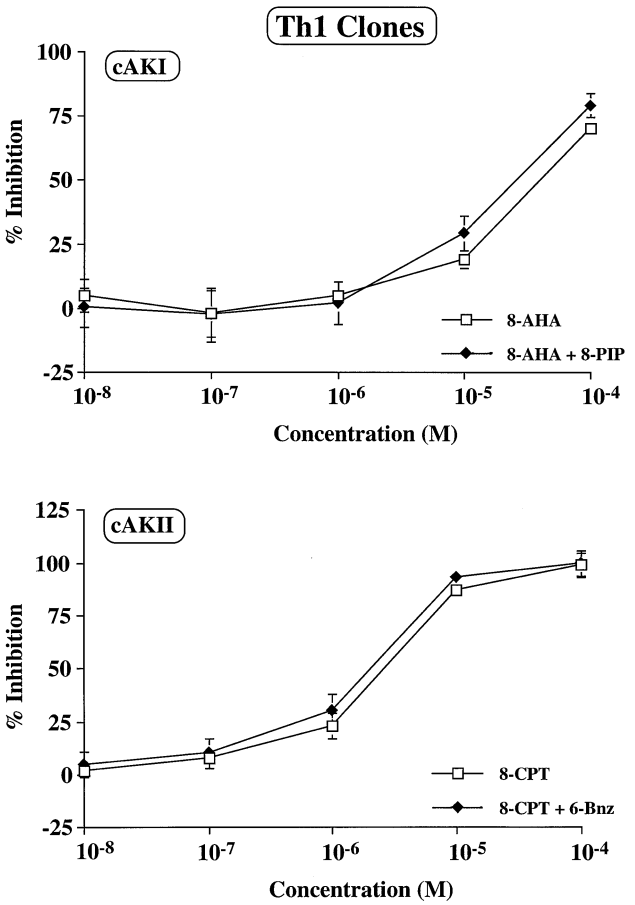

FIG. 3. Modulation of clonal Th1 proliferative responses by cAKI (top) and cAKII (bottom) agonists. Data are presented as percent inhibition ± SEM relative to stimulated, drug-free cultures, corrected for background with medium alone (23,000 ± 2,600 and 1,300 ± 500 cpm, respectively). Each of the clones was used in four separate replicate experiments.

for Th1; $P < 0.05$ at 10^{-7} and 10^{-5} M, $P < 0.01$ at 10^{-6} M for Th2). Differences between Th1 and Th2 clones were not significant. These data suggest co-regulation of antigen-driven T cell proliferation by type I and type II cAK.

Figure 6 depicts the modulation of RW-driven proliferation of Th1 and Th2 clones by single agent, subtype-specific cAK antagonists in the presence or absence of 10^{-5} M rolipram. The concentration of rolipram selected closely approximated its reported IC_{50} value; similar results, relative to the cAK antagonists, were also obtained using 10^{-6} M rolipram (data not shown). The concentrations of the cAKI and cAKII antagonists were each 10^{-5} M; solubility precluded the use of higher concentrations. As shown in Fig. 6, the cAKI antagonist was ineffective in modulating antigen-driven proliferative responses. Moreover, the cAKI antagonist was ineffective in modulating rolipram-induced inhibition of proliferation in either Th1 or Th2 clones. Finally, the cAKII antagonist at 10^{-5} M showed apparent partial agonist activity that has not been reported previously; this effect disappeared at 10^{-6} M. At neither 10^{-5}, nor 10^{-6} M was it effective in modulating the rolipram-induced inhibition of proliferation in either Th1 or Th2 clones. Experiments combining the cAKI and cAKII antagonists were not undertaken due to the presumed partial agonist activity of the cAKII antagonist; to our knowledge, no other specific cAKII antagonists are available.

Antigen-driven Cytokine Generation from Th1 and Th2 Clones

Figure 7 shows the β-actin- and cytokine-specific RT–PCR amplification products from a representative study of Th1 and Th2 clones cultured with antigen and APCs alone (column 1) and in the presence of 10^{-5} M rolipram (column 2), 10^{-5} M rolipram with the cAKI and cAKII antagonists (columns 3 and 4), the cAKI and cAKII agonist: pairs individually (columns 5 and 6), and the cAKI and cAKII agonist pairs together (column 7). All concentrations of cAK agonists and antagonists used in this study were the maximum cited in Table 1; IL-4 from the Th2 clone is shown in row 2, while IFNγ from the Th1 clone is

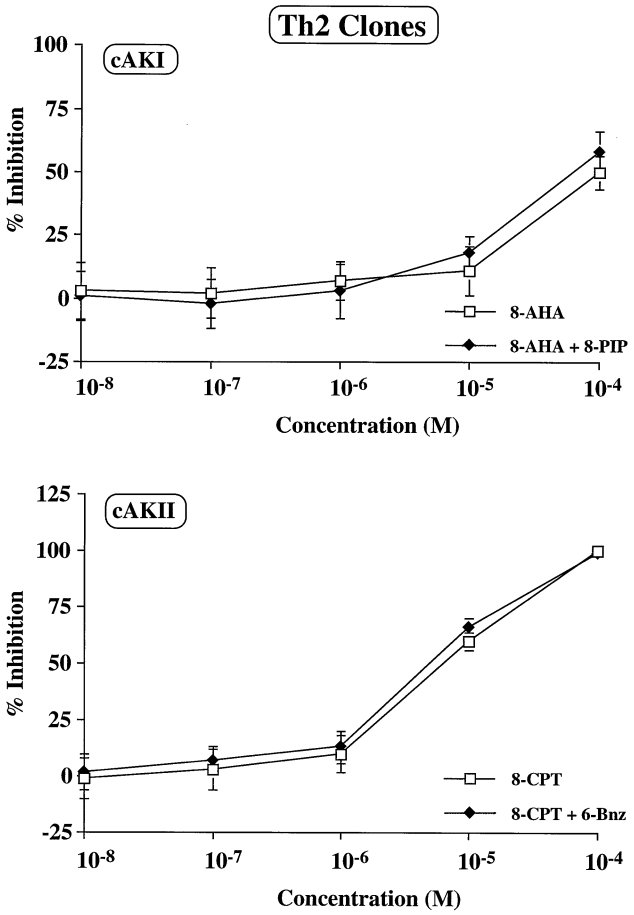

FIG. 4. Modulation of clonal Th2 proliferative responses by cAKI (top) and cAKII (bottom) agonists. Data are presented as percent inhibition ± SEM relative to stimulated, drug-free cultures, corrected for background with medium alone (29,000 ± 2,200 and 430 ± 50 cpm, respectively). Each of the clones was used in four separate replicate experiments.

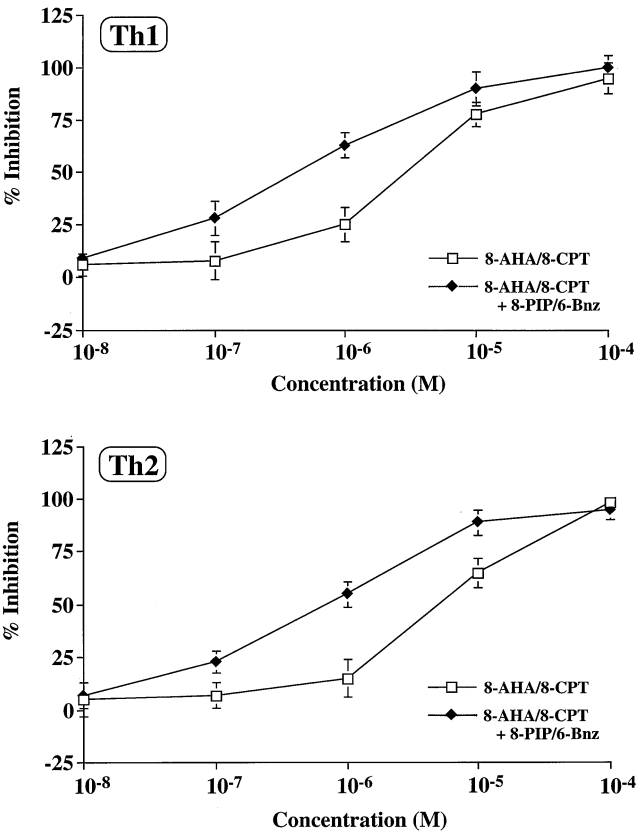

FIG. 5. Modulation of clonal Th1 (top) and Th2 (bottom) proliferative responses by simultaneous use of cAKI and cAKII agonists. Data are presented as percent inhibition ± SEM relative to stimulated, drug-free cultures, corrected for background with medium alone (22,000 ± 1,900 and 710 ± 100, respectively, for Th1; 29,000 ± 2,200 and 670 ± 100 cpm, respectively, for Th2). Each of the clones was used in three separate replicate experiments.

shown in row 4. Resting clonal cells cultured with APCs in the absence of antigen did not express significant message for proinflammatory cytokines; control assays in the absence of reverse transcriptase were uniformly negative (data not shown). Adequate normalization of RNA for each clone was confirmed by the equality of RT–PCR amplification products for β-actin gene expression at subsaturating cycle number (30 cycles; rows 1 and 3, respectively, for Th2 and Th1 clones). Exposure to 10^{-5} M rolipram induced a clear down-regulation of proinflammatory cytokine gene expression that was not reversed by either the cAKI or cAKII antagonist used individually. While neither the cAKI or cAKII agonist pairs, used individually, were efficacious in down-regulating proinflammatory cytokine gene expression, the combination of the agents showed efficacy similar to that of rolipram in this semi-quantitative RT–PCR assay. These data suggest the co-regulation of antigen-driven T cell responses by type I and type II cAK and argue against differential efficacy of these isozymes in Th1 and Th2 responses.

Figure 8 depicts the percent inhibition of cytokine secretion from these same Th1 and Th2 clones cultured with antigen and APCs in the presence of rolipram, the cAK antagonists, and the cAK agonist pairs. The accuracy of individual values for each of the T cell clones was confirmed by both duplicate culture experiments and replicate ELISA assays at different dilutions of the culture supernatants (data not shown). Once again, neither of the cAK antagonists cultured individually with rolipram showed efficacy in reversing the down-regulation of proinflammatory cytokine secretion from either Th1 or Th2 clones. While cAK subtype-specific agonist pairs, used individually, were ineffective in down-regulating cytokine secretion, a combination of these agonists significantly down-regulated cytokine secretion with efficacy comparable to that achieved with rolipram ($P < 0.01$ for both Th1 and Th2 clones). These data closely parallel the results of cytokine gene expression and support the hypothesis that the transduction of cAMP-mediated signals in antigen-driven Th1 and Th2 clones is co-regulated by both type I and type II cAK. Finally, no differential efficacy of these agents on antigen-driven cytokine protein secretion was noted between Th1 and Th2 responses.

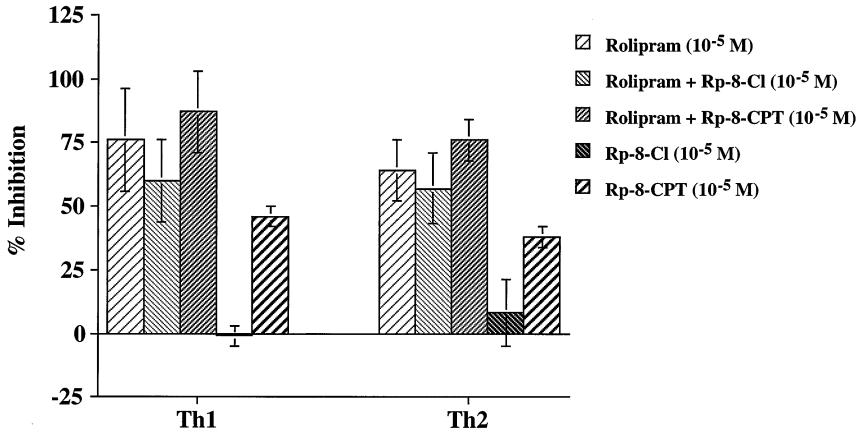

FIG. 6. Modulation of clonal T cell proliferative responses by cAKI and cAKII antagonists. Data are presented as percent inhibition ± SEM relative to stimulated, drug-free cultures, corrected for background with medium alone (19,000 ± 2,500 and 2,000 ± 750 cpm, respectively, for Th1 and 28,000 ± 2,000 and 410 ± 50 cpm, respectively, for Th2). Each of the clones was used in four separate replicate experiments.

DISCUSSION

In this manuscript, we have provided evidence that cAMP-mediated down-regulation of antigen-driven T cell proliferation and cytokine generation requires the activation of both cAKI and cAKII subtypes. These data are the first to investigate this pathway in antigen-driven human T cells; moreover, they are the first to investigate the potential for differential regulation of this pathway in Th1 and Th2 clones. Our results differ from those of other investigators, likely based on study design differences. Skalhegg *et al.* [12], using the identical agonist pairs, showed selective down-regulation of T cell proliferative responses by activation of cAKI, and not cAKII. However, this study utilized purified peripheral blood T cells activated by anti-CD3 in the absence of APCs. We have shown previously that mitogen- and antigen-driven T cell responses differ significantly in their sensitivity to cAMP-mediated signals [1]. Moreover, the lack of APCs obviates the effects of co-stimulatory pathways important in cAMP-mediated signal transduction [19, 20]. Interestingly, Laxminarayana *et al.* [13] have described the preferential activation of cAKI commensurate with the activation of purified human $CD4^+$ T cells with anti-CD3 and rhIL-1α. These data would suggest a regulatory role for the cAKI during T cell activation, in accordance with the ability of the cAK RIα subunit to associate with the T cell receptor/CD3 complex [11]. While cAKII did not appear to mediate proliferative responses in either of these studies, Ostenstad *et al.* [21] have shown that cAMP-mediated modulation of LAK cell cytotoxicity is regulated predominantly by cAKII. Finally, our data confirm and extend the findings of Bauman *et al.* [14] that purified human T cells stimulated with either prostaglandin E_2 or isoproterenol produced equal activation of both cAK subtypes.

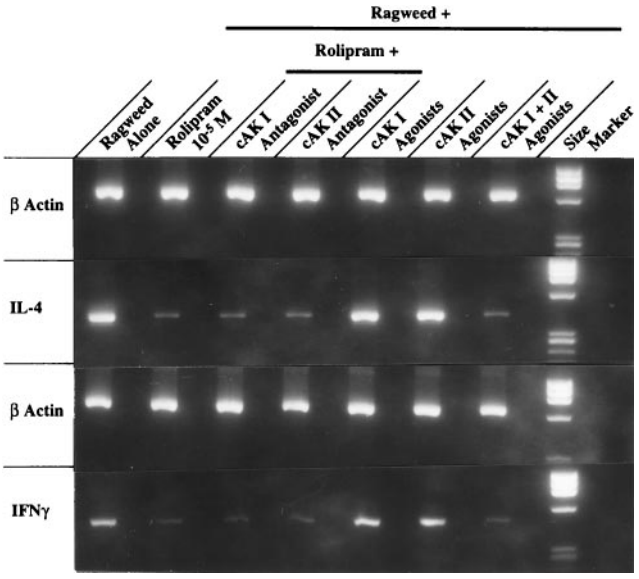

FIG. 7. Modulation of cytokine gene expression by subtype-specific cAK agonists and antagonists in Th1 and Th2 clones. Representative results of RT–PCR products for β-actin, IL-4, and IFNγ are shown for two different antigen-stimulated T cell clones under the specific culture conditions indicated. The top two rows are from a Th2 clone, while the bottom two rows are from a Th1 clone. Normalization by equivalent β-actin gene expression at subsaturating cycle number is depicted for each condition in the first and third rows. The ϕX174 *Hae*III fragment size marker is shown in the eighth column. Each of the clones was used in three separate replicate experiments.

Numerous issues in the design of these studies deserve further attention. First, our hypothesis would suggest that the effects of rolipram on antigen-driven T cell responses should be reversible by a combination of the cAKI and cAKII antagonists. While this would be important to show, the partial agonist activity of the cAKII antagonist precludes our ability to address this issue. The precise mechanism of this partial agonist activity is unclear, but may be mediated by occupancy of both A and B binding sites of the cAKII, with subsequent release of the catalytic subunit. While the use of lower concentrations of the cAKII

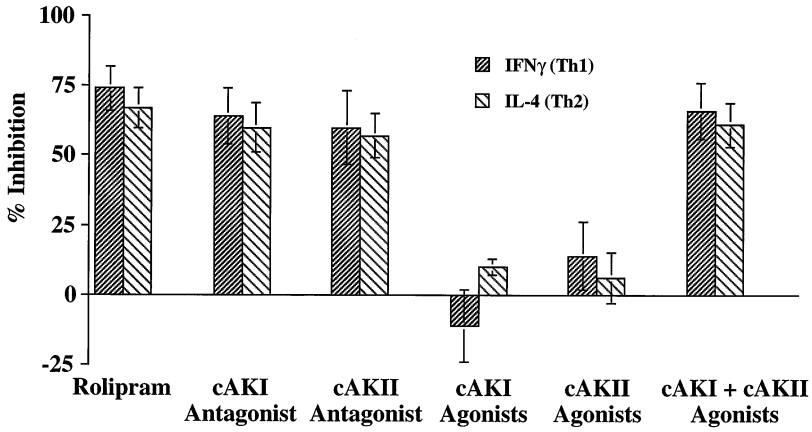

FIG. 8. Modulation of cytokine secretion by subtype-specific cAK agonists and antagonists in Th1 and Th2 clones. Secretion of IL-4 by Th2 clones and IFNγ by Th1 clones is shown for the specific culture conditions indicated. Data are presented as percent inhibition ± SEM relative to stimulated, drug-free cultures (2,600 ± 420 and 3,900 ± 650 pg/mL, respectively, for IL-4 and IFNγ). Each of the clones was used in three separate replicate experiments.

antagonist with the cAKI antagonist did partially reverse the effects of rolipram, this reversal was incomplete, perhaps due to the use of a suboptimal concentration of the cAKII antagonist (data not shown). Finally, antigen-driven T cell activation in our experimental system is dependent upon both the responder T cells and the APCs; these data do not preclude cAK modulation of T cell responses due to effects on APCs. However, we have reported previously that the efficacy of rolipram in modulating antigen-driven proliferation and cytokine gene expression in human PBMCs is mediated exclusively by the effects of rolipram on the lymphocyte fraction [22]. Furthermore, the unresponsiveness of the autologous, irradiated PBMCs to pharmacologic regulation by cAMP modulation militates against this possibility [23]. Thus, we are confident that the effects described in this manuscript are specific for the responder T lymphocytes.

We have reported previously the differential efficacy of PDE4 inhibitors on Th1 and Th2 responses from antigen-driven PBMCs and phenotypically specific T cell clones activated with antigen and APCs [5]; these data would suggest the presence of different cAMP-mediated downstream signaling pathways in these two T cell phenotypes. While the present data delineate the requirement for co-regulation by type I and type II cAK, we have not undertaken studies to determine whether the differential utilization of subtype-specific isoforms is present in Th1 or Th2 clones. Differences in cAMP-mediated signaling may be present at the level of the cAK isoforms or may be a function of differential utilization and/or regulation of cAK targets between these two T cell phenotypes. Future studies from our laboratory will address these issues.

In conclusion, transduction of signaling through the cAMP pathway in antigen-driven human T cells is co-regulated by both cAKI and cAKII subtypes. These cAK subtypes do not exert differential effects on phenotypically specific antigen-driven T cell clones.

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