0026-895X/02/6206-1471–1481$7.00
MOLECULAR PHARMACOLOGY
Copyright © 2002 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 62:1471–1481, 2002

Vol. 62, No. 6
1805/1026493
Printed in U.S.A.

Commitment of Activated T Cells to Secondary Responsiveness Is Enhanced by Signals Mediated by cAMP-Dependent Protein Kinase A-I

MONIKA VIG, ANNA GEORGE, RANJAN SEN, JEANNINE DURDIK, SATYAJIT RATH, and VINEETA BAL

National Institute of Immunology, New Delhi, India (M.V., A.G., S.R., V.B.); Rosenstiel Research Center, Brandeis University, Waltham, Massachusetts (R.S.); and Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas (J.D.)

Received April 18, 2002; accepted September 10, 2002

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

Modalities that induce specific differentiation to T cell memory in immune responses are important for vaccine design, but there is a paucity of well characterized molecular pathways useful to target for this purpose. We have shown previously that pentoxifylline (PF), a phosphodiesterase (PDE) inhibitor in common clinical use, enhances the commitment of in vitro alloprimed human T cells to secondary responsiveness, a characteristic crucial for memory T cells, which are key determinants of the longevity of the immune response. We now show that this effect can also be mediated by activation of adenylate cyclase (AC) and involves PDE4, but not PDE3 or PDE7. PF-mediated enhancement of T-cell priming is inhibited by blocking AC, is specifically signaled via cAMP-dependent protein kinase A (PKA) isoform I, and is probably independent of both nuclear factor-κB and the mitogen-activated protein kinase cascade. Furthermore, known pharmacological inhibitors of AC or PKA by themselves cannot block T-cell priming in the absence of PF or rolipram (Rm), and enhancement of priming requires the presence of PF only relatively late during a 4-day priming in vitro (at 48–96 h), suggesting that pharmacological extension of cAMP-mediated signaling can bring about an event critical for T cell commitment to memory. Furthermore, PF and Rm prevent induction of caspase activation and apoptosis in anti-CD3–activated human T cells. Together, our data suggest that PKA-I–mediated signals triggered by prolonging the half-life of cAMP induced during T-cell priming increase survival of activated T cells and enhance memory T cell commitment.

To deal effectively with infections in vertebrate hosts, various components of the immune system play important roles. Immunological memory, primarily contributed by T and B lymphocytes, helps significantly toward successful elimination of pathogens during re-exposure. For activation of naive T cells, presentation of peptide-MHC complex by professional antigen presenting cells (APCs) along with accessory signals is necessary (Bernard et al., 2002). Differentiation of antigen-exposed T cells into effector phenotypes is crucial for dealing with acute stage of infections, but it also induces death pathways in them, leading to loss of the effector population (Ahmed and Gray, 1996). A residual population of antigen-triggered T cells survives as T cell memory and is essential in mediating long-lived immune protection against re-infection (Swain, 1994; Ahmed and Gray, 1996). The specific pathways leading to effector versus memory T cell differentiation are currently not well understood. Some data support a linear model of differentiation in which all responding T cells become effector T cells, with some surviving as memory (Swain, 1994), whereas other lines of evidence support the possibility that alternate signaling pathways may lead to either effector or memory differentiation (McHeyzer-Williams and Davis, 1995). We have been using a system of in vitro immunization of allo-specific human peripheral T cells by MHC-mismatched stimulator APCs to examine issues relating to T-cell

This work was funded in part by grants from the Department of Science and Technology, Government of India (to A.G., V.B., and S.R.), Department of Biotechnology, Government of India (to S.R.), the Wellcome Trust (to V.B.), and the United States Public Health Service, National Institutes of Health [R15-AG17472-01 (to J.M.D.) and TW00982 (to R.S. and S.R.)]. The National Institute of Immunology is supported by the Department of Biotechnology, Government of India.

ABBREVIATIONS: MHC, major histocompatibility complex; APC, antigen presenting cell; PDE, phosphodiesterase; PF, pentoxifylline; dbcAMP, dibutyryl cAMP; AC, adenylate cyclase; TCR, T cell receptor; PKA, protein kinase A; AKAP, A-kinase anchoring protein; PBMC, peripheral blood mononuclear cell; mAb, monoclonal antibody; Fs, forskolin; Sq, 9-(tetrahydro-2′-furyl)adenine (SQ22536); Rm, rolipram; Tq, trequinsin; Rp-BrcAMPs, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer; RpClcAMPs, 8-chloroadenosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer; RpCPTcAMPs, 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer; pAKAP, peptide inhibitor of PKA-II-AKAP interaction; pCon, control inactive peptide for pAKAP; PD98059, 2′-amino-3′-methoxyflavone; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; VAD-fmk-Flu, Val-Ala-Asp-fluoromethylketone-fluorescein; ERK, extracellular signal-regulated kinase; AICD, activation-induced cell death; NF-κB, nuclear factor-κB.

priming. We have shown previously that priming of T cells in the presence of the PDE inhibitor PF results in enhanced secondary responsiveness of T cells (Gupta et al., 1997). We have also shown that this enhancement in the commitment to secondary responsiveness is also induced by an analog of cAMP, dibutyryl cAMP (dbcAMP) and is caused by increased frequency of surviving allo-specific T cells (Gupta et al., 1999).

As a key second messenger, cAMP regulates a variety of cellular functions. It serves to transduce the action of a wide variety of hormones and neurotransmitters and can modulate signal transduction processes regulated by a variety of growth factors, cytokines and other agents (reviewed in Skalhegg and Tasken, 2000). The intracellular levels of cAMP are regulated by two distinct enzyme superfamilies: the ACs synthesizing cAMP and the cAMP-specific PDEs hydrolyzing it. ACs are associated with the plasma membrane and can be triggered via G-protein-mediated signals in many cell lineages, including T cells (Kammer, 1998). PDEs show a wide variety of isoforms (Beavo, 1995), with prominent expression of the PDE3, -4, and -7 families in T cells (Giembycz et al., 1996). The induction of PDE7 seems to be critical for T cell receptor (TCR)-mediated activation (Li et al., 1999). Downstream, cAMP activates a family of cAMP-dependent kinases, the PKA group, although not all cAMP-driven signals are necessarily mediated through PKA (MacKenzie et al., 1999). Despite its ubiquity, signals mediated by cAMP can be cell-specific, even specific for particular locations within a cell (Zaccolo and Pozzan, 2002), because of differences in both cell type-specific expression and subcellular localization of PKA isoforms and their adapters as well as substrates, possibly involving differential interactions with A-kinase anchoring proteins (AKAPs) (Michel and Scott, 2002). PKA-I is cytosolic but is recruited to the TCR-CD3 complex at the cell membrane upon T-cell activation (Skalhegg et al., 1994a,b), whereas PKA-II is membrane-associated (Tasken et al., 1997). The PKA pathway has been reported to affect T cell activation in a variety of ways, particularly through effects on the MAPK pathway (Beals et al., 1997; Ramstad et al., 2000). We have therefore examined further the role played by the cAMP-mediated signaling pathway in the PF-mediated enhancement of T-cell priming, and we report here that PKA-I–mediated signals are capable of rescuing T cells from apoptosis with a resultant enhancement in T-cell priming.

Materials and Methods

T-Cell Priming and Proliferation Assays. Human peripheral blood was obtained from consenting human leukocyte antigen-mismatched healthy donors under protocols supervised and approved by an Institutional Review Board. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient separation using heparinized blood on Ficoll-Paque (Pharmacia, Uppsala, Sweden). For primary allo-proliferative responses, γ-irradiated (30 Gy) stimulators were added in titrated numbers with or without graded doses of various pharmacological agents as appropriate to responder PBMCs (1×105 cells/well) in RPMI 1640 medium (Invitrogen, Carlsbad, CA) fortified with L-glutamine (Invitrogen), antibiotics (Hi-Media, Mumbai, India), and 10% heat inactivated (56°C, 30 min) responder autologous serum. Where indicated, anti-human leukocyte antigen-DR (L243) or anti-CD4 (OKT4) monoclonal antibodies (mAbs) used as culture supernatants (~1 μg/ml) were added to the cultures. Cultures were maintained at 37°C in 5% CO_2 atmosphere, for 5 to 6 days, pulsed with 0.5 μCi of [^{3}H]thymidine (NEN, Boston, MA) for the last 12 to 16 h of culture and were harvested onto glass fiber filters for scintillation counting (Betaplate; PerkinElmer Wallac, Turku, Finland). The results are expressed as mean cpm ± S.E. for triplicate cultures.

For allo-priming in vitro, responder cells were mixed with irradiated stimulator APCs in a ratio of 2:1 in the presence or absence of various agents as indicated and cultured for 5 days. Where indicated, stimulators were also fixed lightly with 0.003% paraformaldehyde. Viable cells were then harvested and used as responders in re-stimulation assays at a density of 3×10^4/well with titrated irradiated stimulator APCs, cultured for 72 h, and pulsed with [^{3}H]thymidine as above.

The various modulators used were: the AC activator forskolin (Fs) (Calbiochem, Darmstadt, Germany), the AC inhibitor SQ22536 (Sq) (Calbiochem), the PDE inhibitor PF, the PDE4-specific inhibitor rolipram (Rm), the PDE3-specific inhibitor trequinsin (Tq), and the cAMP analog dbcAMP (all from Sigma Chemical Co., St. Louis, MO); predominantly PKA-I inhibitors 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer (RpBrcAMPs; Calbiochem) and 8-chloroadenosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer (RpClcAMPs; Biolog Life Science Institute, Bremen, Germany), and the PKA-II–specific inhibitor 8-(4-chlorophenylthio)adenosine-3′,5′-cyclic monophosphorothioate, *R*p-isomer (RpCPTcAMPs; Biolog) (Gjertsen et al., 1995); the peptide inhibitor of PKA-II-AKAP interaction St.HT31 (pAKAP), and a corresponding inactive control peptide (pCon; Promega, Madison, WI), the MEK-1 inhibitor PD98059 (New England Biolabs, Beverly, MA); and the PDE7-specific phosphorothiolated anti-sense oligonucleotides described previously (Li et al., 1999) (Sigma Genosys, Pampisford, UK).

Analysis of Intracellular cAMP Levels from the Cells. Essentially, the manufacturer's instructions were followed for estimation of intracellular cAMP levels (Biotrak cellular communication assay; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) by enzyme immunoassay. In brief, responder cells were allo-primed in presence of various pharmacological agents for defined periods, and viable cells were separated by density gradient, washed, and lysed using buffer supplied with the kit. Based on the standard curve, amount of cAMP present in each sample in femtomoles per 10^5 cells was calculated.

Western Blot Analysis of NF-κB in Primed T Cells. CD4 T cells were isolated after 5 days of allo-priming in culture by labeling cells with biotinylated anti-CD4 mAb (BD Pharmingen, San Diego, CA) followed by streptavidin-coated immuno-magnetic beads and passage over a magnetic activated cell sorting separation column according to manufacturer's protocols (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of such populations was consistently >90%. Nuclear and cytoplasmic cell extracts were then prepared as described previously (Schreiber et al., 1989). All extracts were stored at −70°C until use. Nuclear or cytoplasmic extracts (4 μg protein/lane) were electrophoresed in SDS-containing 10% polyacrylamide gels along with molecular weight markers, transferred to nitrocellulose membranes, and the membranes probed for rel family proteins using polyclonal antibodies to c-rel and p65 (Santa Cruz Biotechnology, Santa Cruz, CA). Bound antibodies were detected by the enhanced chemiluminescence method following the manufacturer's instructions (Amersham Biosciences).

Activation-Induced T Cell Death in Vitro. PBMCs were stimulated in culture with the anti-CD3ε mAb, OKT3 (0.1 μg/ml), in the presence or absence of the various modulators indicated. After 48 h in culture, the cells were stained with anti-CD4–phycoerythrin, and annexin-V–fluorescein (BD Pharmingen) was used for detecting apoptotic CD4 T cells, whereas caspase induction in these cells was detected using a cell-permeable fluorescent caspase substrate, VAD-fluoromethylketone-fluorescein (VAD-fmk-Flu; Promega). Stained cell samples were subjected to two-color flow cytometric analysis on a BD LSR flow cytometer (BD Biosciences, San Jose, CA) or an Elite

ESP flow cytometer (Beckman Coulter, Fullerton, CA). Data were analyzed using FlowJo software (Treestar, San Carlos, CA).

Results

Activation of AC during T-Cell Priming Enhances Commitment to Secondary Responses, Whereas AC Blockade Prevents PF-Mediated Enhancement of T-Cell Priming. We have used primary allo-specific human T-cell responses and priming in vitro followed by enhanced secondary allo-specific responses as a model system in these experiments, as described previously (Satyaraj et al., 1994). Because AC generates intracellular cAMP, we used an AC activator, Fs, as well as an AC inhibitor, Sq, to examine the effects of endogenously synthesized cAMP on T-cell priming for secondary responsiveness. To ensure that any modulation observed was caused by direct effects on T cells, the irradiated allo-stimulator APCs were lightly fixed with paraformaldehyde. The priming of T cells by allo-stimulator PBMCs as APCs was effective as shown by enhanced recall responses compared with unprimed cells (Fig. 1A). When T cells were allo-primed in the presence of 10 μM Fs, there was an enhancement in the commitment to secondary responsiveness similar to that induced by 360 μM PF (Fig. 1A). In contrast, the presence of 100 μM Sq during priming had no effect on T cell commitment to secondary reactivity (Fig. 1B).

To examine the role of newly triggered cAMP synthesis in the PF-mediated enhancement of T-cell priming, we also examined the effect of Sq on PF-mediated modulation. The presence of the AC inhibitor led to blockade of the PF-mediated enhancement of secondary responsiveness (Fig. 1B), indicating that PF mediates its function through newly synthesized cAMP.

Curiously, the presence of either Fs or Sq during the primary allo-specific proliferative assay inhibited the primary response (Fig. 1, C and D), indicating lack of any correlation between the effects on primary proliferative responses on the one hand and the consequences for secondary responsiveness on the other.

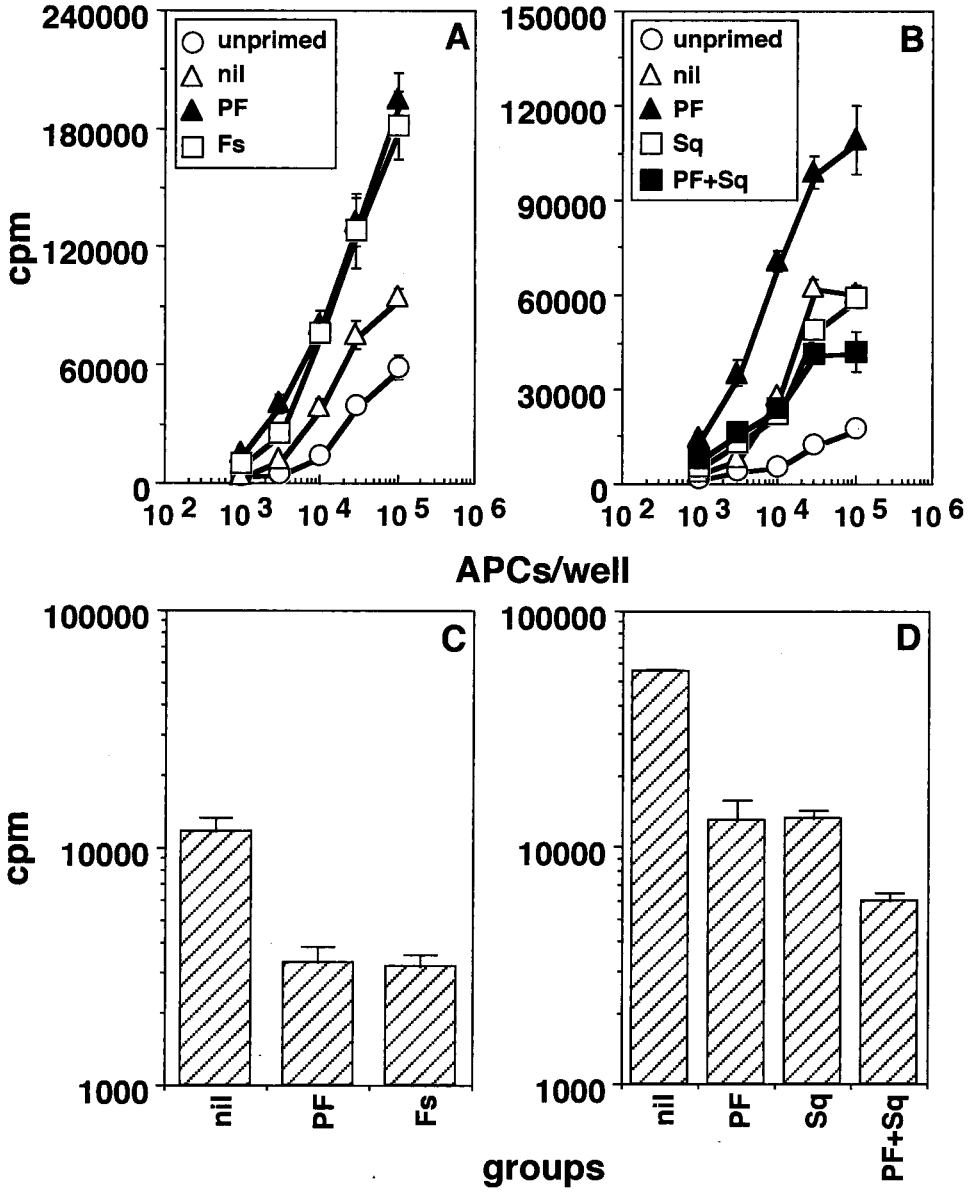

Fig. 1. Activation of AC during priming enhances secondary T cell responses, whereas inhibition of AC blocks PF-mediated enhancement. A and B, secondary proliferative responses of T cells allo-primed in the presence or absence of PF (360 μM), Fs (10 μM), Sq (100 μM), or both PF and Sq and then restimulated with titrated numbers of stimulator APCs. C and D, effects of the same agents on primary allo-proliferative responses. Data represent five independent experiments.

Inhibition of PDE4, but Not PDE3 or PDE7, During Priming of T Cells Enhances Their Commitment to Secondary Responses. The cAMP generated in cells is degraded by PDEs, and PDE3, PDE4, and PDE7 are among the prominent PDE isoforms found in T cells (Giembycz et al., 1996). We therefore tested the effects of specific inhibition of these isoforms during T-cell priming on the commitment to secondary responsiveness. Whereas PDE4 inhibition by 30 μM Rm during allo-priming increased the resultant commitment to secondary response (Fig. 2A), PDE3 inhibition by 10 μM Tq (Fig. 2B) or PDE7 inhibition by specific antisense oligonucleotides (Li et al., 1999) (Fig. 2C) showed no such enhancement of T-cell priming. Although both Rm and Tq inhibited primary proliferative responses (Fig. 2, D and E), the PDE7 antisense oligonucleotides did not (Fig. 2F). Even when lightly fixed APCs were used for priming, the presence of Rm led to enhancement of T-cell priming (Fig. 2G), indicating that the effect of Rm was directly on responding T cells rather than on APCs. The presence of either anti-MHC class II or anti-CD4 mAbs during the proliferative recall responses led to their inhibition (Fig. 2H), confirming that the proliferative allo-specific responses being measured were elicited from CD4 T cells.

Although the efficacy of the PDE4 inhibitor in enhancing secondary allo-responses is clearly evident, it is unclear from these data whether the PDE3 and PDE7 inhibitors used do not in fact enhance cAMP levels, or if enhancement of cAMP levels by these agents does not mediate any increase in secondary allo-responses. Therefore, levels of cAMP were measured at 24, 48, and 72 h in whole-cell extracts of responder T cells allo-primed in presence of various PDE inhibitors and AC regulators at the same respective concentrations used above. Although the unprimed population continued to show low cAMP levels, primed cells showed at least a 3- to 4-fold induction of cAMP. PF, Rm, Tq, the PDE7 antisense oligonucleotide, and Fs all caused further substantial increases (by 5- to 25-fold) in the cAMP levels at both 48 and 72 h. In the presence of Sq or PDE7 control oligonucleotides, the cAMP levels did not increase above those seen in the primed cells (Fig. 3).

Enhancement of T-Cell Priming by PF Is Mediated through cAMP-Dependent PKA-I. An increase in intracellular cAMP levels during T-cell priming resulted in enhanced commitment to secondary reactivity, when brought about by either AC activation or PDE4 inhibition. Because the signaling functions of cAMP are mostly mediated by PKA (Brindle et al., 1995), we next analyzed the effects of PKA agonism and antagonism in this system. Of the two major PKA-isoforms, RpBrcAMPs (30 μM) and RpClcAMPs (30 μM) are considered strong antagonists of PKA-I activity

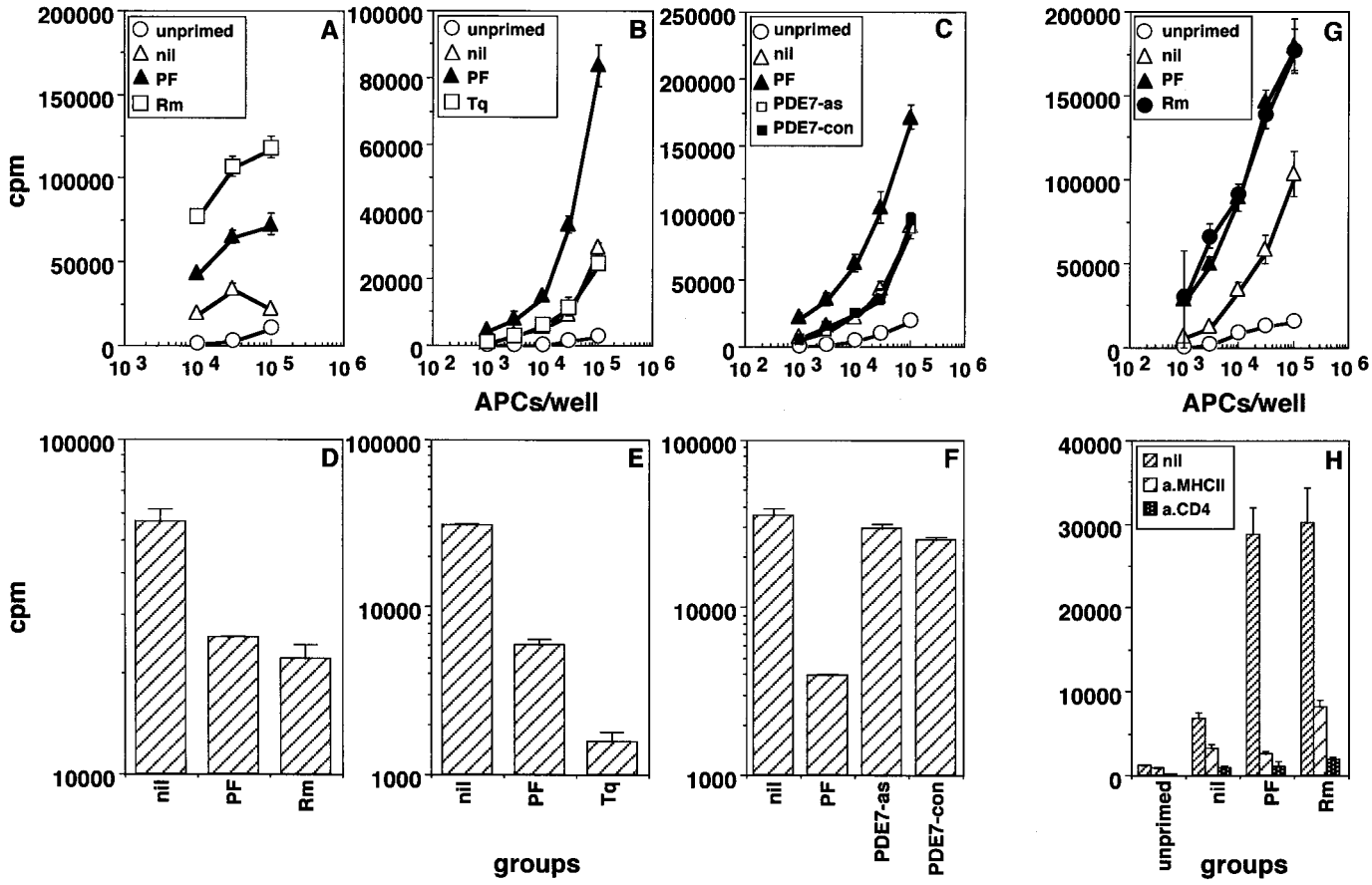

Fig. 2. PDE4 inhibition during priming enhances secondary CD4 T cell responses. A, B, and C show secondary proliferative responses of T cells allo-primed in presence or absence of PF (360 μM), Rm (30 μM), Tq (10 μM), PDE7-antisense oligonucleotide (PDE7-as; 10 μM), or PDE7-control oligonucleotide (PDE7-con; 10 μM) and then restimulated with titrated numbers of stimulator APCs. D, E, and F, primary allo-responses for the same agents. G, secondary proliferative responses of T cells allo-primed in presence or absence of PF (360 μM) or Rm (30 μM) with fixed APCs and then restimulated with titrated numbers of stimulator APCs. H, the secondary proliferative allo-responses elicited in the presence or absence of anti-MHC class II (a.MHCII) or anti-CD4 (a.CD4) mAbs. The data are representative of three to nine separate experiments.

(Gjertsen et al., 1995). The presence of either of these during allo-priming did not alter the efficiency of T cell commitment to secondary reactivity (Fig. 4, A and B). However, their presence along with PF (360 μM) during priming reproducibly blocked the PF-mediated enhancement of secondary response capability (Fig. 4, A and B), indicating that the effect of PF on T-cell priming was mediated through cAMP-dependent PKA. Neither RpBrcAMPs nor RpClcAMPs has any major effect on the primary allo-specific T cell proliferative response on their own at the concentrations used (Fig. 4, D and E).

This observation was also confirmed by using the PDE4-specific inhibitor Rm. When Rm was used during allo-priming, it enhanced secondary allo-responses, but the presence of RpBrcAMPs along with Rm during priming led to block of the Rm effect (Fig. 4C).

PKA-II May Not Play a Significant Role in Enhancing Secondary T-Cell Allo-Responses. We next attempted to analyze whether PKA-II has any role to play in secondary response enhancement brought about by inhibition of PDE4. RpCPTcAMPs is a potent but partial antagonist of PKA-II (Gjertsen et al., 1995). It had no effect on either T-cell priming on its own or in presence of PF (Fig. 5A). We also used peptides that inhibit recruitment of PKA-II to AKAP. Various isoforms of AKAP bind to various regulatory domains of PKA with differing affinities (Herberg et al., 2000), thereby facilitating subcellular localization of PKA function. St.HT31 (pAKAP) was used as an inhibitor of PKA-II-AKAP interaction (Vijayaraghavan et al., 1996) in the T-cell priming assays. Allo-priming in the presence of pAKAP alone did not alter commitment to secondary responses, and pAKAP did not affect the enhancement in T-cell priming brought about by PF (Fig. 5B). The presence of pCon during priming, either alone or with PF, also had no effect on secondary response commitment (Fig. 5B). Unlike PKA-I–specific inhibitors, primary proliferative responses were inhibited by both RpCPT-cAMPs (Fig. 5C) and pAKAP (Fig. 5D), whereas pCon showed no effect. Together, these data indicated that the enhancement of T cell commitment to secondary responses brought about by PF was likely to be mediated primarily through PKA-I.

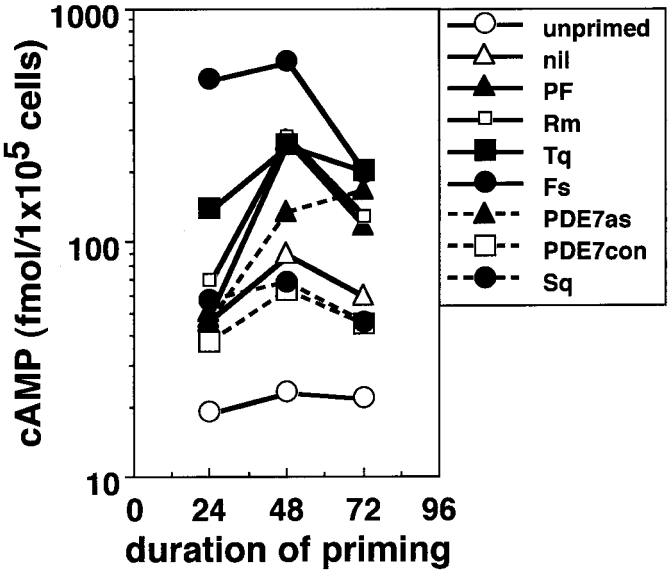

Fig. 3. Intracellular levels of cAMP generated by PDE3, PDE4, and PDE7 inhibitors are not different. Amount of cAMP generated (femtomoles per 10^5 cells) is shown over 24 to 72 h after the initiation of allo-priming in the absence of any drug or in presence of PF (360 μM), Rm (30 μM), Tq (10 μM), PDE7-antisense oligonucleotide (PDE7-as; 10 μM), PDE7-control oligonucleotide (PDE7-con; 10 μM), Fs (10 μM), or Sq (100 μM). Data represent two separate experiments.

Enhancement of T Cell Priming by PF Is not Mediated through Effects on MEK-1 or NF-κB. Kinases of the MAPK cascade, activated by the MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK), are major signaling mediators downstream of cAMP/PKA in many cell types, although the precise effect of cAMP and PKA on MAPK/ERK activation may differ (Busca et al., 2000; Ramstad et al., 2000). We therefore examined whether MEK-1 modulation could modify the PF-mediated enhancement of T-cell priming using a MEK-1 inhibitor, PD98059. The presence of PD98059 during T cell allo-priming, either alone or with PF, did not modify the subsequent secondary T cell responsiveness (Fig. 6A), although PD98059 was capable of inhibiting the primary proliferative allo-response on its own (Fig. 6B), establishing its functionality. These data indicated that the effect of PF on T-cell priming did not involve MEK-1.

The transcriptional regulator c-rel has been shown to be crucial for T cell proliferation (Strasser et al., 1999; Liou et al., 1999), and we have shown previously that PF specifically blocks the induction of c-rel, but not p65, during T-cell activation (Wang et al., 1997). It was therefore possible that the effect of PF via cAMP and PKA on T-cell priming was mediated through blockade of c-rel. To examine this possibility, we tested the effect of PKA inhibition on PF-mediated blockade of c-rel induction. PBMCs were primed in the presence or absence of PF for 5 days, after which CD4 T cells were purified with the use of magnetic activated cell sorting, and nuclear and cytoplasmic extracts were subjected to Western blot analysis using polyclonal anti-c-rel and anti-p65 antibodies. Nuclear extracts from unprimed CD4 cells showed negligible presence of c-rel and p65. The nuclear levels of both c-rel and p65 were markedly enhanced in primed cells (Fig. 7). In cells primed in the presence of PF, induction of c-rel was blocked, whereas p65 up-regulation was less affected (Fig. 7). However, addition of the PKA inhibitor Rp-BrcAMPs along with PF made no difference to PF-mediated blockade of c-rel induction and it also down-modulated p65 induction (Fig. 7). Thus, blockade of c-rel induction is unlikely to be a crucial feature by itself for PF-mediated enhancement of T-cell priming.

PF-Mediated Signal Modulation Is Required Only Late during T Cell Priming for Enhancement. Whereas PF functions via cAMP and PKA to enhance T-cell priming, cAMP-PKA-mediated signals do not normally seem to regulate T cell memory commitment, because inhibition of AC or PKA during T-cell priming does not affect secondary responsiveness in the absence of PF (Figs. 1 and 4). Because it has been shown that the cAMP-PKA–mediated signaling pathway is triggered early during T cell activation (Kammer et al., 1988; Laxminarayana et al., 1993), it was possible that the PF-cAMP-PKA–mediated effect on T-cell priming was caused by modulation of late events in T cell activation. We tested this possibility by adding PF to T-cell priming cultures during restricted time periods. During the 96-h period of T-cell priming in vitro, presence of PF during the first 48 h had no effect on the magnitude of recall responses, whereas

addition of PF late during priming over the 48- to 96-h period showed enhancement of priming equivalent to that seen if PF was present all through the priming period (Fig. 8A). The presence of Rm and dbcAMP during this 48- to 96-h window also enhanced T-cell priming (Fig. 8B).

Because the 48- to 96-h period seems to be crucial to achieve enhanced secondary responses, it was important to determine whether the lack of an effect on allo-priming by the inhibitors of PDE3, PKA-II, or MEK-1 was not a result of their failure to persist in culture until late time points. Tq induces enhanced cAMP levels even at 72 h of culture (Fig. 3), ruling out this possibility. When RpCPTcAMPs (with or without Rm) or the MEK-1 inhibitor was added to the cultures during the 48- to 96-h time window, there was no effect on secondary responsiveness (Fig. 8C). The outcome of secondary response when pAKAP was added at 48 h was also similar to that seen when it was added at 0 h (data not shown), thus further supporting the finding that PDE4–cAMP–PKA-I signaling extends an early event or modulates a late event in T-cell priming to enhance secondary responsiveness and is unlikely to involve MEK1.

PDE4 Inhibition Prevents Apoptosis of Activated T Cells. We have shown previously that death of allo-primed T cells in culture is inhibited by PF (Gupta et al., 1999). It has been reported that activated primary T cells are not susceptible to apoptotic cell death at early time points (Russell et al., 1991). It was therefore possible that the late event in T cell activation affected by PF to enhance T cell memory involved apoptosis. We tested the effect of PF on T cell apoptosis directly by triggering activation-induced cell death

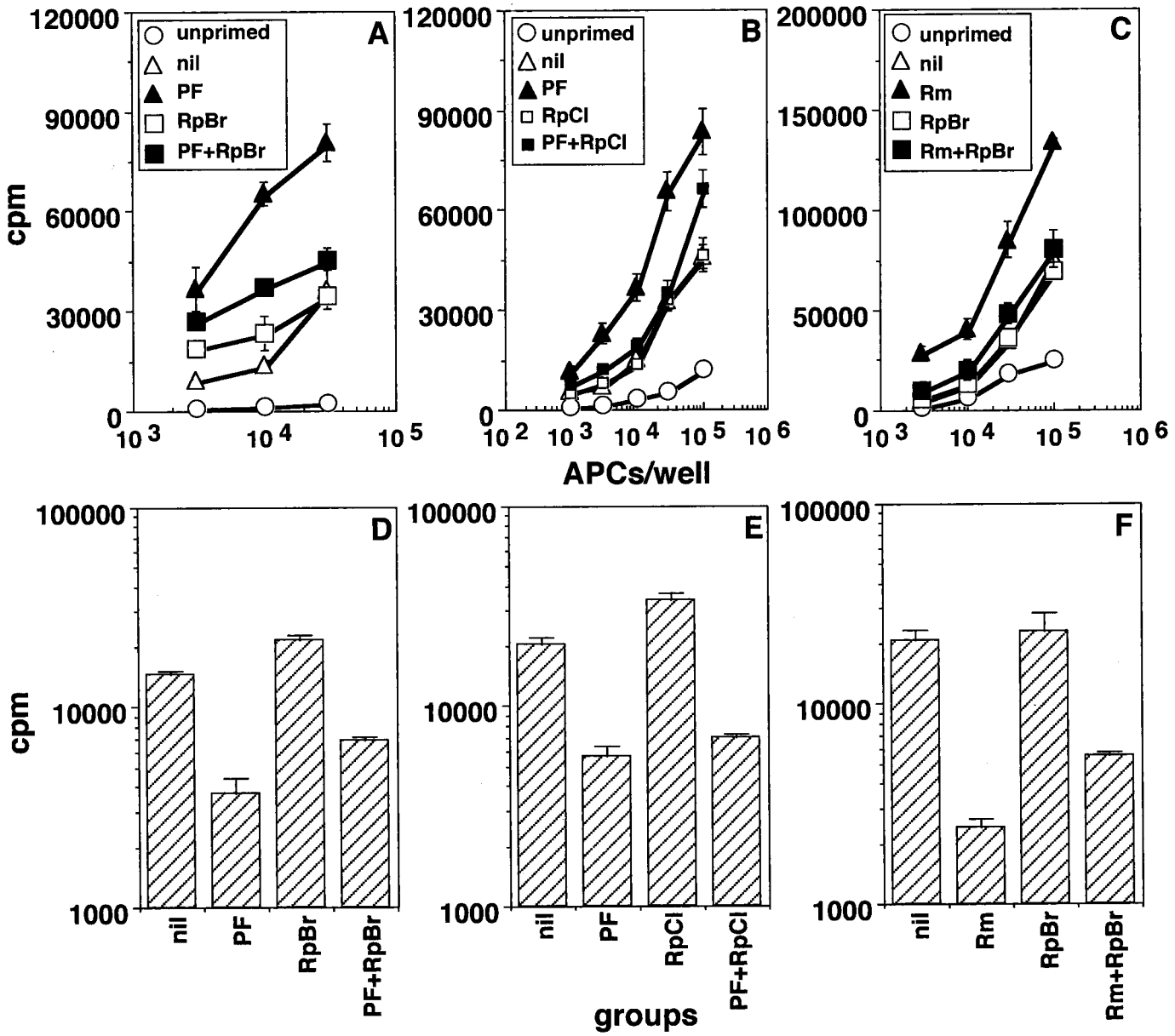

Fig. 4. PDE4-mediated enhancement of T cell commitment to secondary responses is mediated through PKA. A–C, secondary proliferative responses of allo-primed T cells. T cells were allo-primed in presence or absence of PF (360 μM) (A, B), Rm (30 μM) (C), RpBrcAMPs (RpBr; 30 μM) (A, C), RpClcAMPs (RpCl; 30 μM) (B), PF and RpBrcAMPs (A), PF and RpClcAMPs (B), or Rm and RpBrcAMPs (C) and then restimulated with titrated numbers of stimulator APCs. D–F, effects of the same agents on primary allo-proliferative responses. The data are representative of 3 to 5 separate experiments.

(AICD) in T cells with anti-CD3 mAb, and examining the effect of PF on this event. At 48 h after stimulation, anti-CD3–triggered T cells showed induction of caspases, the cysteine aspartyl proteases thought to play a central role in many pathways of apoptosis (Fig. 9A), as well as membrane changes characteristic of apoptosis as indicated by binding of annexin-V (Fig. 9B). The presence of PF substantially inhibited the induction of caspase activation as well as apoptosis (Fig. 9, A and B). However, the simultaneous presence of RpBrcAMPs with PF in culture significantly reversed the antiapoptotic effect of PF (Fig. 9B), confirming that the effects of PF in this instance were also mediated through PKA-I. Similarly, the presence of Rm also prevented anti-CD3–triggered T cell apoptosis (Fig. 9C). Thus, PDE4 inhibition prevented the induction of both caspases and apoptosis (Fig. 9), suggesting that apoptosis is likely to be the event affected by PF during T cell activation for enhancing secondary response commitment.

Discussion

Priming human CD4 T cells with γ-irradiated and lightly fixed allo-PBMCs results in the enhanced secondary proliferative responses characteristic of T cell memory. We have used this system previously to examine the signals involved during T-cell priming in the regulation of the commitment to secondary responsiveness (Satyaraj et al., 1994). We have demonstrated previously that the presence of PF or dbcAMP during priming leads to enhanced secondary T cell response capabilities by decreasing T cell AICD and increasing primed

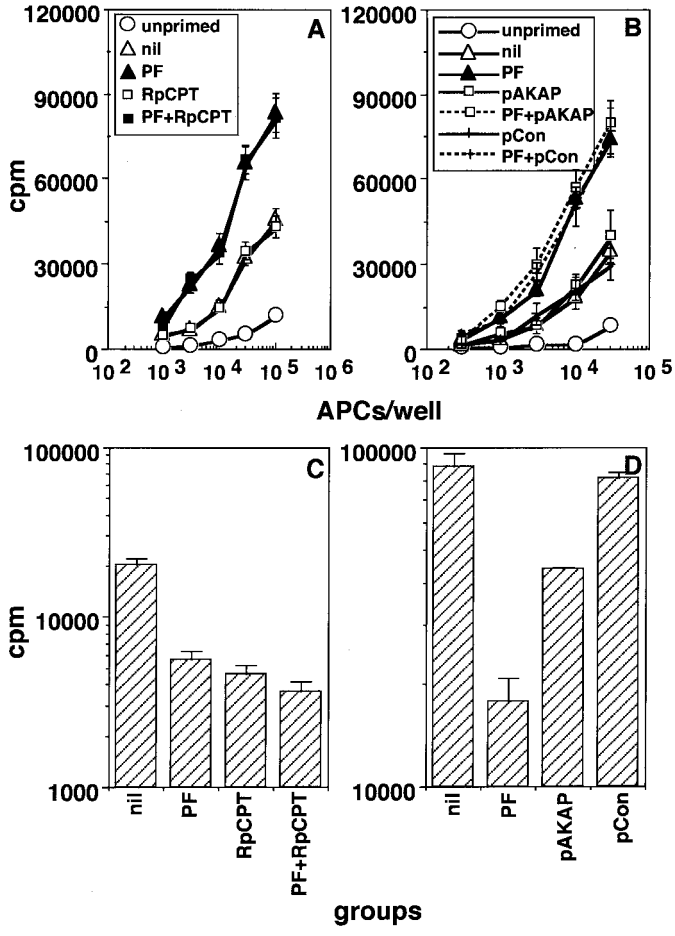

Fig. 5. PF-mediated enhancement of T cell commitment to secondary responses is not mediated through PKA-II. A and B, secondary proliferative responses of T cells allo-primed in presence or absence of PF (360 μM), RpCPTcAMPs (RpCPT; 30 μM), PF and RpCPTcAMPs, pAKAP (30 μM), PF and pAKAP, pCon (30 μM), or PF and pCon and then restimulated with titrated numbers of stimulator APCs. C and D, effects of the same agents on primary allo-proliferative responses. The data are representative of 3 to 5 separate experiments.

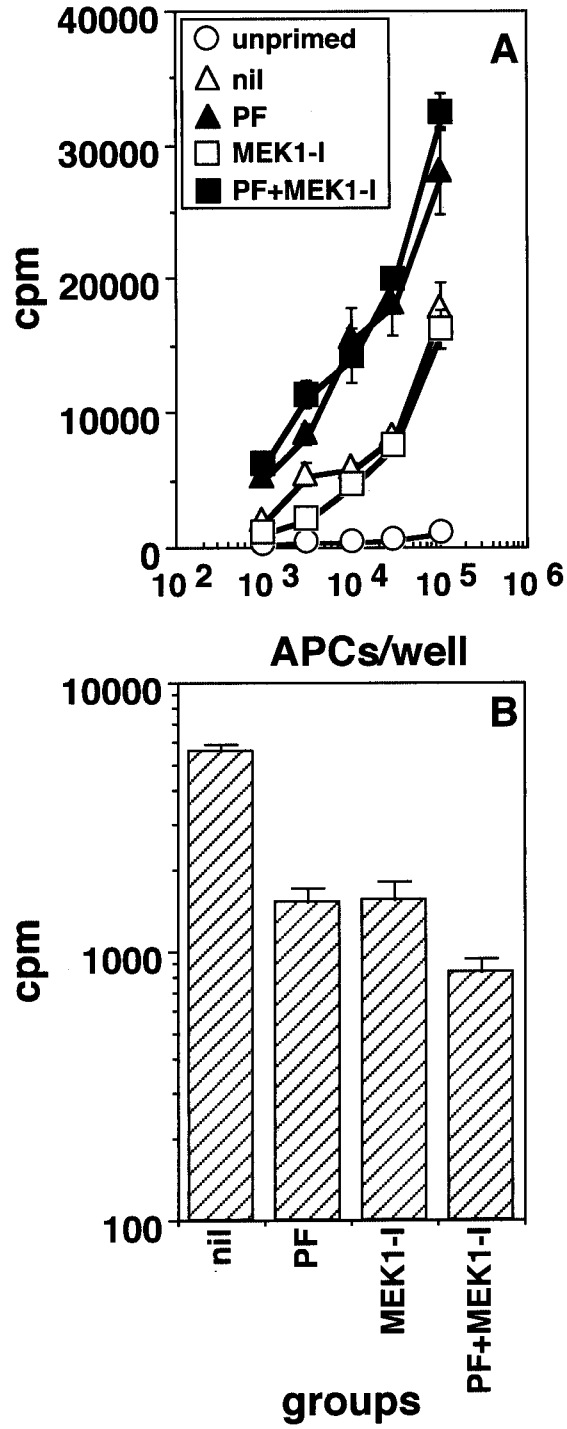

Fig. 6. Inhibition of MEK-1 does not affect PF-mediated enhancement of T-cell priming. A, secondary proliferative responses of T cells allo-primed in presence or absence of PF (360 μM), PD98059 (MEK-1-I; 10 μM), or PF and PD98059 and then restimulated with titrated numbers of stimulator APCs. B, effects of the same agents on primary allo-proliferative responses. The data represent three separate experiments.

responder T cell frequency (Gupta et al., 1997, 1999). We now show that accumulation of newly synthesized cAMP in T cells beyond 48 h of T-cell priming allows PKA-I–mediated signaling that enhances T-cell priming for secondary responsiveness. This signal is independent of NF-κB, and probably functions by inhibiting T cell AICD.

Acquisition of the ability to respond more strongly in a secondary recall response is fundamental in the generation of T cell memory during the initial antigenic priming. The assay system we have used here, which allows us to prime allo-specific T cells in vitro and then recall their response to estimate the efficiency of commitment during priming to secondary responsiveness, is therefore relevant to understanding T cell memory. Measurement of interferon-γ, a secondary cytokine used as an additional indicator of primed secondary T cell functionality, also shows that T cells primed in presence of PF or Rm secrete higher levels of interferon-γ than cells primed in the absence of PF/Rm, commensurate with higher proliferative response observed (data not shown). We have tested the applicability of the conclusions of these experiments in vivo in mice and have observed that transient treatment with PF, dbcAMP and Rm during immunization does in fact enhance the magnitude and persistence of the T cell recall response (Suresh et al., 2002). The present set of data address the signals involved in PF-mediated enhancement of T-cell priming of human T cells in vitro.

All the effects of PF seen in this system are directly on T cells, because PF enhances T-cell priming even when paraformaldehyde-fixed stimulator APCs are used. While PF is a known PDE inhibitor, it was necessary to demonstrate that it is indeed that property of PF which is responsible for its effects on T-cell priming. While we have shown that enhancing cAMP levels by PF-independent means, either with db-cAMP (Gupta et al., 1999) or by activation of AC, mimics the effects of PF on T-cell priming, these data by themselves did not implicate cAMP-mediated signals in the effect of PF on T-cell priming. However, preventing either the generation of cAMP by an AC inhibitor, or the signaling via cAMP by inhibiting cAMP-dependent PKA, blocks the effects of PF. Together with the demonstration that the effects of PF can be mimicked by the PDE4-specific inhibitor Rm, these data establish that the effects of PDE4 inhibition on T-cell priming are mediated through cAMP and PKA.

Most cAMP-mediated effects in cellular signaling involve PKA, although in some instances PKA-independent signals have also been reported (Bryce et al., 1999). Multiple isoforms exist for both the catalytic and regulatory subunits of PKA, and they differentially localize to specific intracellular sites. The regulatory subunits R-I are predominantly cytosolic, while the R-II subunits are essentially found in the particulate fraction due to their interaction with the members of the AKAP family of proteins (Tasken et al., 1997). Our data show that signals primarily mediated via PKA-I are responsible for the effects of PF and Rm on T-cell priming. PKA-I has been shown to be recruited to the immunological synapse during T cell activation (Vang et al., 2001), and our data provide further evidence of its potential to modulate TCR-mediated signaling.

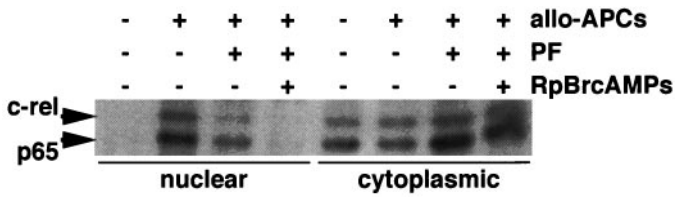

Fig. 7. PF-mediated blockade of c-rel induction is not reversed by PKA inhibition. Western blot analysis of c-rel and p65 levels in nuclear and cytoplasmic extracts of CD4 T cells, either unprimed or allo-primed in the presence or absence of either PF (360 μM) or PF and RpBrcAMPs (RpBr; 30 μM) is shown. Data represent two independent experiments.

However, PKA-II inhibition, either by an antagonist or by an AKAP inhibitor, does not have any effect on the secondary response capability induced in the allo-specific T cells, despite causing an inhibition of the primary proliferative response, an effect possibly due to a specific role of PKA-II in cell cycling (Carlson et al., 2001). It is noteworthy that, while PF and Rm do cause an inhibition of primary proliferative responses, this inhibition is not reversed by PKA-I inhibition, while their enhancing effect on secondary T cell responses as well as their inhibitory effect on T cell death are PKA-I-dependent. Similarly, PDE3, PDE7 and MEK-1 inhibition lead to blockade of primary proliferative responses, but do not enhance secondary reactivity, indicating that inhibition of primary T cell proliferation by many of these agents may be PKA-independent at least in part, and is clearly irrelevant to their effects on T cell survival and memory

With regard to the signaling intermediates involved downstream of PKA-I in the PDE4-mediated effect, we have only negative evidence to offer. While activation of the MAPK/ERK pathway has been shown to be either positively or negatively regulated by cAMP-PKA-mediated signaling, depending on cell type and mode of stimulation (Ramstad et al., 2000; Busca et al., 2000), MEK-1 inhibition does not modulate the effect of PF or Rm, suggesting that MEK-1 may not be involved in PKA-mediated signaling for enhancement of T-cell priming.

PF inhibits all PDE isoform families. However, when isoform-specific PDE inhibitors are used, it is evident that inhibiting PDE3 or PDE7 does not bring about any enhancement of T-cell priming. On the other hand, inhibiting PDE4 alone with Rm is sufficient to mimic the effects of PF. Thus, there is an isoform specificity to the effect of PDE inhibition on T-cell priming. Both PDE3 and PDE7 are present in T cells (Glavas et al., 2001), and in fact, PDE7 is induced within hours in T cells by TCR engagement (Li et al., 1999). Despite this, inhibiting PDE3 or PDE7 does not bring about enhancement of T-cell priming. One possibility was that the amount of cAMP accumulated in presence of isoform-specific inhibitors could be different, which is not the case. Another possibility is that PDE isoforms are located in specific subcellular regions, and that only PDE4 is located in the right region to mediate the effect seen here on priming. Presence of differing levels of cAMP has been demonstrated in subcellular microdomains in cardiac myocytes supporting this notion (Zaccolo and Pozzan, 2002), although there are no reports on such differential localization of PDEs in T cells. We are attempting to address the issue of localized effects of PDE4 in this context. Independently, it has been shown that PDE4 is induced in a durable fashion in human memory T cells (Sun et al., 2000).

There are many indications that timing may be critical in the effect of PF on T-cell priming. Neither AC inhibition nor PKA inhibition can by themselves modify commitment to secondary responsiveness during priming in the absence of

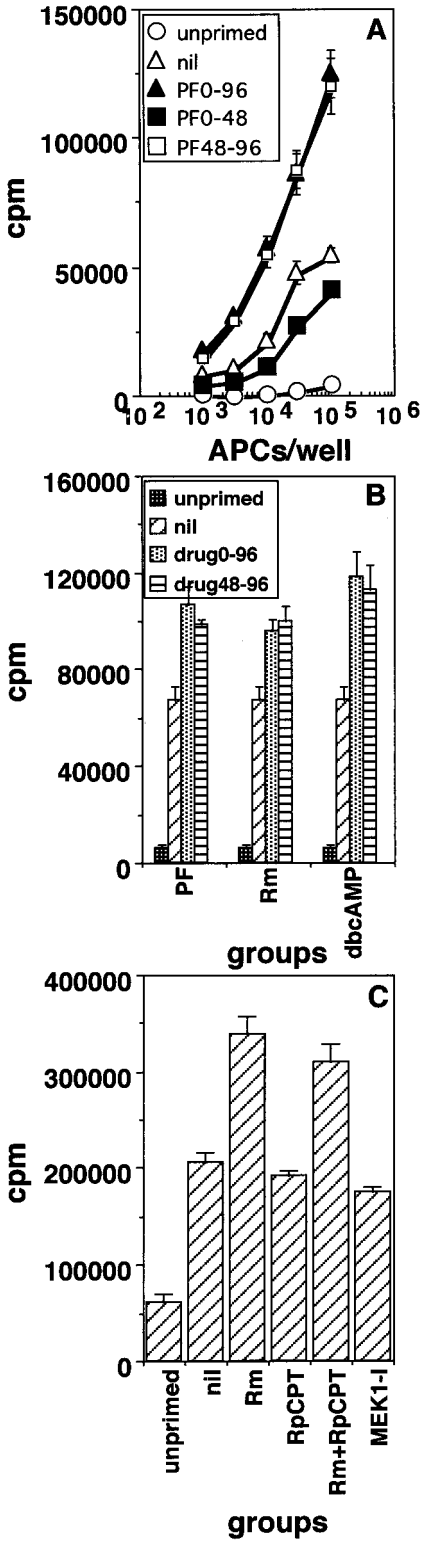

Fig. 8. PF-mediated enhancement of commitment to secondary response is a late event during priming. A, secondary proliferative responses of T cells allo-primed in presence or absence of PF (360 μM) either throughout the 4-day priming period (PF0–96), or only during the first 48 h (PF0–48), or only during the last 48 h (PF48–96) and then restimulated with titrated numbers of stimulator APCs. B, secondary proliferative allo-specific responses when PF (360 μM), Rm (30 μM), or dbcAMP (100 μM) were present during priming only during the last 48 h. C, secondary allo-proliferative responses when Rm, RpCPTcAMP (30 μM), Rm + RpCPTcAMP, MEK1-inhibitor (10 μM) are added at 48 h during priming. The data represent two to five separate experiments.

PF. Furthermore, PF is ineffective if available only at early time points during T-cell priming. It is required to be present late in priming if enhancement of secondary responses is to be achieved. It is therefore possible that, normally, AC-mediated induction of cAMP during T cell activation is also accompanied by an induction of PDE4, leading to rapid loss of

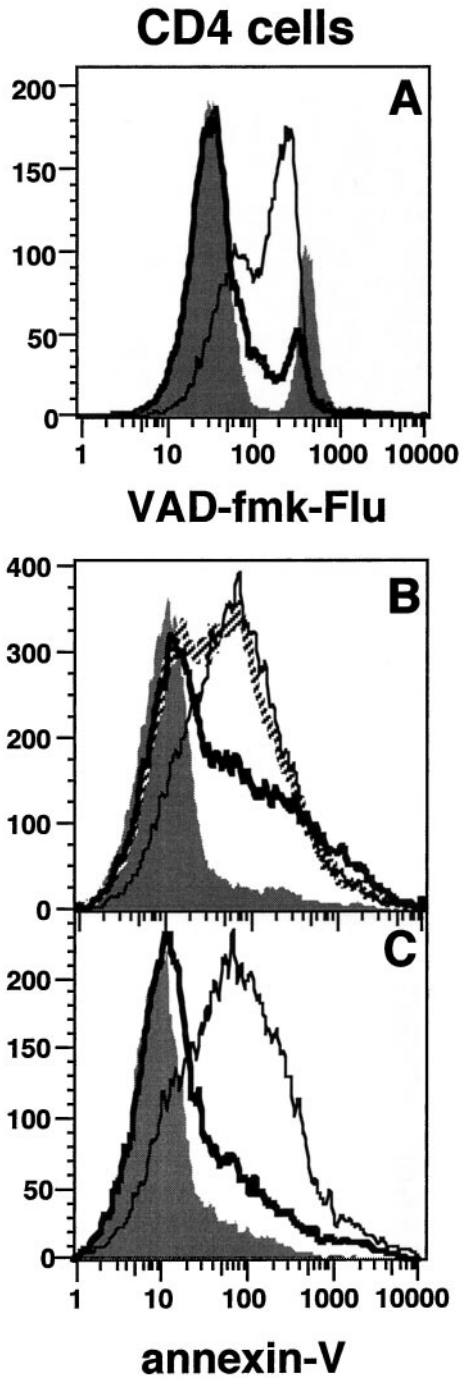

Fig. 9. Blocking PDE4 inhibits anti-CD3–mediated caspase induction and apoptosis in CD4 T cells. PBMCs were activated in vitro with either no stimulus (shaded curves), or with anti-CD3 in the presence (thick lines) or absence (thin lines) of PF (360 μM) (A, B), Rm (30 μM) (C), or PF + RpBrcAMPs (30 μM, broken line) (B) for 48 h. Flow cytometric analysis was done after staining for CD4 versus either VAD-fmk-Flu (A) or annexin-V (B, C). The VAD-fmk-Flu (A) and annexin-V (B, C) profiles of the gated CD4 cells are shown as single-color histograms. The data represent three to five independent experiments.

cAMP. Prolongation of the availability of cAMP late into T cell activation may allow PKA-I-mediated signals to be delivered to modulate late activation events in pharmacological fashion, causing enhancement of secondary responsiveness. Also, we have shown previously that a TCR-mediated signal is required for PF-mediated enhancement in T-cell priming to be seen (Gupta et al., 1997; Gupta et al., 1999). It is therefore probable that some late event triggered by TCR engagement is modulated by PF/Rm via cAMP and PKA to enhance T-cell priming.

What could be the identity of the event through which PF can regulate T-cell priming? We have shown earlier that PF enhances the survival of activated allo-specific T cells in these priming cultures (Gupta et al., 1999), and AICD is a relatively late event during T cell activation. PF and Rm clearly block the induction of AICD in T cells, and this is accompanied by inhibition of the induction of caspases. Apoptosis is a late event in T cell activation and may not normally be affected by cAMP-mediated signaling due to cAMP clearance by PDE4, and prolonged availability of cAMP may lead to PKA-I-mediated inhibition of apoptosis (Kim et al., 2001), resulting in enhanced T cell survival, increased secondary T cell frequencies and improved commitment to T cell memory.

The rel family of proteins comprising the NF-κB group are intimately involved in cell death pathways in a variety of systems as both positive and negative regulators (Chen et al., 2000; Manna et al., 2000), and we have previously shown that the activation of c-rel during T cell stimulation, but not that of p65, is blocked by PF (Wang et al., 1997). However, we find that the inhibition of c-rel induction by PF is PKA-independent, whereas the enhancement of T-cell priming by PF is PKA-dependent. It is therefore unlikely that the blockade of c-rel induction by PF is involved by itself in the rescue of responding T cells from apoptosis. There are numerous possible death mechanisms involved, and we are attempting to identify the crucial pathways so that we can dissect their intersection with PKA-I–mediated signals.

Thus, our data indicate that during T cell activation, AC-mediated cAMP induction takes place and is limited by the PDEs. Inhibition of PDE4 leads to cAMP accumulation that triggers PKA-I at relatively late time points to inhibit activation-mediated T cell death, resulting in increased secondary T cell survival, thus yielding a greater magnitude of secondary T cell responses. Because the effect is PDE4-specific despite the ability of both PDE3 and PDE7 inhibitors to increase cAMP levels, it is possible that subcellular localization of cAMP-PKA signals may be crucial for mediating this effect.

References

Ahmed R and Gray D (1996) Immunological memory and protective immunity: understanding their relation. *Science (Wash DC)* **272:**54–60.

Beals CR, Sheridan CM, Turck CW, Gardner P, and Crabtree GR (1997) Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science (Wash DC)* **275:**1930–1934.

Beavo JA (1995) Cyclic nucleotide phosphodiesterases: functional implications of multiple isoforms. *Physiol Rev* **75:**725–748.

Bernard A, Lamy And L, and Alberti I (2002) The two-signal model of T-cell activation after 30 years. *Transplantation* **73(1 Suppl):**S31–S35.

Brindle P, Nakajima T, and Montminy M (1995) Multiple protein kinase A-regulated events are required for transcriptional induction by cAMP. *Proc Natl Acad Sci USA* **92:**10521–10525.

Bryce PJ, Dascombe MJ, and Hutchinson IV (1999) Immunomodulatory effects of pharmacological elevation of cyclic AMP in T lymphocytes proceed via a protein kinase A independent mechanism. *Immunopharmacology* **41:**139–146.

Busca R, Abbe P, Mantoux F, Aberdam E, Peyssonnaux C, Eychene A, Ortonne JP, and Ballotti R (2000) Ras mediates the cAMP-dependent activation of extracellular signal-regulated (ERKs) in melanocytes. *EMBO (Eur Mol Biol Organ) J* **19:** 2900–2910.

Carlson CR, Witczak O, Vossebein L, Labbe JC, Skalhegg BS, Keryer G, Herberg FW, Collas P, and Tasken K (2001) CDK1-mediated phosphorylation of the RIIalpha regulatory subunit of PKA works as a molecular switch that promotes dissociation of RIIalpha from centrosomes at mitosis. *J Cell Sci* **114:**3243–3254.

Chen C, Edelstein LC, and Gelinas C (2000) The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). *Mol Cell Biol* **20:**2687–2695.

Giembycz MA, Corrigan CJ, Seybold J, Newton R, and Barnes PJ (1996) Identification of cyclic AMP phosphodiesterases 3, 4 and 7 in human CD4+ and CD8+ T-lymphocytes: role in regulating proliferation and the biosynthesis of interleukin-2. *Br J Pharmacol* **118:**1945–1958.

Gjertsen BT, Mellgren G, Otten A, Maronde E, Genieser HG, Jastorff B, Vintermyr OK, McKnight GS, and Doskeland SO (1995) Novel (Rp)-cAMPS analogs as tools for inhibition of cAMP-kinase in cell culture. Basal cAMP-kinase activity modulates interleukin-1 beta action. *J Biol Chem* **270:**20599–607.

Glavas NA, Ostenson C, Schaefer JB, Vasta V, and Beavo JA (2001) T cell activation up-regulates cyclic nucleotide phosphodiesterases 8A1 and 7A3. *Proc Natl Acad Sci USA* **98:**6319–6324.

Gupta M, Satyaraj E, Durdik JM, Rath S, and Bal V (1997) Differential regulation of T cell activation for primary versus secondary proliferative responses. *J Immunol* **158:**4113–4121.

Gupta M, George A, Sen R, Rath S, Durdik JM, and Bal V (1999) Presence of pentoxifylline during T cell priming increases clonal frequencies in secondary proliferative responses and inhibits apoptosis. *J Immunol* **162:**689–695.

Herberg FW, Maleszka A, Eide T, Vossebein L, and Tasken K (2000) Analysis of A-kinase anchoring protein (AKAP) interaction with protein kinase A (PKA) regulatory subunits: PKA isoform specificity in AKAP binding. *J Mol Biol* **298:** 329–339.

Kammer GM (1998) The adenylate cyclase-cAMP-protein kinase A pathway and regulation of the immune response. *Immunol Today* **9:**222–229.

Kammer GM, Boehm CA, Rudolph SA, and Schultz LA (1988) Mobility of human T lymphocyte surface molecules CD3, CD4 and CD8: regulation by a cAMP-dependent pathway. *Proc Natl Acad Sci USA* **85:**792–796.

Kim SN, Ahn YH, Kim SG, Park SD, Cho-Chung YS, and Hong SH (2001) 8-Cl-cAMP induces cell cycle-specific apoptosis in human cancer cells. *Int J Cancer* **93:**33–41.

Laxminarayana D, Berrada A, and Kammer GM (1993) Early events of human T lymphocyte activation are associated with type I protein kinase A activity. *J Clin Invest* **92:**2207–2214.

Li L, Yee C, and Beavo JA (1999) CD3- and CD28-dependent induction of PDE7 required for T cell activation. *Science (Wash DC)* **283:**848–851.

Liou HC, Zhuang J, Tumang J, Andjelic S, Smith KA, and Liou ML (1999) c-Rel is crucial for lymphocyte proliferation but dispensable for T cell effector function. *Int Immunol* **11:**361–371.

MacKenzie WM, Hoskin DW, and Blay J (1999) The adhesion of anti-CD3-activated mouse T cells to syngeneic colon adenocarcinoma cells is differentially regulated by protein tyrosine kinase-, protein kinase C- and cAMP-dependent pathways in the effector cell. *Biochem Biophys Res Commun* **255:**460–465.

Manna SK, Haridas V, and Aggarwal BB (2000) Bcl-x(L) suppresses TNF-mediated apoptosis and activation of nuclear factor-kappaB, activation protein-1 and c-Jun N-terminal kinase. *J Interferon Cytokine Res* **20:**725–735.

McHeyzer-Williams MG, and Davis MM (1995) Antigen-specific development of primary and memory T cells *in vivo*. *Science (Wash DC)* **268:**106–111.

Michel JJ and Scott JD (2002) Akap mediated signal transduction. *Annu Rev Pharmacol Toxicol* **42:**235–257.

Ramstad C, Sundvold V, Johansen HK, and Lea T (2000) cAMP-dependent protein kinase (PKA) inhibits T cell activation by phosphorylating ser-43 of raf-1 in the MAPK/ERK pathway. *Cell Signal* **12:**557–563.

Russell JH, White CL, Loh DY, and Meleedy-Rey P (1991) Receptor-stimulated death pathway is opened by antigen in mature T cells. *Proc Natl Acad Sci USA* **88:**2151–2155.

Satyaraj E, Rath S, and Bal V (1994) Induction of tolerance in freshly isolated alloreactive CD4+ T Cells by activated T cell stimulators. *Eur J Immunol* **24:** 2457–2461.

Schreiber E, Matthias P, Muller MM, and Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17:**6419.

Skalhegg BS, Rasmussen AM, Tasken K, Hansson V, Jahnsen T, and Lea T (1994a) Cyclic AMP sensitive signalling by the CD28 marker requires concomitant stimulation by the T-cell antigen receptor (TCR/CD3) complex. *Scand J Immunol* **40:**201–208.

Skalhegg BS, Tasken K, Hansson V, Huitfeldt HS, Jahnsen T, and Lea T (1994b) Location of cAMP-dependent protein kinase type I with the TCR-CD3 complex. *Science (Wash DC)* **263:**84–87.

Skalhegg BS and Tasken K (2000) Specificity in the cAMP/PKA signaling pathway. Differential expression, regulation and subcellular localization of subunits of PKA. *Front Biosci* **5:**D678–D693.

Strasser A, Grumont RJ, Stanley ML, and Gerondakis S (1999) The transcriptional regulator Rel is essential for antigen receptor-mediated stimulation of mature T cells but dispensable for positive and negative selection of thymocytes and T cell apoptosis. *Eur J Immunol* **29:**928–935.

Suresh R, Vig M, Bhatia S, Goodspeed EPB, John B, Kandpal U, Srivastava S, George A, Sen R, Bal R, et al. (2002) Pentoxyphylline functions as an adjuvant in vivo to enhance T cell immune responses by inhibiting activation-induced cell death. *J Immunol* **169:**4262–4272.

Swain SL (1994) Generation and *in vivo* persistence of polarized Th1 and Th2 memory cells. *Immunity* **7:**543–552.

Sun Y, Li L, Lau F, Beavo JA, and Clark EA (2000) Infection of CD4+ memory T cells by HIV-1 requires expression of phosphodiesterase 4. *J Immunol* **165:**1755–1761.
Tasken K, Skalhegg BS, Tasken KA, Solberg R, Knutsen HK, Levy FO, Sandberg M, Orstavik S, Larsen T, Johansen AK, et al. (1997) Structure, function and regulation of human cAMP-dependent protein kinases. *Adv Second Messenger Phosphoprotein Res* **31:**191–204.
Vang T, Torgersen KM, Sundvold V, Saxena M, Levy FO, Skalhegg BS, Hansson V, Mustelin T, and Tasken K (2001) Activation of the COOH-terminal Src kinase (Csk) by cAMP-dependent protein kinase inhibits signaling through the T cell receptor. *J Exp Med* **193:**497–507.
Vijayaraghavan S, Stephens DT, Trautman K, Smith GD, Khatra B, da Cruz e Silva EF, and Greengard P (1996) Sperm motility development in the epididymis is associated with decreased glycogen synthase kinase-3 and protein phosphatase 1 activity. *Biol Reprod* **5:**709–718.
Wang W, Tam WF, Hughes CC, Rath S, and Sen S (1997) c-Rel is a target of pentoxifylline-mediated inhibition of T lymphocyte activation. *Immunity* **6:**165–174.
Zaccolo M and Pozzan T (2002) Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. *Science (Wash DC)* **295:**1711–1715.

Address correspondence to: Vineeta Bal, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi 110 067 India. E-mail: vineeta@nii.res.in