TCR- and CD28-Mediated Recruitment of Phosphodiesterase 4 to Lipid Rafts Potentiates TCR Signaling

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cAMP, generated by G-protein-mediated (G_{s}) activation of adenylyl cyclase (AC), is a common and versatile second messenger controlling numerous cellular processes including inhibition of mitogenic responses in fibroblasts and T cells. Although cAMP is known to activate protein kinase A (PKA) (1), Epac (exchange protein directly activated by cAMP) (2) and cyclic nucleotide-gated ion channels (3), PKA is likely to be the major target of cAMP in T cells because neither Epac (our unpublished data and Ref. 4) nor cyclic nucleotide-gated ion channels are expressed in lymphocytes (3). Compartmentalization of receptors, cyclases, and PKA by A-kinase-anchoring proteins (5, 6) as well as generation of local pools of cAMP within the cell by the action of anchored cAMP phosphodiesterase (PDE) isoforms (7) underpin the high degree of specificity of action in PKA-mediated signaling.

It has previously been demonstrated that stimulation of the T cell Ag receptor (TCR) elevates cAMP (8). However, increased cAMP levels in T cells inhibit T cell function and proliferation (9). and, as a consequence, TCR-mediated cAMP production must be regulated for T cell activation to occur. Since the only known way of reducing intracellular cAMP levels is through activation of the large family of cAMP PDEs (10–12), they are poised to play a key regulatory role in regulation of T cell function. The majority of the cAMP-hydrolyzing activity in T cells is known to be mediated by the PDE3 and PDE4 cAMP PDE families (13–15). Indeed, there is currently considerable interest in the PDE4 family as selective PDE4 inhibitors that exert a profound anti-inflammatory action are being developed to treat asthma and chronic obstructive pulmonary disease (16–18), where T cells are thought to provide one of the key cell targets for their action. Furthermore, there is now considerable evidence demonstrating that individual PDE4 isoforms display distinct patterns of intracellular targeting, indicating that they are likely to play a key role in compartmentalized cAMP signaling (10, 11). Indeed, PDE4 enzymes have very recently been shown to interact with the signaling scaffold protein β-arrestin (19) that together serve to regulate signaling through β2-adrenergic receptors. However, the functional role of PDE4 in the proximal TCR signaling in primary T cells is not well known, although it was recently demonstrated that PDE4B2 stably transfected into Jurkat cells localizes to the immunological synapse upon activation (20), suggesting a role for PDE4 in proximal T cell signaling.

It is generally accepted that proximal TCR-mediated signaling is initiated in specialized sphingolipid- and cholesterol-enriched microdomains in the cell membrane called lipid rafts (21). Lipid rafts function as signaling platforms that are comprised of or recruit protein complexes involved in the proximal signal transduction in T cells (22). The importance of such membrane microdomains has been demonstrated in T cells, as the integrity of lipid rafts is necessary for propagation of TCR-induced signaling to occur (23). One of the most proximal events taking place in T cells after engagement of the TCR is activation of the Src family protein tyrosine kinases, in particular Lck, and phosphorylation of the ITAMs present in the CD3 subunits (for review, see Ref. 24). This process is inhibited by C-terminal Src kinase (Csk) (25, 26). The molecular mechanism for the inhibitory effect of cAMP on proximal T cell signaling involves PKA-mediated phosphorylation of
serine 364 in Csk, resulting in Csk activation and subsequent inhibition of Lck (27). Furthermore, PGE₂ and other inputs leading to elevated levels of cAMP before stimulation of the TCR inhibit signaling through the TCR due to increased association between Csk and Csk-binding protein/protein associated with glycosphin-golipid-enriched microdomains residing in lipid rafts (28). However, it is not known to what extent cAMP and PKA may inhibit T cell activation during TCR triggering in the absence of concomi-

tant ligand-operated stimuli through G protein-coupled receptors such as, for example, PGE₂ that drive the generation of cAMP.

In this study, we investigate TCR-mediated cAMP production and subsequent PKA activation in primary T cells and the role of PDE4 in the propagation of T cell signaling in the absence and presence of CD28 coreceptor stimuli. We show that the TCR-mediat

ed increase in cAMP levels in rafts is generated by recruitment and dissociation of G proteins and that PKA activation serves to down-modulate signal transduction through the TCR. We show here for the first time that concurrent TCR and CD28 stimulation acts to increase raft-localized PDE4 enzymes, enhancing the degrada-
tion of cAMP and, thereby, potentiating T cell activation. Thus, recruitment of PDE4 isoforms to lipid rafts is required and necessary for a maximal T cell immune response to occur.

Materials and Methods

Cells and transfections

Human peripheral blood T cells were purified by negative selection (95–
98% pure) as described previously (29) and transected in accordance with the manufacturer’s instructions (Amaxa, Cologne, Germany) nucleofector kit for peripheral T cells, catalogue no. VPA-1002. The human leukemic T cell line Jurkat TAg was cultured and transfected by electroporation as previously described (30) (10⁻⁴ μg DNA for each transfection). Unless otherwise indicated, all experiments were conducted in normal primary peripheral blood T cells.

Reagents and Abs

Rolipram (R-6520), Brij 98 (P-5641), PMA (P-8139), ionomycin (I-0634), inFLAG M2 Ab (F-3165), and pFLAG Ab (F-7425) were purchased from Sigma-Aldrich (St. Louis, MO). n-octyl-β-D-glucoside (19775) was ob-
tained from USB (Cleveland, OH). 8-Bromoadenosine-3'-5'-cyclic mono-
phosphorothioate (Rp-8-Br-cAMPs) was obtained from BIOLOG Life Sci-
ence Institute (Bremen, Germany). Anti-Fc F(ab')₂, for cross-ligation of bound Ab and peroxidase-conjugated secondary reagents were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). HI-89 (371963) was obtained from Calbiochem (San Diego, CA). Protein A/G PLUS agarose and Abs toward Lck (sc-433), CD3-ζ (sc-1239), G_s (sc-
823), G_q (sc-393), and G_12a (sc-7276) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD3ε (OKT3) Ab was affinity-purified from a hybridoma cell line from American Type Culture Collection (CRL-8001; Manassas, VA). Magnetic beads coated with anti-CD14 (Dynabeads M-450 CD14, 111.12), anti-CD19 (Dynabeads M-450 CD19, 111.04), and anti-CD3/anti-CD28 Abs (Dynabeads CD3/CD28 T cell exp-
ander, 111.31) were obtained from Dynal Biotech (Oslo, Norway). mAb toward CD28 (clone CD28.2) was purchased from Immunotech (Marseille, France). Anti-linker for activation of T cells (LAT; 06H807), anti-p44/42 MAPK (06-182), and anti-Tyr(P) mAb (4G10) were purchased from Up-
state Biotechnology (Lake Placid, NY). Abs recognizing phospho-416 Src family (phospho-394 Lck/phospho-417 Fyn⁺) (2101), p42/p44 MAPK (9106), and PKA substrate phospho-specific Ab (abbreviated anti-RRXS/PS/PY, 9621) were purchased from Cell Signalling Technology (Beverly, MA). Abs toward PDE4A, PDE4B, PDE4D, and β-arrestin were as previously described (19, 31–33).

Purification and stimulation of lipid rafts

Isolation of Triton X-100 (0.7%) and Brij 98 (1%)–insoluble rafts were performed as described in detail elsewhere (34, 35) except that cells were lysed in standard lysis buffer (30). Brij 98 lysis was performed at 77°C for 7 min. After lysis both Triton X-100 lysates and Brij 98 lysates were completely homogenized in a Dounce homogenizer, mixed with 80% sucrose, loaded at the bottom of a 40–55% sucrose gradient (in 25 mM MES (pH 6.5), 5 mM EDTA, and 150 mM NaCl), centrifuged at 200,000 × g for 20 h at 4°C, and fractionated into 12 fractions from the top. Western blot with LAT Abs revealed that fractions 2–5 normally contained the majority of LAT and were used as a measurement for successful separation. Peak raft fractions were made by mixing fractions 2–5. For all cAMP measurements, fractions were prepared with Brij 98 as detergent. All other separa-
tions were done in the presence of Triton X-100. After Brij 98 cell sepa-
ration, all fractions were stimulated with anti-CD3 (5 μg/ml) alone or with anti-CD3 in combination with anti-CD28 (1 μg/ml) in the presence of ATP (1 mM) and MgCl₂ (15 mM). After 1 min of Ab incubation F(ab')₂ (20 μg/ml) were added for Ab cross-ligation. F(ab')₂ (20 μg/ml) were also added to controls. Reactions were stopped in reaction termination buffer supplied with the cAMP radioimmunoassay. Activity was normalized for protein content.

Stimulation of T cells for time course studies

PBL were incubated at 37°C for 10 min and then stimulated with anti-CD3 alone (5 μg/ml) or anti-CD3/anti-CD28 (1 μg/ml). Thereafter, F(ab')₂ (20 μg/ml) were added for Ab cross-ligation and incubation proceeded for 1–10 min as described in relevant figures. Reactions were ended by adding ice-cold RPMI 1640 followed by cell pelleting and lysis in standard lysis buffer (30) containing n-octyl-β-D-glucoside (50 mM). Equal cell numbers were used at each time point for lipid raft purification after stimulation.

Immunoprecipitations were as before (30).

Densitometric scanning analysis

Scanned x-ray films were subjected to densitometric analysis with the pro-

gram Scion image (www.scioncorp.com).

PDE activity assay

After ultracentrifugation and lipid raft separation, the collected fractions were analyzed for PDE activity (36). Assays were conducted in duplicate with the presence and absence of the PDE4 selective inhibitor rolipram (10 μM) to determine the rolipram-dependent PDE4 activity. PDE4 activity was similarly assessed but in this instance through chemical ablation in the presence of the PDE3 selective inhibitor cilostamide (1 μM, C 79781; Sigma-Aldrich). Protein concentrations were quantified according to the method of Bradford using BSA as a standard. Activity was normalized for primary protein content.

IL-2 assay and cAMP assay

For IL-2 production, T cells were stimulated with beads coated with anti-
CD3 and anti-CD28 Abs for 18 h (two beads per cell). For inhibition of PKA or PDE4, cells were treated with 10 μM H-89 or 10 μM rolipram, respectively, for 20 min at 37°C before stimulation. Standard IL-2 assay (S2050; R&D Systems, Minneapolis, MN) was used to measure secreted IL-2. cAMP radioimmunoassays (cAMP kit from NEN, Boston, MA, cata-

logue no. SP004) were performed in accordance with the manufacturer's instructions.

NFAT-AP-1 luciferase assay

Jurkat TAg cells were transfected with NFAT-AP-1 luciferase reporter con-

struct (10 μg) and Renilla-TK luciferase (1 μg) in combination with vec-
tors encoding β-arrestin 2, PDE4B2, PDE4A4, PDE4D1, or empty vector (20 μg) as indicated in Fig. 5D. Sixteen hours after transfection cells were either left unstimulated, stimulated with anti-CD3 (2.5 μg/ml) alone, stimu-
alted with a combination of anti-CD3/anti-CD28 (2.5 μg/ml/0.5 μg/ml), or stimulated with PMA (40 nM) and ionomycin (10 μM). Six hours after stimulation, cells were harvested and lysed. Thereafter, luciferase activity was measured by a dual luciferase reporter assay system from Promega (E1960; Madison, WI). NFAT-AP-1 luciferase activity in any sample was normalized against Renilla luciferase activity in the same sample. Measurements were done in triplicates. NFAT-AP-1 activity in each series was calculated as percentage of control (PMA/ionomycin) that was found to be unaffected by increased cAMP levels (see Fig. 5C).

Intracellular staining and FACS analysis

Three hours after transfection, primary T cells were stimulated with anti-
CD3 (5 μg/ml/anti-CD28 (1 μg/ml) Abs (or not) and cross-linked with F(ab')₂ (10 μg/ml), or a mix of PMA (25 nM) and ionomycin (10 μM). Stimulation proceeded for 6 h in a 96-well plate. Pelleted cells were fixed, stained and analyzed as previously described (37).

Constructs

FLAG β-arrestin 2 (19), PDE4A4 (38), PDE4B2 (32), PDE4D1 (39), Csk-wt and Csk-SH3-SH2 (30) were as previously described.
Results

**TCR-mediated cAMP production is increased upon 3-isobutyl-1-methylxanthine (IBMX) treatment and may occur in lipid rafts**

The TCR-induced elevation of cAMP levels has been observed previously in T cells (8). We demonstrate here that TCR stimulation in the absence of PDE inhibitors resulted in a modest but consistent cAMP production (Fig. 1A). However, in the presence of the nonselective PDE inhibitor IBMX, both basal and TCR-induced cAMP levels were further increased (Fig. 1A). In contrast, in T cells activated by TCR and CD28 cross-ligation, cAMP levels actually decreased (Fig. 1B). An increase in cAMP levels was only observed in the presence of IBMX (Fig. 1B). Since the cAMP levels in total cell lysates were low, we next explored the possibility that the TCR-stimulated accumulation of cAMP might be localized to lipid rafts since these membrane domains have been shown to provide a key focus for initiation of TCR-mediated signaling in T cells (23). To evaluate this, we purified lipid rafts from primary T cells using Brij 98 as a detergent. As previously shown, lysis of cells with Brij 98 at 37°C before sucrose gradient ultracentrifugation prevents the TCR-CD3 complex from being dissolved from rafts (35). Upon lipid raft purification with Brij 98, we identified CD3ζ, Gs (Fig. 1C), and low levels of the coreceptor CD28 (data not shown) in the fractions corresponding to lipid rafts. In comparison, lipid raft purification with Triton X-100 dissolved the TCR-CD3ζ from the rafts (data not shown). The fact that CD3 and Gs copurifies with these purified cell-free fractions indicates that at least part of the cAMP production machinery may already be present in lipid rafts, without the need for recruitment of other components. Indeed, when lipid raft fractions isolated by sucrose gradient centrifugation were reconstituted with Mg²⁺/ATP and subsequently stimulated with anti-CD3/anti-CD28, high levels of cAMP production were detected in lipid raft fractions (up to 3-fold above control in 1 min) (Fig. 1D). This reflects increased AC activity occurring in isolated lipid rafts in response to TCR stimulation in purified raft fractions. Comparable results were obtained by in vitro TCR stimulation by anti-CD3 treatment alone (data not shown). Furthermore, PAKA-mediated phosphorylation of putative raft-localized PKA substrates was detected immediately after TCR stimulation of primary T cells (Fig. 1E). Densitometric analysis revealed a 4- to 5-fold increase in PAKA-mediated phosphorylation of a protein of ~80 kDa. Phosphorylation of the same protein as shown in Fig. 1E was reduced upon H-89 pretreatment (data not shown). This provides further evidence of the activation of the cAMP signaling pathway upon T cell activation.

Gs, Gs, and AC have been reported to segregate into lipid rafts (40, 41). Therefore, we next examined whether these G proteins played a role in the raft-associated cAMP increase. In resting T cells, Gs was detected both in lipid rafts as well as in nonraft fractions (Fig. 1, C and F). However, the level of Gs in rafts was rapidly and transiently increased upon anti-CD3/anti-CD28 cross-ligation of whole cells, with a decline to basal levels occurring after 2 min (Fig. 1F, first panel). Conversely, the G protein Gs, which acts to inhibit AC, was present at high levels in rafts from resting T cells but dissociated rapidly from rafts following TCR stimulation (Fig. 1F, second panel). As shown previously by others (42), the G protein Gs is recruited to rafts upon engagement of the TCR (Fig. 1F, third panel). However, this G protein is not considered to be directly involved in regulating cAMP signaling (43). Since both AC and its activator, Gs, are present in lipid rafts, we next set out to hamper the anti-CD3/anti-CD28-induced cAMP production in vitro by using specific blocking Abs that inhibit Gs function by interfering with effector coupling (44). Pretreatment of the isolated lipid raft fractions with these inhibitory Gs Abs before stimulation reduced the in vitro anti-CD3/anti-CD28-induced cAMP production almost back to control levels (Fig. 1G). Conversely, incubation with Ab blocking Gs effector coupling increased both the basal and stimulated cAMP levels to 1.6 and 2 pmol cAMP/10⁶ cells, respectively, demonstrating the specificity of action of these reagents (data not shown). Together these data indicate that in vitro TCR stimulation induces cAMP production in lipid rafts through a Gs-mediated process. Additionally, in intact cells, generation of cAMP seems to be increased by concurrent recruitment of additional Gs to rafts and dissociation of inhibitory Gs from rafts. Furthermore, by blocking PDE activity using the PDE inhibitor IBMX, TCR-induced cAMP production is increased identifying a key role of PDEs in regulation of cAMP levels upon T cell activation.

**T cell stimulation increases PDE4 activity in lipid rafts**

Localization of PDE isoforms to specific cellular compartments (10) has been shown to provide a pivotal contribution to the generation of local intracellular cAMP gradients in various cell types (7, 45). A number of PDE isoforms are expressed in T cells (PDE3B, 4A, 4B, 4D, 7A1, 7A3, 8A), of which PDE3 and PDE4 provide the majority of cAMP PDE activity (13, 15, 46). The fact that the nonselective PDE inhibitor IBMX augmented cAMP production upon T cell stimulation indicates a role for PDEs in regulating cAMP levels in T cells (Fig. 1, A and B). Given that PDE4 selective inhibitors are considered to mediate part of their therapeutic potential by acting on T cells and that a key feature of PDE4 isoforms is their intracellular targeting (10, 11), we set out to determine whether PDE4 activity was localized to lipid rafts where T cell signaling is initiated (47) and where the majority of cAMP seems to be produced. Doing this we see that although anti-CD3 stimulation induces PDE4 activity in fractions corresponding to lipid rafts (Fig. 2, A and A’), CD28 costimulation leads to a profound raft-associated increase in PDE4 activity (Fig. 2, B and B’). In marked contrast to this, lipid raft fractions even from T cells subjected to anti-CD3/anti-CD28 stimulation contained little or no PDE3 activity (Fig. 2, C and C’).

**Enhanced recruitment of PDE4 and β-arrestin to lipid rafts after TCR and CD28 costimulation**

The specific increase in PDE4 activity in lipid raft fractions upon TCR/CD28 engagement might suggest that temporal changes in PDE4 activity can play a key role in tuning intracellular activation-induced gradients of cAMP in T cell lipid rafts and thereby increase signal propagation upon costimulation. Since a key feature of T cell activation is to induce the redistribution of components both to and from lipid rafts, we next explored whether there was any specific recruitment of PDE4 enzymes to lipid raft fractions upon concomitant TCR and CD28 engagement. The PDE4 family consists of four subfamilies (A, B, C, and D), each of which generates a series of isoforms by the use of distinct promoters and alternative mRNA splicing (10, 11). However, within any one subfamily, the C-terminal regions of all active isoforms are identical. This has been effectively exploited to generate antisera that are not only specific for each subfamily but thereby identify all isoforms within a particular subfamily (39). Using antisera specific for the four PDE4 subfamilies, we found that the long PDE4A4 isoform, the short PDE4B2 isoform, and the short PDE4D1/2 isoforms were all rapidly and concomitantly recruited to lipid rafts upon TCR and CD28 costimulation (Fig. 3A). This suggests that the increased PDE4 activity seen in lipid rafts upon TCR/CD28 stimulation at least in part is a result of increased levels of PDE4 in membrane microdomains.
FIGURE 1. cAMP levels increase upon T cell activation. A, Increase in total cellular cAMP levels upon TCR stimulation. Purified primary T cells were stimulated with either F(ab')₂ as a control or with anti-CD3 and F(ab')₂ in the absence and presence of IBMX. Subsequently, cAMP levels were measured by radioimmunoassay. The experiment is representative of three similar experiments. B, TCR-induced cAMP levels in T cells are reduced upon cross-linkage of the TCR with CD28. Primary T cells were stimulated with F(ab')₂ or with anti-CD3/anti-CD28 and F(ab')₂ in the absence and presence of IBMX. cAMP levels were measured as in A. CD3ζ and Gₛ, are localized in T cell lipid rafts. Insoluble membranes from T cells were prepared using Brij 98 as detergent. Lysates from the 11 first fractions were analyzed with the indicated Abs. D, Anti-CD3/anti-CD28-mediated cAMP increase occurs in lipid rafts. Insoluble membranes from T cells were prepared as in C (Brij 98), whereupon the TCR is not dissolved from rafts. Each fraction harvested from the sucrose density gradient centrifugation was reconstituted with Mg/ATP and stimulated with anti-CD3, anti-CD28, and F(ab')₂ at 37°C for 1 min. Subsequently, cAMP production was measured by radioimmunoassay. Protein content in each fraction was measured. The data shown are representative of three representative experiments. E, T cell activation increases PKA activity in rafts. Primary T cells were stimulated with anti-CD3 and F(ab')₂ and subsequently subjected to sucrose gradient separation with Triton X-100. Peak raft fractions were analyzed for proteins phosphorylated by PKA (Anti-RXXPS/PT). The blot shown is representative of four experiments. Densitometric scanning revealed about a 4-fold induction in PKA activity (average, 4.2 ± 1.2, n = 4). F, Concomitant reduction of Gᵢₛ levels and increased Gₛ levels in lipid rafts upon anti-CD3/anti-CD28 stimulation. T cells were stimulated and separated as in E. Peak raft fractions were analyzed for the presence of Gₛ, Gᵢₛ, Gₛ as well as LAT. G, Preincubation of raft fractions with inhibitory Abs toward Gₛ reduces anti-CD3/anti-CD28-induced cAMP production. T cell lysates were subjected to sucrose gradient fractionation with Brij 98 as in A. Raft fractions were reconstituted with Mg/ATP, not pretreated or pretreated with Abs inhibiting Gₛ, stimulated as indicated, and cAMP production compared with control was calculated.
It has recently been shown that upon β_{2}-adrenergic stimulation of HEK293 cells a cytoplasmic complex consisting of β-arrestin and PDE4 is recruited to membranes where the phosphorylated β_{2}-adrenergic receptor is localized (19, 48). Intriguingly, we found here that anti-CD3/anti-CD28 stimulation caused a clear recruitment of β-arrestin to T cell lipid raft fractions concurrently with PDE4 (Fig. 3A). β-Arrestin serves as a cytosolic scaffold protein that can bind a variety of signaling molecules and recruit them to the plasma membrane upon appropriate receptor stimulation (49).

One such species is the MAPK/ERK (50–52) and we demonstrate here (Fig. 3A) that anti-CD3/anti-CD28 stimulation also serves to concomitantly recruit ERK along with β-arrestin to lipid rafts in T cells. Furthermore, the fraction of ERK present in lipid rafts was evidently active, as determined by phospho-specific Abs toward Thr(P){sup 202} and Tyr(P){sup 204} in ERK (Fig. 3A).

To examine whether PDE4 and β-arrestin exist in a complex both before and after T cell stimulation, we transfected T cells with a vector encoding a FLAG-tagged β-arrestin, and immunoprecipitated β-arrestin from both unstimulated cells and cells stimulated with anti-CD3/anti-CD28. Interestingly, β-arrestin and PDE4D co-immunoprecipitated from both unstimulated and stimulated cells (Fig. 3B), indicating that they exist in a preassembled complex in T cells as has been shown to occur in other cell types (19). Other PDE4 isofoms were not tested here, as they have been described to coimmunoprecipitate with β-arrestin before.

Stimulation using anti-CD3 alone was not as effective as CD28 costimulation in increasing PDE4 activity in lipid raft fractions (Fig. 2). Indeed, compared with anti-CD3 stimulated cells, 2.5-fold more PDE4/β-arrestin was recruited upon costimulation with CD28 (Fig. 3C). This led us to evaluate the impact of costimulation on recruitment of the PDE4/β-arrestin complex to lipid rafts. Indeed, cross-linking of CD3 alone was far less effective in recruiting both β-arrestin and ERK to lipid rafts compared with costimulation with anti-CD3/anti-CD28 (Fig. 3C). In the same experiment, PDE4 activity was lower upon CD3 stimulation compared with CD3/CD28 stimulation (data not shown) as described in Fig. 2.

The diterpene forskolin interacts directly with the catalytic unit of AC to cause its activation. Treating T cells with this agent clearly fails to show any recruitment of PDE4 to rafts (Fig. 3D), although forskolin always gave a robust cellular increase in cAMP compared with CD3 stimulation (Fig. 3E). This demonstrates that PDE4 recruitment is not elicited through any increase in intracellular cAMP levels and argues toward a specific role for the TCR and CD28-mediated signaling in recruitment of PDE4 to rafts. Anti-CD3 stimulation was used as a control in these experiments as only low levels of PDE4 are recruited upon anti-CD3 stimulation, allowing detection of potentially low PDE4 recruitment upon forskolin treatment.

Only a few TCRs are concomitantly ligated under normal circumstances in vivo, generating incomplete activation events that eventually lead to anergy or cell death (53). However, CD28 costimulation amplifies the weak TCR-induced signals leading to full T cell activation and clonal expansion in vivo. When T cells were stimulated with anti-CD28 Abs alone here, we found that this was sufficient to induce recruitment of β-arrestin to lipid rafts (Fig. 3F).
A PDE4/β-arrestin complex is recruited to T cell lipid rafts following T cell activation. A, The levels of PDE4, β-arrestin, and ERK in lipid rafts are increased upon anti-CD3/anti-CD28 stimulation. After stimulation with anti-CD3, anti-CD28 and F(ab’)_2 human peripheral T cells were lysed and subjected to sucrose gradient ultracentrifugation with Triton X-100. Peak raft fractions (fractions 2–5) from cells activated for various periods of time were analyzed with the indicated Abs. B, Jurkat TAg cells were transfected with empty vector or with a construct coding for FLAG-β-arrestin, stimulated as indicated, and lysed in standard lysis buffer. Immune complexes were precipitated with Abs toward FLAG and analyzed for the presence of PDE4D and FLAG/β-arrestin. C, Enhanced recruitment of PDE4 and β-arrestin to T cell lipid rafts after anti-CD28 costimulation. T cells were stimulated either with anti-CD3 and F(ab’)_2 alone or in combination with anti-CD28, peak raft fractions were analyzed for the recruitment of β-arrestin and ERK at the indicated times of activation. Recruitment of β-arrestin to rafts with anti-CD3/anti-CD28 compared with anti-CD3 alone was 2.5 ± 0.2-fold (average ± SEM, n = 3). D, Increased cAMP induced by forskolin is not sufficient for PDE4 recruitment to rafts. T cells were stimulated either with forskolin (3 min) or anti-CD3 and F(ab’)_2 (3 min) that give low levels of PDE4 in rafts and thereafter subjected to sucrose gradient ultracentrifugation. Peak raft fractions (fractions 2–5) were analyzed with the indicated Abs. E, Forskolin induces a robust cAMP increase. T cells were stimulated with forskolin (10 μM) or anti-CD3 and F(ab’)_2 for 3 min. Thereafter, cAMP was measured. F, Anti-CD28 stimulation of T cells is sufficient for β-arrestin recruitment to lipid rafts. T cells were stimulated with indicated combinations of anti-CD3, anti-CD28, and anti-CD4 and analyzed with Abs detecting β-arrestin and LAT.
In contrast, upon cross-ligation of the TCR or CD28 with CD4, a T cell surface marker to which the Src kinase Lck binds, no additional effect on recruitment of β-arrestin was found (Fig. 3F).

Altogether, these results suggest that signals generated upon CD28 stimulation are critical for enhancing the recruitment of the PDE4 and β-arrestin to lipid rafts in a cAMP-independent fashion.

**PKA is activated upon TCR stimulation and inhibits proximal T cell signaling**

The fact that PDE4 selective inhibitors inhibit T cell functioning therapeutically (17, 54) might suggest that our novel demonstration of the recruitment of PDE4 isoforms to lipid raft fractions upon T cell activation and regulation of raft-associated cAMP signaling has particular functional significance. We thus set out to gain insight into the possible physiological consequences of TCR-induced cAMP production by analyzing the effect of PKA-mediated signaling on proximal TCR activation in T cells. As a start, we began to analyze whether TCR stimulation increases PKA-mediated signaling. It has previously been demonstrated that the phosphatase HePTP is directly phosphorylated at serine 23 by PKA in T cells (55), making serine 23 phosphorylation of HePTP a good readout for PKA activity.

**FIGURE 4.** PKA is activated following T cell activation and regulates proximal T cell signaling events. A, TCR stimulation induced phosphorylation of the phosphatase HePTP on Ser23, a known PKA phosphorylation site. Jurkat TAg cells were transfected with hemagglutinin-tagged HePTP and stimulated with C305 (IgM), that cross-ligates the TCR on Jurkat cells. Phosphorylation of HePTP, a good readout for PKA activation in lymphocytes, was analyzed with an Ab with specificity toward phospho-Ser23 in HePTP. B, PKA inhibition by H-89 or the cAMP antagonist Rp-8-Br-cAMPs decreases TCR-induced CREB phosphorylation. Purified human T lymphocytes were pretreated with H-89 (upper panel) or Rp-8-Br-cAMPs (lower panel) and subsequently stimulated with anti-CD3 and Fab′3 for the indicated time periods and thereafter analyzed for CREB phosphorylation by immunoblotting. C, PKA is activated following TCR stimulation and is involved in down-regulation of the TCR-induced signal. Purified human T lymphocytes were left untreated or pretreated with H-89 and subsequently stimulated with anti-CD3 for the indicated time periods. TCR-induced T cell activation upon PKA inhibition was assessed by detection of ζ-chain phosphorylation (4G10) and autophosphorylation of Lck and Fyn (Anti-PY394Lck/anti-PY417Fyn). D, PKA inhibition increases ζ-chain phosphorylation 2- to 4-fold. Densitometric scanning analysis of ζ-chain phosphorylation (normalized for the amount of ζ) for three similar experiments as shown in A is presented as fold induction over control (average ± SEM, n = 3). E, β-arrestin and PDE4A4 potentiate proximal T cell signaling. T cells were transfected with 2 μg vector, β-arrestin, or PDE4A4. Sixteen hours after transfection, cells were stimulated with anti-CD3 (2.5 μg/ml) and F(ab′)2 (20 μg/ml) for the indicated times. Lysates were analyzed for ζ-chain phosphorylation.

In contrast, upon cross-ligation of the TCR or CD28 with CD4, a T cell surface marker to which the Src kinase Lck binds, no additional effect on recruitment of β-arrestin was found (Fig. 3F). Altogether, these results suggest that signals generated upon CD28 stimulation are critical for enhancing the recruitment of the PDE4 and β-arrestin to lipid rafts in a cAMP-independent fashion.

**PKA is activated upon TCR stimulation and inhibits proximal T cell signaling**

The fact that PDE4 selective inhibitors inhibit T cell functioning therapeutically (17, 54) might suggest that our novel demonstration of the recruitment of PDE4 isoforms to lipid raft fractions upon T cell activation and regulation of raft-associated cAMP signaling has particular functional significance. We thus set out to gain insight into the possible physiological consequences of TCR-induced cAMP production by analyzing the effect of PKA-mediated signaling on proximal TCR activation in T cells. As a start, we began to analyze whether TCR stimulation increases PKA-mediated signaling. It has previously been demonstrated that the phosphatase HePTP is directly phosphorylated at serine 23 by PKA in T cells (55), making serine 23 phosphorylation of HePTP a good readout for PKA activity. Stimulation of Jurkat cells with
FIGURE 5. PKA and PDE4 are activated following T cell activation and play opposite regulatory roles in titrating T cell immune functions. A, IL-2 secretion is impaired upon rolipram and H-89 pretreatment. Primary T cells pretreated with rolipram or H-89 were left unstimulated, stimulated with beads coated with anti-CD3 and anti-CD28, or with a mix of PMA and ionomycin. Twenty hours after stimulation IL-2 secreted from the cells was measured with ELISA (average ± SEM, n = 3). B, Expression of PDE4B2 and β-arrestin increases IL-2 production in T cells. Primary T cells were transfected with indicated constructs. At 3 h after transfection, cells were stimulated with anti-CD3/anti-CD28-coated beads and 18 h after stimulation cells were pelleted and IL-2 secreted was analyzed by ELISA (average ± half range, n = 2). C, Increased cAMP does not affect PMA/ionomycin-induced proximal IL-2 promoter (NFAT-AP-1-luc) activity. Jurkat cells were transfected with NFAT-AP-1 luciferase and with Renilla luciferase. Eighteen hours after transfection, cells were pretreated with forskolin (15 min) and subsequently stimulated with PMA and ionomycin for 6 h and then analyzed for luciferase activity. D, Increased IFN-γ production in PDE4-expressing and β-arrestin-expressing T cells. Primary T cells were transfected with indicated constructs. Cells were stimulated at 3 h after transfection with anti-CD3/anti-CD28 and F(ab')2, harvested 6 h after stimulation (5 h in the presence of brefeldin A), fixed, and permeabilized, then stained with Abs detecting intracellular IFN-γ. FACS analysis was subsequently performed to determine (Figure legend continues)
anti-CD3-IgM (C305) resulted in rapid phosphorylation of serine 23 in this protein (Fig. 4A), clearly demonstrating TCR-induced PKA activation. Next, we studied phosphorylation of CREB (56), another direct target of PKA, through a pathway that was largely inhibited by pretreatment with H-89 (Fig. 4B, upper panel). Likewise, pretreatment of primary T cells with the PKA antagonist Rp-8-Br-cAMPS reduced the activation-induced CREB phosphorylation (Fig. 4B, lower panel). Altogether, this demonstrates TCR-induced PKA activation in primary T cells.

Next, we explored what functional role TCR-induced PKA activity plays in the proximal TCR signaling. One of the most proximal events taking place upon TCR stimulation is activation of the Src family kinases Lck and Fyn and phosphorylation of the CD3 ζ-chains (57). The phosphorylated ζ-chains function as docking sites for the Src homology (SH) 2 domain of the Syk family member ZAP-70 (58), which upon Lck phosphorylation mediates the phosphorylation of the important adapter molecule linker for activated T cells (LAT) (for review, see Ref. 24). Phosphoryrosines on LAT in turn attract complexes of adapters and signaling molecules like SH2 domain-containing leukocyte protein of 76 kDa (SLP-76) necessary to mediate a functional T cell response (59). Indeed, upon pretreatment of T cells with the PKA inhibitor H-89, early T cell activation markers such as ζ-chain phosphorylation and phosphorylation in the catalytic domain of Lck/Fyn were increased (Fig. 4C). Densitometric analysis revealed that in the presence of H-89, TCR-induced ζ-chain phosphorylation was increased 2- to 4-fold compared with control cells (Fig. 4D). Furthermore, phosphorylation of other molecules important for T cell activation such as LAT and SLP-76 (24) were also increased under conditions of low PKA activity (data not shown). Lastly, we

percent IFN-γ-producing cells in the transfected GFP-positive population. Two hundred thousand GFP-positive cells were analyzed for each transfection. The experiment shown is representative of two independent experiments from two different blood donors. E. IFN-γ production in PDE4-expressing and β-arrestin-expressing T cells. Graphic presentation from the experiment shown in Fig. 5D. Viable, GFP-positive cells were gated, and the IFN-γ production was calculated as percentage of PMA/ionomycin induced for each transfection. F. Increased transcription from the proximal IL-2 promoter (NFAT-AP-1-luc) in Jurkat TAg cells expressing PDE4 isoforms and β-arrestin. Jurkat TAg cells were transfected with the constructs indicated, Renilla luciferase and NFAT-AP-1-luciferase reporter constructs. Eighteen hours after transfection, cells were stimulated with anti-CD3, anti-CD3/anti-CD28, or PMA/ionomycin for 6 h and then analyzed for luciferase activity. NFAT-AP-1 luciferase activity was normalized against Renilla luciferase in the same sample. The experiment shown has triplicate measurements (average ± SD, n = 3) and is representative of three independent experiments.
wanted to investigate whether expression of β-arrestin or PDE4A4 influenced the proximal signaling events as well. As shown in Fig. 4E, β-arrestin clearly potentiated proximal events like ζ-chain phosphorylation (Fig. 4E). Overexpression of PDE4A4 also potentiated ζ-chain phosphorylation although the induction was not as evident as when β-arrestin was expressed (Fig. 4E).

**PKA and PDE4 control T cell function**

Since β-arrestin and PDE4 expression potentiate proximal signaling events in T cells, we next wanted to assess the effects of PKA and PDE4 on downstream T cell function. Upon full T cell activation, in vivo T cells proliferate and produce different growth-promoting cytokines like IL-2 and IFN-γ. To assess the effect of PDE4 and PKA activity on T cell function, we treated T cells with either rolipram or H-89 and measured IL-2 secretion. Pretreatment of cells with rolipram reduced TCR-induced IL-2 production to ~50% of control (Fig. 5A), whereas H-89-mediated inhibition of PKA had the opposite effect (2-fold increase compared with control; Fig. 5A). Similarly, IFN-γ production was also reduced by rolipram and increased by H-89 (data not shown). This is in accordance with previous reports demonstrating that increased levels of cAMP reduce IFN-γ production in T cells (37).

Because we found that β-arrestin and PDE4 are recruited to lipid rafts as a complex, we next investigated the effect of overexpression of β-arrestin and various PDE4 isoforms on IL-2 secretion and IFN-γ production. Overexpression of PDE4B2 and β-arrestin augmented anti-CD3/anti-CD28-mediated IL-2 production (Fig. 5B). Overexpression of Csk-SH3-SH2, an interfering mutant of Csk which is known to potentiate TCR-induced signaling (30), is shown as a control (Fig. 5B). Maximal T cell activation can be accomplished through full PKC activation, full Ca2+ response, and MAPK activation and can be induced upon concomitant treatment with phorbol ester and Ca2+ ionophore (PMA and ionomycin). Such treatment bypasses the inhibitory effect of cAMP (Fig. 5C) and was therefore used as a measure of maximal activation of cells that had received various treatments (e.g., different transfections).

The effects in Fig. 5B are comparably small because only up to 50% of the primary T cells were transfected. However, assessing IFN-γ production in transfected GFP-positive primary T cells by flow cytometry, we found a clear potentiating effect of β-arrestin, PDE4A4, and PDE4B2 (Fig. 5D). We also saw a potentiating effect of PDE4A4 and PDE4D1 (Fig. 5E). Again, overexpression of dominant negative Csk-SH3-SH2 or wild-type Csk are used as control, potentiating and inhibiting TCR-induced signaling, respectively (30) (Fig. 5E). Similar results were also obtained with a proximal IL-2 promoter reporter construct containing the NFAT and AP-1 elements of the IL-2 promoter (NFAT-AP-1-luciferase; Fig. 5F). Taken together, these data suggest that the activities of both PKA and PDE4 are important for regulation of TCR-induced signaling and T cell function. We suggest that recruitment of β-arrestin and PDE4 to T cell lipid rafts upon concomitant TCR and CD28 stimulation down-regulates the inhibitory effect of cAMP induced upon TCR stimulation. Through this mechanism β-arrestin and PDE4 potentiate T cell signaling upon CD28 costimulation (Fig. 6).

**Discussion**

cAMP plays an inhibitory role in regulating proximal TCR-induced T cell signaling (28). On this basis, one might expect the intracellular level of cAMP to fall upon T cell activation in the compartment relevant for regulation of proximal T cell signaling. Interestingly, however, increased intracellular cAMP levels following T cell activation in the presence of the PDE inhibitor IBMX was reported >15 years ago (8, 60). Despite this, the significance of activation-induced cAMP production has been ill-understood to date, as has the location in the cell where cAMP is generated. In this study, we show that T cell activation rapidly induces the production of the immunoinhibitory signaling molecule cAMP in lipid rafts, resulting in raft-associated PKA activation. Our data indicate that the concomitant recruitment to lipid rafts of the stimulatory G protein, Gi, and dissociation of the inhibitory G protein, Gb, play a key role in the activation of AC that occurs upon T cell activation. These data imply that a localized increase in cAMP is generated in T cells upon activation and that this appears to be concentrated in lipid rafts.

Gαi-coupled receptors, such as the β2-adrenergic receptor, invariably generate transient increases in cAMP (61). A key regulator of receptor-stimulated AC activity is the recruitment of β-arrestin from the cytosol to the plasma membrane-associated receptor (61). This has the effect of causing the uncoupling of Gi from receptor stimulation, leading to desensitization of AC. However, regulation of intracellular cAMP levels is not solely the prerogative of AC action, but is also crucially regulated by the action of PDEs, which provide the sole route of cAMP degradation in cells (10, 12). Indeed, although β-arrestin may serve to terminate AC activation, it is only through the action of PDEs that cAMP levels can return to their basal state. The importance of PDE action in T cells is clearly evident from both previous studies by other investigators (13–15, 20) and also from our experiments (Fig. 1, A and B) showing that PDE inhibition augments the increase in cAMP caused by T cell activation. Not only are PDE4 enzymes the main contributors of cAMP-PDE activity on T cell function, but PKA and PDE4 are important for regulation of TCR-induced T cell signaling (9, 28). Presumably it is expected that functioning PDE4 activity would serve to promote T cell signaling, perhaps by reducing cAMP levels in an appropriate compartment. It is interesting then to find that TCR stimulation actually leads to the activation of AC and the generation of the second messenger cAMP that plays an inhibitory role in regulating proximal TCR-induced T cell signaling (9, 28). Presumably it is important that T cells have a mechanism to ensure that cAMP levels do not become chronically elevated, which would thereby inhibit T cell functioning. In this study, we have shown that the elevation of cAMP is ablated subsequent to costimulatory activation of T cells. We suggest that an important component in defining cAMP levels is recruitment of PDE4 enzymes to lipid raft fractions, a source for compartmentalized cAMP generation in T cells. Interestingly, such recruitment of PDE4 to rafts is apparently accompanied by that of β-arrestin, which in various other cell types has been shown to play a pivotal role in not only uncoupling the receptor-mediated stimulation of Gi (71) but also in allowing the receptor-mediated recruited of PDE4 isoforms (19, 48).

Although we clearly identified the recruitment of PDE4 isoforms to rafts upon T cell stimulation, it is also possible that phosphorylation could further contribute to the increased PDE4 activity in lipid raft fractions. Thus, the long PDE4A4 isoform has been shown to be activated by ~25% through phosphorylation by PKA (62, 63) and the short PDE4B2 and PDE4D1 isoforms can be similarly activated through the action of ERK (33), which becomes activated upon TCR stimulation (64). However, the paucity of PDE4 protein in rafts of these cells militates against a direct demonstration of this using either direct phosphorylation or the use of phospho-Abs. There are other PDEs found in T cells (14, 65); however, no change in the activity of PDE3 was detected in lipid rafts upon TCR costimulation (Fig. 2, C and C*). PDE7 has been suggested to be important for T cell proliferation with its expression up-regulated during the first 8 h of T cell activation. However,
it is absent from resting T cells (65) and T cell functioning has recently been shown to be normal in knockout mice (66), indicating that PDE7 is unlikely to be involved in the regulation of the initial T cell signaling events.

Stimulation of the TCR is known to induce a signal that is too weak to fully activate T cells (67). The signal can, however, be amplified by CD28 costimulation and together these two signals can induce full activation and clonal expansion (68). In this regard, we show here that β-arrestin recruitment appears to be mainly induced by CD28 stimulation and this may play a key role in constraining the inhibitory consequence of TCR-induced AC activation through the recruitment of PDE4. The recruited β-arrestin may also serve an uncoupling role, although the demonstration of this and the identification of its partner allowing recruitment will be a challenge for future studies. Since the combination of anti-CD3 and anti-CD28 stimulation recruits and activates PDE4 to a greater extent than anti-CD3 stimulation alone, signal amplification by costimulation may be mediated through activation of unknown molecules in addition to identified species such as PI3K, Fak, and Vav-1 (67). We suggest an additional role for CD28 as a molecular amplifier of TCR-induced signals whereby CD28-mediated PDE4 recruitment to lipid rafts serves to down-modulate inhibitory cAMP signals. The inhibitory function of cAMP might, for example, be mediated through the action of PKA on the Csk-Lck pathway as previously demonstrated (27, 28). Upon TCR stimulation alone however, PDE4 recruitment may be too low to fully reduce the cAMP levels and therefore maximal T cell activation cannot occur.

In conclusion, our results suggest opposing functions of PKA and PDE4 isoforms during proximal T cell signaling, thereby tamping the activation-induced response. In this study, we then propose a novel facet of the CD28 costimulation effect, namely, in achieving down-modulation of TCR-induced cAMP-mediated inhibitory signals through recruitment of PDE4 to lipid rafts.

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References


