PKCθ and PKA are antagonistic partners in the NF-AT transactivation pathway of primary mouse CD3+ T lymphocytes

Natascha Hermann-Kleiter1, Nikolaus Thuille1, Christa Pfeifhofer1, Thomas Gruber1, Michaela Schäfer2, Christof Zitt2, Armin Hatzelmann2, Christian Schudt2, Michael Leitges3 and Gottfried Baier1*

1Department for Medical Genetics, Molecular and Clinical Pharmacology, Innsbruck Medical University, Austria
2ALTANA Pharma AG, Konstanz, Germany
3Medical University, Hanover, Germany

Total word/character count: 4,566/25,924 excluding title page, figure legends and references

*Corresponding author:
A.Univ.-Prof. Dr. Gottfried Baier, Innsbruck Medical University, Schoepfstraße 41, A-6020 Innsbruck, Austria/Europe.
Tel: +43 (512)/507-3451, Fax: +43 (512)/507-2861,
Email: Gottfried.Baier@i-med.ac.at

Running title: PKA and PKC0 crosstalk
Key words: PKC0/cAMP/PKA/NF-AT/Interleukin-2/T cells
Scientific category: Immunobiology

This work was supported by grants of the FWF (P16229 & SFB021) and by a cooperation agreement with Altana Pharma (Konstanz, Germany).
ABSTRACT (200 words):

We here investigate the crosstalk of PKC and PKA signalling during primary CD3+ T lymphocyte activation using pharmacological inhibitors and activators in combination with our established panel of PKC isotype-deficient mouse T cells in vitro. PKCθ and PKA inversely affect the CD3/CD28-induced IL-2 expression, whereas other PKC isotypes are dispensable in this signalling pathway. Gene ablation of PKC0 selectively results in a profound reduction of IL-2 production, however, complete abrogation of IL-2 production in these PKC0−/− T cells was only achieved by simultaneous co-activation of the cAMP/PKA pathway in CD3+ T cells. Conversely, the reduced IL-2 production in PKC inhibitor treated T cells can be rescued by inhibition of the cAMP/PKA pathway in wild-type but not in PKC0−/− T cells. Mechanistically, the cAMP/PKA and PKC0 pathways converge at the level of NF-AT, as shown by DNA binding analysis. The combined increase in PKA and decrease in PKC0 activity leads to an enhanced inhibition of nuclear NF-AT translocation. This PKC0/PKA crosstalk does neither significantly affect the NF-κB, the AP-1 nor the CREB pathways. Taken together, this opposite effect between the positive PKC0 and the negative cAMP/PKA signalling pathways appears rate limiting for NF-AT transactivation and IL-2 secretion responses of CD3+ T lymphocytes.
INTRODUCTION:

Following T cell receptor (TCR) stimulation, lymphocyte activation threshold is coordinated by a complex interplay of distinct signal transduction pathways. The "fine-tuning" of signalling cascades is considered as a crucial step during T cell activation in order to result in adequate amplitude of an immune response. The molecular processes that regulate the precise level of immune activation and their complex inter-pathway integration are, however, still poorly understood. Among several signalling pathways, Protein kinase C (PKC) plays an important role in the cellular activation pathway downstream of the antigen receptor. PKC\(\theta\), in particular, is known to translocate to the immunological synapse \(^1\). Once activated, PKC\(\theta\) plays a critical role in TCR signalling \(^2\). This is confirmed by analysis of CD3\(^+\) T cells of mice lacking PKC\(\theta\). Ex vivo, PKC\(\theta\)-deficient T cells are mostly TCR-unresponsive and deficient in the production of interleukin 2 (IL-2) due to an impaired transactivation of NF-\(\kappa\)B, AP-1 and NF-AT \(^3\)-\(^5\). In strict contrast, the cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA) pathway represents a negative regulatory mechanism that controls IL-2 expression in T cells. cAMP itself has been demonstrated to activate directly a class of cyclic nucleotide ion channels \(^6\),\(^7\) as well as the Rap1 guanine exchange factors Epac1 and Epac2 \(^8\). The principle cAMP receptor in lymphoid cells, however, is PKA \(^9\).

cAMP is produced after TCR stimulation in T lymphocytes, suggesting the cAMP-mediated repression of T cell activation may represent a physiological negative feedback control mechanism \(^10\). Suppression of cAMP signalling is thus required for T-cell activation \(^11\). Among other mechanisms, cAMP inhibition is mediated by de-novo transcriptional induction of phosphodiesterases (PDEs) upon T cell activation. In T cells, PDE4 seems to play a particularly important role \(^12\),\(^13\). Increased PDE4 expression in T cells is associated with increased IL-2 production via its hydrolysis of cAMP \(^12\). Inversely, PDE4 blockade is shown to augment cAMP levels and thus known to abrogate cytokine secretion and proliferation in T cells through inhibition of NF-\(\kappa\)B and NF-AT and activation of CREB \(^13\). Several direct targets of cAMP/PKA signalling have been identified, such as CREB \(^14\), COOH-terminal Src kinase (Csk) \(^15\) and NF-AT \(^16\). The detailed interplay and potential crosstalks of PKA with other signalling pathways during physiological T cell activation, however, remain mostly unresolved.

Employing cell-permanent pharmacological inhibitors and activators of PKA and PKC, a
critical functional crosstalk of PKA and PKC derived signals during T cell activation has been elaborated\textsuperscript{17,18}. A number of compounds have been reported to inhibit the PKC family, and thus have been used extensively. However, the specificity of these compounds has not been tested rigorously in cell-based assays. Among them, Ro 318220 and related bisindoylmaleimides are the most selective PKC inhibitors, however, these compounds inhibit at least two other entirely distinct protein kinase families with similar potency \textsuperscript{19}. Thus, conclusions drawn from their use in intact cells might have been, at least in part, misleading. Additionally, the PKC isotype-specific roles and selective downstream signalling pathways could not be resolved in these pharmacological panPKC inhibition studies.

In this study, we carefully investigate the crosstalk of PKA and PKC derived signals during primary CD3\textsuperscript{+} T cell activation using pharmacological inhibitors and activators of PKA, PDE4 and PKC plus our established panel of PKC knock out mice \textsuperscript{4,20-24}. Our results reveal a non-redundant physiological counteraction between the positive PKC\textsubscript{0} isotype and the negative cAMP/PKA signalling pathways in regulating the threshold of T cell activation. Importantly, multiple inhibitory and activatory systems result in consistent data of the critical roles of PKA and PKC\textsubscript{0}: PKA activation (via both PKA activator, Sp-8-Br-cAMP, and PDE4 inhibitor RP73401/piclamilast) plus PKC\textsubscript{0} inhibition (by PKC\textsubscript{0} gene ablation) augment T cell immuno-suppression as measured in TCR/CD28-induced IL-2 secretion responses of T lymphocytes \textit{in vitro}. Conversely, the reduced activation in panPKC inhibitor treated T cells can be rescued by pharmacological inhibition of the cAMP/PKA pathway. Mechanistically, our data resolve that this yin/yang PKA/PKC\textsubscript{0} signalling crosstalk selectively regulates NF-AT but not the distinct AP-1, CREB and NF-\kappa B transactivation pathways.
MATERIALS AND METHODS

Pharmacological inhibitors
8-pCPT-2’-O-Me-cAMP was purchased from Sigma-Aldrich and H89, Rp-8-Br-cAMP, Sp-8-Br-cAMP from Calbiochem, respectively. PDE4 LMWI RP73401/piclamilast, R0-31-8220, GF109203X and panPKC LMWI were provided by AltanaPharma, Konstanz. Antibodies against murine CD69, CD44, and CD25 were obtained from BD PharMingen, respectively.

Protein Kinase Scintillation Proximity Assay
Activity of recombinant overexpressed PKCθ constructs was assessed as described in Thuille et al., 2005. Baculovirus-expressed recombinant proteins were incubated in kinase assay buffer (40mM Tris pH 7.5, 40mM MgCl₂, 0.2mM HEPES pH 7.4, 0.2mM DTT, 0.0002% Triton X-100, 0.3mg/ml BSA) containing 1µM ATP, 2µCi [³³P-ATP], 3 µM of N-terminal biotinylated peptide (NH₂-RKRQRSMRRRVH-COOH), 1µM PDBu, 160µM phosphatidylserine. After 40min at room temperature, radioactivity was counted with the Wallac Micro β1450 (PerkinElmer).

Analysis of T cell Proliferation
Naive CD3⁺ T cells were purified from pooled spleen and lymph nodes with mouse T cell enrichment columns (R&D Systems). T cell populations were typically 95% CD3⁺, as determined by staining and flow cytometry. For anti-CD3 stimulations, T cells (5x10⁵) in 200µl proliferation medium (RPMI supplemented with 10% FCS, 2 mM L-glutamine and 50 units ml⁻¹ penicillin/streptomycin) were added in duplicates to 96-well plates precoated with anti-CD3 antibody (clone 2C11, 10 µg ml⁻¹). Alternatively PDBu (10 ng ml⁻¹; Sigma) plus Ca²⁺ ionophore (ionomycin, 125 ng ml⁻¹; Sigma) was used. Where indicated soluble anti-CD28 (1 µg ml⁻¹; BD Bioscience) was added. Cells were harvested at 64 hrs after a 16 hrs pulse with [³H]thymidine (1 µCi/well) on filters and the incorporation of [³H]thymidine was measured with a Matrix 96 direct β counter system. Results shown were the mean ± SD of at least three independent experiments.

Analysis of IL-2 Production
IL-2 produced from the proliferating CD3+ cell cultures (see above) was determined by ELISA (QuantikineM; R&D Systems) from the supernatant. Results shown were the mean ± SD of at least three independent experiments.

Western Blot Analysis

T cells were stimulated with medium alone (control), solid-phase hamster anti-CD3 (clone 2C11, 10 µg ml⁻¹), with or without hamster anti-CD28 (1 µg ml⁻¹; clone 37.51; BD Biosciences) at 37°C for various time periods. Protein lysates were subjected to Western blotting as previously described ⁴, using antibodies against PKCθ (BD/Signal Transduction), NF-ATc (Affinity BioReagents), CREB, pCREB (Cell Signalling), DNA Polymerase δ (Santa Cruz). All experiments have been performed at least three times with similar outcomes.

Gel Mobility Shift Assays

Nuclear extracts were harvested from 2x10⁷ cells according to standard protocols as previously described ⁴. Extract proteins (2 µg) were incubated in binding buffer with [³²P]-labeled, double-stranded oligonucleotide probes (NF-κB: 5'-GCC ATG GGG GGA TCC CCG AAG TCC-3'; AP-1: 5'-CGC TTG ATG ACT CAG CCG GAA-3'; NF-AT 5'-GCC CAA AGA GGA AAA TTT GTT TCA TAC AG-3';) (Nushift; Active Motif). All experiments have been performed at least three times with similar outcomes.

Statistical analysis

Statistical analysis was done with the statistic environment R (www.cran.r-project.org) as described by ⁵⁵,⁵⁶.

RESULTS:

Activation of resting T lymphocytes (resulting in IL-2 cytokine secretion and subsequent proliferation) is known to require stimulation of the TCR/CD3 complex (plus the CD28 auxiliary receptor) or treatment with phorbol ester (a potent PKC agonist) plus Ca²⁺ ionophore. In this study we investigated potential crosstalk between the distinct second messenger systems, DAG and cAMP, with regard to the activation of CD3+ T lymphocytes in vitro.
Pharmacological targeting of the PKA and the PKC pathways during CD3⁺ T cell activation. For evaluating the PKC functions, we initially used panPKC low molecular weight inhibitors (LMWI) (18 and not shown). The obtained IC₅₀ values on the baculo-expressed isolated enzymes in vitro were listed in Figure 1A in direct comparison to the commonly used panPKC LMWI GF-109203X and Ro-31-8220. Consistently with published reports, the panPKC LMWI was able to significantly reduce activation-induced proliferation in purified mature mouse CD3⁺ T cells, isolated from spleen and lymph nodes at a concentration of 500 nM (Figure 1B). The decrease in proliferation, observed in panPKC LMWI treated T cells, stimulated with anti-CD3 plus anti-CD28 was accompanied by a nearly complete reduction in the level of secreted IL-2 (Figure 1C) Thus, and similar to the PKCθ⁻/⁻ T cells 3-5, pharmacological inhibition of PKC results in partially defective IL-2 cytokine production and cell cycle progression in vitro (Figure 1 and 18).

In order to investigate the cAMP/PKA and cAMP/Epac pathways we used the established inhibitor (Rp-8-Br-cAMP, 27) and two specific activators (Sp-8-Br-cAMP and PDE4 LMWI/piclamilast, which is significantly more potent than rolipram 28) of the cAMP/PKA pathway. Sp cAMP, an established cAMP agonist of the cAMP/PKA pathway 8, thereby simply bypasses the generation of endogenous cAMP. As reported previously 17, the Sp cAMP isomer was able to abrogate activation induced proliferation and IL-2 secretion in response to anti-CD3 and anti-CD28 stimulation of primary CD3⁺ T cells (Figure 2 A&B) at the standard concentration of 250 μM. Treatment with the potent PDE4 LMWI at a concentration of 5 nM, which more physiologically increases endogenous cAMP levels in intact cells, also significantly repressed T cell proliferation and IL-2 production (Figure 2 A&B). In contrast, Rp cAMP, a direct competitor of cAMP, that antagonistically interacts with PKA, had no inhibitory effect at the standard concentration of 1000 μM, but rescued the effect of the PDE4 LMWI in proliferation and IL-2 secretion (Figure 2 A&B). This was consistent with the function of the Rp cAMP isomer as potent PKA inhibitor. H89, an extensively used enzymatic PKA inhibitor, was not able to rescue PDE4 LMWI effects, indicating lack of this PKA inhibition in intact cells (data not shown, 29). Therefore Rp cAMP but not H89 was used in this study.

Additive effects of PKC and PKA modulations in primary CD3⁺ T cells. Each, the PKA activating compounds (Sp-8 cAMP isomer and PDE4 LMWI) as well as the panPKC LMWI significantly reduced proliferation and IL-2 production in activated CD3⁺ T cells (see Figure 1
Yet, when applied in combination, PDE4 LMWI and panPKC LMWI together acted marginally more effective in inhibiting activation of primary CD3\(^+\) T cells than each compound by itself (Figure 3 A&B). This indicated that inhibition of PKC and activation of PKA signalling may additively augment CD3\(^+\) T cell inhibition processes.

Since such a potential PKC/PKA crosstalk has been delineated earlier employing pharmacological inhibition studies, we here now aimed to investigate TCR/CD3 signalling functions in our established panel of PKC isotype-deficient T cells in combination with the very selective PDE4 LMWI\(^{28}\) as cAMP/PKA pathway activator. Both, PKC\(\theta\)\(^{4}\) and, as very recently observed by us PKC\(\alpha\)\(^{24}\), are shown to take non-redundant and critical parts in signalling pathways necessary for full antigen receptor mediated T cell activation \textit{in vitro} and T lymphocyte-dependent immunity \textit{in vivo}. In contrast, PKC\(\beta\), \(\varepsilon\), \(\delta\) and \(\zeta\) had been recently shown by us to be dispensable in T cell signalling\(^{20-23}\).

PKC\(0^{-/-}\) T cells already intrinsically demonstrate reduced IL-2 secretion\(^{4}\). Therefore and for direct comparison of CD3/CD28 induced IL-2 production, the DMSO control of wild-type (wt) and PKC\(0^{-/-}\) deficient T cells was normalized to each other and set as 100%. The titration curve of PDE4 LMWI demonstrates a strong additive effect of PDE4 LMWI in PKC\(0^{-/-}\) deficient T cells (Figure 4 A). This was especially obvious at suboptimal concentration of PDE4 LMWI. Consistently, when using even such suboptimal concentration as 0.4 nM, PDE4 LMWI this selectively augmented the defects in IL-2 production of PKC\(0^{-/-}\) deficient T cells (Figure 4 D&E). Activation-induced IL-2 secretion was completely abrogated in the PKC\(0^{-/-}\) deficient CD3\(^+\) T cells, once tested in combination with the PDE4 LMWI at 0.4 nM, while no significant effect could be observed in the wild-type T cell controls. Of note, CD28 costimulation not only enhanced the level of anti-CD3 mAb-induced IL-2 secretion but also mostly overcame the immunosuppressive effects of the cAMP elevating agents in wild-type T cells. The order of potency of the stimulatory signals in reversing the inhibitory effects of cAMP-elevating agents on induced IL-2 secretion was anti-CD3/anti-CD28 (app. 5% inhibition) >> anti-CD3 (app. 85% inhibition) (Figure 4 and not shown). Thus, this difference in the ability of the wild-type versus PKC\(\theta^{-/-}\) T cells to be inhibited by PDE4 LMWI was only observed in physiologically more relevant anti-CD3/anti-CD28 double stimulation conditions. Activation-induced proliferation rates in both genotypes, however, were less significantly affected by the simultaneous combination with the PDE4 LMWI (Figure 4 B&C). Taken together, the
complete abrogation of CD3/CD28-induced IL-2 secretion in PKCθ-deficient CD3+ T cells was achieved by a simultaneous co-activation of the cAMP/PKA pathway.

TCR-induced S-phase entry of resting T cells is known to promote transcriptional upregulation of both IL-2 but also IL-2 receptor α-chain (CD25) genes, thereby constituting the autocrine cycle of IL-2 cytokine and its high-affinity receptor. However, albeit treatment with PDE4 LMWI slightly reduces CD25 and CD69 expression in activated CD3+ T cells, no additive effect of PDE4 LMWI and PKCθ gene ablation was observed. PDE4 LMWI treatment of PKCθ-deficient T cells induced comparable CD25 and CD69 surface expression when compared to wild-type T cells (Figure 5 A&B and data not shown). Consistently, CD3/CD28-ligation induced expression of CD25 (as well as the activation marker CD69, not shown) was reported to be normal in PKCθ-/- T cells. This indicated that the PKA/PKCθ crosstalk acts in a unique pathway specific for TCR-mediated IL-2 expression that is distinct from pathways utilized for CD25 and CD69 upregulation.

PKA activation in PKCθ-deficient T cells resulted in pronounced decrease of IL-2 secretion (Figure 4 D&E above). Importantly, however, no such combinatorial effect can be observed when employing the PKCα-deficient T cells. In response to either of the stimuli, neither proliferation nor IL-2 secretion of CD3+ T cells from the PKCα-/- mice was further reduced in the presence of PDE4 LMWI relative to that of wild-type T cells (Figure 5 C-D). The normalized effects of PDE4 LMWI in % induction of CD3/CD28 induced proliferation and IL-2 production of CD3+ wild-type and PKCθ or PKCα deficient T cells (DMSO control was set as 100% in each genotype for direct comparison purposes) is shown. Similarly to PKCα-/- T cells, no significant additive PDE4 LMWI defect in neither proliferation nor IL-2 secretion were obtained with our PKCβ-/- T cells (22 and not shown). Consistently, when testing PKCe, δ and ζ KO lines (data not shown), none of these other PKC isotype were non-redundantly involved in the counter regulation of cAMP/PKA-mediated suppression of IL-2 responses. These genetic data clearly excluded any functional crosstalk of PKCθ, except PKCθ, with PKA in these pathways (Figure 4, 5 and not shown).

**Counteraction between the positive PKCθ and the negative cAMP/PKA pathways in primary CD3+ T cells.** cAMP/PKA and PKC pathways have been discussed as direct antagonists maintaining a balance for signalling responses downstream of the TCR.
Consistently, the effect of the panPKC LMWI was significantly rescued by an PKA inhibitor (Rp cAMP) (Figure 6 A&C), indicating that once the negative signals from PKA were missing the activated T cells can mostly overcome pharmacological PKC inhibition. However, Rp cAMP treatment by itself had no additional effect (see Figure 2 and not shown). Yet in PKC0−/− T cells, where no PKC0 protein is expressed, the PKA inhibitor Rp cAMP failed to rescue activation-induced IL-2 secretion (Figure 6 B&D). The PKA inhibitor Rp cAMP is only able to rescue the effect in panPKC LMWI treated wild-type, but not in the PKC0-deficient CD3+ T cells. This finding again clearly indicate that among the PKC family members in T cells, the PKC0 isotype plays the critical and non-redundant role in counteracting the cAMP/PKA negative signalling during T cell activation.

**PKC0/PKA crosstalk converge at the level of TCR-induced NF-AT activation.** To further elucidate the molecular basis of the functional PKC0/PKA interaction, we analyzed the NF-κB, AP-1, CREB and NF-AT pathways, known to be critical in TCR/CD28-induced IL-2 cytokine expression 30. As reported, electrophoretic mobility shift assays (EMSA) demonstrated a significant decrease in NF-κB, AP-1 and NF-AT activation in PKC0−/− T cells 3-5. Consistently, CD3/CD28 cross-linking failed to fully activate the NF-κB, AP-1 (Figure 7 A&B) and NF-AT pathways (Figure 8 A) in PKC0-deficient CD3+ cells. Yet, when employing five-fold more of nuclear extracts from the PKC0-deficient CD3+ cells, relative to the wild-type extracts, the residual CD3/CD28-induced DNA binding was clearly detectable (Figure 7 C for AP-1 and Figure 8 B for NF-AT).

As result, activation of the cAMP/PKA pathway (via PDE4 LMWI) did not augment defects of NF-κB (Figure 7 A) nor did it significantly enhance AP-1 transactivation defects in PKC0-deficient CD3+ T cells (Figure 7 C). Consistently, western blot analysis revealed no profound differences in activated phospho-ERK1/2 in wild-type and PKC0-deficient CD3+ T cells during PDE4 LMWI co-treatment (not shown). Yet, upon PDE4 LMWI treatment, an enhanced defect of NF-AT binding in PKC0−/− T cells had been reproducibly observed (Figure 8 B): PDE4 LMWI treatment reduced NF-AT transactivation below the detection limit only in PKC0-deficient CD3+ T cells. Consistently, upon PDE4 LMWI treatment nuclear translocation of NF-AT was further impaired in PKC0−/− T cells (Figure 8 C). The activation-dependent induction of the three prominent isoforms of NF-ATc in whole cell extracts were not altered in wild-type and PKC0−/− T cells, treated either with PDE4 LMWI or DMSO buffer control (not
shown), excluding signalling defects caused by simply different NF-AT expression levels. Taken together, this additive transactivation defect on NF-AT signalling was consistent with the reported role of PKA to modulate nuclear entry of NF-AT \(^{16}\) and with the observed additive effects of PKA activation in PKC\(^{0/–}\) T cells (Figure 5 & 6 above).

The PDE4 LMWI activates the cAMP/PKA plus the cAMP/Epac/Rap1 pathway. In order to address which of the two distinct pathways is affected, we used the cAMP isomer agonists, Sp-8Br-cAMP and 8-pCPT-2′-O-Me-cAMP, which selectively activate the cAMP/PKA or the cAMP/Epac/Rap1 pathways \(^{31}\), respectively. As result, the NF-AT transactivation of activated wild-type and PKC0-deficient T cells appears to be independent of the cAMP/Epac/Rap1 pathway under the experimental conditions used: When analysing Epac cAMP isomer (8-pCPT-2′-O-Me-cAMP) treated cells for TCR-induced NF-AT activation, no effect could be seen with this cAMP isomer (Figure 8 D and not shown), while NF-AT DNA binding was strongly reduced by the Sp cAMP isomer in parallel samples. As further control, we defined the phosphostatus of CREB as the marker of PKA activation employing immunoblotting: phosphorylation of CREB was found to be inducible upon CD3/CD28 stimulation (Figure 8 E) as well as by the treatment with Sp cAMP (but not Epac cAMP) in CD3\(^+\) T cells, consistent with an selective activation of the cAMP/PKA pathway. Importantly for our hypothesis, phosphorylation of CREB was found to be similarly induced in wild-type and PKC0-deficient T cells upon CD3/CD28 ligation (Figure 8 E). Sp cAMP (but not Epac cAMP) slightly enhanced the stimulus-induced phosphorylation of CREB, confirming the selective activity of these distinct cAMP isomers, Sp cAMP and Epac cAMP. Taken together, these experiments validate the cAMP/PKA crosstalk as the negative cAMP signalling pathways physiologically counteracting the antigen receptor induced NF-AT but not NF-κB, the AP-1 nor the CREB pathways.

**DISCUSSION**

On the basis of pleiotropic pharmacological inhibition studies, several examples of crosstalk between the cAMP/PKA and PKC pathways are described \(^{32-34}\). It appears strictly cell-type dependent, whether the crosstalk between PKA and PKC thereby counteracted or potentiate each other: In platelets and neutrophils, the PKC pathway activates cellular functions, such as
proliferation, but the cAMP signalling system antagonizes this activation. Conversely, in lymphoma cells, PKC activation potentiates cAMP production and stimulation of PKC and the cAMP/PKA pathway strongly activate binding of CREB to its DNA binding site, CRE. This enhancer element then positively modulates the expression of CREB-mediated activation genes. In primary T lymphocytes, however, cAMP is produced after stimulation, suggesting the cAMP-mediated repression of T cell activation following TCR/CD3 ligation represents a negative feedback control mechanism. Consequently, suppression of cAMP signalling is strictly required for a biological T-cell response. cAMP levels in the T cell are the results of adenylate cyclases which synthesize cAMP and phosphodiesterases (PDEs) that degrade cAMP. The PDE4 isotype thereby mainly controls cAMP levels during T cell activation.

The most intriguing result of our study is, that during TCR activation of primary CD3+ T cells, the positive and the negative signalling cascades of PKC\(\theta\) and PKA act in an yin/yang mode on the regulation of IL-2 expression. Consistently, combined increase in PKA and decrease in PKC0 activity (via gene ablation of PKC0, Figure 4), completely abrogated the IL-2 production in primary CD3+ T cells. Thus activating the cAMP/PKA pathway has an enhanced inhibitory effect on T cells, once the signalling cascade from the TCR is specifically disturbed by PKC0 loss-of-function. Conversely, panPKC LMWI-inhibited T cells, PKA inhibition via Rp cAMP mostly rescues IL-2 expression (Figure 6 C), since the residual PKC0 activity (inaccessible for the PKC inhibitor) then allows a sufficient signalling threshold to occur in the absence of the PKA antagonism. However and importantly, once no PKC0 at all is present in the activated cell (as in the T cells derived from the PKC0-KO mouse line), no positive PKC signalling is available. As a consequence, inhibition of the negative PKA signal then has no rescue effect (Figure 6 D). This is interpreted as strong evidence that PKC0 (but no other PKC isotype) non-redundantly attenuates PKA-mediated negative signalling in T-cells. Consistently, when testing other PKC KO lines (Figure 5 C-E and data not shown), we were able to conclude that no other PKC isotype, is non-redundantly involved in the counter regulation of cAMP/PKA-mediated suppression of IL-2 responses.

NF-AT is an established site of integration of multiple signalling pathways. Thus NF-AT ensures correct responses to complex biological stimuli. The cAMP/PKA type I pathway thereby is known to induce Csk activation, the major negative regulator of the Src-PTK Lck. Additionally, PKA has been reported to directly phosphorylate NF-AT and thus inhibits nuclear entry of NF-AT via association with 14-3-3 proteins. Consistently, overexpression of
NF-AT antagonizes the inhibitory effect of PKA on IL-2 gene expression. NF-AT is negatively regulated by several NF-AT kinases such as glycogen synthase kinase 3 (GSK3). These are constitutive NF-AT protein kinases that promote NF-AT nuclear export \(^{36,37}\), however, phosphorylation by GSK3 requires prior phosphorylation by a priming kinase such as PKA. Consistently, NF-AT mutants of the PKA phosphorylation sites lost 14-3-3 binding, translocated to the nucleus and became transcriptionally active \(^{38}\). In contrast, upon TCR stimulation, NF-AT is dephosphorylated by calcineurin, which is established to functionally cooperate with PKC\(^\theta\) \(^{39,40}\) in this pathway. This Ca\(^{2+}\)/Calcineurin/ PKC\(^\theta\) pathway leads to activation and nuclear entry of NF-AT and subsequent IL-2 production of the activated T cells. Thus, cAMP/PKA-mediated Csk activation (and subsequent abrogation of the PLC\(\gamma\)1-derived calcium fluxes, an upstream requirement of the Ca\(^{2+}\)/calcineurin/NF-AT activation) as well as the direct NF-AT protein phosphorylation (as priming kinase for GSK3), provide molecular mechanisms for the counteraction of the Ca\(^{2+}\)/Calcineurin/ PKC0/NF-AT signalling pathways.

Consistent with the negative role for cAMP/PKA in TCR signalling (reviewed in \(^9\)), PKC0 inhibition acts additive in the modulation of NF-AT (Figure 8). Combined increase in PKA (via PDE4 LMWI) and decrease in PKC0 activity (via gene ablation of PKC0), abrogated NF-AT nuclear translocation (Figure 8 C) and DNA-binding (Figure 8 B) in primary CD3\(^+\) T cells. As inhibitory effects of pharmacological elevations in cAMP in T cells may not strictly require PKA activity \(^{41}\), we tested the possibility that the cAMP-Epac-Rap1 pathway is responsible for a reduced NF-AT binding and subsequent reduced IL-2 production in wild-type CD3\(^+\) T cells (Figure 8 D). However, we could not observe a decreased NF-AT binding using this specific Epac cAMP isomer, 8-pCPT-2\'-O-Me-cAMP \(^{31}\). NF-AT binding was only reduced using the PDE4 LMWI or the specific PKA cAMP isomer, Sp-8-Br-cAMP. Conversely, the PKA/PKC0 signalling crosstalk had no significant effect on NF-\(\kappa\)B (Figure 7 A) and CREB transactivation pathways (Figure 8 E). Opposite effects on T cell signalling thresholds have also been correlated with opposite effects of PKA and PKC on the Raf/MEK/ERK pathway \(^{31}\). However, when analysing primary mouse CD3\(^+\) cells, no profound differences in activated phospho-ERK1/2 and AP-1 DNA binding activity (data not shown and Figure 7 C) in wild-type and PKC0-deficient CD3\(^+\) T cells during PDE4 LMWI co-treatment was revealed.

PKC0 and PKA, unlike the more proximally acting Lck and ZAP70 protein tyrosine kinases, may not serve as triggers of the immune response but instead may further refine lymphocyte signalling by acting as critical signal threshold regulators of NF-AT activation, i.e. as
rate-limiting steps in triggering a full T cell IL-2 production during antigenic stimulation. Full and sustained NF-AT must be maintained to induce an irreversible commitment to T cell activation, particularly at lower antigenic peptide concentrations. Along this line of argumentation, PKCθ-deficient T cells generate still detectable, albeit significantly reduced nuclear localization of NF-ATc family members (Figure 8 C). Given the importance of the sustained NF-AT translocation, this partial reduction appears to correlate with an inability of the stimulus to induce full IL-2 gene expression and T cell proliferation in these T cells.

Consistently, agents that raise PKA activity in T cells, such as PDE4 LMWI and Sp cAMP counteract PKCθ activation signalling by this mechanism (Figure 2 & 6). Given that upstream regulatory regions of many genes contain NF-AT binding sites, the described PKCθ and PKA inter-pathway integration may modulate a number of genes during an effective immune response. In deed, other cytokines, in particular IL-4 and IL-10, were found to be inhibited by PKA activation in these T cells (data not shown). This is consistent with recent reports that PKCθ is required for the development of a robust immune response controlled by TH2 cells.

Even so the antagonistic roles of PKA and PKCθ dependent signalling cascades are here defined, the exact biochemical mechanism of how positive PKCθ and the negative PKA signalling pathways regulate the fine-tuning of the critical NF-AT responses, however, is still speculative.

Conclusion:

In primary mouse CD3+ T cells, a bidirectional PKA/PKC control model exists. The isotype-selective combination of PKCθ inhibition and PKA activation completely abrogates the level of TCR/CD28-induced IL-2 expression. Mechanistically, activation of the cAMP/PKA pathway in combination with PKCθ gene ablation resulted in a selective block of NF-AT transactivation. Thus, PKCθ is the essential part of a physiological signalling cascade that is necessary to counteract the inhibitory cAMP/PKA pathway during sustained clonotypic T cell expansion. This antagonistic crosstalk between the PKCθ derived positive signals and the PKA derived negative signals may represent one mechanism of how the antigen-receptor dependent fine-tuning of the amplitude of T lymphocyte activation works. Consequently, a combined increase in PKA and decrease in PKCθ activity might serve as an innovative combinatorial drug treatment concept in order to manifest improved efficacy and selectivity in therapeutic options for different immune disorders.
Acknowledgments:

We are grateful to H. Dietrich, N. Krumböck and G. Böck (all from Innsbruck) for animal house keeping, expert technical assistance including FACS analysis, respectively. All experiments comply with the current laws of Austria. Correspondence should be addressed to G.B. (e-mail: Gottfried.Baier@uibk.ac.at).

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Figure legends:

**Figure 1. Impaired T-cell activation in panPKC LMWI treated cells:**

A) IC50 values of panPKC LMWI on isolated PKC isotypes in kinase assays and established controls as Ro-31-8220, GF109203x. B) Proliferative response and C) IL-2 production of purified naive CD3+ T cells in the presence of the panPKC LMWI inhibitor which reduces the proliferation (p = 0.016) and IL-2 secretion (p = 0.003) significantly. Cells were left unstimulated or were stimulated with anti-CD3 (precoated at a concentration of 10 µg ml-1), plus soluble anti-CD28 (1 µg ml⁻¹), as indicated, and analysis was done by using standard procedures. Results shown are the mean ± SD of at least three independent experiments.

**Figure 2. T-cell activation in cAMP isomer and PDE4 LMWI treated cells:**

A) Proliferative response and B) IL-2 production of purified mature CD3+ T cells in the presence of the inhibitors as specified. The PKA activators (SP-8-Br-cAMP 250 µM, PDE4 LMWI 5 nM) reduce the proliferation and the IL-2 production significantly p < 0.05. Cells were left unstimulated or were stimulated with anti-CD3 (precoated at a concentration of 10 µg ml⁻¹), plus soluble anti-CD28 (1 µg ml⁻¹), as indicated. Note the significant (p = 0.0005 in the proliferation assay; p = 0.015 in the IL-2 assay) rescue of the PDE4 LMWI effect by the Rp-8-Br-cAMP (the PKA inhibitor used at 1000 µM). Results shown are the mean ± SD of at least three independent experiments.

**Figure 3. Impaired Proliferation and IL-2 secretion in pharmacological co-treatment conditions:**

A) Proliferative response and B) IL-2 production of purified mature CD3+ T cells in the presence of inhibitors as specified. The panPKC LMWI and the PKA activator PDE4 LMWI reduce the proliferative and the IL-2 response significantly (p< 0.05). In combination the additive effect on proliferation of the PDE4 LMWI and the panPKC LMWI (p = 0.067) is even stronger but not significantly different to the single compounds. Cells were left unstimulated or were stimulated with anti-CD3 (precoated at a concentration of 10 µg ml⁻¹), plus soluble anti-CD28 (1 µg ml⁻¹) or PDBu/ionomycin as indicated. Results shown are the mean ± SD of at least three independent experiments.

**Figure 4. T-cell activation in PDE4 LMWI treated wild-type and PKCθ-/- T cells:**
A) Titration curve of the PDE4 LMWI with normalised % of CD3/CD28 induced IL-2 production of CD3+ wild-type and PKC0-deficient T cells (DMSO control was set as 100% in each genotype). B+C) Proliferative response and D+E) IL-2 production of purified mature CD3+ T cells in the presence or absence of PDE4 LMWI inhibitor as specified. Cells were left unstimulated or were stimulated with anti-CD3 (precoated at a concentration of 10 µg ml⁻¹), plus soluble anti-CD28 (1 µg ml⁻¹) or PDBu/ionomycin as indicated, and analysis was done by using standard procedures. Results shown are the mean ± SD of at least three independent experiments. Inset: Western blot of CD3+ T cell lysates were immunostained for the endogenous PKC0 isotype and fyn, as indicated. Note the complete breakdown of IL-2 production in the PKC0−/− T cells when stimulated with anti CD3 plus anti CD28. The suboptimal low concentration of 0.4 nM of PDE4 LMWI reduces neither the proliferation nor the IL-2 production in the wild-type significantly, but does so in the PKC0−/− T cells (p = 0.0097 in the proliferation assay; p = 0.0015 in the IL-2 assay; t-test).

Figure 5. CD25 surface expression in PKCθ−/− and T-cell activation in PKCα−/− T cells is not affected by PDE4 LMWI:
A+B) Flow cytometric analysis of expression of CD25 wild-type PKCθ+/+ and PKCθ−/− T cells: Single-cell suspensions of purified mature CD3+ T cells, stimulated or not for 16 hrs with anti-CD3 and anti-CD28, were stained with anti-CD25. Percentages of positive cells are indicated. Experiments were repeated at least three times with similar results. C+D) IL-2 production of purified mature CD3+ T cells in the presence of inhibitors as specified. Cells were left unstimulated or were stimulated with anti-CD3 (precoated at a concentration of 10 µg ml⁻¹), plus soluble anti-CD28 (1 µg ml⁻¹) or PDBu/ionomycin as indicated. Results shown are the mean ± SD of at least three independent experiments. Inset: Western blot of CD3+ T cell lysates were immunostained for the endogenous PKCα isotype and fyn, as indicated. E) Effects of PDE4 LMWI (at 5 nM) on relative % of CD3/CD28 induced proliferation and IL-2 production of CD3+ wild-type and PKCθ or PKCα deficient T cells (DMSO control was set as 100% in each genotype for direct comparison purposes). Basically similar results have been obtained with PDE4 LMWI at 0.4 nM (not shown). Statistical analysis of three experiments is shown.

Figure 6. Proliferation and IL-2 secretion in co-treatment conditions in wild-type and PKCθ−/− T cells:
A+B) Proliferative response and C+D) IL-2 production of purified mature CD3+ T cells in the presence of inhibitors as specified. The panPKC LMWI reduces the proliferation of wild-type
but not PKCθ⁻/⁻ T cells significantly (p < 0.05), whereas the IL-2 production is significantly reduced in both genotypes. Cells were left unstimulated or were stimulated with anti-CD3 (precoated at a concentration of 10 µg ml⁻¹), plus soluble anti-CD28 (1 µg ml⁻¹), as indicated. Arrows mark the rescue result occurring in wild-type PKCθ⁺/⁺ (significantly in the IL-2 assay; p = 0.037) but not PKCθ⁻/⁻ T cells. Results shown are the mean ± SD of at least three independent experiments.

Figure 7. EMSA analysis of NF-κB & AP-1 in wild-type and PKCθ⁻/⁻ T cells:
A) Nuclear extracts were prepared from purified mature CD3⁺ wild-type and PKCθ⁻/⁻ T cells, stimulated for 16 hrs with medium alone or plate-bound anti-CD3 plus soluble anti-CD28 as indicated. Gel mobility shift assays were performed using radiolabeled probes containing either (A) NF-κB or (B+C) AP-1 binding site sequences. (C) To study potential additive effects in PKCθ-deficient T cells, five-fold more PKCθ⁻/⁻ nuclear extracts had been employed. The specificity of p50 NF-κB as well as fos was confirmed by supershifting the electrophoretic mobility shift with antibodies, as indicated by the arrow. Experiments were repeated at least three times with similar results.

Figure 8. EMSA and nuclear translocation analysis of NF-AT in wild-type and PKCθ⁻/⁻ T cells:
Nuclear extracts were prepared from purified mature CD3⁺ wild-type and PKCθ⁻/⁻ T cells, stimulated for 16 hrs with medium alone or plate-bound anti-CD3 plus soluble anti-CD28 in combination with the PDE4 LMWI, the Sp-8-Br-cAMP and the Epac specific activator (8-pCPT-2'-O-Me-cAMP), as indicated. In EMSA (A, B, D), the specificity of NF-ATc was confirmed by supershifting the electrophoretic mobility shift with antibodies, as indicated by the arrow. To study potential additive effects employing EMSA methodology in PKCθ-deficient T cells, five-fold more PKCθ⁻/⁻ nuclear extracts had been employed (B), as PKCθ gene ablation already strongly reduced NFAT levels (A). Activation-induced translocation of NF-AT or phosphorylation of CREB was determined by immunoblotting of nuclear extracts (C and E) for NF-ATc, (p)CREB and panCREB, respectively, as indicated. Experiments were repeated at least three times with similar results.
Abbreviations

cAMP; cyclic adenosine monophosphate
DMSO, dimethyl sulfoxide;
EMSA, electrophoretic mobility shift assays;
LMWI, low molecular weight inhibitor;
IL-2, interleukin 2;
PDBu, phorbol-12,13-dibutyrate;
PDE, phosphodiesterase;
PKA, protein kinase A;
PKC, protein kinase C;
TCR, T cell receptor;
wtt, wild-type.
Figure 1

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<th>PKC-α IC50 (μM)</th>
<th>PKC-β IC50 (μM)</th>
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A

B

C

\[ \text{DMSO Contr.} \]

\[ \text{PanPKC LMW1 500 nM} \]
Figure 2

A

[^H] thymidine uptake [pmol x 10^6]

DMSO Contr.
RP cAMP 1000 μM
SP cAMP 250 μM
PDE4 LMWI 5 nM
PDE4 LMWI + RP cAMP

Medium CD3 + CD28

B

pg/ml IL-2

DMSO Contr.
RP cAMP 1000 μM
SP cAMP 250 μM
PDE4 LMWI 5 nM
PDE4 LMWI + RP cAMP

Medium CD3 + CD28
Figure 4

A

B

PKCθ+/+

3H thymidine uptake

Bq/mg x 10^6

Medium CD3 CD3 +

PKDθ+/+

CD28 PDBu + IONO

PKCθ-/-

C

Medium CD3 CD3 +

CD28 PDBu + IONO

D

PKCθ+/+

Il-2

Kids/mg

Medium CD3 CD3 +

CD28 PDBu + IONO

PKCθ-/-

E

Medium CD3 CD3 +

CD28 PDBu + IONO

PKDθ+/+

PKDθ-/-

DMSO Ctrl.

PDE4 LMW 0.4 nM

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Figure 5

**A**

PKCε<sup>+/+</sup>

- % of CD25<sup>+</sup> cells
- X-axis: Medium, CD3 + CD28, PDBu + IONO
- Y-axis: 0 to 100

**B**

PKCε<sup>−/−</sup>

- % of CD25<sup>+</sup> cells
- X-axis: Medium, CD3 + CD28, PDBu + IONO
- Y-axis: 0 to 100

**C**

PKCα<sup>+/+</sup>

- pg/ml IL-2
- X-axis: Medium, CD3, CD3 + CD28
- Y-axis: 0 to 4000

**D**

PKCα<sup>−/−</sup>

- pg/ml IL-2
- X-axis: Medium, CD3, CD3 + CD28
- Y-axis: 0 to 4000

**E**

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A

- p50 supershift
- NFκ-B

B

- AP-1

C

- fos supershift
- AP-1

CD3/CD28: - + + + - + + + +
Figure 8

Hermann-Kleiter et al.

A

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NFATc supershift
NFAT

CD3/CD28
- + + - + +

B

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NFATc supershift
NFAT

CD3/CD28
- + + - + +

C

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NFAT

CD3/CD28
- + + + +

D

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NFATc supershift
NFAT

CD3/CD28
- + + +

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(μ)CREB
pan CREB

CD3/CD28
- + + - + +
PKCθ and PKA are antagonistic partners in the NF-AT transactivation pathway of primary mouse CD3+ T lymphocytes

Natascha Hermann-Kleiter, Nikolaus Thuille, Christa Pfeifhofer, Thomas Gruber, Michaela Schafer, Christof Zitt, Armin Hatzelmann, Christian Schudt, Michael Leitges and Gottfried Baier