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<td>Q1</td>
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<td>Kitamura and his group (1993) was changed to Kitamura and his group. Please check and correct if necessary.</td>
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Adrenomedullin receptors on human T cells are glucocorticoid-sensitive

Elisabetta Liverani *, Julie D. McLeod, Carolyn Paul

Faculty of Health and Life Sciences, Centre for Research in Biosciences, University of the West of England, Coldharbour Lane, Fenchay, Bristol, UK

► Examination of AM1 and AM2 receptor expression by human T lymphocytes. ► T cell receptor expression was affected by stimulation state. ► AM signaling pathways differed between T cell activation states. ► Glucocorticoids further polarize the stimulation-dependent AM receptor presentation in T cells. ► Glucocorticoids exerted greater control over AM receptor expression than AM.

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Adrenomedullin receptors on human T cells are glucocorticoid-sensitive

Elisabetta Liverani *, Julie D. McLeod, Carolyn Paul

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1. Introduction

Adrenomedullin (AM) is a novel vasodilatory peptide originally isolated from human pheochromocytoma by Kitamura and his group [9] that circulates in the plasma. Although AM is well known for its cardiovascular effects, AM production has been found to be high in the brain and the cerebral endothelia cells have been identified as a major source [1]. Indeed, AM has subsequently been classified as a neuropeptide [2,3], recognizing the peptide’s influence within the brain and its regulatory capacity at the blood–brain barrier [1].

AM effects are mediated through a G-protein-coupled receptor, calcitonin receptor-like receptor (CLR) [4], associated with receptor-activity-modifying protein (RAMP) 2 or 3. The CLR/RAMP2 receptor or AM1, is characterized by approximately 100-fold greater affinity for AM over other members of the peptide family [5], on the contrary CLR/RAMP3, or AM2, appears to discriminate less between AM and related peptides. RAMPs have been shown to play an important role not only in determining the ligand specificity of CLR, but also in mediating translocation of CLR from the endoplasmic reticulum to the cell surface [6,7]. Following AM binding to the AM receptor, adenylate cyclase protein kinase pathways are activated resulting in elevation of intracellular cAMP [8,9]. However, alternative signaling events such as elevated Ca2+ [9,10] and activation of endothelial NO synthase have been demonstrated [11]. Although there have been no reports showing differences in intracellular signaling via the two AM receptors, tissue distribution of RAMP2 and RAMP3 differs, as well as cell gene expression under physiological and pathological conditions, suggesting a separate role played by AM1 and AM2 [12].

Increases in plasma concentrations of AM are well documented in association with inflammatory and infectious disease states. Indeed, endothelial cells (EC) and vascular smooth muscle cells, as well as macrophages, monocytes and neutrophils augment AM production when exposed to IL-1, TNF-α and LPS [13]. Similarly, astrocytes, which can secrete AM under normal conditions, were shown to increase AM production after cytokine treatment (TNF-α, IL-1 and INF-γ) [14]. All of the above sources will have likely contributed to the inflammatory properties have also been attributed to this peptide: Wong et al. (2005) reported that AM markedly increased IL-6 expression in fibroblasts, although this was in contrast with Kubo et al. (1998), who reported a reduction in IL-6 production by LPS-activated macrophages following AM treatment, indicating a cell-dependent effect [16,17]. However, AM could clearly influence other macrophage cytokine expression, down-regulating its own inducer TNF-α, indicating a further anti-inflammatory effect during inflammation [18]. Importantly, AM has also shown its ability to reduce inflammation level, in a variety of animal models: in experimental arthritis where it successfully reduced both incidence and severity of disease [19] and in two different models of sepsis by decreasing levels of immuno-inflammatory mediators [20].

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Glucocorticoids (GC) are the best-known immunosuppressant, exerting an important role during the inflammatory process [21]. Interestingly, an interaction between AM and GC has been proven in a variety of cell types including cultured rat ventricular myocytes [22], human vascular EC [23] and T98G human glioblastoma cells [24]. Treatment with the synthetic GC dexamethasone (Dex), increased the secretion of AM in both vascular EC and glioblastoma cells in a dose-dependent and time-dependent manner. Interestingly, a dose-dependent GC-mediated up-regulation of AM concentration and expression was observed in the lung [25]. Also hormones have been shown to influence AM and AM receptor levels such as for example thyroid hormone which appears to directly up-regulate AM mRNA expression in rat EC and vascular smooth muscle cells [26]. However, no previous findings have analyzed how GCs affect AM, AM1, and AM2 expression and hence AM-sensitivity in T cells upon stimulation.

Previous studies detected RAMP2 and CLR mRNA expression in the Jurkat leukemia cell line and primary T cells [27], but no further investigations were conducted on RAMP3 or on these receptor components at a protein level. In order to clarify AM’s role during inflammation, the purpose of our research has been to assess the protein expression of AM receptor components in T cells. To accomplish this aim, expression of AM receptor proteins RAMP2, RAMP3 and CLR was investigated in a T cell line and human primary CD3+T cells before and following activation. Furthermore, we assessed AMP2, RAMP2, and CLR sensitivity to AM and GC exposure. Our results underline the importance of AM in the inflammatory process, suggesting that AM1 and AM2 expression and functionality are closely related to the T cell activation state, as is the influence exerted by GC’s on T cell AM-sensitivity.

2. Materials and methods

2.1. Cell culture

Fresh PBMCs were prepared from heparinized blood of healthy volunteers by Ficoll density gradient centrifugation [Axis-Shield PoC AS] and CD14+ PBMCs were isolated using a monocyte isolation kit [Miltenyi Biotech] with magnetic separation. CD14+ PBMC were maintained at 37 °C and 5% CO2 in RPMI 1640 media [Sigma-Aldrich], fully supplemented with penicillin-streptomycin (0.8 mM) [Sigma-Aldrich], Amphotericin B (0.03 μM) [Sigma-Aldrich] and glutamine (2 mM) [Sigma-Aldrich]. For activation, the T cell fraction (1×10⁶ cells/ml) was incubated with 5 μg/ml Phytotemagglutinin (PHA) [Sigma-Aldrich] for 48 h. The Jurkat T cell line was maintained in fully supplemented RPMI media at 37 °C and 5% CO2.

2.2. Treatments

Cells were treated with human Adrenomedullin (AM – 10⁻⁶ M) [Bachem] or Dexamethasone (Dex – 10⁻⁶ M) [Sigma-Aldrich] or AM/Dex (10⁻⁵/10⁻⁷ M respectively) or AM plus AM antagonist (human AM 22–52 [Bachem] 10⁻⁵/10⁻⁶ M respectively) in fully supplemented media and incubated for 24 h. Control cells received an equivalent amount of vehicle.

2.3. Flow cytometry analysis

Unstimulated and PHA-stimulated T cells were stained for T cell surface marker CD3 plus either RAMP2, RAMP3 or CLR using antibodies successfully applied previously [5,22]. Cells were firstly incubated with anti-CD3 antibody directly conjugated with Phycoerythrin (PE) [eBiosciences] and then fixed with 1% paraformaldehyde in PBS with addition of 0.1% saponin [Sigma-Aldrich], if permeabilized. Thereafter, cells were incubated with either primary antibody anti-RAMP2, anti-RAMP3 or anti-CLR [1:100 dilution; Santa Cruz Biotech] followed by a Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-goat IgG [1:50 dilution; DAKO]. Cells were acquired using a Becton Dickinson FACS Vantage cell sorter and analyzed with CellQuest software. Values are expressed as geometric mean of fluorescence intensity (GMFI).

2.4. Measurement of cAMP levels

Activation of adenylate cyclase was assessed by detecting the levels of cAMP using the direct cAMP enzyme immunoassay kit [Sigma-Aldrich]. Briefly, cells were incubated at 37 °C in the presence or absence of AM, Dex or AM–Dex co-treatment in supplemented media for 15 min and then lysed using 0.1 M HCl for 10 min, centrifuged at 600 g at room temperature, and the supernatant used directly in the assay. All samples were acetylated with the acetylating reagent and aliquoted into a 96-well plate, neutralized with the neutralizing reagent and treated with cAMP conjugate and CAMP antibody. After incubation at room temperature for 2 h, wells were washed three times, followed by incubation with substrate for 1 h at room temperature. The reaction was stopped, read at 405 nm and the measured optical density was used to calculate the concentration of CAMP.

2.5. Calcium mobilization assay

Cells were incubated with 2 M Fure2-AM ( Molecular Probes, Invitrogen) in assay buffer (13 mM Glucose, 10 mM Heps, 147 mM NaCl, 2 mM KCl, 1 mM MgCl2, 2 mM CaCl2, pH 7.3) supplemented with pleuronic acid (1 M, Invitrogen) at 37 °C for 1 h in the dark. Subsequently, cells were washed and AM (10⁻³ M) and AM/AM antagonist (equal concentrations 10⁻⁷/10⁻⁸ M respectively, as previously reported [1,23]). Ionomycin (1 M, Sigma-Aldrich) was used as a positive control. Mobilization of intracellular calcium was measured by recording the ratio of fluorescence emission at 510 nm after sequential excitation at 340 and 380 nm using NOVOSTar (BMG labtech, Aylesbury) microplate reader. Results were expressed as a % of the positive control response.

2.6. Real-time PCR amplification

Total RNA was isolated using Trizol [Invitrogen] and quantified by optical density at 260 nm. All primers were designed using Gene Fisher software package and synthesized by TAGN Ltd [Gateshead] except the primer for the housekeeping gene GAPDH which was synthesized by MWG-Biotech AG Oligo Production (Ebersberg) (Table 1). Primer pair annealing temperatures had been optimized during a series of preliminary studies (Table 1). RT-PCR was performed to obtain cDNA. The reaction was set up in a total volume of 10 μl containing 1× buffer (50 mM KCl, 10 M Tris–HCl pH 9 0.01% triton X-100), 25 mM MgCl2, 1 U Rnasin [Promega], 5 U MMLV [Promega], 0.5 mM dNTP [Promega], 0.5 g oligo dT per μg RNA and “common” sequence (5 μg per 1 μg of RNA; 5'- NNNNNNTTATTT-3') [Tagn], Thermal parameters were 23 °C for 5 min, 42 °C for 1 h, 37 °C for 1 h, 99 °C for 5 min and 4 °C for 5 min. Real-time PCRs were conducted using detection of IQ™ SYBR Green supermix [BioRad] fluorescence on a BioRad iCycler real-time PCR platform. Each real-time reaction contained primers (500 nM in each)

Table 1

<table>
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<tr>
<th>Gene</th>
<th>Primer sequences (5′-3′)</th>
<th>PCR product (bp)</th>
<th>Annealing T (°C)</th>
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<tr>
<td>AM</td>
<td>Sense: GCCACAGCAGACTCTACA</td>
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<tr>
<td></td>
<td>Antisense: CTTGTTGCTTAGACA</td>
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<td>RAMP2</td>
<td>Sense: CCAGATCCACTTTGCCAA</td>
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<tr>
<td></td>
<td>Antisense: CTTGTCCTTTCTCCA</td>
<td>150</td>
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<td>Sense: AGACAGCACTTGAGCA</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Antisense: TGGACGTGACCTGCCGTCA</td>
<td>160</td>
<td>59–61</td>
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2.5 µl each) (Table 1), BioRad Supermix (6.5 µl) and 50 ng of cDNA for a total volume of 13 µl. They were performed in Thermo-Fast semi-skirted 96-well microplates [Abgene] capped with optical caps [Abgene]. A single fluorescence measurement was taken at the end of the 72 °C for 20 s segment (amplification and quantitation step) and continuous fluorescence measurements were taken during the annealing step (50 °C for 30 s) and melting step (95 °C for 30 s).

The amount of cDNA was calculated relative to the fluorescence intensity of the amplified housekeeping gene GAPDH. Data were analyzed with the iCycler™ iQ, Optical System software [BioRad], by comparing the threshold cycle (Ct), at which the reporter dye emission intensities rose above background noise. The real-time amplified products were also analyzed by electrophoresis through a 2% Agarose [Geneflow Limited], containing ethidium bromide (20 ng/ml) and compared to 50 bp DNA marker [Invitrogen]. Gels were visualized on a 650 nm ultraviolet transilluminator and images taken with Gel Capture software (Siveton Scientific).

2.7. Statistics

Data was normally distributed, therefore statistical analysis was carried out using Student’s t-test, with p value less than or equal to 0.05 being taken as significant. All data are expressed as means ±/− standard error of the mean (SEM).

3. Results

3.1. RAMP2 and RAMP3 expression in Jurkat leukemic cell line

In order to better understand the T cell’s response to AM, expression of RAMP2 and RAMP3 was initially assessed in Jurkat leukemic cells, using flow cytometry and Real-time PCR. Cells were analyzed in permeabilized and unpermeabilized states, in order to discriminate between cytosolic and membrane locations. Jurkat cells demonstrated a higher expression of RAMP3 than RAMP2 on the cell surface (Fig. 1, p ≤ 0.05), while an increase in RAMP3 expression was noted intracellularly, although not significant. It is also worth noting that RAMP2 expression on the cell surface was significantly lower than intracellularly (Fig. 1, p ≤ 0.05) although a similar profile of mRNA expression for RAMP2 (Ct: 31.2 ± 0.6) and RAMP3 (Ct: 35.6 ± 0.8) was reported at a basal level, compared with the housekeeping gene GAPDH (Ct: 20.5 ± 0.8).

Changes in T cell sensitivity to AM through receptor expression was analyzed, following either AM or Dex exposure for 24 h (Fig. 3). AM treatments (10−6 M) significantly decrease RAMP2 expression on the cell surface in PHA-stimulated T cells (Fig. 3A, p ≤ 0.05) while intracellularly, a decrease in RAMP3 was observed in unstimulated cells (p ≤ 0.05). No differences were noted in CLR for any of the conditions analyzed.

Dex exposure (10−6 M) affected the cell surface expression of all AM receptor components analyzed showing opposite effects upon PHA stimulation (Fig. 3B). Indeed, an increase in RAMP2, RAMP3 and CLR was observed in unstimulated T cells (p ≤ 0.05), while conversely a decrease in all proteins was noted for treated stimulated T cells (p ≤ 0.05). Intracellularly, only RAMP3 expression was altered by exposure to the GC, demonstrating an increase for stimulated cells. A single

3.4. AM stimulation of cAMP production and Calcium mobilization in T cells

In order to gauge AM receptor functionality, a preliminary assessment of cAMP production was conducted in response to AM (10−6 M), Dex (10−6 M) or AM-Dex (both 10−6 M) exposure for 15 min in unstimulated and PHA-stimulated T cells. In unstimulated T cells (Fig. 4A), Dex and AM-Dex co-treatments elicited cAMP outputs that were significantly lower than control (n = 3, p ≤ 0.05), but not different to each other. Indeed AM (10−6 M) alone produced no significant change from control cAMP. However, in stimulated T cells AM administration elevated cAMP production above control levels, signifying that stimulation alters AM signaling capabilities in T cells (Fig. 4B) (n = 3, p ≤ 0.05). Both AM and Dex appeared to increase cAMP production to a similar degree and no further augmentation was observed when co-administered.

Further to this, Ca2+ mobilization was measured following AM (10−6 M) treatment alone and when co-administered with its antagonist, AM 22−52 (10−6 M) (Fig. 4C). Results are shown as a percentage of the values observed after Ionomycin (1 M) addition. After AM exposure, Ca2+ release appeared to be significantly higher than when AM and AM antagonist were added at the same time (p ≤ 0.05). Assessment of calcium mobilization determined that AM (10−6 M) generated a large calcium response within both stimulated and unstimulated T cells (Fig. 4C), which was greater in those PHA-stimulated than when AM and AM antagonist were added at the same time (p ≤ 0.05). Moreover, this calcium response was significantly attenuated by co-administration of peptide antagonist AM 22−52 (p ≤ 0.05).

4. Discussion

In order to pursue our aims, we firstly characterized all the AM receptor component expression in T cells, which was accomplished by

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Although intracellular RAMP levels are not hence CLR, RAMP2 and RAMP3 production in primary human CD3+ T cells has a known ability to regulate its receptor components [26, 30], while in an alternative model of renal failure [31] RAMP2 and RAMP3 expression of RAMP3 and CLR was lower than that of healthy kidneys compared to the remnant kidneys of rat with mass ablation where the expression cells were exposed to [12]. For example, in calcified VSMC [29], compared to the remnant kidneys of rat with mass ablation where the expression of RAMP3 and CLR was lower than that of healthy kidneys [30], while in an alternative model of renal failure [31] RAMP2 and CLR were shown to be strongly up-regulated.

AM has a known ability to regulate its receptor components [26], hence CLR, RAMP2 and RAMP3 production in primary human CD3+ T cells was investigated following 24 h treatment with AM. Considering increased AM levels during inflammation [13, 32] and hypoxia conditions [17, 27], a pathological concentration of 10^{-6} M was selected in line with previous experiments on the blood–brain barrier [33] and cerebral endothelial cells [1]. A significant decrease in CLR and RAMP3 surface expression followed 24 h PHA stimulation, while intracellular reductions in receptor components were not significant (n = 4; \( p \leq 0.05 \) compared to unstimulated cells). GMFI of the secondary antibody control for unpermeabilized and permeabilized cells was respectively 73±7.1 and 119±10.1. Real-time PCR studies indicated RAMP3 mRNA levels to be lower than RAMP2, AM and the housekeeping gene in T cells both before and following stimulation (n = 3).

<table>
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<tr>
<th>Marker</th>
<th>GAPDH</th>
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<th>RAMP3</th>
<th>AM</th>
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<td>24.8± 1.1</td>
<td>26.3± 0.8</td>
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<td>Ct-PHA-stimulated ± SEM</td>
<td>23.1± 0.9</td>
<td>24.1± 0.3</td>
<td>27.4± 1.3</td>
<td>27.4± 1.5</td>
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### Q3

Firstly analyzing RAMP2, and RAMP3 at mRNA and protein levels in the Jurkat T cell line and in primary human CD3+ T cells before and after PHA stimulation under normoxic conditions. Our results suggest a different distribution for AM receptors in Jurkat T cells with AM1 being primarily located intracellularly while AM2 is situated on the cell membrane, as previously reported for astrocytes [28] and cerebral endothelial cells [1]. Although intracellular RAMP levels are not significantly different, data suggest AM2 as the predominant AM receptor in Jurkat cells, with a tendency towards increased intracellular expression, which may be biologically important. On the contrary, in primary human T cells a differential expression for RAMP2 and 3 was observed upon PHA stimulation of primary T cells with a reduction being seen only on the cell surface. When mRNA investigation were carried out, we could not detect any difference in RAMP mRNA levels between PHA-stimulated and unstimulated cells, suggesting that receptor expression is regulated locally. This observed decrease in AM2 receptor indicates a decrease in AM-sensitivity that appears to distinguish stimulated T cell phenotype from their unstimulated counterpart. Previous investigations on alternate cell systems have also clearly shown differences in RAMPs and CLR expression depending on the condition cells were exposed to [12]. For example, in calcified VSMC all AM were up-regulated in calcified versus control VSMC [29], compared to the remnant kidneys of rat with mass ablation where the expression of RAMP3 and CLR was lower than that of healthy kidneys [30], while in an alternative model of renal failure [31] RAMP2 and CLR were shown to be strongly up-regulated.

AM has a known ability to regulate its receptor components [26], hence CLR, RAMP2 and RAMP3 production in primary human CD3+ T cells was investigated following 24 h treatment with AM. Considering increased AM levels during inflammation [13, 32] and hypoxia conditions [17, 27], a pathological concentration of 10^{-6} M was selected in line with previous experiments on the blood–brain barrier [33] and cerebral endothelial cells [1]. Our study revealed a modest activation-dependent down-regulation of RAMP2 and RAMP3 following exposure to AM, while RAMP2 altered intracellularly in unstimulated cells. The relevance of these subtle RAMP2 and 3 changes in response to elevated AM in the cellular environment requires investigation, however altered sensitivity to AM may assist cells in recognizing an inflammatory environment [15, 34] or contribute to a protective autocrine mechanism [27, 35]. Furthermore, the apparent association of certain RAMPs, and hence receptors, with particular stimulation states is of interest, as investigated in other cell types and conditions [12] such as up-regulation of only RAMP3 was reported in rat lungs [36], while up-regulation of CLR, RAMP2 and RAMP3 was detected in rat heart [37].

GCs have always played an active part in the physiological homoeostatic response to inflammation, being a fundamental component of the recovery phase. Interestingly CLR, RAMP2 and RAMP3 were down-regulated in PHA-stimulated cells following Dex treatment, indicating that cells were rendered much less responsive to AM and hence possible changes in their environment. On the other hand, GCs could up-regulate both AM1 and AM2 in the non-stimulated cell population, drastically increasing their AM-sensitivity. Therefore, GCs seem to further polarize the AM receptor profile of the unstimulated and stimulated T cell populations, whereby stimulation, and more so GC-modulation of stimulated cells, reduces the availability of AM receptors on the cell membrane. Such GC-sensitive AM receptor presentation...
is in agreement with previous studies, as for example in osteoblastic cells where RAMP2 and RAMP3 changes were reported following incubation with Dex for 10 h [38]. Considering AM anti-inflammatory properties previously shown [19,20], it is surprising to notice that these anti-suppressants would decrease T cell sensitivity to the peptide. However, other work attributes both pro- and anti-inflammatory effects to AM most likely based on the peptide’s concentration [17], indicating that AM also plays a role in regulating inflammation rather than only enhancing or suppressing it. Moreover, studies by Makino et al. (2003) have clearly demonstrated AM’s contribution to protect T cell ability to perform under hypoxic conditions [27]. Therefore, by reducing the available AM receptors in sensitized T cells, GCs may be acting to limit this protective function. All considered, we believe that influencing cell sensitivity to AM via receptor availability rather than its concentration could be a mechanism through which it is possible to regulate the inflammatory process. Furthermore, decreasing sensitivity may help to start the recovery phase, as GCs could do in this case. Hence a strong relationship between T cell activation state and the GC-mediated changes in AM receptor expression on the cell may point to an interesting and novel anti-inflammatory action of GCs.

AM has been shown to exert its effect through two independent signaling transduction pathways: cAMP accumulation after adenylate cyclase activation [9] and Ca²⁺ mobilization inducing Akt phosphorylation [39]. Our data showed for the first time that AM treatment could increase cAMP cellular levels in PHA-stimulated versus unstimulated T cells, in accordance with signaling mechanisms reported in endothelial cells.
In our study while Dex caused an increase in PHA-stimulated cells, it decreased further the already low cAMP levels in unstimulated cells, probably indicating apoptosis induction in stimulated cells (immunosuppressive activity) but not in their unstimulated counterparts. The ability of Dex to increase cAMP cellular levels in stimulated T cells supports previous observations that indicate an increase in cAMP levels as a mechanism through which Dex causes apoptosis in T cells, hence how it exerts its immunosuppressive activity [41,42]. Furthermore, co-treatment with AM and Dex did not augment increased cAMP levels in stimulated T cells or reduced cAMP levels in unstimulated T cells beyond that seen with individual treatments, suggesting either a possible competition for signaling cascades between the two mediators or that the cAMP responses elicited by the single treatments are already at peak levels and thus cannot be further increased.

In conclusion, our studies show key differences between stimulated and unstimulated T cells firstly in terms of their presentation of cell surface AM receptor proteins and secondly regarding the signaling functionality of those receptors and their responsiveness to external mediators. In particular, AM receptor presentation in T cells is GC-sensitive, which is highly dependent on stimulation state. The importance of the activation state-dependent sensitivity of the human T cell to this peptide and how this links to its protective capabilities under hypoxic conditions on the one hand and to the known anti-inflammatory properties of AM on the other, will require further consideration and provides an intriguing paradox to resolve.

Acknowledgments

We would like to thank all the donors who participated in this study, David Corry for his technical assistance in the flow cytometry experiments and Dr Ruth Morse for her help with molecular biology assays. We also thank Professor Mauro Perretti for hosting the calcium mobilization studies and Dr Vincenzo Brancalone and Stefania Bena for their assistance.

References


