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Stimulatory and suppressive signal transduction regulates vasoactive intestinal peptide receptor-1 (VPAC-1) in primary mouse CD4 T cells

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ABSTRACT

Vasoactive intestinal peptide receptor-1 (VPAC-1) is an anti-proliferative, G-protein coupled receptor that is highly expressed on naïve T cells, and has been reported to be downregulated upon T cell activation. The T cell signaling molecules involved in mediating low VPAC-1 levels have not been identified. Therefore, to gain a greater understanding into this regulation, this study investigated the signaling pathways that regulate (VPAC-1) in murine, primary CD4 T cells. To this end, murine, splenic CD4 T cells were pretreated separately with 10 different pharmacological inhibitors and incubated +/− anti-CD3 for 24 h. Total RNA was isolated, and VPAC-1 mRNA levels were measured by qPCR. Our results support that JNK kinases, downstream from the protein kinase, Zap70, are involved in suppressive regulation of VPAC-1 steady-state mRNA levels after anti-CD3 treatment. In contrast, inhibitors against PKC, ERK, p38, Zap70 and Rac1 supported a stimulatory influence in VPAC-1 regulation in the absence of T cell signaling. By studying the signaling pathways that regulate VPAC-1 in T cells, we can gain greater insight into the role of this anti-inflammatory receptor in autoimmunity and infectious diseases.

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1. Introduction

Vasoactive intestinal peptide receptor-1 (VPAC-1) and VPAC-2 are members of the glucagon/PACAP/secretin (GPS), class IIA, G-protein coupled receptor (GPCR) superfamily (Nussdorfer and Malendowicz, 1998). VPAC receptors are widely expressed in mammalian tissues, including the immune compartment (Dorsam et al., 2000; Johnson et al., 1996). Two GPS superfamily peptides bind two different VIP receptors (K_d = 1–3 nM), termed vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP), and couple through G_s, $G_{i/o}$, and G_q (O'Dorisio et al., 1981; Dorsam et al., 2000; Xia et al., 1996; Delporte et al., 1995; McCulloch et al., 2000, reviewed by Delgado et al. 2004). In T cells, VIP and PACAP are involved in various cellular functions including, proliferation (Ottaway and Greenberg, 1984; Wang et al., 2000), trafficking (Ottaway, 1984), cytokine expression (Tang et al., 1996; Martinez et al., 1996; Voice et al., 2001), apoptosis (Delgado et al., 2002) and adhesion (Johnston et al., 1994; Xia et al., 1996; reviewed by Delgado et al., 2004). During CD4 T cell activation, it has been shown that VPAC-1 expression is significantly downregulated (Lara-Marquez et al., 2000, 2001).

CD4 T cell activation is initiated through engagement of the T cell receptor (TCR). Antigen presenting cells (APC) activate CD4 T cells by binding the TCR with foreign peptide antigen presented within the major histocompatability complex (Kane et al., 2000). After TCR engagement, Src kinases Fyn and Lck, are the most proximal signaling molecules activated that phosphorylate ζ-chain Tyr residues within the TCR/CD3 complex. Hyperphosphorylation of these immunoreceptor tyrosine-activation motifs, in turn, recruits and activates additional kinases (Zap70), and adaptor molecules (LAT, SLP76) to the immunological synapse (Gupta et al., 2003). From this point, activation of several signaling cascades are prompted, including Ras (reticular activating system protein), PKC (protein kinase C), MAPK (mitogen-activated protein kinase) pathways (ERK, JNK and p38), calcium-dependent cascades, G-proteins (Rac1 and CDC42) and PI3-K (phosphatidylinositol 3-kinase; Clements et al., 1999; Samelson, 2002).

We have contemporaneously shown that VPAC-1 mRNA expression levels are negatively regulated by TCR signaling in primary CD4 T cells (Vomhof-DeKrey, et al., 2008). However, the TCR-signaling cascades responsible for regulating VPAC-1 expression are not known. Thus, if the TCR-signaling pathways that control VPAC-1 are determined, then relevant candidate trans-acting factors activated by these pathways might be identified that bind to and regulate the VPAC-1 gene. Importantly, phorbol esters have been observed to mimic the negative effect of TCR signaling on VPAC-1 expression in CD4 T cells and may suggest a PKC mediated mechanism (Lara-Marquez et al., 2000, 2001). This observation was used as a starting point for investigating the TCR-signaling cascades responsible for VPAC-1 regulation in T cells.

In this study, we have collected pharmacological evidence which supports a Zap70 → JNK signal transduction pathway as relevant contributors for negative VPAC-1 regulation during T cell activation.

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Also, there was a suppressive tendency for inhibitors against PKC, Rac1, and PI3-K toward VPAC-1 regulation. Surprisingly, we collected data supporting a lack of Ras involvement in VPAC-1 regulation during TCR signaling. In contrast, inhibitors against PKC as well as other signaling molecules including Zap70, Rac1, ERK, and p38 also supported a stimulatory regulation of VPAC-1 expression in the absence of TCR signaling. Collectively, these data have pharmacologically identified signaling pathways that positively and negatively regulate the anti-inflammatory VPAC-1 mRNA expression levels dependent on the presence of TCR signaling in CD4 T cells.

2. Methods

2.1. Reagents

1× PBS (Ca^{2+} and Mg^{2+} free, PBS), pyrogen free water, defined fetal bovine serum, 1 M Hepes, 40% Glucose, 1 M sodium pyruvate and penicillin/streptomycin/ampotericin B were purchased from Hyclone and RPMI 1640 media was from Cellgro. DNAse I, QIAshredder, RNeasy kits were obtained from Qiagen. Magnetic columns, 30 μM sieves, anti-CD4-labeled magnetic beads were purchased from Miltenyi. Antibodies against mouse CD3 and CD28 were obtained from Biolegend. DNA oligo primers and fluorescent probes were from Integrated DNA Technologies. Taqman 2X Universal Master Mix was obtained from Applied Biosystems Inc. DNAse-Free kits came from Ambion, and real time plates and covers from Fisher Scientific. Nuclease free water was purchased from Invitrogen. M-MLV reverse transcriptase, deoxyribonucleotides and random primers were purchased from Promega. Phorbol 12-myristate 13-acetate (PMA) and all pharmacological inhibitors were bought from Calbiochem. Phytohemagglutinin (M form; PHA-m) and all other reagents used were obtained from Sigma.

2.2. Mice

Wild type C57BL/6J mice were purchased from Charles River Hollister (Hollister, CA) or Jackson Laboratories (Bar Harbor, ME) and bred in a mouse facility at North Dakota State University. Mice were housed in a ventilated Nalgene ventilated cabinet (VWR) as described by the manufacturer. Mice cages, water bottles and steel lids were purchased from Jackson Labs. Spleens were harvested by standard dissection techniques in a clean UV irradiated PCR hood. All mouse protocols were approved by the NDSU IACUC board and met all federal guidelines.

2.3. T cell isolation, activation and pharmacological inhibitor studies

Each independent experiment used four to six male or female mice between the ages of 6–32 weeks. Mice were euthanized by CO_2 narcotization followed by rapid cervical dislocation. Harvested spleens were minced in PBS at RT, and dispersed splenocytes were passed through a 30 μm sieve. Erythrocytes were lysed with 1 ml/spleen of lysis solution (0.155 M ammonium chloride, 0.01 M potassium carbonate and 0.1 mM EDTA) for 1 min, diluted to 50 ml with PBS and centrifuged at 500*g* for 5 min. To remove adherent cells, splenocytes were placed in complete media (86% RPMI 1640, 10% FBS, 10 mM Hepes, 0.4% glucose, 1 mM sodium pyruvate and 1× penicillin/streptomycin/amphotericin B) for 1 h at 37 °C, 5% CO_2/95% air in a humidified incubator. Non-adherent splenocytes were passed through a 30 μm sieve, centrifuged as above, resuspended in 93 μl of PBS/0.5% BSA with 7 μl of anti-mouse CD4 magnetic beads/1 × 10^7 cells and refrigerated (4–8 °C) for 20 min. CD4 T cells were purified by a Miltenyi Auto-MACs instrument using the positive selection option. Cells were counted with a hemacytometer using 0.2% Trypan blue that showed ⩾90% cell survival and ⩾95% pure by FACS. Activation and pharmacological inhibitor studies used 1 or 4 × 10^6 CD4 T cells/ml/well in 24-well tissue culture plates using media alone, ⩽0.1% DMSO (vehicle control), 0.81–8.1 nM PMA, 4 μg/ml plate-bound anti-CD3 + 8.1 nM PMA, 1 or 4 μg/ml plate-bound anti-CD3 +/− indicated inhibitor concentration range unless otherwise noted, or 4 μg/ml plate-bound anti-CD3 + 2.5 μg/ml anti-CD28 +/− indicated inhibitor concentration. Cells treated with pharmacological inhibitors (see Table 1 for concentrations) were pretreated 15–30 min prior to anti-CD3 treatment. Cells were seeded at 1 × 10^6 cells/ml were plated in quadruplicate wells and pooled after incubation, while cells seeded at 4 × 10^6 cells/ml in a single well gave similar VPAC-1 data. Plate-bound anti-CD3 was prepared by incubating with complete media for ⩾2 h at 37 °C. After 24 h, cells were collected, centrifuged as above and used for total RNA isolation and qPCR analysis.

2.4. Fluorometric-based kinetic RT-PCR (qPCR)

Total RNA was isolated by sequential passes through a QIAshredder spin column followed by a Mini Prep RNeasy kit with on-column DNase I treatment as described by the manufacturer (Qiagen). Following total RNA elution using nuclease-free water, a second DNAse I treatment was performed using the DNA-Free kit or by a slightly modified procedure. Briefly, to each 50 μl total RNA eluant, 1 μl of DNase I, 6 μl of 10× PCR buffer and 3 μl of nuclease-free water was added and incubated for 45 min at 37 °C. DNase I was inactivated by a 69 °C incubation for 20 min. RNA was precipitated by the addition of 2.5 volumes of 100% ethanol and a 1/10 dilution of a 3 M sodium acetate solution. Samples were incubated at −80 °C for ⩾20 min, centrifuged at 18,000*g* at 4 °C for 15 min, washed with 1 ml 70% EtOH, air dried and reconstituted in 20 μl nuclease-free water. Some samples were purified by a second RNeasy column instead of EtOH precipitation as described by the manufacturer with similar results. Purified total RNA (⩽1–4 × 10^6 cells) was used to generate cDNA using reverse transcriptase and random primers as described by the manufacturer. Real time reactions contained 10 μl of cDNA template with 15 μl of a 2× master mix containing: 2× ABI master mix, 500 nM primers (mVPAC-1, 1888–1212 bp, forward, 5′- TTG GAG TTC ACT ATG TCA TGT TTG C-3′; mVPAC-1, 1245–1268 bp, reverse, 5′-CTA CGA CGA GTT CAA AGA CCA TTT-3′; mHPRT, 636–655 bp, forward, 5′-CTG GTG AAA AGG ACC TCT CG-3′; mHPRT, 719–744 bp, reverse, 5′-TGA AGT ACT CAT TAT AGC AAG GGC A-3′) and 400 nM of a 5′-labeled 6-carboxyfluorescein (FAM) and 3′-labeled quencher dye 6-carboxytetramethylrhodamine (TAMRA) labeled probe (mVPAC-1, 1218–1242 bp, 5′-FAM-TTG TGG TGG CCA TCC TCT ACT GCT TCC- TAMRA-3′; mHPRT, 659–687 bp, 5′-FAM-TGT TGG ATA CAG GCC AGA CTT TGT TGG AT- TAMRA-3′). Primer and probe sequences for VPAC-1 and HPRT were determined by PrimerExpress software. The VPAC-1 (Accession No. NM_011703) and HPRT (Accession No. NM_013556) qPCR amplicons were sequenced to confirm authenticity. Some reactions were conducted with total RNA in a one-step procedure with similar results. Reactions for both amplicons were run with nuclease-free water alone, and in the absence of reverse transcriptase to ensure ⩾6 cycle thresholds compared to reactions in the presence of reverse transcriptase. This would verify ⩽1.6% genomic DNA contamination in reactions as described by the manufacturer. The qPCR reaction was conducted using a 7500 ABI instrument with the following parameters: 2 min at 48 °C, 10 min at 94 °C, followed by 40 cycles of 15 s at 94 °C and 60 s at 60 °C. Three serially diluted cDNA samples (1/4) were measured in duplicate for mVPAC-1 and mHPRT to ensure similar amplification efficiency for all samples analyzed, and relative VPAC-1 levels calculated by the $\Delta\Delta C_t$ method. Relative $\Delta\Delta C_t$ values were used (highest and lowest values excluded) to obtain average ± SEM of relative mVPAC-1 levels normalized to mHPRT.

2.5. Statistical analysis

All data are presented as means ± SEM and experiments were conducted at least three independent times unless otherwise noted in the figure legend. Statistical significant values ($p \leqslant 0.05$) are noted in the figure legends by asterisk symbols. A two way *t*-test analysis was performed by the Origin® graphical software program to determine statistical significance.

3. Results

3.1. Negative VPAC-1 regulation by anti-CD3 or PMA treatment is not reversed by an inhibitor against Ras

TCR-signaling downstream from Fyn/Lck are numerous with a large degree of crosstalk. Fig. 1 graphically illustrates several major TCR-signaling pathways with the names of the signaling molecules, their relative position within their signal transduction pathways and the names of the pharmacological inhibitors used in this study that selectively inhibit their respective target protein. Table 1 summarizes the IC_{50}, range of concentrations and selected references for these inhibitors used in this study.

A 24 h ex vivo incubation of purified CD4 T cells incubated with anti-CD3 or anti-CD3 plus anti-CD28 resulted in what appeared to be a negative regulation of VPAC-1 steady-state mRNA levels compared to complete media (Fig. 2A). Surprisingly, splenic CD4 T cells used immediately after isolation (naïve) had 80% lower VPAC-1 levels than cells incubated in complete media for 24 h. In contrast, in the presence of TCR-signaling induced by anti-CD3 alone +/− anti-CD28, resulted in VPAC-1 levels remaining at naïve levels. Therefore, these data show stimulatory and suppressive influences on VPAC-1 regulation in the absence and presence of TCR signaling.

The first evidence for a PKC mediated regulatory mechanism of VPAC-1 in T cells was suggested by O'Dorisio's laboratory when they observed phorbol esters mimic TCR-induced negative regulation of VPAC-1 expression (Lara-Marquez et al., 2001). We also observed a concentration-dependent inhibition in VPAC-1 levels with phorbol ester treatment between a range of 0.81–8.1 nM, which

Table 1
Pharmacological inhibitors used in this study

Inhibitor name	Signaling molecule affected	IC50	Vehicle	Range used	References
PP2	Fyn/Lck	5 nM	PBS	0.7–3.3 μM	Hanke et al. (1996)
Staurosporine	PKC	700 pM	DMSO	0.14–3.5 nM	Bruno et al. (1992)
Calcineurin autoinhibitory peptide	Calcineurin	10 μM	DMSO	2–50 μM	Perrino et al. (1995), Hashimoto et al. (1990)
Wortmannin	PI3-K	5 nM	DMSO	0.01–100 nM	Nakamura et al. (1995), Arcaro and Wymann (1993)
FTase inhibitor II	Ras	50 nM	Water	1.25–2.5 μM	Song and White (2003)
ERK activation inhibitor peptide I	ERK	2.5 μM	PBS	2.5–62.5 μM	Kelemen et al. (2002)
JNK inhibitor V	JNK	220 nM	DMSO	1.1–13.4 μM	Gaillard et al. (2005)
SC-68376	p38	3.5 μM	DMSO	3.5–21.2 μM	Guan et al. (1998, 1997)
Rac1 inhibitor	Rac1	50 μM	Water	2–50 μM	Clayburgh et al. (2005), Gao et al. (2004)
Piceatannol	Zap70	10 μM	DMSO	20–100 μM	Ticchioni et al. (2002), Soede et al. (1999)

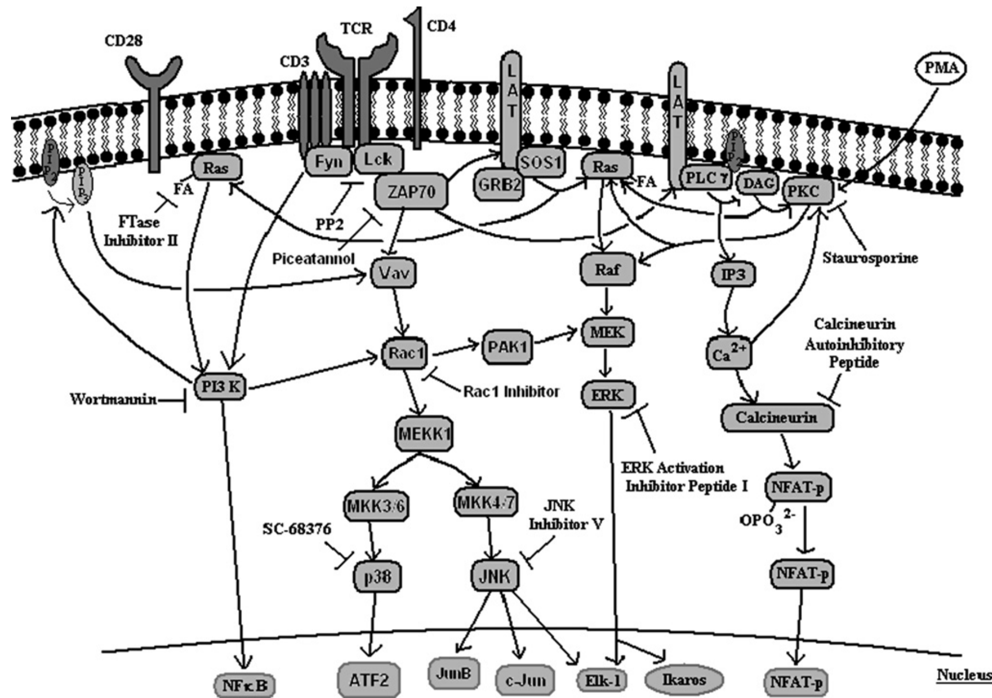

Fig. 1. Schematic representation of T cell receptor evoked signal transduction. Several major pathways are represented downstream of the Src-kinases, Fyn/Lck, including the PLCγ, PKC, Ras, PI3-K, Vav/Rac1, ERK, JNK and p38 MAPK pathways. Candidate transcription factors that each pathway is known to activate are included. Names of the pharmacological inhibitors used in this study and their selective protein target are represented. PIP_2 = phosphatidylinositol phosphate, Fyn/Lck = Src family kinases, PP2 = 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine, PLCγ = phospholipase Cγ, DAG = diacylglyceride, PKC = protein kinase C, IP_3 = inositolphosphate-3, PI3-K = phosphatidylinositol-3-kinase, PMA = phorbol 12-myristate 13-acetate, NFAT-p = nuclear factor of activated T cells, AKT = v-Akt murine thymoma viral oncogene, Ras = reticular activating system protein, Raf = a serine/threonine-selective protein kinase, MEK = MAP/ERK kinase, ERK = extracellular signal regulated kinase, NFκB = nuclear factor κB, Zap70 = ξ associated protein 70, LAT = linker for activation of T cells, GRB2 = growth factor receptor binding protein 2, SOS1 = sons of sevenless, Vav = oncogene Vav, Rac1 = Ras-related C3 botulinum toxin substrate, MEKK1 = MAP/ERK kinase kinase 1, MKK3/6 = MAP kinase kinase 3/6, MKK4/7 = MAP kinase kinase 4/7, p38 = also known as SAPK = stress activated protein kinase, JNK = c-Jun NH2 terminal kinase, ATF2 = activating transcription factor 2, FA = farnesyl.

was insensitive to PP2 treatment, as expected (Fig. 2B). Interestingly, we did not see any detectable restoration of VPAC-1 levels when a PKC inhibitor (0.14–3.5 nM stauorsporine) or a Ras inhibitor (farnylsylation inhibitor, 1.25 and 2.5 μM) were used, but rather an enhancement with staurosporine (Fig. 2A). Similarly, there was no statistically significant evidence of PKC or Ras involvement in VPAC-1 regulation from anti-CD3 treated cells (Fig. 2C and D) although, staurosporine showed a reproducible, but not statistically significant restorative effect. In contrast, there was significant blunting of VPAC-1 mRNA levels by staurosporine when used alone (Fig. 2B, stauro) supports its biological activity, and suggested that PKC positively contributes to basal VPAC-1 regulation. In summary, these data support a stimulatory role for PKC in VPAC-1 expression in the absence of TCR signaling, and interestingly, an additive or a weak restorative effect, although not statistically significant, from PMA and anti-CD3 treatment (Fig. 2B and 2C).

3.2. JNK, but not p38 or ERK MAPK pathways, contribute to the inhibition of VPAC-1 upregulation

A previous report has shown VPAC-1 levels are inversely proportional to secreted IL-2 levels (Lara-Marquez et al., 2001), and therefore its expression might be controlled by signaling cascades that promote cell cycle progression. In addition, others have shown that signaling through VPAC-1 negatively regulates TCR signaling and IL-2 upregulation (Wang et al., 2000; Teresi et al., 1996), and therefore it is reasonable to assume that T cells must ensure low VPAC-1 expression to optimally express IL-2 and proliferate (Lara-Marquez et al., 2001). The MAPK signaling molecule, c-jun N-terminal kinase (JNK), is a good candidate to mediate negative regulation of VPAC-1 during T cell signaling as it upregulates a number of pro-inflammatory cytokines including TNF-α and IL-8 (Zhong and Kyriakis, 2007). Moreover, Ras contribution to TCR-signaling regulation of VPAC-1 (Fig. 2D) would support little

involvement for ERK, as Ras signals directly into ERK via Raf → MEK (Fig. 3A; Hughes-Fulford et al., 2005). To cast a wide net, we used inhibitors to three most downstream MAPK enzymes: ERK, p38 and JNK. We observed no statistically significant differences in VPAC-1 levels with ERK and p38 inhibitors across several concentrations compared to anti-CD3 alone (Fig. 3A and C). Whereas, the inhibitor for JNK activity showed a concentration-dependent derepression of VPAC-1 mRNA levels compared to anti-CD3 alone (Fig. 3B). It is noteworthy to report that the two MAPK inhibitors against ERK and p38 that did not affect VPAC-1 levels compared to anti-CD3 alone, in contrast showed a suppressive effect for basal VPAC-1 regulation. This indicates that although these two MAPK pathways do not affect VPAC-1 regulation during T cell signaling, they both positively contribute to VPAC-1 regulation in naïve T cells. Intriguingly, the JNK inhibitor V was unable to restore VPAC-1 levels from anti-CD3 plus anti-CD28 treatment and indicates that co-stimulation signaling renders VPAC-1 suppression insensitive and perhaps additive suppression, in the presence of the JNK inhibitor V.

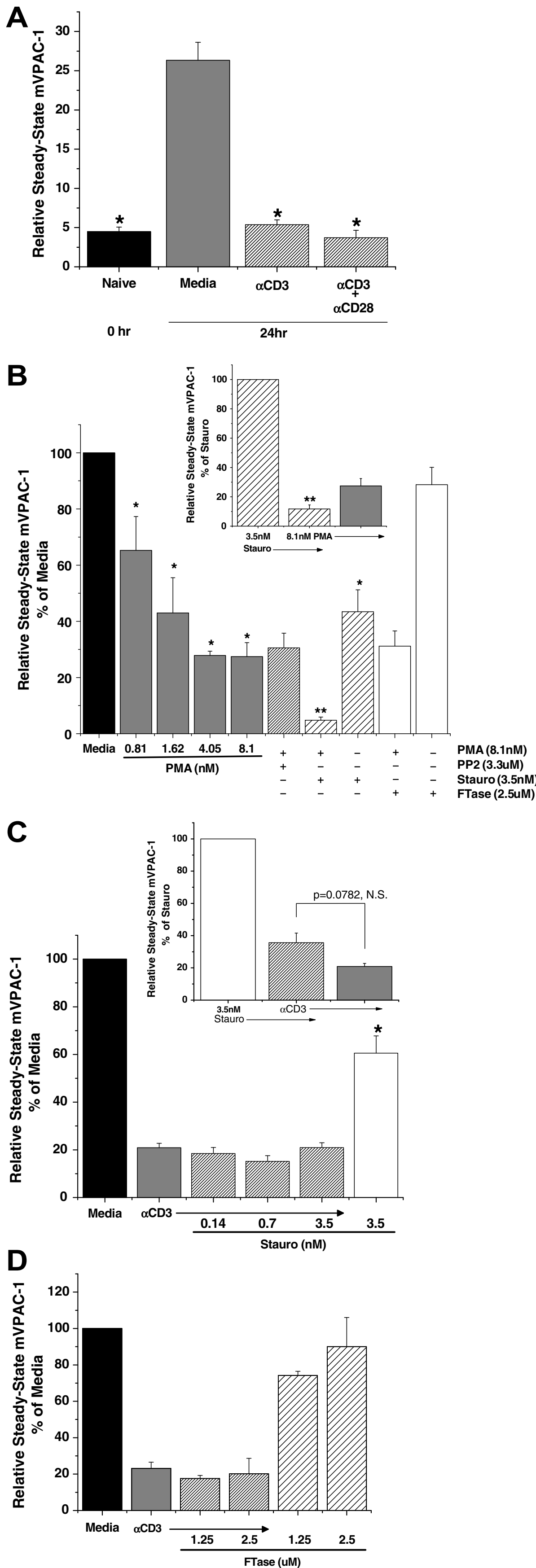

Fig. 2. PMA and anti-CD3 treatment induces low levels of VPAC-1 that is not reversed by inhibitors against PKC or Ras. Purified CD4 T cells were seeded at 1 or 4×10^6 cells/ml for 24 h, total RNA isolated and relative VPAC-1 mRNA levels measured by qPCR (Section 2). VPAC-1 levels from media alone were arbitrarily set to 100% ($^{*}p \leqslant 0.05$ as compared to media alone; $^{**}p \leqslant 0.05$ as compared to 5 ng/ml PMA). (A) Cells used immediately (naïve) or incubated for 24 h +/− anti-CD3 (4 μg/ml) or anti-CD3/anti-CD28 (2.5 μg/ml). (B) Cells treated with increasing concentrations of phorbol 12-myristate 13-acetate (PMA) as indicated, or with 8.1 μM PMA +/− various inhibitor concentrations as indicated against Fyn/Lck (PP2), PKC (staurosporine; Stauro) or Ras (FTase Inhibitor II; FTase). (C) Cells were treated with media control, inhibitor alone (Stauro) or plate-bound 4 μg/ml anti-CD3 (α-CD3) with increasing concentrations of staurosporine or (D) FTase Inhibitor II. Inset graphs are inhibitor alone normalized to 100%, and inhibitor alone plus αCD3 in an attempt to compare relative changes in VPAC-1 levels compared to α-CD3 alone (N.S., not significant).

3.3. A strong calcium-dependent mechanism was observed for blunting VPAC-1 upregulation

The enzymatic activity of phospholipase C generates a lipid soluble diacylglycerol secondary messenger that activates PKC (van Rheenen et al., 2007). However, we collected no evidence for a major role of PKC or Ras on the negative regulation of VPAC-1 levels by TCR signaling. We therefore focused on the water soluble PLC product inositol 1,4,5-triphosphate (IP_3) that induces a large cytoplasmic calcium flux activating many Ca^{2+}-dependent molecules, including calcineurin (Fig. 1; Samelson, 2002). We showed a robust blunting of basal VPAC-1 regulation by ionomycin (Fig. 4) that strongly suggested Ca^{2+} as being suppressive for VPAC-1 regulation in the absence of TCR signaling. However, the selective, cell-permeable, and competitive inhibitor, calcineurin autoinhibitory peptide, was ineffective at altering VPAC-1 levels when treated with anti-CD3 (Fig. 4). Also, this autoinhibitor did not alter basal VPAC-1 regulation when used alone. In summary, we conclude that there is most likely a Ca^{2+}-dependent mechanism for inhibiting media-induced upregulation of VPAC-1, but upon TCR engagement, the Ca^{2+}-dependent calcineurin protein is not playing a significant role.

3.4. Evidence for ZAP-70 involvement in VPAC-1 regulation

An important signaling molecule that becomes phosphorylated by Src-kinases is Zap70 (Latour and Veillette, 2001). This protein propagates TCR signaling to downstream molecules, such as Vav, a pleckstrin-domain containing GEF for Rac1 (Fig. 3). These signaling molecules can potentate downstream signal transduction pathways such as MEKK1 → MEK4/7 → JNK (Gelkop et al., 2005). We therefore asked whether Zap70, Rac1 (no available inhibitors for Vav) and PI3-K (catalyzes PIP_3 to which the pleckstrin-domain

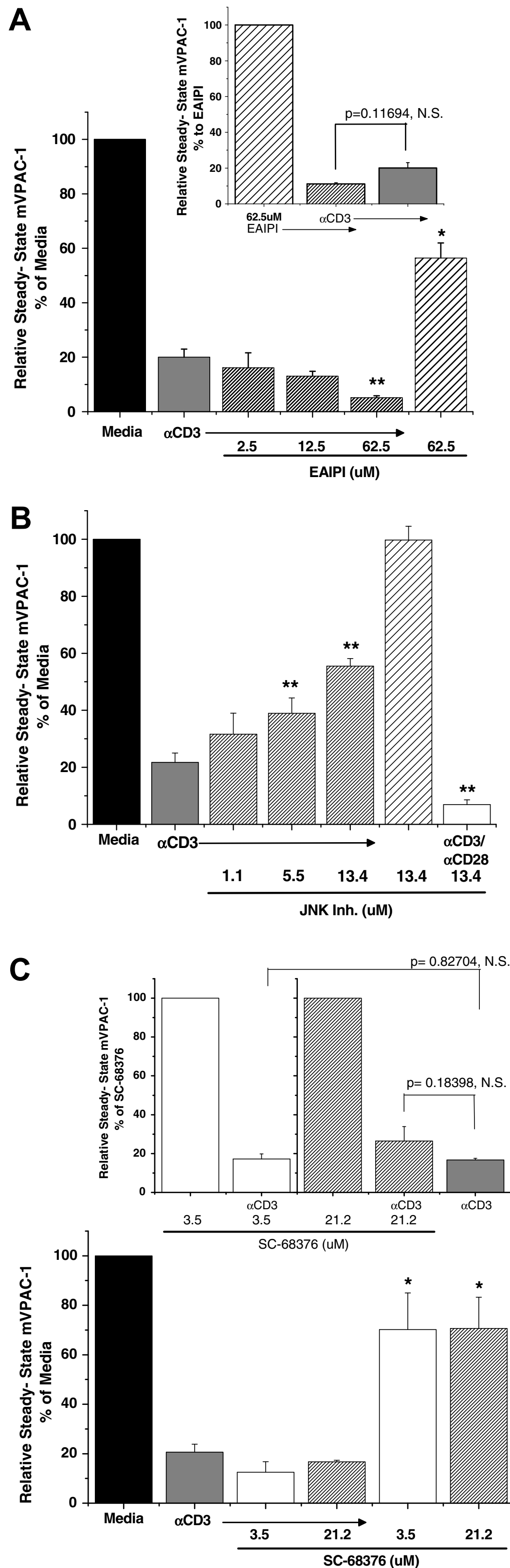

Fig. 3. JNK inhibitor V reverses anti-CD3, but not anti-CD3/anti-CD28, effect on VPAC-1 regulation. Purified CD4 T cells were incubated for 24 h, total RNA isolated and VPAC-1 levels assessed by qPCR (Section 2). Media VPAC-1 levels were arbitrarily set to 100% ($^{*}p \leqslant 0.05$ as compared to media alone; $^{**}p \leqslant 0.05$ as compared to anti-CD3). Cells were seeded at 4×10^6 cells/ml in media alone, inhibitor alone or with 4 μg/ml anti-CD3 (αCD3) +/− increasing concentrations of (A) ERK activation inhibitor peptide I, (B) JNK inhibitor V and (C) the p38 inhibitor SC-68376. Inset graphs are inhibitor alone normalized to 100%, and inhibitor alone plus αCD3 in an attempt to compare relative changes in VPAC-1 levels compared to α-CD3 alone (N.S., not significant).

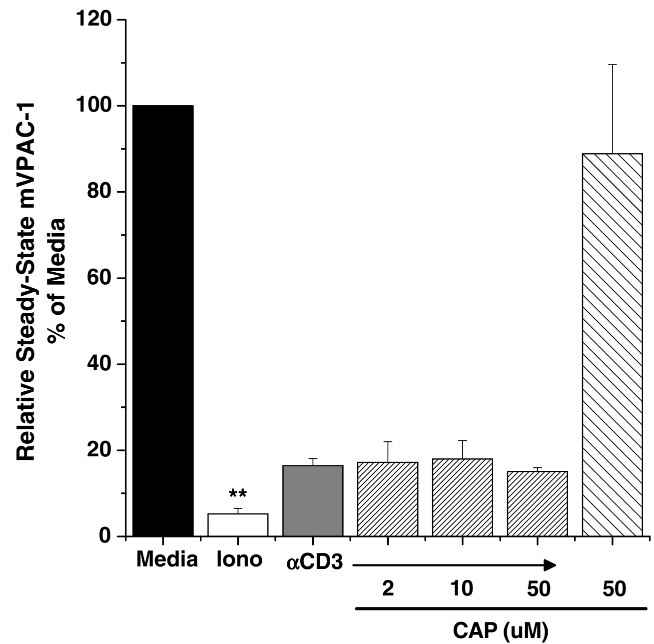

Fig. 4. Calcineurin does not mediate TCR-induced negative regulation during T cell activation. Purified CD4 T cells were incubated for 24 h, total RNA isolated and VPAC-1 levels assessed by qPCR (Section 2). Media VPAC-1 levels were arbitrarily set to 100%. Cells were seeded at 1 or 4×10^6 cells/ml and incubated in media alone or 1 μg/ml ionomycin, or inhibitor alone (CAP (calcineurin autoinhibitory peptide) or anti-CD3 (αCD3) +/− increasing concentrations of calcineurin autoinhibitory peptide.

binds) were involved in VPAC-1 regulation, thus linking the Src-kinases to JNK activation. Fig. 5A illustrates a derepression in VPAC-1 mRNA levels for a Zap70 inhibitor (piceatannol) in a concentration-dependent fashion but not significantly for inhibitors against Rac1 or PI3-K (Fig. 5B and C). Nonetheless, there was a modest tendency for both Rac1 and PI3-K involvement in VPAC-1 regulation. In the absence of TCR signaling, Zap70 and Rac1 were potent inhibitors for basal VPAC-1 expression, thus suggesting a stimulatory role for these signaling molecules in naïve T cells.

4. Discussion

The present study has identified a plausible TCR-signaling pathway evoked by anti-CD3 treatment that ensures steady-state VPAC-1 mRNA is maintained at low levels consistent to those found in naïve, splenic CD4 T cells. A working proposed signaling pathway consists of the Src-kinases Fyn and Lck (Vomhof-DeKrey, et al., 2008), the kinase Zap70 and the MAPK JNK. Although not statistically significant, inhibitors against Rac1, PI3-K and PKC may also modestly contribute to the negative regulation of VPAC-1 during T cell signaling.

A panel of pharmacological inhibitors was used in this study (Table 1). Based on their effect on VPAC-1 mRNA steady-state levels in basal versus anti-CD3 treated T cells, these inhibitors could be classified into four distinct groups (Table 2). The first group did not have any effect on VPAC-1 expression in media alone but did derepress VPAC-1 levels in anti-CD3 treated T cells (inhibitors against Fyn/Lck (Vomhof-DeKrey, et al., 2008) and JNK). The second and largest group of inhibitors blunted basal VPAC-1 regulation, while not showing a statistical change in VPAC-1 expression with anti-CD3 treatment (inhibitors against PKC, Ras, Rac1, ERK and p38). The third and fourth groups influenced basal and TCR-signaling induced VPAC-1 expression (inhibitor against Zap70), or had no affect in either treatment condition (inhibitors against calcineurin

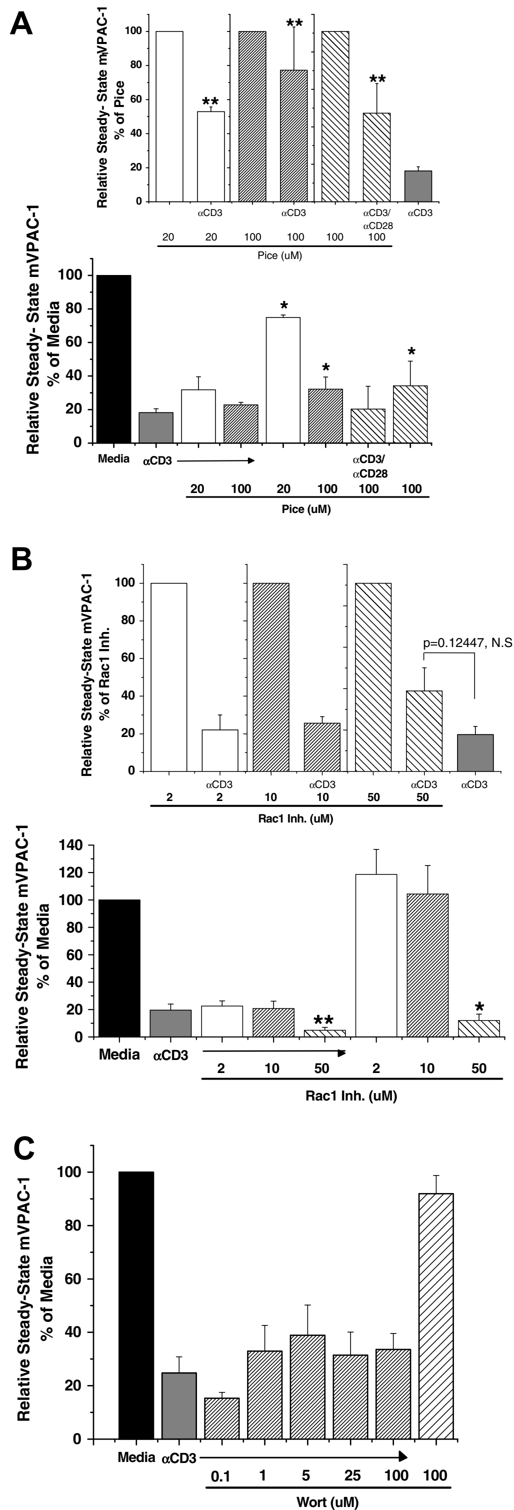

Fig. 5. A pharmacological inhibitor against Zap70 regulates VPAC-1 in an opposite manner dependent on TCR signaling. Purified CD4 T cells were incubated for 24 h, total RNA isolated and VPAC-1 levels were assessed by qPCR (Section 2). Media VPAC-1 levels were arbitrarily set to 100% ($^{*}p \leqslant 0.05$ as compared to media alone; $^{**}p \leqslant 0.05$ as compared to anti-CD3). Cells were seeded at 1 or 4 × 10^6 cells/ml and incubated for 24 h in media alone, inhibitors alone or with α-CD3 (1 or 4 μg/ml) +/− increasing concentrations of (A) piceatannol, and or +/− αCD3/αCD28, (B) Rac1 inhibitor or (C) wortmannin. Inset graphs are inhibitor alone normalized to 100%, and inhibitor alone plus αCD3 in an attempt to compare relative changes in VPAC-1 levels compared to α-CD3 alone (N.S., not significant).

and PI3-K; Table 2). These observations suggest that there is a division of labor between positive and negative signaling cascades regulating VPAC-1 expression between signaling molecules in groups I and II dependent on the presence of TCR signaling. Group III signaling molecule(s) affected VPAC-1 levels in an opposite manner dependent on the presence of TCR signaling. We conclude that Zap70 (and to a lesser extent PI3-K, Rac1, and PKC) propagates positive signals to the nucleus that elevates VPAC-1 expression in the absence of TCR signaling (Fig. 2A, Media, 24 h), but switches to propagating suppressive signals during T cell signaling that maintains VPAC-1 at low levels (Fig. 2A, anti-CD3 and anti-CD3/anti-CD28, 24 h). Our data supports that TCR signaling (Src kinase activation) overcomes any stimulatory influence on VPAC-1 mRNA levels by signaling molecules from group II, while signaling molecule(s) in group III serve as a molecular switch with respect to their effect on VPAC-1 regulation. Evidence to support the latter switching mechanism has been previously demonstrated in that Rac1 is a bona-fide "switching station" between the various MAPK modules (Olson et al., 1995).

Our data demonstrate a positive regulation by staurosporine of basal VPAC-1 expression as it shows a 40% decrease when cultured in media (Fig. 2B). Staurosporine, a fairly promiscuous inhibitor, was used at 5× its IC50 concentration (or lower) in an attempt to reduce known off-target effects (Combadière et al., 1993). Intriguingly, the effects on VPAC-1 regulation was opposite when used in conjunction with PMA verses anti-CD3 (Fig. 2B versus 2C). With PMA, there was an enhanced suppressive effect (additive). In stark contrast, in the presence of TCR signaling, staurosporine did not cause a greater decrease but instead weakly restored VPAC-1 levels by about twofold; albeit not in a statistically significant manner (Fig. 2C). It is well accepted that PMA activates PKC activity by binding and recruiting it to the cytoplasmic leaflet of the plasma membrane for optimal kinase activity (Mosior and Newton, 1995). In contrast, staurosporine has been shown to be a pan PKC enzymatic inhibitor, although several studies have shown off-target effects (Combadière et al., 1993). Thus, we struggled with the paradox of how VPAC-1 expression can be further downregulated by simultaneous PKC activation (PMA) and PKC inhibition (staurosporine). There are several ways to interpret these data. Perhaps, an off-target effect of staurosporine, rather than inhibition of PKC, causes these effects. Alternatively, Combadière et al. (1993) showed that staurosporine causes elevated PMA/PKC binding sites at the plasma membrane of polymorphonuclear leukocytes. Moreover, staurosporine does not elevate diacylglycerol (DAG)/PKC binding sites (Mosior and Newton, 1995), the natural ligand for PKC activation during TCR signaling, which does not bind as tightly to PKC compared to PMA. Thus, membrane bound PKC/PMA or PKC/DAG appears to suppress VPAC-1 expression, but tight rather than weak membrane association of PKC induced by staurosporine could explain the discordant results. Said another way, staurosporine can weakly restore TCR-signaling induced negative regulation of VPAC-1 if PKC is weakly associated with the plasma membrane, but becomes less effective at inhibiting tightly bound PKC in the presence of PMA. Either way, it seems reasonable to conclude that PMA and TCR-signaling activate PKC that results in VPAC-1 suppression. We would therefore predict that staurosporine would enhance the suppressive effect on VPAC-1 expression if treated in conjunction with anti-CD3 plus PMA.

Our data supports a dual role for Zap70 in VPAC-1 regulation dependent on T cell signaling. In the absence of anti-CD3 treatment, the Zap70 inhibitor, piceatannol, blunted VPAC-1 upregulation in a concentration-dependent fashion in media. In contrast, T cells treated with anti-CD3 (and with anti-CD28) and piceatannol restored negative VPAC-1 regulation at 20 and 100 μM (Fig. 4A). It is noteworthy to state that 200 μM of piceatannol resulted in little RNA recovery in 1 of 2 experiments indicating a possible toxic effect to the cells at that concentration. It is reasonable to conclude that Zap70 is participating in suppressive regulation of VPAC-1

Table 2
Division of pharmacological inhibitor categories

Group I	Group II	Group III	Group IV
Inhibitors that effect TCR signaling regulation of VPAC-1	Inhibitors that effect media induced upregulation of VPAC-1	Inhibitors that effect both	Inhibitors that have little effect
• PP2 • JNK inhibitor	• Staurosporine • FTase inhibitor II • Rac1 inhibitor • ERK activation inhibitor peptide I • SC-68376	• Piceatannol	• Calcineurin autoinhibitory peptide • Wortmannin

after TCR crosslinking with anti-CD3 as it is directly phosphorylated by Fyn and Lck (Béné, 2006). In the absence of TCR engagement, our data supports Zap70 contributing to media-induced upregulation of VPAC-1. Crosstalk between non-T cell receptors leading to Zap70 activation has been documented as the chemokine receptor, CXCR4, signals through Zap70 contributing to trans-endothelial migration of activated T cells (Ticchioni et al., 2002). Such evidence supports Zap70 activation from media (e.g. serum factors) that may lead to an elevation in VPAC-1 expression. Nonetheless, any stimulatory effect by Zap70 signaling on VPAC-1 expression is counteracted by TCR signaling.

Rac1 involvement in basal VPAC-1 expression is supported as the Rac1 inhibitor suppressed VPAC-1 in a concentration-dependent manner by up to 88%. Rac1 signaling is required for actin polymerization and cell-shape changes that are crucial for successful diapedesis of T cells (Ferreira et al., 2006). If proper trafficking of naïve T cells require elevated VPAC-1 levels (Ottaway, 1984), it would follow that a cell-shape modulating signaling molecule, Rac1, would be involved. Rac1 involvement in negative regulation of VPAC-1 levels by TCR signaling, however, is less certain as the Rac1 inhibitor did not show a statistical change in VPAC-1 levels compared to anti-CD3 alone. Rac1 is activated by PI3-K activity and a class of proteins called guanine nucleotide exchange factors (GEF) (Samstag and Nebl, 2005). One such GEF, termed Vav, contains a pleckstrin domain (PH) that selectively binds the product of PI3-K activity, phosphatidylinositol 3,4,5 triphosphate (PIP_3) and increases its GEF activity. Both inhibitors for PI3-K and Rac1 showed a modest trend in derepressing TCR-signaling suppression of VPAC-1. It would therefore be enticing to speculate a role for PI3-K and Rac1 in negative VPAC-1 regulation during TCR signaling. Perhaps our present ex vivo system is not sensitive enough to statistically measure such a mechanism. It is a major future goal, therefore, to repeat our studies using Vav-deficient T cell (Dennehy et al., 2007). The only inhibitor used in the present study that had little effect on VPAC-1 levels in the presence or absence of TCR signaling was calcineurin autoinhibitory peptide. This demonstrated that calcineurin and presumably nuclear factor of activated T cells (NFAT) is most likely not playing a major role in TCR-signaling induced regulation of VPAC-1. Interestingly, ionomycin alone had a marked effect (Fig. 4A). This suggests that intracellular calcium has a strong negative regulatory influence on VPAC-1 regulation but is independent of calcineurin signaling. Possible mediators of this negative Ca^{2+} influence could be a direct affect by calmodulin (recently reviewed by Gwack et al., 2007).

The role for c-jun N-terminal kinase (JNK) in TCR mediated regulation of VPAC-1 is consistent with its role as a pro-inflammatory signaling molecule. In T cells, JNK knockout mice demonstrated that it is required for Th1 differentiation and cytokine expression (Dong et al., 2001). We conclude that a negative JNK regulatory influence in TCR mediated VPAC-1 regulation is reasonable as signaling from this GPCR is considered to be anti-inflammatory. Naïve T cells do not express high levels of JNK protein but upregulate JNK within hours after T cell activation, with maximal enzymatic activity after 24–36 h (Weiss et al., 2000; Dong et al., 2001). This is consistent with our data showing an inhibitory delay of VPAC-1 levels with anti-CD3 within the first 6 h but completely restoring low VPAC-1 levels at 24 h equal to those measured at time zero (Vomhof-DeKrey, et al., 2008). In activated T cells, JNK activity is known to phosphorylate c-jun and thereby activate one of a family of proteins that dimerize to generate the transcription factor activator protein-1 (AP-1). Wang et al. (2000) have very convincingly shown that VIP suppresses IL-2 upregulation by changing the AP-1 heterodimeric combination from c-jun/c-fos to junB/c-fos. This same group has suggested that cAMP-dependent VPAC-1 signaling mediates this effect and is caused by the re-translocation of JunB into the nucleus. These observations coupled with our identification of JNK negatively regulating VPAC-1 during TCR signaling may imply an inhibitory loop where VPAC-1 expression levels can be maintained by sustained VPAC-1 signaling through $G\alpha_s$/cAMP resulting in high nuclear JunB levels. We would further hypothesize that the AP-1 heterodimer that represses IL-2, junB/c-fos, also binds and positively contributes to VPAC-1 expression.

Utilization of pharmacological inhibitors has revolutionized the ability to identify and distinguish between signaling transduction molecules. Such strategies, while very insightful, can result in unwanted off-target effects. This present study has focused on the use of such inhibitory molecules, some of which are more selective than others, to preliminarily identify the TCR-signaling cascade that regulates VPAC-1 expression during T cell activation. We intend to expand on these pharmacological results with the use of more sophisticated experimental procedures, including the use of knockout mice and overexpression of dominant negative forms of signaling proteins identified in this study to gain a greater understanding and confirmation of VPAC-1 regulation in T cells.

In summary, these data have demonstrated that CD4 T cells express VPAC-1 at differing levels dependent on TCR signaling. The regulation of VPAC-1 in the absence of TCR signaling is (present study and Vomhof-DeKrey, et al., 2008) mediated, in part, by Zap70, Rac1, PKC, ERK and p38 MAPK pathways, as these inhibitors showed suppression of basal VPAC-1 levels. TCR-signaling appears to override the positive influence on VPAC-1 regulation by activating Src kinases, Fyn and Lck, and switches Zap70 to an inhibitory signaling molecule regarding VPAC-1 regulation. Finally, our data suggests JNK phosphorylation activity negatively affects VPAC-1 expression levels during TCR signaling (anti-CD3), in the absence but not presence of a co-stimulating second signal (anti-CD28). Perhaps, T cell tolerance necessitates low levels of VPAC-1 expression mediated by JNK activity. Additional research is necessary to fully understand the regulatory mechanisms of VPAC-1 in T cells. Such research will be essential to fill in a fundamental gap in knowledge regarding TCR-signaling regulation of the anti-inflammatory GPCR, VPAC-1, in T cells.

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