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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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1. Introduction

Vasoactive intestinal peptide receptor-1 (VPAC-1) and VPACR-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., 2001, 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 histocompatibility 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 S 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 (Rac-1 and CDC42) and PI3-K (phosphatidylinositol 3-kinase; Clements et al., 1999; Samelson 2002).

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We have contemporaneously shown that VPAC-1 mRNA expression levels are negatively regulated by TCR signaling in primary CD4 T cells (data recently submitted to BBI). 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 transacting 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. 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 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

RPMI 1640 media, 1X 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. 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. Nuclease-free water and DNase-Free kits came from Ambion, and real time plates and covers from Fisher Scientific. M-MLV reverse transcriptase, deoxynucleotides 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 1ml/spleen of lysis solution (0.155 M ammonium

chloride, 0.01 M potassium carbonate and 0.1 mM EDTA) for 1 minute, diluted to 50 ml with PBS and centrifuged at $500 \times g$ for 5 minutes. To remove adherent cells, splenocytes were placed in complete media (85% RPMI 1640, 10% FBS, 10 mM Hepes, 0.4% Glucose, 1 mM sodium pyruvate and 1X penicillin/streptomycin/amphotericin B) for 1 hour at 37°C, 5% CO₂/95% 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 minutes. 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 $\geq 90\%$ cell survival and $\geq 95\%$ 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, $\leq 0.1\%$ DMSO (vehicle control), 5 ng/ml PMA, 4 µg/ml plate-bound anti-CD3 + 5ng/ml 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 + 2.5µg/ml anti-CD28 +/- indicated inhibitor concentration. Cells treated with pharmacological inhibitors were pretreated 15-30 minutes 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 hours at 37°C. After 24 hours, 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 10X PCR buffer and 3 µl of nuclease-free water was added and incubated for 45 minutes at 37°C. DNase I was inactivated by a 69°C incubation for 20 minutes. RNA was precipitated by the addition of 2.5 volumes of 100% ethanol and a 1/10 dilution of a 3M sodium acetate solution. Samples were incubated at -80°C for ≥ 20 minutes, centrifuged at $18,000 \times g$ at 4°C for 15 minutes, 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 (≤ 2 ug) 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 2X master mix containing: 2X ABI master mix, 500 nM primers (mVPAC-1, 1888-1212bp, forward, 5'-TTG GAG TTC ACT ATG TCA TGT TTG C-3'; mVPAC-1, 1245-1268bp, reverse, 5'-CTA CGA CGA GTT CAA AGA CCA TTT-3'; mHPRT, 636-655bp, forward, 5'-CTG GTG AAA AGG ACC TCT CG-3'; mHPRT, 719-744bp, 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, 1218-1242bp, 5'-FAM-TTG TGG TGG CCA TCC TCT ACT GCT TCC- TAMRA-3'; mHPRT, 659-687bp, 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 Number NM_011703) and HPRT (Accession Number 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 $\leq 1.6\%$ 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 $\pm$ SEM of relative mVPAC-1 levels normalized to mHPRT.

2.5 Statistical analysis

All data are presented as means $\pm$ SEM and experiments were conducted at least three independent times unless otherwise noted in the figure legend. Statistical significant values ($p \leq 0.05$) 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 inhibitors against Ras

TCR signaling downstream from Fyn/Lck are numerous with a large degree of crosstalk. Figure 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.

A 24 hour *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 hours. 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 0.5-5 ng/ml, which was insensitive to PP2 treatment, as expected (Fig. 2B). Interestingly, we did not see any detectable restoration of VPAC-1 levels with a PKC inhibitor (0.14 - 3.5nM Staurosporine) or a Ras inhibitor (farnesylation inhibitor, 1.25 and 2.5uM) 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 (Figs. 2C and 2D) although, staurosporine showed a reproducible trend seen with the suppressive TCR signaling. In contrast, there was significant blunting of VPAC-1 mRNA levels by staurosporine when used alone (Fig. 2B, stauro) that confirmed its biological activity, and suggested that PKC positively contributes to basal VPAC-1 regulation. In summary, these data support a weak stimulatory role for PKC in VPAC-1 expression in the absence of TCR signaling, and interestingly, a weak suppressive (statistically significant) role as well during TCR signaling.

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-Marquex 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-Marquex 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 proinflammatory cytokines including TNF- α and IL-8 (Zhong et al., 2007). Moreover, Ras contribution to TCR signaling regulation of VPAC-1 (Fig. 2) would support little involvement for ERK, as Ras signals directly into ERK via Raf $\rightarrow$ MEK (Fig. 3A; Hughes-Fulford 2005). To cast a wide net, we used inhibitors to three most downstream MAPK enzymes: ERK, p38 and JNK. We observed no statistically significant difference in VPAC-1 levels with ERK and p38 inhibitors across several concentrations compared to anti-CD3 alone (Fig. 3A and 3C). 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 to JNK inhibitor V.

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 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 (IP₃) that induces a large cytoplasmic calcium flux activating many Ca²⁺-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 Ca²⁺ as being suppressive for VPAC-1 regulation. 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 Ca²⁺-dependent mechanism for inhibiting media-induced upregulation of VPAC-1, but upon TCR engagement, the Ca²⁺-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 Rac-1 (Fig. 3). These signaling molecules can potentate downstream signal transduction pathways such as MEKK1 $\rightarrow$ MEK4/7 $\rightarrow$ JNK (Gelkop et al., 2005). We therefore asked whether Zap70, Rac1 (no available inhibitors for Vav) and PI3-K (catalyzes PIP₃ to which the pleckstrin-domain binds) were involved in VPAC-1 regulation, thus linking the Src-kinases to JNK activation. Figure 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 5C).

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 (data recently submitted to BBI), 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 naïve 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 (recently submitted to BBI) 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 VPAC-1 expression (inhibitor against Zap70), or had no effect in either treatment condition (inhibitors against calcineurin 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. Furthermore, 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, but switches to propagating suppressive signals during T cell signaling that maintains VPAC-1 at low levels. 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 molecules 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 (Olsen et al., 1995).

Our data demonstrates a positive role for staurosporine as it shows a 40% decrease in basal VPAC-1 expression when cultured in complete medium (Fig. 2B). Staurosporine, a fairly promiscuous inhibitor, was used at 5X 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 versus anti-CD3. 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 two-fold; 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 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, if PKC is being inhibited by staurosporine, then another explanation must

explain these results. 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 even more 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 derepressed negative VPAC-1 regulation at 20 and 100 uM (Fig. 4A). It is noteworthy to state that 200 uM 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 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 transendothelial 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 Rac 1 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 (PIP₃) 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 cells, (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 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 proinflammatory 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 hours (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 hours but completely restoring low VPAC-1 levels at 24 hours equal to those measured at time zero (recently submitted to BBI). 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 $\text{G}\alpha_s/\text{cAMP}$ 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 recently submitted to BBI) mediated, in part, by Zap70, Rac1, PKC, ERK and p38 MAPK pathways, as these inhibitors showed suppression. 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). A possible mechanism for this might be due to JNK translocation to the nucleus that skews the AP-1 heterodimer to favor c-jun/c-fos that acts in an inhibitory fashion on VPAC-1 during T cell activation (Wang *et al.*, 2000). 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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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 PLC γ , 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. PIP₂= phosphatidylinositol phosphate, Fyn/Lck= Src family kinases PP2= 4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-d]pyrimidine PLC γ = phospholipase C γ , DAG= diacylglyceride, PKC= protein kinase C, IP₃= 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, NF κ B= nuclear factor κ B, 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.

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 hours, total RNA isolated and relative VPAC-1 mRNA levels measured by qPCR (Materials and Methods). VPAC-1 levels from media alone were arbitrarily set to 100% (* = $p \leq 0.05$ as compared to media alone; ** = $p \leq 0.05$ as compared to 5ng/ml PMA). **A.** Cells used immediately (naïve) or incubated for 24 hrs +/- anti-CD3 (4 μ g/ml) or anti-CD3/anti-CD28 (2.5 μ g/ml) **B.** Cells treated with increasing concentrations of phorbol 12-myristate 13-acetate (PMA) as indicated, or with 8.1uM PMA $\pm$ 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 μ g/ml anti-CD3 (α -CD3) with increasing concentrations of staurosporine or **D.** FTase Inhibitor II. *Inset:* Insert graphs are inhibitor alone normalized to 100%, and inhibitor alone plus α -CD3 in an attempt to compare relative changes in VPAC-1 levels compared to α -CD3.

Fig. 3.

JNK inhibitor V reverses anti-CD3, but anti-CD3/anti-CD28, induced low levels of VPAC-1. Purified CD4 T cells were incubated for 24 hours, total RNA isolated and VPAC-1 levels assessed by qPCR (Materials and Methods). Media VPAC-1 levels were arbitrarily set to 100% (* = $p \leq 0.05$ as compared to media alone; ** = $p \leq 0.05$ as compared to anti-CD3). Cells were seeded at 4×10^6 cells/ml in media alone, inhibitor alone or with 4 μ g/ml anti-CD3 (α CD3) +/- increasing concentrations of **A.** ERK activation inhibitor peptide I, **B.** JNK inhibitor V and **C.** the p38 inhibitor SC-68376. *Inset:* Insert graphs are inhibitor alone normalized to 100%, and inhibitor alone plus α CD3 in an attempt to compare relative changes in VPAC-1 levels compared to α -CD3.

Fig. 4.

Calcineurin does not mediate TCR induced negative regulation during T cell activation. Purified CD4 T cells were incubated for 24 hours, total RNA isolated and VPAC-1 levels assessed by qPCR (Materials and Methods). 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 $1 \mu\text{g/ml}$ ionomycin, or incubated with media alone, inhibitor alone (CAP (Calcineurin Autoinhibitory Peptide) or anti-CD3 (αCD3) +/- increasing concentrations of calcineurin autoinhibitory peptide.

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 hours, total RNA isolated and VPAC-1 levels were assessed by qPCR (Materials and Methods). Media VPAC-1 levels were arbitrarily set to 100% (* = $p \leq 0.05$ as compared to media alone; ** = $p \leq 0.05$ as compared to anti-CD3). Cells were seeded at 1 or 4×10^6 cells/ml and incubated for 24 hours in media alone, inhibitors alone or with α -CD3 (1 or $4 \mu\text{g/ml}$) +/- increasing concentrations of **A.** piceatannol, and +/- α CD3/ α CD28, **B.** Rac1 inhibitor or **C.** wortmanin. *Inset:* Insert graphs are inhibitor alone normalized to 100%, and inhibitor alone plus α CD3 in an attempt to compare relative changes in VPAC-1 levels compared to α -CD3.

Table 1

Inhibitor Name	Signaling Molecule Affected	IC ₅₀	Vehicle	Range Used	References
PP2	Fyn/Lck	5nM	PBS	0.7-3.3 μ M	Hanke et al., 1996
Staurosporine	PKC	700pM	DMSO	0.14-3.5 nM	Bruno et al., 1992
Calcineurin Autoinhibitory Peptide	Calcineurin	10nM	DMSO	2-50 μ M	Perrino et al., 1995 Hashimoto et al., 1990
Wortmannin	PI3-K	5nM	DMSO	0.01-100 nM	Nakamura, et al., 1995 Arcaro et al., 1993
FTase Inhibitor II	Ras	50nM	Water	1.25-2.5 μ M	Song and White, 2003
ERK Activation Inhibitor Peptide I	ERK	2.5nM	PBS	2.5-62.5 μ M	Kellemen et al., 2002
JNK Inhibitor V	JNK	220nM	DMSO	1.1-13.4 μ M	Gaillard et al., 2005
SC-68376	p38	3.5nM	DMSO	3.5-21.2 μ M	Guan et al., 1998 Guan et al., 1997
Rac1 Inhibitor	Rac1	50nM	Water	2-50 μ M	Clayburgh et al., 2005 Gao et al., 2004
Piceatannol	Zap70	10nM	DMSO	20-100 μ M	Ticchioni et al, 2002 Soede et al., 1999

Table 2

Inhibitors that affect TCR signaling regulation of VPAC-1	Inhibitors that affect media induced upregulation of VPAC-1	Inhibitors that affect both	Inhibitors that have little effect
PP2	Staurosporine	Piceatannol	Calcineurin Autoinhibitory Peptide
JNK Inhibitor	FTase Inhibitor II		Wortmannin
	Rac1 Inhibitor		
	ERK Activation Inhibitor Peptide I		
	SC-68376		