Differential Signaling of T Cell Generation of IL-4 by Wild-Type and Short-Deletion Variant of Type 2 G Protein-Coupled Receptor for Vasoactive Intestinal Peptide (VPAC$_2$)[1]

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Abstract

Vasoactive intestinal peptide (VIP) released from some neurons and T cells affects T cell migration, cytokine generation, and other functions by binding to constitutively expressed type 1 G protein-coupled receptor (VPAC$_1$) or activation-induced type 2 G protein-coupled receptor (VPAC$_2$). Recently, a short-deletion (SD) splice variant of mouse VPAC$_2$ that lacks 14 amino acids at the end of the last transmembrane domain has been identified in T cells and shown to resemble wild-type (WT) VPAC$_2$ in affinity of VIP binding but to differ by lack of signaling of T cell adenylyl cyclase, migration, and IL-2 secretion. As Th2 cells are the principal source of immune VIP and have the greatest functional responses to VIP, the differences in signals transduced by WT and SD VPAC$_2$ were studied in VPAC$_2$–low D10G4.1 model Th2 cell transfectants individually expressing the respective types of VPAC$_2$ equally. WT and SD VPAC$_2$ Th2 cell transfectants secreted equal amounts of VIP. WT VPAC$_2$ transfectants generated more IL-4 than did SD VPAC$_2$ transfectants, and this increment was dependent on endogenous VIP. Exogenous VIP further increased IL-4 production by WT VPAC$_2$ transfectants but decreased IL-4 production by SD VPAC$_2$ transfectants. Cotransfection of the two constructs diminished VIP enhancement of IL-4 production seen with WT VPAC$_2$ alone by preventing increases in nuclear levels of the requisite transcription factors c-Maf and Jun B. Thus the ratio of two forms of T cell VPAC$_2$ determines the net effect of VIP on IL-4 generation by activated Th2 cells.

Vasoactive intestinal peptide (VIP)[3] is generated and secreted by several sets of neurons and by T cells, of which the Th2 subset of CD4 helper-inducer T cells is the most prominent producer (1–3). VIP has broad physiological effects in many organ systems and regulates diverse functions of macrophages, dendritic cells, and B cells as well as T cells in immunity (4,5). The principal activities of T cells that are mediated or regulated by VIP include differentiation, migration, proliferation, survival, and cytokine generation. Normal CD4 T cells of the Th2 subset not only produce much more VIP than other types of lymphocytes, but also

[3] Abbreviations used in this paper: VIP, vasoactive intestinal peptide; VPAC$_1$, VIP type 1 receptor; VPAC$_2$, VIP type 2 receptor; TG, transgenic; KO, knockout; DTH, delayed-type hypersensitivity; HPRT, hypoxanthine phosphoribosyltransferase; WT, wild type; SD, short deletion.

are more responsive to effects of VIP, which results in deviation toward Th2-driven inflammatory and allergic pathways (3,6,7). Of the two types of native G protein-coupled receptors for VIP, type 1 ($VPAC_1$) greatly predominates on unstimulated T cells, but its expression declines after T cell activation, and the level of type 2 ($VPAC_2$) increases concurrently (8,9). Results of studies of immune responses in two lines of C57BL/6 mice with T cell-selective transgenic (TG) overexpression of $VPAC_2$ and genetic deletion or knockout (KO) of $VPAC_2$ have shown highly abnormal immunophenotypes, which are consistent with the preferential effects of VIP on isolated Th2 cells. The $VPAC_2$ TG mouse has increased IgE Ab responses, blood eosinophilia, heightened immediate-hypersensitivity skin testing, and reduced delayed-type hypersensitivity (DTH) (10). The $VPAC_2$ KO mouse has markedly increased DTH and depressed allergic reactions (11). Both abnormal phenotypes are attributable to a ratio of Th2/Th1 cytokines that is different from that of wild-type (WT) mice.

Recently, it has been found that mouse T cells express an alternatively spliced form of $VPAC_2$, as well as WT $VPAC_2$, which has a 14-aa deletion in the seventh transmembrane domain near the cytoplasmic surface (12). This short-deletion (SD) variant of mouse $VPAC_2$, termed $VPAC_2$de367–380, results from a loss of exon 12. For initial studies of differences between the functions of $VPAC_2$de367–380 and WT mouse $VPAC_2$, each was expressed at the same level in a human $VPAC_2$–low subline of Jurkat human T cell transfectants, as assessed by real-time PCR quantification of mRNA and Western blots of extracted $VPAC_2$ membrane protein. Each form of $VPAC_2$ bound VIP with a similar affinity, but only mouse WT $VPAC_2$ transduced VIP mediation of increases in intracellular concentration of cAMP, chemotaxis, and decreases in IL-2 generation in Jurkat T cell transfectants (12).

As the highest levels of production of endogenous VIP and the greatest effects of VIP on immune responses are observed in Th2-type CD4 T cells, WT $VPAC_2$ and SD $VPAC_2$de367–380 were expressed equally in mouse Th2 cells of the $VPAC_2$–low D10G4.1 line for the present investigations of their respective functions. Ab stimulation of the TCR of D10G4.1 cells now is shown to increase both the level of mRNA encoding IL-4 and the concentration of secreted IL-4 much more in WT $VPAC_2$ transfectants than in SD $VPAC_2$de367–380 transfectants. This is explained entirely by differences in transduction of signals from endogenous VIP through WT $VPAC_2$, as contrasted with SD $VPAC_2$, because the level of VIP generation after TCR stimulation is the same for both lines of transfectants. Dependence on endogenous VIP is confirmed by the capacity of an IgG VIPase to suppress the enhancement of levels of mRNA encoding IL-4 in WT $VPAC_2$ transfectants, at concentrations that eliminated > 95% of all endogenous VIP. Further, additions of 10^{-9} M to 3×10^{-7} M exogenous VIP increase levels of secreted IL-4 in WT $VPAC_2$ transfectants, whereas the opposite effect of exogenous VIP was observed in SD $VPAC_2$ transfectants. Analyses of transcriptional mechanisms showed that SD $VPAC_2$ fails to recruit nuclear c-Maf or Jun B, in contrast with WT $VPAC_2$, and prevents WT $VPAC_2$ from engaging these transcriptional factors.

Materials and Methods

Genetic constructs

The full-length cDNAs encoding mouse WT $VPAC_2$ and the SD variant of mouse $VPAC_2$ ($VPAC_2$de367–380) were introduced into pCMV-Tag4 vector (Stratagene), which adds a FLAG epitope tag at the C terminus. The correctness of each sequence was confirmed (ELIM Biopharmaceuticals) before large-scale production and purification (Qiagen).

Cells, nucleofection procedures, and Abs

A mouse Th2 cell line designated D10G4.1 was obtained from American Type Culture Collection. D10G4.1 cells were cultured in complete RPMI 1640 medium (Cell Culture Facility, University of California, San Francisco) containing 10% heat-treated FBS, 10% rat T-STIM factor with Con A (BD Biosciences), 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/ L glucose, 1.5 g/L sodium bicarbonate, 50 μM 2-mercaptoethanol, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 10 pg/ml IL-1α (Biolegend). Nucleofection was conducted with the Nucleofector kit V (Amaxa Biosystems), a total of up to 3 μg of plasmid DNA per 2.5×10^6 D10G4.1 T cells, and program T-01. Viability was $\geq 90\%$ at 24 h after the procedure.

Anti-FLAG M2 Ab (Stratagene) is a mouse IgG1 anti-DYKDDDDK mAb, which was used for Western blots at a concentration of 2 μg/ml. Rabbit anti-mouse $VPAC_2$ Abs, which recognize both native $VPAC_2$ and SD $VPAC_2$ ($VPAC_2$de367–380), were generated with the 23-aa synthetic peptide SIHPECRFHLEIQEEETKCAELL, representing a substituent mid-sequence of the aminoterminus of $VPAC_2$, which is the same in mouse and human $VPAC_2$. This Ab detected only one major 48- to 55-kDa protein in Western blots of 10 μg of cellular proteins extracted from mouse WT $VPAC_2$-human Jurkat T cell transfectants, but none in the proteins extracted from mouse $VPAC_1$-human Jurkat T cell transfectants. Rabbit anti-$VPAC_2$ Abs also detected one protein band of the same size in Western blots of 5 μg of selectively extracted membrane proteins from anti-CD3 plus anti-CD28 mAb-activated mouse splenic T cells (Mem-PER membrane protein extraction kit and PAGE prep Advance kit; Pierce). Other Abs were: rabbit anti-c-Maf (M-153) IgG (Santa Cruz Biotechnology), rabbit anti-Jun B (N-17) IgG (Santa Cruz Biotechnology), and mouse monoclonal anti-lamin B1 Ab (Abcam) used as a control for loading of Western blots with equal quantities of nuclear proteins. Western blots were performed and developed as described (13).

Clone c23.5 is a murine IgG2aκ with VIPase catalytic activity, which cleaves extracellular VIP with an efficiency level that removes ~95% of T cell-derived VIP, whereas at the same concentration, highly purified clone UPC10 isotype control has no effect (14,15).

Quantification of expression of $VPAC_2$ receptors by D10G4.1 transfectants

Total RNA was extracted from each sample with Tri-Reagent (Molecular Research Center), treated with RNase-free DNase I (Qiagen) for 30 min, and isolated on RNeasy columns (Qiagen). The mRNAs encoding mouse $VPAC_2$ receptors and the standard mouse hypoxanthine phosphoribosyl-transferase (HPRT) were amplified in triplicate using the TaqMan reagents: 5′-AAGCAGCCAAACGGAGAATC-3′ and 5′-GGCAGGGCACTGT GACAGTT-3′ primers and 5′-FAM-CTGCAGCGGTGTCTGGGA CAACA-TAMRA-3′ - probe for m$VPAC_2$ and 5′ CTGGTGAAAAGGAC CTCTCG-3′ and 5′- TGAAGTACTCATTATAGTCAAGGGCA-3′ primers and 5′-TET- TGTTGGATACAGGCCAGACTTTGTTGGAT TAMRA-3′ probe for mouse HPRT (Integrated DNA Technologies) as described (16). A Prism 9700 sequence detection system (Applied Biosystems) with the recommended optimal reagents and conditions provided interanalysis coefficients of variation < 4%. The value for each unknown was determined from its optimized threshold cycle (C_T) and was normalized with reference to the C_T value of HPRT using the comparative C_T method (User Bulletin No. 2 of ABI Prism 9700 sequence detection system). Western blots of 2–10 μg of transfectant cellular proteins, which were extracted as described (10), were developed separately with rabbit anti-mouse $VPAC_2$ Abs and anti-FLAG M2 Ab.

Assessment of generation of VIP by D10G4.1 transfectants

Replicate suspensions of $4–5 \times 10^6$ D10G4.1 transfectants per ml of RPMI 1640 with 10% FBS, which expressed mouse WT $VPAC_2$ or SD $VPAC_2$ ($-367–380$), were incubated for 24–

72 h in 24-well plates in which each well had been coated with either 0.5 or 2 μg of anti-CD3 and of anti-CD28 mAbs (BD Pharmingen). After centrifugation of the plate at $1500 \times g$ for 15 min at 4°C, medium samples were transferred to clean test tubes, treated with Halt protease inhibitor mixture (1/100, v/v; Pierce) for 30 min at 37°C, acidified to pH 4.5 with acetic acid, and applied to C18 reversed-phase Sep-Pak columns (Waters) for extraction of VIP, as described (15). Methanol eluates were dried in vacuo and reconstituted in 0.1 ml of PBS each for duplicate quantification of VIP by radioimmunoassay (Phoenix Pharmaceuticals).

Quantification of cellular and fluid-phase concentration of IL-4 and of mRNA encoding IL-4

Replicate suspensions of $0.5–0.8 \times 10^6$ D10G4.1 transfectants per 0.6 ml of RPMI 1640 with 10% FBS, which expressed mouse WT VPAC$_2$ or SD VPAC$_2$ (VPAC$_2$de367–380), were preincubated either with or without 1 μM IgG VIPase or isotype control IgG for 30 min at 37°C and incubated for 24–48 h in 24-well plates in which each well had been coated with 1 μg of anti-CD3 and 0.5 μg of anti-CD28 mAbs (15). VIP at 10^{-9} M to 10^{-6} M was added every 12 h to some wells. IL-4 in media supernatants was assayed with an ELISA (Endogen; Pierce Biotechnology). In some studies, cells were washed twice with cold PBS, incubated for 30 min at 37°C with Halt protease inhibitor mixture (1/100 in PBS, v/v; Pierce), resuspended in 0.1 ml of PBS with 1/100 Halt and 1% Nonidet P40 (v/v), and subjected to two cycles of freezing and thawing before centrifugation and removal of supernatants for ELISAs of cellular IL-4. Real-time PCR quantification of mRNA encoding IL-4 was conducted exactly as for VPAC$_2$ mRNA with the primers 5′-CGCCATGCACGGAGATG-3′ (forward) and 5′-ACGAGCT-CACTCTCTGTGGTGTT (reverse), and probe 5′-Fam-TGCCAACGTCCTCACAGCAACG-TAm-3′ (Integrated DNA Technologies). After calculating the quantities of IL-4 mRNA in all samples with the C_T method and normalizing them to that for HPRT mRNA, relative values were assigned to each based on setting that of IL-4 mRNA in WT VPAC$_2$ transfectants to 1 and effects of anti-TCR Ab stimulation were represented by increments in these relative values.

Transcription factor assays

Replicate suspensions of D10G4.1 transfectants were pretreated and incubated as in studies of IL-4 generation for 4 h at 37°C. Then nuclear proteins were extracted with NE-PER nuclear and cytoplasmic reagents (Pierce). Western blots were performed as described using 5–20 μg of total nuclear protein for electrophoresis and developing blots with rabbit anti-mouse c-Maf and anti-mouse Jun B Abs (Santa Cruz Biotechnology) (16). Levels of active Jun B in replicate 5 μg samples of nuclear proteins were quantified with the TransAM AP-1 kit (Active Motif) in 96-well plates coated with consensus sequence oligonucleotide and using specific Ab to detect active Jun B bound to the oligonucleotide, as described (16).

Results

All studies of differences between mouse WT and SD VPAC$_2$ in transducing signals from VIP to effects on generation of IL-4 by the VPAC$_2$-low D10G4.1 mouse Th2 model cell line were conducted within 48 h of nucleofection with equal amounts of the corresponding expression plasmids. Real time-PCR quantification of mRNA, extracted from the respective transfectants, which encodes each type of VPAC$_2$ GPCR showed similar high levels at 24–48 h (Fig. 1). As for the course of expression of mRNA, membrane proteins extracted from the same sets of transfectants showed similarly high levels of expression of WT and SD VPAC$_2$ for up to 48 h, which encompasses the times of study of IL-4 generation (Fig. 2). The illustration is for samples obtained at 48 h and detected with mouse anti-FLAG M2 mAb to the epitope tag on VPAC$_2$, but results were identical with rabbit anti-VPAC$_2$ Abs, and the levels of WT and SD VPAC$_2$ also were identically high at 24 h with both Abs.

The Th2 subset of CD4 T cells is the principal source of T cell-derived VIP, which affects diverse functions of Th2 cells as an autocrine mediator and other immune cells through paracrine mechanisms. Therefore, it was important to document that D10G4.1 mouse Th2 cells generate VIP, and that both WT and SD VPAC$_2$ transfectants produce identical quantities of VIP. It also was of interest to relate these quantities of VIP to the amounts produced by mouse native Th2 cells. The levels of generation of VIP by WT and SD VPAC$_2$-transfectants of D10G4.1 mouse Th2 cells were indistinguishable after 24 h (Fig. 3) and 48 h (not shown) of incubation without and with TCR-directed Ab activation. Vector-only D10G4.1 mouse Th2 cell transfectants produced 1.3 ± 0.4 ng per 10^6 cells of VIP without stimulation and 2.1 ± 0.6 ng per 10^6 cells with 2 μg of anti-CD3 plus anti-CD-28 Abs for 24 h. The levels of VIP production by both types of D10G4.1 mouse Th2 cell transfectants were higher than that by mouse native splenic mixed CD4 T cells without stimulation but were not increased significantly by TCR-directed Ab activation, unlike the prominent increases observed with native mixed CD4 T cells (15). The identification of radioimmunoassay-detected VIP as authentic was confirmed by reversed-phase HPLC isolation and repeat RIA quantification.

The level of IL-4 secreted by D10G4.1 mouse Th2 cells transfected with WT VPAC$_2$ was consistently higher than that secreted by D10G4.1 mouse Th2 cells transfected with SD VPAC$_2$ after stimulation with adherent anti-CD3 plus anti-CD28 Abs. The mean level $\pm$ S.D. of IL-4 secreted by 10^6 D10G4.1 mouse Th2 cells 24 h after TCR-directed Ab activation was 8.9 ± 2.1 ng for WT VPAC$_2$ transfectants and 5.0 ± 1.5 ng for SD VPAC$_2$ transfectants ($p =$ 0.0046; paired t test). A lack of autocrine IL-4-enhancing signal from SD VPAC$_2$ occupied by endogenous VIP, as contrasted with an effective signal from VIP-occupied WT VPAC$_2$, was considered the most likely explanation for this significant difference. To examine this possibility, suspensions of both types of transfectants were preincubated with an optimally active concentration of 1 μM IgG VIPase for 60 min before stimulation with adherent anti-CD3 plus anti-CD28 Abs in the continued presence of VIPase for 24 h. The level of IL-4 secreted by WT VPAC$_2$ transfectants decreased significantly by 79% from a mean of 11.3 ng/10^6 D10G4.1 Th2 cells with isotype control IgG to 3.2 ng/10^6 D10G4.1 in the presence of IgG VIPase ($p < 0.01$), whereas the lower mean level secreted by SD VPAC$_2$ transfectants of 8.1 ng/10^6 D10G4.1 Th2 cells with isotype control IgG was not significantly changed by IgG VIPase (28%) at 6.4 ng/10^6 D10G4.1 Th2 cells. Quantification of IL-4 mRNA by real-time PCR in the same VIPase protocol similarly showed a significant decrease in WT VPAC$_2$ transfectants as a result of VIPase, whereas the significantly lower level of IL-4 mRNA in SD VPAC$_2$ transfectants was unchanged by VIPase (Fig. 4).

As the concentrations of endogenous VIP attained in T cell cultures are in the low nanomolar range (Fig. 3), but those in compartmental immune reactions that reflect neural and immune sources in vivo may be up to two orders of magnitude higher, effects of a broad range of concentrations of exogenous VIP on IL-4 production were investigated in D10G4.1 mouse Th2 cell transfectants. IL-4 production by WT VPAC$_2$ transfectants stimulated with adherent anti-CD3 plus anti-CD28 Abs for 24 h was enhanced significantly by 10 nM VIP up to a maximum level at 100 nM VIP that was nearly 3-fold higher than that of controls in the absence of VIP (Fig. 5). In contrast, IL-4 production by SD VPAC$_2$ transfectants stimulated with adherent anti-CD3 plus anti-CD28 Abs for 24 h was suppressed significantly by 30 nM VIP up to a maximum at 300 nM VIP that was a mean of ~50% of control (Fig. 5). That VIP signaling through SD VPAC$_2$ alone decreased IL-4 generation by D10G4.1 mouse Th2 cells suggested that co-expression of SD VPAC$_2$ with WT VPAC$_2$ might suppress the IL-4-enhancing effect of the latter receptor. At a ratio of SD VPAC$_2$ to WT VPAC$_2$ of 0.5 and through a ratio of 4, the IL-4-enhancing activity of WT VPAC$_2$ was suppressed significantly by two concentrations of exogenous VIP (Fig. 6). Thus, both the individual levels and the ratio of levels of expression of these two native forms of VPAC$_2$ determine the net regulatory action of VIP on T cell generation of IL-4.

As c-Maf and Jun B transcription factors have been implicated in VIP-WT $VPAC_2$ signaling of enhanced IL-4 generation, nuclear levels of these proteins were quantified to elucidate mechanisms underlying the differences between the effects of SD $VPAC_2$ and WT $VPAC_2$ on IL-4 generation.

Western blots were completed with nuclear proteins extracted from D10G4.1 mouse Th2 cells transfected with WT $VPAC_2$ and SD $VPAC_2$ alone and in combination and stimulated with anti-CD3 Ab in the absence and presence of an optimally effective concentration of exogenous VIP. These analyses revealed substantial increases in the levels of both c-Maf and Jun B when WT $VPAC_2$ transfectants were exposed to VIP, but not for SD $VPAC_2$ transfectants with VIP (Fig. 7, A and B). Furthermore, the coexpression of SD $VPAC_2$ with WT $VPAC_2$ prevented these VIP-induced increases in transcription factors. Additional evidence for the central involvement of active Jun B in WT $VPAC_2$ signaling of enhanced IL-4 generation and the lack of recruitment of active Jun B by SD $VPAC_2$ came from results of consensus oligonucleotide based-antibody detection assays of its nuclear levels (Fig. 8). In WT $VPAC_2$ transfectants, the nuclear level of active Jun B was increased by a mean of 59% with 10^{-7} M VIP ($p < 0.01$), but was not altered significantly by 10^{-7} M VIP in SD $VPAC_2$ transfectants or mock-transfected control D10G4.1 mouse Th2 cells. In WT plus SD $VPAC_2$ cotransfectants (1:2), 10^{-7} M VIP enhanced the increase in active Jun B less than in WT-only $VPAC_2$ transfectants, and this 23% enhancement was not significant (Fig. 8).

Discussion

The first stage of elucidation of pathways by which the VIP–$VPAC_2$ neuroimmune axis regulates the nature of tissue compartmental immune responses consisted of in vitro studies of effects of VIP on numerous functions of cultured immune cells (5). Recognition of the capacity of the VIP–$VPAC_2$ axis to alter the normal ratio of Th1/Th2 activities, and thereby subsequent effector events, led to the development of T cell-selective $VPAC_2$ TG mice and $VPAC_2$-null (KO) mice (10,11). The findings that $VPAC_2$ TG mice had an increased Th2:Th1 ratio, leading to high serum levels of IgE, heightened IgE Ab responses, eosinophilia, and elevated immediate-type hypersensitivity reactions, whereas $VPAC_2$ KO mice had a decreased Th2:Th1 ratio, resulting in strikingly augmented DTH and diminished immediate-type hypersensitivity strongly supported a central role for the VIP–$VPAC_2$ axis as a major determinant of the nature of immune effector responses. What remained to be established was whether and how regulation of secretion of VIP by Th2 cells and of $VPAC_2$ signal transduction in Th2 cells contributed substantially to the immune effector outcome. The present studies were designed to begin to answer these questions and to delineate possible contributions to net $VPAC_2$ signal transduction in T cells of a newly recognized and functionally different T cell SD splice-variant of mouse $VPAC_2$.

These initial investigations were conducted in a model Th2-type D10G4.1 cell with low background expression of WT $VPAC_2$, because native Th2 cells are the main source of immune VIP and show the greatest functional responses to $VPAC_2$-transduced signals (3). Transfectants were created that consistently expressed similar levels of mRNA and membrane protein for WT and SD mouse $VPAC_2$ (Figs. 1 and 2). The first point demonstrated in this system was that both types of transfectants generated and secreted similar levels of VIP that established nanomolar concentrations of extracellular VIP (Fig. 3). The remaining studies thus focused on transduction of VIP signals by the two forms of mouse $VPAC_2$. That the VIP secreted by D10G4.1 Th2 cells expressing WT $VPAC_2$ acts as an autocrine and possibly juxtacrine stimulus capable of signaling through WT $VPAC_2$ to enhance IL-4 generation was shown by the very significant suppression of IL-4 secretion when VIPase eliminated endogenous VIP (Fig. 4). In contrast, IL-4 generation by D10G4.1 Th2 cells expressing SD $VPAC_2$ was significantly lower than that by the WT $VPAC_2$ transfectants and did not decrease as a result

of VIPase treatment, indicating a lack of influence of the VIP–SD VPAC$_2$ axis that apparently is attributable to ineffective transduction of stimulatory VIP signals by the SD VPAC$_2$. To examine this difference further at VIP concentrations exceeding those attainable from the Th2 cells but documented in some immunological inflammatory states, the effects of signals from WT and SD VPAC$_2$ were examined in two different protocols. In the first, 10^{-8} M to 3×10^{-7} M exogenous VIP enhanced significantly secretion of IL-4 by WT VPAC$_2$ transfectants, whereas 3×10^{-8} M to 3×10^{-7} M exogenous VIP inhibited secretion of IL-4 by SD VPAC$_2$ transfectants (Fig. 5). In the second protocol, IL-4 generation by anti-CD3 plus anti-CD28-stimulated transfectants expressing WT and SD VPAC$_2$ together at different ratios was enhanced by WT VPAC$_2$-predominant mixtures, not enhanced (unchanged) at WT to SD VPAC$_2$ ratios of 0.5 to 2, and suppressed slightly by the higher concentration of VIP when the ratio was 4 (Fig. 6). Thus the SD splice-variant of VPAC$_2$ suppresses IL-4-enhancing VIP signals from WT VPAC$_2$ but does not suppress IL-4 generation as much below control levels in a mixture as it did when expressed alone.

To begin delineation of the signaling pathways used by SD VPAC$_2$, as well as WT VPAC$_2$, to regulate IL-4 generation, the transcription factors c-Maf and Jun B that are known to be involved in VIP enhancement of IL-4 generation (16) were examined in a series of VPAC$_2$ transfectants. The VIP–WT VPAC$_2$ axis, but not the VIP–SD VPAC$_2$ axis, increased nuclear levels of c-Maf and Jun B proteins and of active Jun B as quantified respectively by Western blots and a plate-bound consensus oligonucleotide-active Jun B-anti-Jun B Ab assay (Figs. 7 and 8). In WT plus SD VPAC$_2$ cotransfectants, VIP had no apparent effect on nuclear levels of c-Maf and Jun B proteins, and much less effect on nuclear levels of active Jun B than in WT VPAC$_2$-only transfectants, suggesting competitive interactions between the two forms of VPAC$_2$ in the same signaling pathway.

The next logical step to complete these investigations will be to quantify separately the expression of WT and SD VPAC$_2$ in native CD4 T cells under a range of conditions representing states in which the VIP–VPAC$_2$ axis significantly influences T cell-directed immune effector reactions. These studies also could include CD4 T cells from VPAC$_2$ TG and VPAC$_2$-null (KO) mice to assess, respectively, any influence of elevated levels of WT VPAC$_2$ on those of SD VPAC$_2$ and dependence of expression of SD VPAC$_2$ on that of WT VPAC$_2$. As the ratio of these two forms of VPAC$_2$ appears to determine the net effect of VIP, mechanisms controlling expression of the SD VPAC$_2$ are important targets of research and possibly therapeutic intervention. Initial quantification of the two forms of VPAC$_2$ relied on real-time PCR and Western blots of cellular proteins, but it would be desirable also to analyze the orientation of SD VPAC$_2$ in plasma membranes. That only a 14-aa deletion in a transmembrane domain distinguished one form from the other suggested a daunting problem for immunochemical quantification. However, recent results of structural studies indicate that the C-terminal tail of the SD VPAC$_2$ is extracellular rather than cytoplasmic as for the WT VPAC$_2$. Thus, application of Abs that recognize the native C-terminal tail or a C-terminal epitope tag will permit studies of expression of each form separately in intact cells by labeling without and with permeabilization of the plasma membrane. In addition, the possibility must be considered that signal-transducing proteins interact with a different domain of SD VPAC$_2$ than WT VPAC$_2$. All such data will be integrated into a tentative model depicting differences between T cell SD VPAC$_2$ and WT VPAC$_2$.

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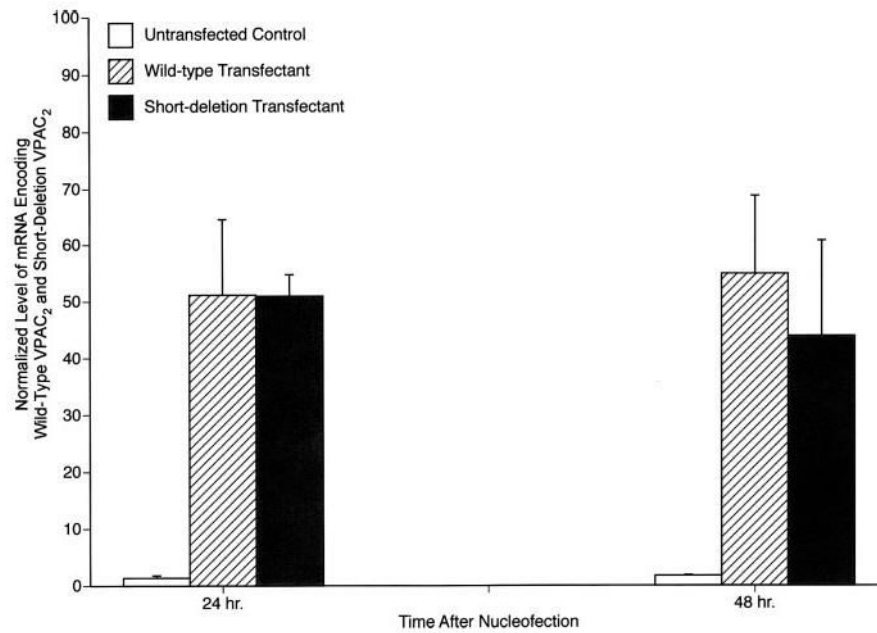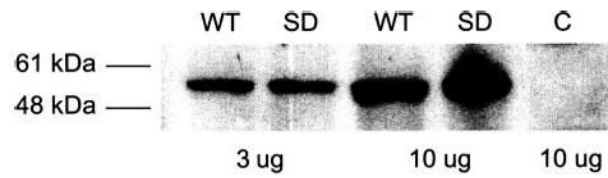
FIGURE 1.
Quantification of the levels of mRNA encoding WT and SD VPAC$_2$ in D10G4.1 cell transfectants. Each column and bar depicts the mean ± SD of the results of three individual determinations. The corresponding values for mock-transfected D10G4.1 Th2 cells were marginally detectable and were indistinguishable from those of untransfected D10G4.1 Th2 cells. Peaks of mRNA encoding each type of VPAC$_2$ receptor are evident at 24 h and persist without significant change at 48 h. None of the differences between levels of mRNA encoding WT and SD VPAC$_2$ were significant.

FIGURE 2.
Western blot of membrane proteins extracted from mouse D10G4.1 Th2 cells transfected 48 h before with plasmids encoding mouse WT and SD VPAC$_2$. The primary reagent for development was mouseanti-FLAG M2 mAb. The first pair of WT-SD lanes received 3 μg of extracted proteins, and the second pair 10 μg of extracted proteins; 10 μg of extract of control (C) untransfected D10G4.1 T cells were loaded into the last lane.
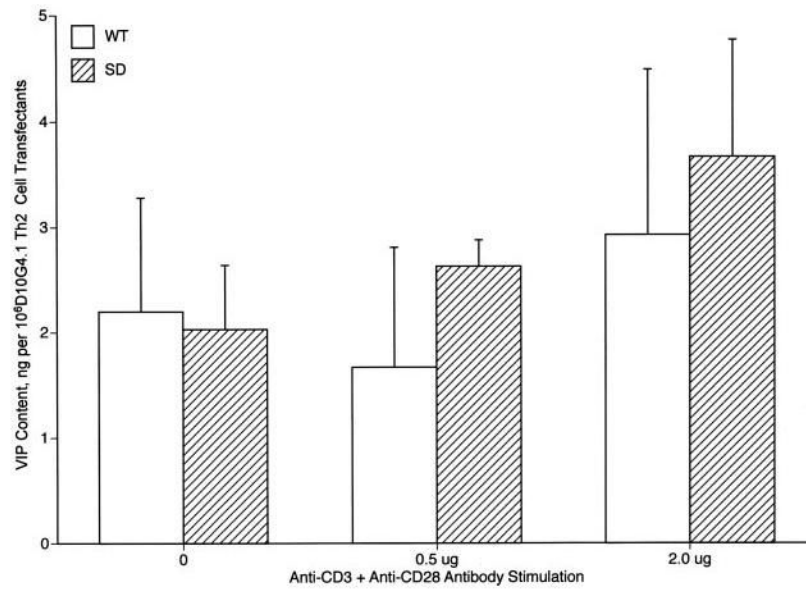
FIGURE 3.
Generation of VIP by mouse D10G4.1 Th2 cell transfectants. Each column and bar represents the mean ± SD of results from two studies analyzed in duplicate. None of the differences between levels of VIP generated by WT and SD transfectants was significant.
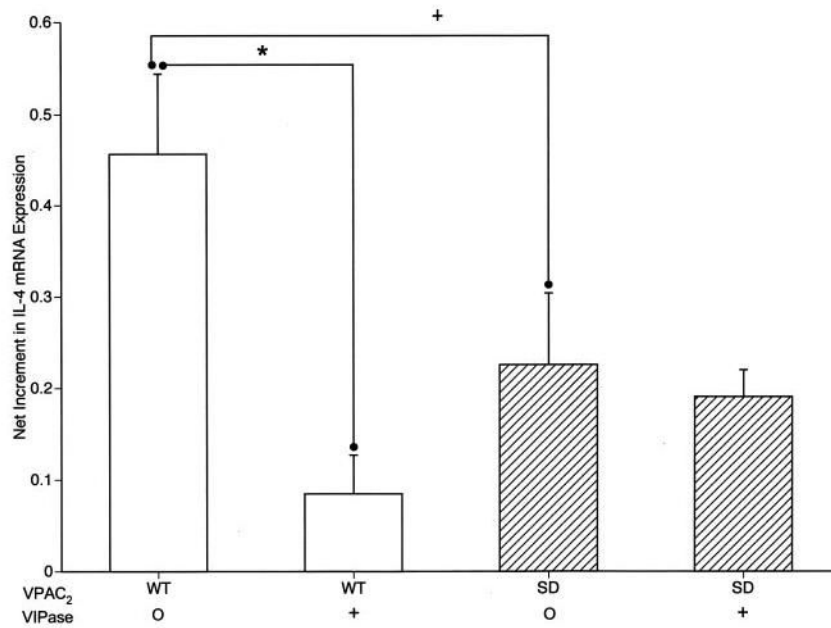
FIGURE 4.
Dependence of the level of expression of mRNA encoding IL-4 by mouse D10G4.1 Th2 cell transfectants on endogenous VIP signaling through WT and SD $VPAC_2$. The difference in corrected levels of mRNA encoding IL-4 in stimulated WT and SD $VPAC_2$ transfectants without VIPase is significant with $p < 0.05$ (+) and that between WT transfectants without and with VIPase is significant with *, $p < 0.01$; $n = 3$, paired Student's t test.
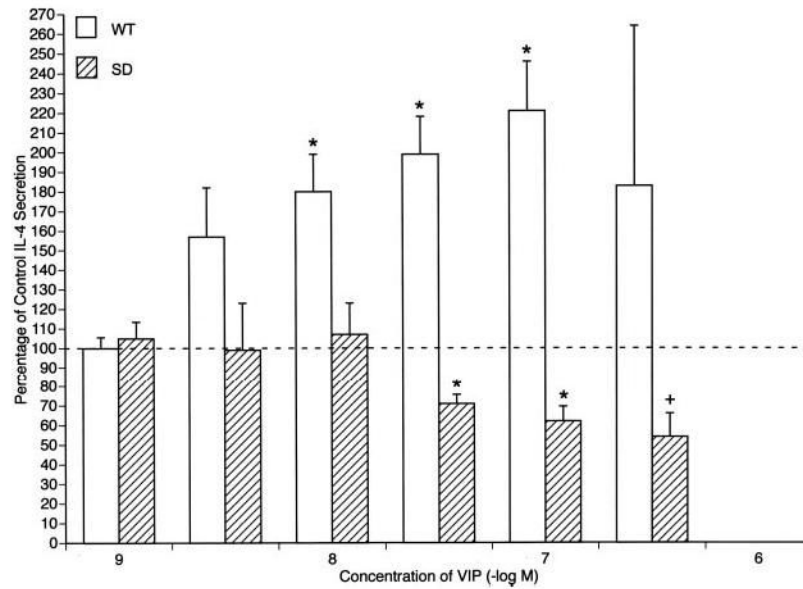
FIGURE 5.
Opposite signals from WT and SD VPAC$_2$ to IL-4 secretion by D10G4.1 Th2 cell transfectants stimulated by adherent anti-CD3 plus anti-CD28 mAbs. Control secretion of immunoreactive IL-4 by transfectants in 24 h without VIP was set at 100%. Each column and bar depicts the mean ± SD of the results of three or more studies. The concentrations of VIP between those marked were 3×10^{-9}, 3×10^{-8}, and 3×10^{-7} M. The significance of differences between IL-4 secretion in the presence and absence of exogenous VIP for each set of transfectants was calculated by a standard paired t test;+, $p < 0.05$; and *, $p < 0.01$.
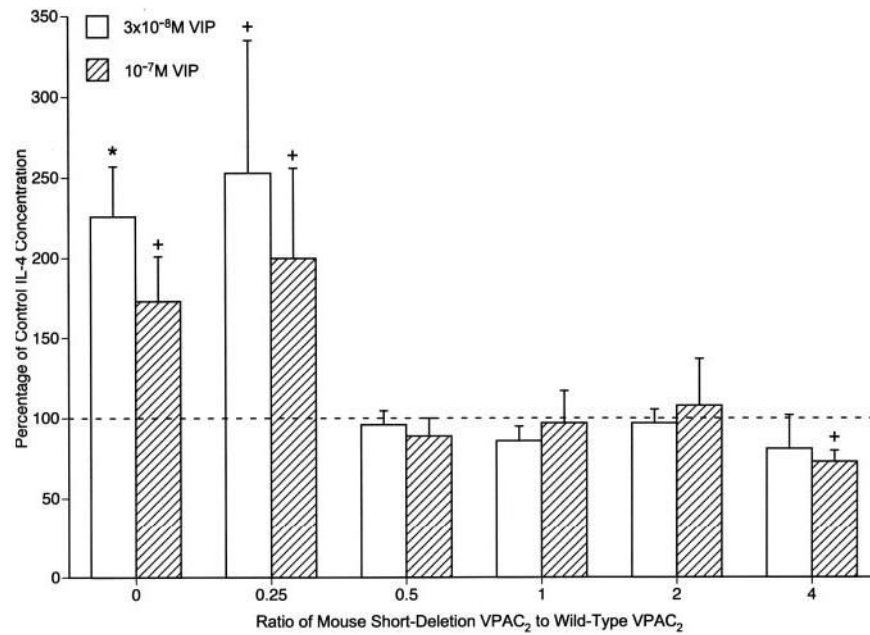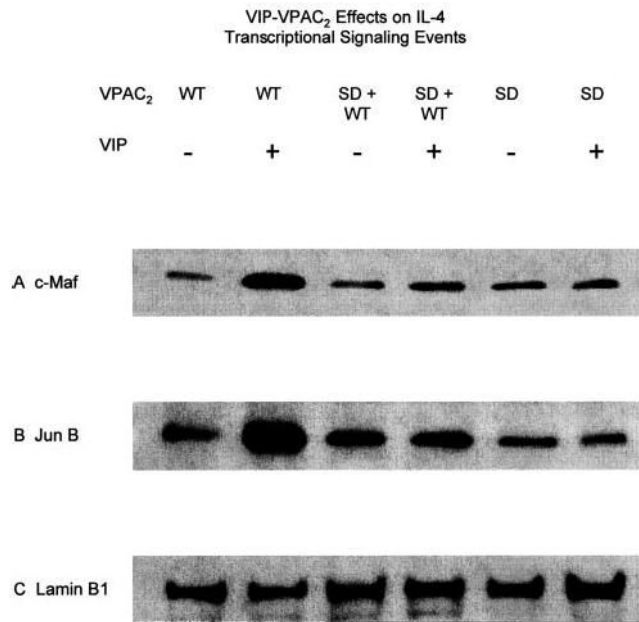
FIGURE 6.
Antagonism of VIP-WT VPAC$_2$ stimulation of IL-4 secretion by VIP-SD VPAC$_2$ in double transfectants stimulated by adherent anti-CD3 plus anti-CD28 mAbs. Control secretion of immunoreactive IL-4 by transfectants in the absence of VIP was set at 100%. Each column and bar depicts the mean ± SD of the results of three studies. The significance of differences between IL-4 secretion in the presence and absence of exogenous VIP was calculated and shown as in Fig. 5.

FIGURE 7.
Western blot analyses of effects of WT VPAC$_2$ and SD VPAC$_2$ signals on nuclear levels of transcription factors required for VIP enhancement of generation of IL-4 and the Th2 phenotype. The six lanes shown in each frame of this figure are analyses of nuclear proteins from transfectants expressing: WT VPAC$_2$ without and with 10^{-7} M VIP, respectively, SD VPAC$_2$ plus WT VPAC$_2$ (2:1) without and with 10^{-7} M VIP, and SD VPAC$_2$ alone without and with 10^{-7} M VIP. *A*, c-Maf detected by rabbit anti-c-Maf IgG Ab. *B*, Jun B detected by rabbit anti-Jun B IgG Ab. *C*, Nuclear protein lamin B1 loading control marker detected by mouse anti-lamin B1 mAb.
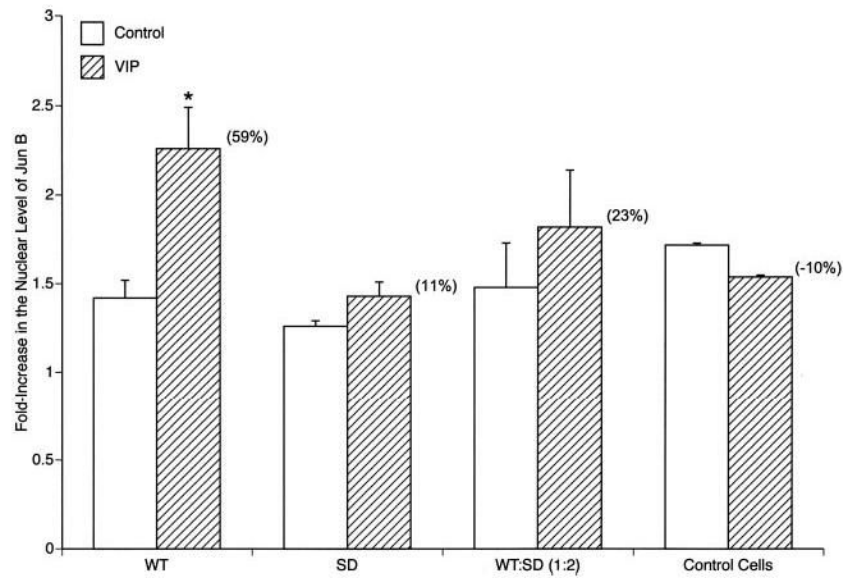
FIGURE 8.
Effect of VIP on anti-CD3 stimulated increases in the nuclear level of active Jun B protein in D10G4.1 Th2 cell transfectants. Each column and bar represents the mean ± range of two studies in duplicate. Suspensions of 10^6 transfectants in 0.6 ml of RPMI 1640 with 10% FBS were preincubated 60 min without or with 10^{-7} M VIP and then incubated with 1 μg of anti-CD3 Ab for 4 h. Each number in parentheses is the mean increase (+) or decrease (−) in active nuclear Jun B attributable to VIP. The significance of differences in levels of active nuclear Jun B between samples without and with VIP was calculated by a paired t test; *, $p < 0.01$.