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Novel association between vasoactive intestinal peptide and CRTH2 receptor in recruiting eosinophils: A possible biochemical mechanism for allergic eosinophilic inflammation of the airways

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Running Title: *Novel association between vasoactive intestinal peptide and CRTH2 receptor*

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Background: ligand receptor ligation regulates immune-inflammatory cells chemotaxis.
Results: Vasoactive intestinal peptide and Prostaglandin D2 share CRTH2 receptor in inducing eosinophil chemotaxis.
Conclusion: Strong association between VIP and CRTH2 in eosinophil chemotaxis.
Significance: This is the first evidence that may indicate that CRTH2 could modulate the neuro-immuno-regulatory axis in allergic eosinophil inflammation.

SUMMARY

We explored the relation between vasoactive intestinal peptide (VIP), CRTH2, and eosinophil recruitment. It is shown that CRTH2 expression by eosinophils from allergic rhinitis (AR) patients and eosinophil cell line (Eol-1 cells) was up-regulated by VIP treatment. This was functional and resulted into exaggerated migratory response of cells against PGD2. Nasal challenge of AR patients resulted into significant increase of VIP contents in nasal secretion (ELISA), and the immunohistochemical studies of allergic nasal tissues, showed significant expression of VIP in association with intense eosinophil recruitment. Biochemical assays showed that VIP-induced eosinophil chemotaxis from AR patients and Eol-1 cells, was mediated through CRTH2 receptor. Cells migration against VIP was sensitive to protein kinase C (PKC) and protein kinase A (PKA) inhibition, but not to tyrosine kinase or P38 MAP-kinase inhibition, or calcium chelation. Western blot demonstrated a novel CRTH2 mediated cytosol to membrane translocation of PKC-ε, PKC-δ and PKA-α, γ and IIα reg in Eol-1 cells upon stimulation with VIP. Confocal images and FACS demonstrated a strong association and co-localization between VIP peptide and CRTH2 molecules. Further, VIP induced PGD2 secretion from eosinophils. Our results demonstrate the first evidence of association between VIP and CRTH2 in recruiting eosinophils.

The allergic inflamed airway contains a pool of mediators that competes as chemoattractive signals to eosinophils. Neuropeptides secreted from the sensory neurones have been reported as chemoattractants for eosinophils (1-4). Accumulating evidences indicate an important neuro-immune interaction between the rich expression of VIP on the allergic nasal tissue and bronchial smooth muscle bundle and inflammatory cells recruitment (5-7). Furthermore, eosinophils from intestinal mucosa store and secrete VIP (8). All these evidences indicate an important neuro-immuno-inflammatory axis between VIP and eosinophils.

VIP is a 28 amino acid polypeptide, which exists in the parasympathetic nerves and to a lesser extent in the sensory fibers, is one of the most abundant of the neuropeptides found in the upper and lower airways (9, 10). Although VIP induces its biological activity through its specific receptors (11), we earlier failed to demonstrate a VPAC1 receptor on human eosinophil when compared to lymphocytes (4). Therefore, at the time we proposed that VIP may activate human eosinophil through non-specific phospholipids receptors. Recently, the novel chemoattractant receptor-homologous molecule expressed by T_{H2} cells, basophils and eosinophils (CRTH2) gained a lot of attention as promoter for PGD2 induced eosinophilia in allergic airway diseases (12-16). In an allergic rhinitis murine model it has been demonstrated that the PGD2-CRTH2 interaction is elevated following pollen sensitization. This resulted into specific IgE and IgG1 production, nasal eosinophilia and IL-4 & IL-5 production by submandibular lymph node cells. Additionally, CRTH2 mRNA in nasal mucosa was significantly elevated in Cry j 1-sensitized mice (17). Moreover, in nasal tissue, ligation of PGD2 to CRTH2 appeared to be selectively involved in eosinophil recruitment (18).

In addition to its expression on leukocytes, CRTH2 is also richly expressed in the different parts of the brain (19), which may further indicate a relation of this

receptor to neuropeptides. Molecularly, CRTH2 is a seven-transmembrane G-protein-coupled receptor (GPCR) that is composed of 395 amino acids residues with lower homology to other protanoid receptors (13, 20), but to date no other agent is reported to utilize this receptor for eosinophil chemotaxis, except for its ligand PGD2.

Accordingly, the current study was designed to explore the relation between CRTH2 and VIP in airway eosinophilic inflammation and to investigate the molecular events involved in this scenario. Eol-1 cell line that is ideal to study cellular proteins and have the ability of differentiation to mature eosinophils by n-butyrate, allowed us to explore these aims. Immunohistochemical analysis of nasal tissue from allergic chronic rhinosinusitis (ACRS) patients and nasal provocation challenges allowed us to validate our biochemical results and to have an applied in vivo correlation.

EXPERIMENTAL PROCEDURES

VIP contents in nasal secretions-ELISA. The content of VIP was measured in 10 patients with AR and 7 control healthy subjects after nasal provocation with the aeroallergen. Aeroallergens were chosen according to the results of skin test sensitivity and radioallergosorbant test (RAST) of the patients. The control subjects were challenged with histamine. None of them were taking antihistaminics or nasal/systemic cortisone therapy. After obtaining their consent, the purified and standardized allergen dilutions (stallorgenes 100IR/ml) was introduced into the nose. After 1-2 min the patients started to blow their nose and were asked to continue collecting the secretion during 15 min. Saline nasal irrigation was then done 2-3 times and the patients' vital signs were monitored for at least 30 min after challenge before being discharged from the clinic. VIP levels in collected nasal secretion were measured using VIP EIA KIT (PHOENIX PHARMACEUTICALS, INC.) according to the manufacturer's recommendations. The sensitivity of our assay was 0.04 ng/ml. All nasal secretions were used at dilution 1:50 for the EIA.

Eosinophil purification. Eosinophils were purified by percoll solution separation from patients suffering from AR. Briefly, 60 ml of heparin-anti-coagulated peripheral blood were obtained by venopuncture. The blood was diluted with Phosphate-buffered saline (PBS) containing 2% FCS in the ratio of 1:1. The percoll solution at concentration of 66% was then placed carefully by a pipette in the bottom of the tube. After centrifugation 30 min at 20^0C and 1500-RPM, a band and a pellet were obtained. The band is composed of mononuclear cells while the pellet is a mixture of eosinophils and neutrophils. Sedemented red blood cells were removed by hypodense lysis. Eosinophils were further purified by immunomagnetic cell separation (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), using anti-CD16 as described previously [4]. Eosinophil purity was >98%.

Eol-1 cell line. Human eosinophilic leukaemia (Eol-1) cell line (Riken BioResource Center, Japan) was used in parts of the current biochemical study. Cells differentiation into mature eosinophils was induced by histone deacetylase inhibitors, n-butyrate precisely as described earlier (21).

Flow cytometry analysis (FACS). CRTH2 surface expression on eosinophils and Eol-1 cells was analysed by FACS (FACS CANTO II BD Systems). Briefly, after 30 min, stimulated (VIP) or not stimulated (buffer) cells were fixed with 4% paraformaldehyde for 15 minutes. The cells were then washed and incubated with the CRTH2 antibody (BD-Pharmingen), for 60 min in the dark, on ice. After two additional washes, cells (10^5 cells/FACS plot) were conserved in paraformaldehyde 1% and then analyzed for their fluorescence intensity.

RT-PCR. Reverse transcriptase products were PCR-amplified with specific primers for HPRT, VIP, VPAC1 or VPAC2 as follows:

HPRT forward	5'-GTT GGA TAT AAG CCA GAC TTT GTT G-3'	177 bp
HPRT reverse	5'-CAG ATG TTT CCA AAC TCA ACT TGA A-3'	
VIP forward	5' CCA GGC ATG CTG ATG GAG TTT TC 3'	227 bp
VIP reverse	5' CCT CTT TCC ATT CAG AAT TGA GTT 3'	
VPAC1 forward	5' CTT CTG GTC GCC ACA GCT ATC CTG 3'	534 bp
VPAC1 reverse	5' ACT GCT GTC ACT CTT CCT GAT ATC 3'	
VPAC2 forward	5' CGT CAC GGT GCC CTG CCC AAA AGT 3'	462 bp
VPAC2 reverse	5' CCC TCC ACC AGC AGC CAG AAG A 3'	

The PCR conditions for HPRT were: initial denaturation at 95°C for 5 min followed by 35 cycles of 45 s at 95°C, 45 s at 60°C and 45 s at 72°C. The PCR conditions for VIP were: denaturation at 94°C for 5 min followed by 40 cycles of 30 s at 94°C, 30 s at 57°C and 45s at 72°C and then a final cycle of 5 min at 72°C. The PCR conditions for VPAC1 were similar to those for VIP, 30 s at 94°C, 30 s at 60°C, and 45 s at 72°C. In the case of VIPAC2 the PCR conditions were: denaturation at 94°C for 5 min, and 40 cycles of 30 s at 94°C, 30 s at 58°C and 45 s at 72°C and then a final cycle of 5 min at 72°C.

RT-PCR products were assayed on 1.8% - agarose gel electrophoresis and visualized by staining with ethidium bromide.

Immunohistochemistry. Sections of nasal specimens underwent immunoperoxidase staining using antibodies directed against VIP (1:50) (ab8556, Abcam). The sections were deparaffinized in xylene and rehydrated in methanol. Endogenous peroxidases were blocked by 5% H_2O_2 treatment. Samples were then washed with PBS. A second treatment for 5 min with H_2O_2 (Dako) was then applied. The samples were washed again in PBS before being blocked for 10 min with Dako blocking reagent. Samples were then incubated with the primary antibody at room temperature overnight. After washings, the revelation was performed with the use of appropriate secondary antibodies and the LSAB2 system (VIP; Dako A/S) according to the supplier's recommendations. Immunoreactivity was visualized by a treatment with diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA), and the slides were counterstained with Mayer's hematoxylin. For staining intensity, (-) represented samples in which the staining was undetectable, whereas (+) and (++) denoted samples with weak and strong staining, respectively.

Chemotaxis assays. Chemotaxis assays were performed in triplicate in a 48-well microchemotaxis Boyden chamber incubated in 5% CO*2* at 37°C for 90 min. Aliquots of 29µl of the chemotactic agent eotaxin (R&D Systems; Minneapolis, MN), VIP (Phoenix Pharmaceuticals), or PGD2 (Cayman chemical) were placed in the lower wells and 50µl of either peripheral purified eosinophils or Eol-1 suspension (10^6cells/ml) were placed in the upper wells. The two chambers were separated by a 5.0µm pore polycarbonate membrane (Nuclepore, Whatman, Middlesex, UK). The controls consisted of a solution of Hank's balanced salt solution (HBSS). After 90 min incubation at 37^0C, the membrane was removed, fixed in methanol and stained with

Diff-Quick (Baxter Scientific; Miami, FL.). Migrated cells adherent to the lower surface were counted in 5 selected high power fields/well under a light microscope (5hpf; X400). As for the blocking experiments, the cells were pretreated with VIPR1 (Sigma) the VIP receptor antagonist, antihuman CRTH2 receptor antibody (R&D systems), H-89 Dihydrochloride (VWR-CALBIOCHEM) a PKA inhibitor, Bisindolylmaleimide (VWR-CALBIOCHEM) a PKC inhibitor, SB203580 (VWR-CALBIOCHEM) a P38 MAP-Kinase inhibitor or Genistein (Sigma) a tyrosine kinase inhibitor, for 60 min at 37^0C. Cells were then washed twice, re-suspended in buffer medium and their chemotaxis was checked as stated above.

Ca^{2+} depleted cells. Ca^{2+} depleted cells were obtained by incubating 10^7cells/ml with 30 µmol/L of the calcium chelating agent BAPTA-AM (CALBIOCHEM) in test medium [130mmol/L NaCl, 5mmol/L NaHCO3, 4.6 mmol/L KCl, 5mmol/L glucose, 2mmol/L ethyleneglycol-bis (ß-aminoethyl ether)-N,N,N´,N´-tetraacetic acid (EGTA) and 20 mmol/L N-2-hydroxyethylpiperazine-N´-2-ethanesulfonic acid (HEPES)], for 30 min at 37^oC.

Assessment of actin reorganization with phalloidin-FITC and the cytoskeleton changes. 30 µl of cell suspensions at 10^6 cells/ml were placed in 1 *µ-Slide* VI coated (collagen IV) cell microscopy chamber (Ibidi integrated BioDiagnostics, Munich-Germany) and left to adhere for 30 min. Stimulation with buffer, eotaxin or VIP were then performed for 15 min. After two washes with PBS the cells were fixed in 4% formaldehyde for 20 min and permeabilized with 0.1% saponin, for another 30 min. The cells were then stained with Alexa fluor 488® – Phalloidin diluted 40X (Invitrogen Molecular probes®) for 30 min, in the dark, on ice. After two washes with PBS, the cells were conserved in prolong® Gold antifade with DAPI (Invitrogen Molecular Probes Eugene Oregon. USA.) and were then analyzed by confocal microscopy (Leica).

Preparation of cell extracts for western blotting. For preparation of whole cell extracts, cells were pelleted by centrifugation and washed twice with PBS. The cell pellets were re-suspended in cold RIPA lysis buffer (Tris HCl pH7.6 25mM; NaCl 150mM; NP40 1%; sodium deoxycholate 1%; sodium dodecyl sulfate 0.1%; Pierce) supplemented with protease inhibitors (Complete, Roche) and subsequently swirled for 10 min on ice. The extracts were then centrifugated at 14000 g for 15 min at 4°C. The supernatants were analysed for protein content by the Bio-Rad protein assay based on Bradford method (Bio-Rad). While for preparation of membrane extracts, Mem-PER eukaryotic membrane protein extraction reagent kit was used (Pierce). In accordance to the manufacturer's protocol, cells were pelleted by centrifugation and washed twice with PBS. The cell pellets were re-suspended in reagent A supplemented with protease inhibitors (Complete, Roche) and subsequently incubated for 10 min at room temperature. The suspensions were placed on ice and diluted reagent C was added for 30 min. After centrifugation at 10000 g for 3 min at 4°C, supernatant were incubated 10 min at 37°C and after a second centrifugation at 10000 g for 2 min at room temperature, membrane proteins were isolated. The supernatants were analysed for protein concentration by the Bradford method (Bio-Rad).

Antibodies— The antibodies used for Western blot were rabbit anti-PKCδ antibody (C-17, sc-213), rabbit anti-PKCε antibody (C-15, sc-214), rabbit anti-PKAα cat antibody (C-20, sc-903), rabbit anti-PKAγ cat antibody (C-20, sc-905), rabbit anti-PKA IIα reg antibody (C-20, sc-908), all were purchased from Santa Cruz Biotechnology. Anti-CRTH2 rabbit polyclonal antibody was obtained from ABCAM (ab59382). The blocking antibody used is anti-CRTH2 rat monoclonal antibody (BM16) purchased from BD Biosciences.

*Western blotting experiments.*Western blot analysis was performed on proteins extracted after 30 min or 24h of treatment with VIP at 10^{-7}M (Phoenix Pharmaceuticals) as indicated and with or without pretreatment for 1h with the blocking antibody. Samples were separated on a 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Roche). The primary antibodies were used at a 1:200 dilution. The secondary anti-rabbit antibodies coupled with horseradish peroxidase (Amersham) at a 1:3000 dilution were detected by chemiluminescence with the ECL system (Pierce). For fig. 1D and fig. 4, blots were scanned and quantified using ImageJ software, using GAPDH or Coomassie blue staining as loading control, respectively.

Fluorescence-labelled VIP & CRTH2 binding studies. A nonradioactive technique utilising 1µM concentration of Cy3-Ahx-VIP (PiCHEM-Austria), Cy3-Ahx-HSDAVFTDNYTRLRKQMAVKKYLNSI LN-NH2, was used. Eol-1 cells were incubated with Cy3-Ahx-VIP for 30 min in the presence or absence of 10^{-7}M anti-VIP (Phoenix Pharmaceuticals), 10µg/ml anti-CRTH2 blocking antibody, or 10^{-5}M anti-VIP receptor antagonist (VIPR1). After two washes, cells were fixed and subjected immediately to confocal laser scanning microscopy and FACS analysis. As for the co-localization experiments, cells were first stained with anti-CRTH2 Alexa fluor-conjugated Ab (B&D pharmingen) for 30 min, washed and then stained with the labelled Cy3-Ahx-VIP as described above. To control for peptide unrelated staining the cells were incubated with Cy3 alone (PiCHEM-Austria).

PGD2 secretion by eosinophils-ELISA. PGD2 level in the supernatant of cultured eosinophils from 3 allergic patients, to poly-aeroallergens, was checked utilising ELISA kit (Cayman chemical), according to the manufacturer's recommendations. The stimulation of eosinophils was for 30 min and 24h with either buffer only, 10^{-7}M VIP only or 10^{-7}M VIP in presence of 0.1 µg/ml anti-VIP.

Statistical analysis

Results are expressed as the mean ± SEM. Statistical significance was analyzed by paired student's *t*-test and ANOVA. A $P<0.05$ was considered to be statistically significant

RESULTS

Relationship between VIP-eosinophil-CRTH2 in AR and allergic chronic rhinosinusitis (ACRS)-To investigate the in vivo amount of VIP secreted by the healthy and allergic airway in response to nasal provocation, we measured VIP amounts by ELISA in the nasal secretions from AR patients and compared them to controls. As seen in fig 1A, there was significantly higher content of VIP in nasal secretions from allergic subjects when compared to the controls. Nasal cytology from AR patients' nasal secretions showed eosinophilia (Data not shown). We have recently reported by immunohistochemical studies of nasal tissue obtained from the middle turbinate as a part of the surgical procedure from patients undergoing endoscopic sinus surgery for ACRS and nonallergic CRS, a positive expression of CRTH2 in population of infiltrating eosinophils and lymphocytes, respectively when compared to controls operated for reduction of the inferior turbinates, highlighting the importance of CRTH2 in inflammatory cells recruitment to the inflamed nose (22). Therefore, we next investigated whether VIP could modulate the expression of CRTH2 on human eosinophils from AR patients. As demonstrated in fig 1B, eosinophils treatment with 10^{-7}M VIP for 24h, resulted into up-regulation of the expression of CRTH2. The mean fluorescence intensity (MFI) of CRTH2 was 56±10 and 69±5 for the spontaneous expression from AR patients, after 24h culture in buffer medium

alone, or in the presence of 10^{-7}M VIP, respectively.

Interestingly, all histograms demonstrated a double or triple population of CRTH2 expression in eosinophils and Eol-1 cells as seen in fig.1B. To further investigate these heterogeneous populations of CRTH2 expression we doubles stained the cells with anti-CRTH2 and anti-CD16. As seen in fig. 1C less than 3% of peripheral blood eosinophils expressed CD16 and none of Eol-1 cells did. The VIP treatment of eosinophils and Eol-1 cells with VIP did not modulate the percentage of CD16 positive cells, but increased the expression and total protein content of CRTH2 (fig. 1D). This up-regulation was functional with exaggerated eosinophil chemotaxis against sub-optimal dose of 10^{-9}M PGD2 (fig 1E). Further, VIP immunohistochemical analysis from nasal middle turbinate mucosa of patients with ACRS demonstrated significant expression by the epithelial layer and lamina propria (fig 2 ii-v) over controls (nasal tissue obtained from the inferior turbinate for turbinate reduction). The expression of VIP was associated with intense eosinophils infiltration (fig. 2vi). All the above data point to a possible in vivo association between VIP-Eosinophil-CRTH2, in the pathophysiology of allergy of the upper airway.

Eosinophilotactic activity of VIP-To investigate whether VIP attracts human eosinophil from AR patients in a different pattern than what we reported earlier from normal peripheral blood eosinophils (4), the following chemotaxis assays were performed. As can be seen from fig. 3A VIP at a wide range of doses (10^{-5}-10^{-9}M) significantly chemoattracted eosinophils from AR patients and lost its significant chemotactic activity at 10^{-10}M. The efficacy of VIP eosinophilotactic activity was comparable to the positive control, eotaxin, but the chemotaxis index was less than eotaxin (CI=4.7 for eotaxin and 3 for VIP). Also VIP dose response curve was different than classical chemokines showing neither dose dependency nor the classical bell shape curve of chemokines. Checker board analysis confirmed a mainly chemotactic effect with a lesser chemokinetic effect (data not shown). These results indicate the ability of VIP to attract normal eosinophils and eosinophils from AR patients in similar fashion.

In agreement with our earlier report from normal subjects (4), eosinophils chemotaxis from AR patients against VIP was not VIP receptor mediated as seen in fig 3B. Intriguingly, pretreatment of eosinophils from AR patients with 10μg/ml CRTH2 receptor antibody before inducing eosinophils chemotaxis against VIP did significantly inhibit VIP induced eosinophil chemotaxis (fig 3B). These results pointed to a possible association between VIP and CRTH2 in mediating eosinophil chemotaxis and the lack of expression of VPAC1 by human eosinophils. To further explore this possibility a search for VIP receptors in eosinophils was performed. As demonstrated by RT-PCR in fig. 3F eosinophils expressed VIP protein but did not express VPAC1 or VPAC2. Further, control experiments on human lymphocytes demonstrated the ability of VPAC1 in dose dependency to mediate VIP-induced lymphocytes chemotaxis as shown in fig. 3C. Next we performed a competition assay between VIP and PGD2 the only known ligand to date for CRTH2. Five min stimulation of eosinophils with PGD2 resulted into quick internalization of CRTH2 as shown in fig.3D and significantly reduced VIP-induced eosinophils chemotaxis as shown in fig 3E.

Involvement of Ca^{2+} independent PKC and PKA in the signal transduction of VIP induced eosinophils chemotaxis-First we tested several protein kinases inhibitors ability to modify VIP-induced eosinophil chemotaxis. As seen in table1, PKC (Bisindolylmaleimide) and PKA (H-89) inhibitors, did block VIP-induced eosinophil chemotaxis but neither P38-MAP Kinase (SB203580), nor tyrosine kinase (Genistein) inhibitors, did block VIP-induced eosinophil chemotaxis. The latter rather increased the

VIP-induced eosinophil chemotaxis through up-regulation of CRTH2 surface expression (supplementary fig.3). Under Ca^{2+} intact or Ca^{2+} depleted conditions VIP induced-eosinophil chemotaxis was equal (table 1). Our results indicate the involvement of Ca^{2+} independent PKC and PKA activity in eosinophil's CRTH2 stimulation by VIP. There are two recognized isoforms of PKC that are Ca^{2+} independent, namely PKCδ & PKCε. To further study the signal transduction mechanism(s) involved in VIP stimulation of CRTH2, we utilized eosinophil cell line (Eol-1) that expresses CRTH2 receptor (22) and thus was ideal for the signal transduction studies. We performed western blot experiments to confirm the involvement of PKC and PKA residues activation in Eol-1 cells following stimulation by VIP.

VIP increases the PKCδ, PKCε, PKAα, PKAαIIreg and PKAγ membranous level through CRTH2- We performed western blots on whole cell extracts (WCE) and membranous extracts (MemE) of Eol-1 cells at low and high passages treated or not with VIP for 30 min. Data obtained on Eol-1 cells at high passages are represented in figure 4. Results showed that VIP did not modulate the CRTH2 level in the whole cell extracts after 30 min of stimulation. Similar results were observed in the Eol-1 cells at low passages (data not shown). However, the level of CRTH2 was higher in Eol-1 at high passages in comparison with Eol-1 at low passages (data not shown). The presence of CRTH2 was also studied in membranous extracts but the protein could not be detected. As observed in figure 4A, VIP did not modify the absolute level of the different PKCs and PKAs studied (see WCE). However, VIP clearly increased the level of these proteins in membranous extracts, particularly PKCε, PKA□ and PKAαIIreg (Fig. 4A, compare lane 2 to lane 1). These increases are significant as shown in the graphs in figure 4B (quantification of membranous extracts). These effects seemed to be transient because at 24 hours of treatment with VIP, the level of the different PKCs and PKAs stayed similar to the untreated cells (data not shown). Thus, it appears that VIP is able to induce the recruitment of the different PKCs and PKAs to the membrane after a short time of treatment of Eol-1 cells at high passages. Interestingly, in Eol-1 cells at low passages, the membranous localization of the different PKCs and PKAs was not modulated by the VIP treatment. This could be correlated with a lower level of CRTH2 protein in these cells in comparison with Eol-1 cells at high passages.

In order to identify the potential implication of CRTH2 in these events, we pretreated cells with a blocking antibody against CRTH2 before treatment with VIP. The pretreatment with this blocking antibody clearly blocked the effect of VIP on all PKCs and PKAs (compare lane 4 to lane 2). Thus, these results indicate that CRTH2 seems to be partially involved in the membranous recruitment of PKCδ, PKCε, PKAα, PKAαIIreg and PKAγ induced by VIP.

VIP-induced eosinophil cytoskeletal changes-Cell migration following exposure to chemoattractants is preceded by many processes, including cytoskeletal reorganization and cell shape changes. Rapid and reversible polymerization of globular monomeric actin into filamentous polymeric actin (F-actin) initiates shape changes. Therefore we performed further experiments to check these cellular events. As demonstrated in fig. 5, cells adherent to the collagen type 4 coated slides changed its shape with F-actin reorganization and increase in their content from mean fluorescence of 347 for the control stimulation to 465 in response to 10^{-7}M VIP stimulation, as judged by FACS analysis. Similar results were obtained with other tested doses of VIP (10^{-5}-10^{-9}M). Blockage of CRTH2 receptor but not VIP-R1, inhibited eosinophil shape changes and F-action reorganization and reduced the F-actin contents to 250. A similar reduction of F–actin contents to near basal levels were also observed with blockage of PKA and

PKC (fig 5). Taken collectively, our data further support a specific chemotaxis signal for VIP in eosinophil that is mediated through CRTH2 receptor and involves PKA and PKC pathways. To this end the issue of VIP receptors is in continuous evolution and recently another variant of 5 transmembrane isoform of VIP receptors was identified [23]. To the best of our knowledge this is the first article to show a signal transduction of chemoattractant other than PGD2 and its derivative, that may utilizes CRTH2 receptor on human eosinophil. To further elaborate on the exact fashion of ligation between VIP and CRTH2, we explored in the following experiments, the possibilities of direct physical co-localization and binding as well as the indirect possibility through liberation of PGD2 by eosinophils in response to VIP.

Strong co-localization between VIP and CRTH2 with reduction of VIP binding to Eol-1 cells by anti-CRTH2 blocking Ab-Confocal images in fig 6A demonstrated strong association and co-localization of VIP and CRTH2 molecules. This was further supported by FACS analysis (fig. 6B&C) and confocal images (fig. 6D) that demonstrated significant reduction of VIP binding to Eol-1 in presence of anti-CRTH2 receptor blocking Ab. Of note no modulation of VIP binding was observed in the presence of VIP-R1 (fig. 6B&C). These results points to strong physical ligation between VIP and CRTH2 in eosinophils, and may indicate specific binding of VIP to CRTH2 on human eosinophils.

VIP induces PGD2 secretion by eosinophils-Finally, to gain further insight about the possible indirect mechanisms by which VIP stimulates eosinophil chemotaxis through CRTH2, we cultured eosinophils from atopic subjects (n=3) for 30 min and 24 h, with 10^{-7}M VIP. Supernatants were then collected and the amount of PGD2 secreted was measured by ELISA. Interestingly, the mean percentage of PGD2 secreted by eosinophils increased upon stimulation with VIP for 30 min and 24 hr from 100% to 105.19% and 140.17%, respectively. These results may indicate the ability of VIP to stimulate CRTH2 through its PGD2 secretagouge activity in human eosinophil.

DISCUSSION

Eosinophilia is a hallmark in AR and is blamed for the chronicity of the disease. Therefore, the current interest in airways eosinophilic inflammation is to discover the molecular events resulting in eosinophil recruitment and hence to develop an effective mode of therapy. The biological effects of VIP presented herein on eosinophil chemotaxis and previous reports on its effect on mast cell chemotaxis, degranulation and cytokines production (5, 24) indicate an important role for VIP in allergic inflammation of the airway. We showed that the allergic nasal tissue secretes VIP in doses that attracted human eosinophil in Boyden chambers. Further, the allergic nasal tissue infiltrated with eosinophils, significantly express VIP. Eosinophils treatment with VIP for 24h, up-regulated CRTH2, on human eosinophils and increased the amount of total CRTH2 protein. This was VPAC1 &VPAC2 independent and seems to be an autostimulation of CRTH2 in response to VIP ligation. This up-regulation was functional and resulted into exaggerated eosinophilotactic response against the sub-optimal dose of PGD2 (22). This indicates that VIP is a potent primer for PGD2-induced eosinophil chemotaxis. This priming effect seems to be different than the priming effect of IFN-γ and TNF-α that we reported earlier (22), that was through the functional up-regulation of CRTH2 expression, since neither IFN-γ nor TNF-α did modulate the total amount of CRTH2 protein.

The expression patterns of CRTH2 by human eosinophils showed heterogeneous populations; with some population expressing higher amounts of the receptor than others (fig 1B&C). To exclude the possibility of either contamination with

other cell types or cells being more activated than others, we doubled stained eosinophils with CRTH2 and CD16. The latter can be induced in vitro by mediators stimulation and is temporary expressed in asthmatics following allergen challenge. Our FACS results excluded any effect of CD16 acquired expression with this heterogeneous CRTH2 expression in absence or presence of VIP. Further the lack of expression of CD16 by Eol-1 cells also excluded such possibilities. We excluded also apoptosis/necrosis (data not shown) or cells aggregation to be a reason for this heterogeneous patterns (supplementary fig. 2c). We induced also the expression of CD48; the subtype of CD2 Ig superfamily, by eotaxin stimulation. These molecules are up-regulated in allergy of the airways (25-28) and are inducible in eosinophils by eotaxin stimulation in vitro. The expression of CD48 molecules were clearly up-regulated in stimulated cells (supplementary fig. 2A). Although both eosinophils' subpopulation expressed CD48 molecules, the fluorescence intensity of these molecules was higher in the sub-population of eosinophils expressing more amounts of CRTH2 (Supplementary fig. 2C). These results indicate that eosinophil's population expressing more amounts of CRTH2 receptor is more primed to subsequent stimuli. On the other hand the ability of genistein to induce CRTH2 up-regulation, with 3 different heterogeneous populations in Eol-1 cells (supplementary fig.3), may indicate an interesting regulation of the rate of synthesis and/or recycling of CRTH2 by tyrosine kinases and/or phosphatases activity.

Chemokine-mediated signal transduction is believed to involve (i) Ca^{2+} mobilization, protein kinase C and heterotrimeric GTP-binding proteins in a classical view (29), and (ii) kinases and phosphatases, adaptor proteins, and a small GTP-binding proteins in an alternate view (30-33). Our data indicate that VIP induced eosinophil chemotaxis was mediated throught CRTH2 receptor, and that calcium per se was not involved as a second messenger in the signal transduction. This VIP-CRTH2 ligation involved a novel PKCε, PKCδ, PKAα, PKAαIIreg and PKAγ cytosol to membrane translocation without altering these proteins total contents. To this end the increased migration of eosinophils against VIP in the presence of genistein treatment is in consistent with our earlier study that indicated modulation of eosinophil migration against different group of chemoattractants by herbimycin A, erbstatin and pervanadate (34). Further, genistein was able to increase the surface expression of CRTH2 (supplementary fig.3) on Eol-1 cells that may explain the increased migration of eosinophils against VIP in genistein presence, and support our signal transduction results that pointed to association between VIP and CRTH2 in inducing eosinophils migration.

We next explored the possible mechanisms of association between VIP and CRTH2. Our results of confocal images and FACS analysis indicate a strong physical association and co-localization between VIP and CRTH2 but not VIP-R1 receptor. This is supported by the fact that eosinophils lack the expression of VPAC1 &2 and that anti-CRTH2 Ab treatment of Eol-1 cells but not VIP-R1, resulted into significant reduction of VIP binding. Further, PGD2 and VIP seemed to be competitive to human eosinophil in chemotaxis assays. On the other hand, VIP induced PGD2 secretion from human eosinophils. This effect was as early as 30 min and lasted up to 24h and was blocked by anti-VIP Ab indicating a specific secretagouge activity. This may further provide an indirect pathway of activating CRTH2 on human eosinophil by VIP, and may explain the lack of the characteristic bell shape dose response of VIP-induced eosinophils chemotaxis shown in fig. 3A.

To conclude, to our best knowledge this is the first article that links a neuropeptide (VIP) signaling to CRTH2 on human eosinophil. Several separate lines of evidence point towards such association between VIP and CRTH2 in inducing eosinophil chemotaxis as follows:

1. The ability of anti-CRTH2 blocking Ab to inhibit VIP-induced human eosinophils cytoskeletal changes and chemotaxis.
2. The ability of anti-CRTH2 blocking Ab to block VIP- induced PKCs and PKAs membrane translocation in Eol-1 cells.
3. The ability of anti-CRTH2 blocking Ab to significantly reduce VIP binding to eosinophils.
4. The ability of VIP to induce protein synthesis of CRTH2 and its surface expression.
5. The ability of VIP to induce PGD2 sceretion by eosinophils.

While it seems certain that CRTH2 modulated the biochemical events of VIP-induced eosinophils chemotaxis, it remains unclear what is the exact mode of VIP and CRTH2 interactions. However, our results open channels for other researchers to further explore the possibility of specific binding of VIP to CRTH2 through further advanced binding assays such as FRET that is beyond the scope of the authors.

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CONFLICT OF INTEREST

The authors claim no conflict of interest with the current work.

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FIGURE LEGENDS

Figure 1: (A) Quantity of VIP recovered from nasal secretion of AR patients following allergen challenge (n=17, 7 healthy subjects and 10 patients with AR). Asterisks indicate $p<0.05$. (B) FACS analysis of CRTH2 receptor surface expression from peripheral blood eosinophils. White histogram represent CRTH2 expression while black histogram represents CRTH2 expression after stimulation of cells by 10^{-7}M VIP for 24 h. Histograms are representative of 12 patients showing similar results. (C) Expression of CRTH2 and CD16 in eosinophils and Eol-1 cells. Left panel represents control markers, middle panel represents CRTH2 and CD16 expression in absence of VIP treatment and the right panel represents CRTH2 and CD16 expression after VIP treatment (D) Expression of CRTH2 in Eol-1 cells and in eosinophils with (+) or without (-) a 24h VIP treatment. Western blot were performed on whole cell extracts; GAPDH was used as control. Graphic represents quantification of 3 experiments.*, $p<0.05$. (E) Priming effect of 10^{-7}M VIP on 10^{-9} M PGD2-induced eosinophils chemotaxis. Results are the mean ±SEM of 3 independent experiments performed in triplicate.

Figure 2: Relationship between eosinophils infiltration and VIP expression within nasal tissue. Brown color represents VIP expression. (i) Control subject, (ii-vi) Patients with ACRS. Note the expression of VIP by the epithelial cells (ii), some stromal cells (ii-vi) and the infiltrating eosinophils (vi). Staining intensity: (-) for the control, in epithelium and subepithelium layer, and (++) for ACRS in the epithelium and subepithelium layer. Magnification X200 for (i-v) images and X400 for (vi) image. Scale bars equal 25µm (i to v) and 50µm in figure (vi).

Figure 3: (A) VIP eosinophilotactic activity compared to eotaxin. Results are ± SEM of 10 independent experiments performed in triplicate. Asterisks indicate $p<0.05$. (B) Modulatory effects of VIP-R1 and anti- CRTH2 blocking antibody on 10^{-7}M VIP eosinophilotactic activity. Results are ± SEM of 6 independent experiments performed in triplicate. The culture medium consisted of a solution of Hank's balanced salt solution (HBSS) for all treatments (buffer only, VIP-R1 and anti-CRTH2) during the chemotaxis assays (C) Blocking effect of VIPR1 on 10^{-7}M VIP induced lymphocytes chemotaxis. Results are ± SEM of 5 independent experiments performed in triplicate. Asterisks indicate $P<0.05$. (D) Internalization of surface CRTH2 receptors in human eosinophils after 5 min stimulation with 10^{-7}M PGD2. Grey histogram represents CRTH2 spontaneous expression while white histogram represents CRTH2 expresssion after stimulation with 10^{-7}M PGD2 for 5 min. (E) The effect of the treatment of eosinophils with 10^{-7}M PGD2, 5 min before and during the chemotaxis assay, against 10^{-7}M VIP (± SEM of 5 independent experiments performed in triplicate). White bars represents migration in absence of PGD2 while black bars represent migration in presence of PGD2 (F) RT-PCR showing expression of VPAC1 & VPAC2 by human lymphocytes but not human eosinophils, while VIP protein was expressed by both cell types.

Figure 4: Expression of CRTH2 and different PKA and PKC in Eol-1 cells after VIP treatment. (A) Western blot were performed on either whole cell extracts (WCE) or membranous extracts (MemE) of Eol-1 cells treated (+) or not (-) with VIP for 30 minutes, with (+) or without (-) pretreatment with CRTH2 blocking antibody. (B) Graphs representing the quantification of western blot experiments on membranous extracts, Coomassie blue staining was used as loading control. *, $p<0.05$

Figure 5: Confocal images of peripheral blood eosinophil's shape changes from AR patients. Green color=F-actin. Stimulants concentrations were as follows: VIP (10^{-7}M), VIP-R1 (10^{-5}M) and anti-CRTH2 antibody 10 μg/ml. Similar images were obtained with other VIP concentrations (10^{-5}-10^{-9}M) and VIP-R1 (10^{-5}-10^{-7}M). Images are from one experiment representative of 3, all showing similar results.

Figure 6: (A) Confocal images of one experiment representative of 3 all showing similar images. (i) Eol-1 stained with labeled-VIP (in green), (ii) Eol-1 stained with anti-CRTH2 (in red), or (iii) both markers i &ii. Of note the controls with unlabeled VIP and Cy3 dye alone did not show any fluorescence (Supplementary fig. 1). The Pearson's coefficient for the presented images was 0.2 as assessed by Microsoft co-localization software. (B) Histogram from one experiment representative of 3, showing labeled-VIP on Eol-1 cell surface in presence and absence of anti-CRTH2 Ab and anti-VIP receptor antagonist. (i) Grey color= control, dotted line= VIP fluorescence and solid line= VIP fluorescence in presence of anti-CRTH2 blocking Ab. (ii) Grey color= control, dotted line= VIP fluorescence and solid line= VIP fluorescence in presence of anti-VIP receptor antagonist (VIP-R1) (C) MFI of labeled-VIP on the surface of Eol-1 cells. Results are the mean ± SEM of 3 independent experiments. (D) confocal images of labeled VIP binding to Eol-1 cells in absence (i) or presence (ii) of blocking anti-CRTH2 Ab.

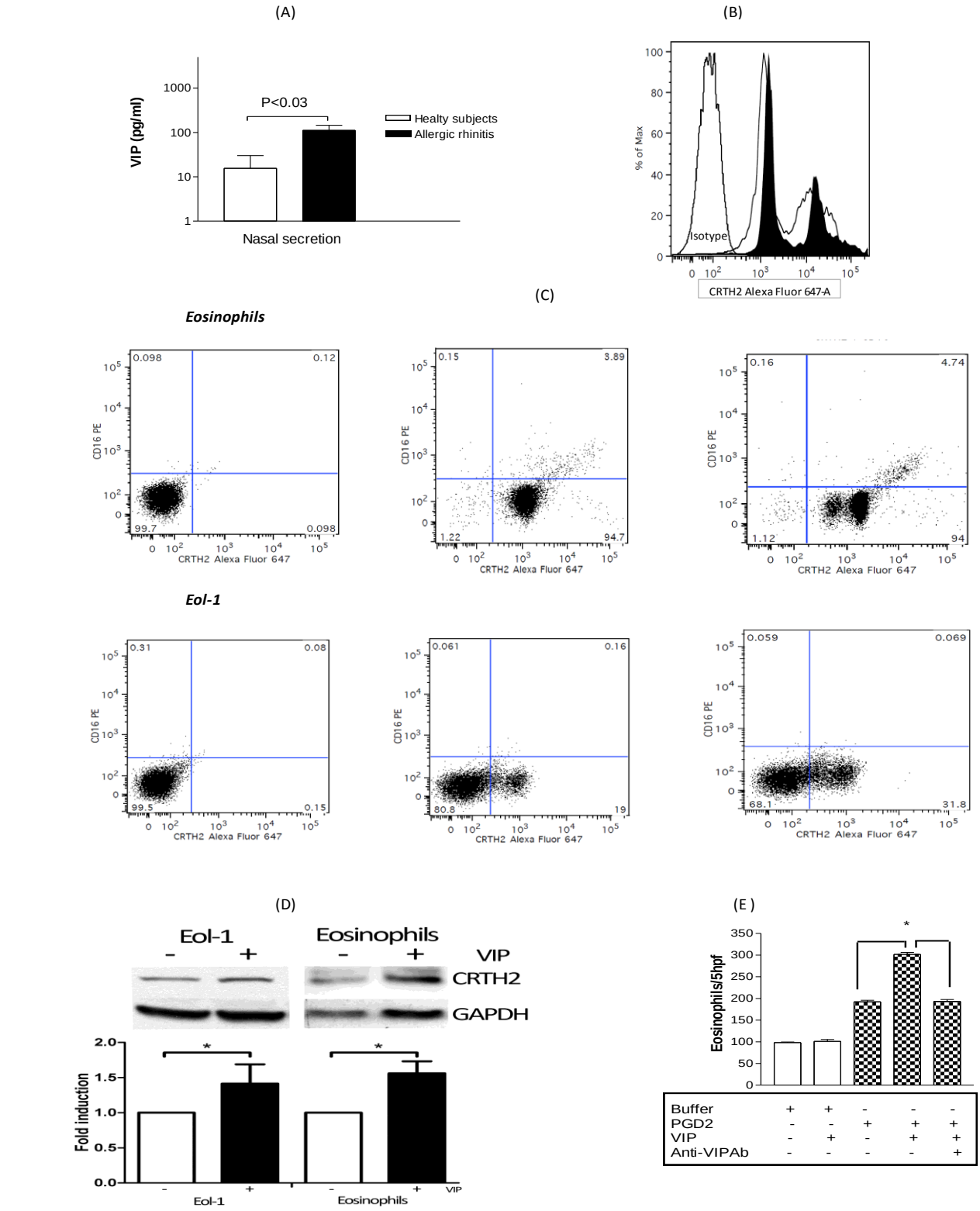

(A)
VIP (pg/ml)
1000
100
10
1
P<0.03
Healty subjects
Allergic rhinitis
Nasal secretion
(B)
% of Max
Isotype
CRTH2 Alexa Fluor 647-A
(C)
Eosinophils
CD16 PE
CRTH2 Alexa Fluor 647
0.098
0.12
99.7
0.098
0.15
3.89
1.22
94.7
0.16
4.74
1.12
94
Eol-1
0.31
0.08
99.5
0.15
0.061
0.16
80.8
19
0.059
0.069
68.1
31.8
(D)
Eol-1
Eosinophils
VIP
CRTH2
GAPDH
Fold induction
(E)
Eosinophils/5hpf
Buffer
PGD2
VIP
Anti-VIPAb

Figure 1

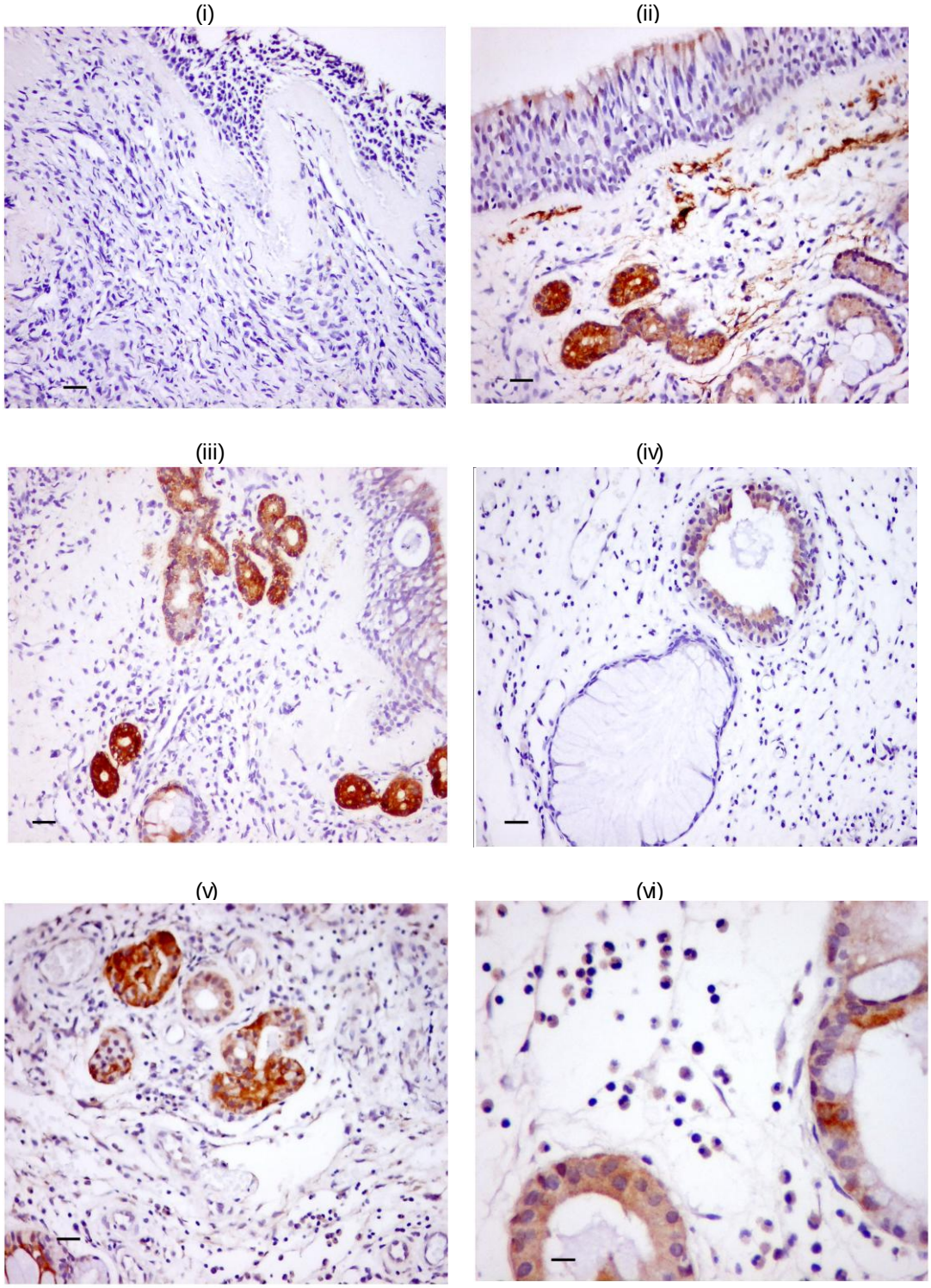


Figure 2

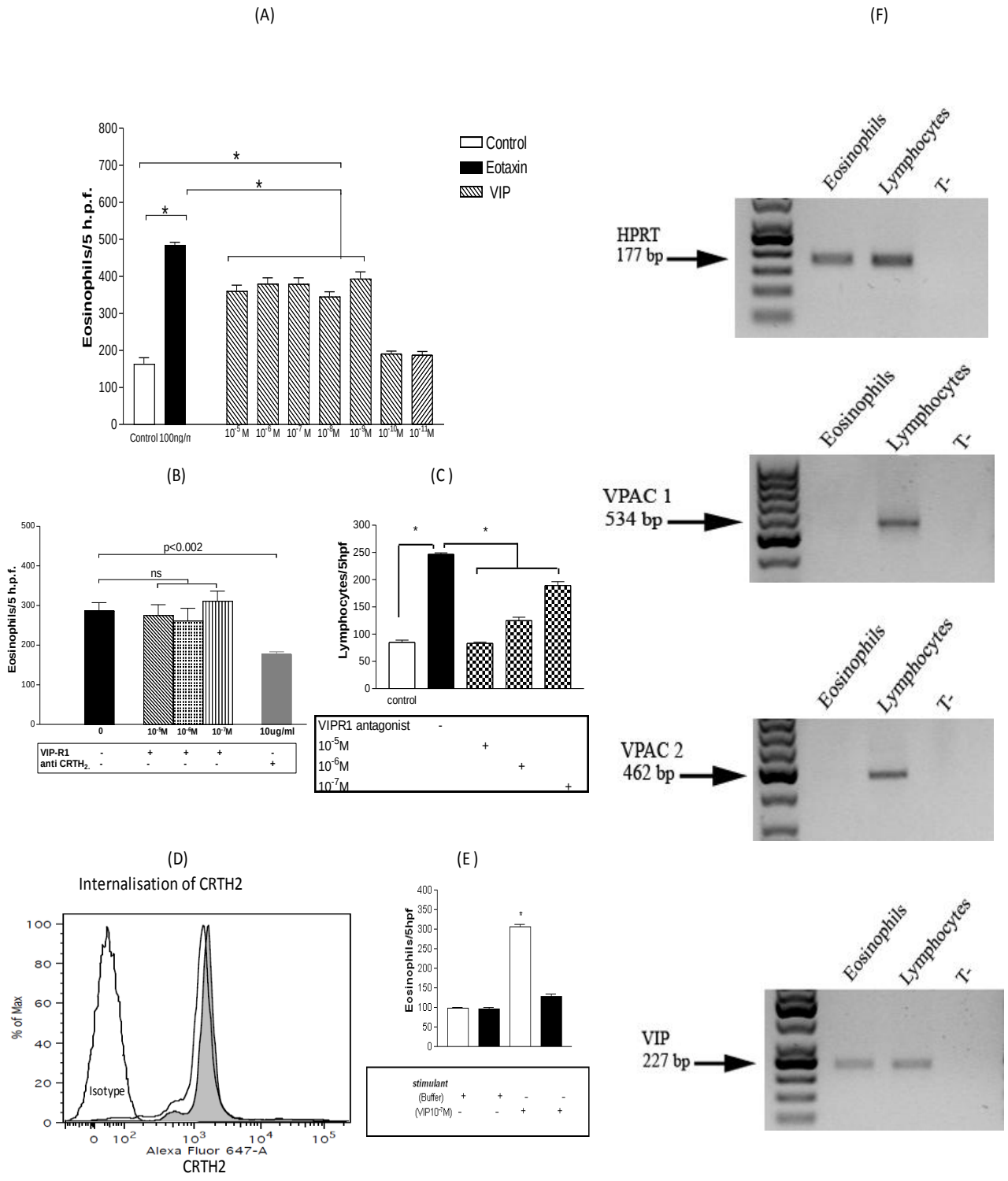

(A)
(F)
Eosinophils/5 h.p.f.
Control
Eotaxin
VIP
Control 100ng/n
HPRT
177 bp
Eosinophils
Lymphocytes
T-
(B)
(C)
p<0.002
ns
VIP-R1
anti CRTH2.
Lymphocytes/5hpf
control
VIPR1 antagonist
VPAC 1
534 bp
VPAC 2
462 bp
(D)
Internalisation of CRTH2
% of Max
Isotype
Alexa Fluor 647-A
CRTH2
(E)
Eosinophils/5hpf
stimulant
(Buffer)
VIP
227 bp

Figure 3

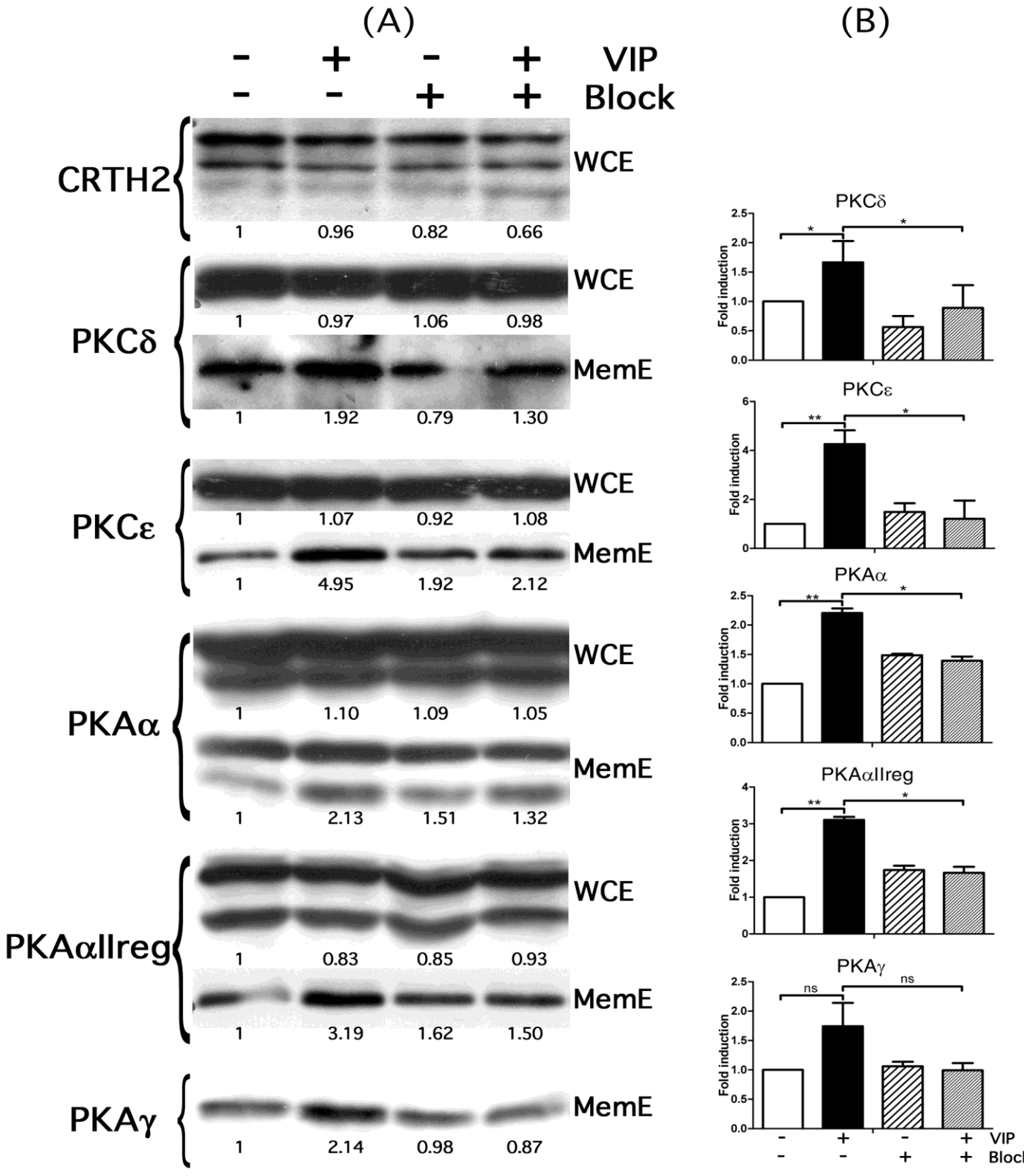
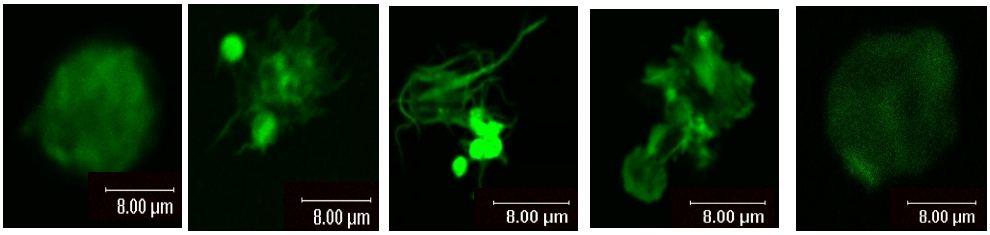

(A)
(B)
- + - + VIP
- - + + Block
CRTH2
WCE
1 0.96 0.82 0.66
PKCδ
WCE
1 0.97 1.06 0.98
MemE
1 1.92 0.79 1.30
PKCε
WCE
1 1.07 0.92 1.08
MemE
1 4.95 1.92 2.12
PKAα
WCE
1 1.10 1.09 1.05
MemE
1 2.13 1.51 1.32
PKAαIIreg
WCE
1 0.83 0.85 0.93
MemE
1 3.19 1.62 1.50
PKAγ
MemE
1 2.14 0.98 0.87
PKCδ
PKCε
PKAα
PKAαIIreg
PKAγ
Fold induction
ns
- + - + VIP
- - + + Block

Figure 4

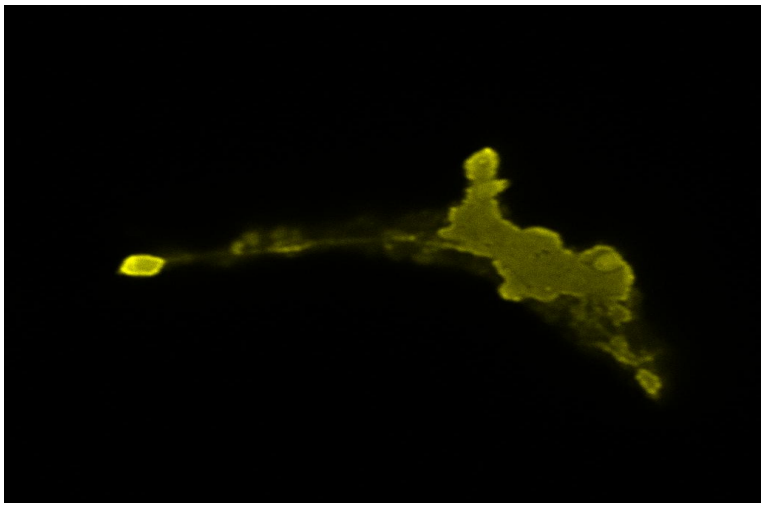


Agent	F -actin mean flouresence
Control	347
VIP 10^{-7}M	465
VIP 10^{-7}M + **Inhibitor**	
CRTH2 blocking antibody 10ug/ml	250
PKA 10^{-5}M	211
PKC 10^{-5}M	251

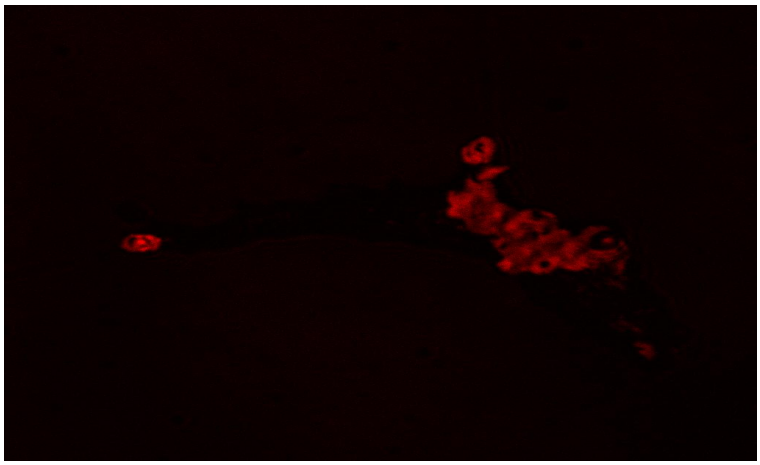
Figure 5

(A)

(i)



(ii)



(iii)

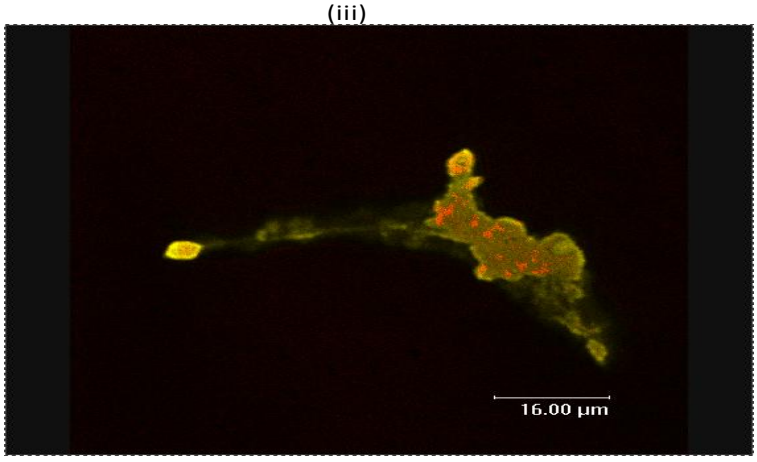


Figure 6 A

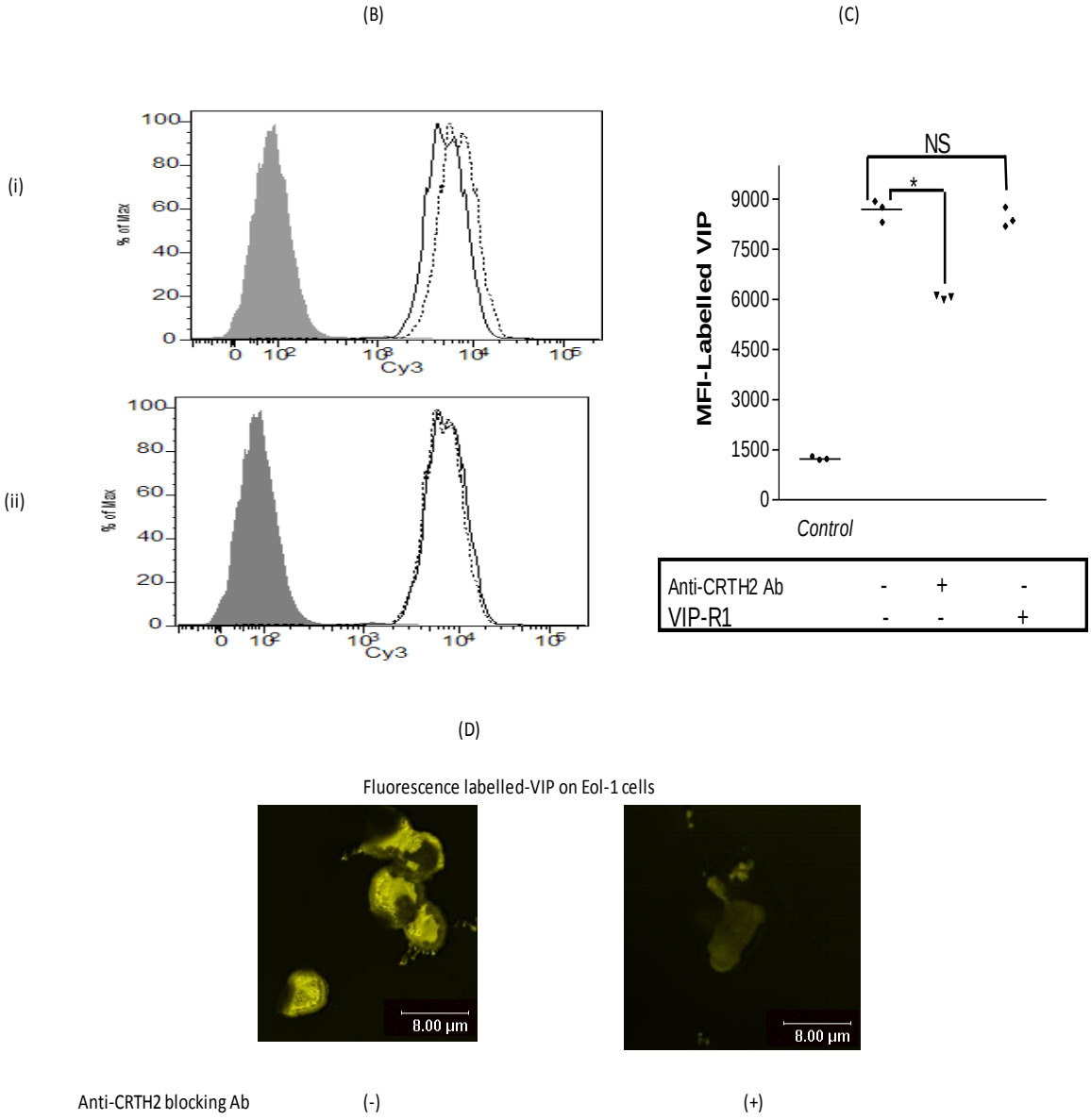

(B)
(C)
(i)
(ii)
% of Max
Cy3
MFI-Labelled VIP
NS
*
Control
Anti-CRTH2 Ab - + -
VIP-R1 - - +
(D)
Fluorescence labelled-VIP on Eol-1 cells
8.00 µm
8.00 µm
Anti-CRTH2 blocking Ab
(-)
(+)

Figure 6 (B, C& D)

Table 1: Effect of different protein kinases inhibitors on VIP-induced eosinophils chemotaxis

Ca2+	+	+		-	-	
INHIBITOR (10-5M)	-	+	p value	-	+	p value
H-89 Dihydrochloride	329 ± 14	193 ± 21	0.0005	316 ± 12	169 ± 5	0.022
Bisindo-lylmaleimide	329 ± 14	174 ± 9	<0.0001	316 ± 12	150 ± 3	0.022
SB203580	329 ± 14	341 ± 16	NS	316 ± 12	304 ± 4	NS
GENISTEIN	329 ± 14	381 ± 9	0.041	316 ± 12	321 ± 7	NS

Table 2: Time course of the percentage of PGD2 secreted in pg/ml by human eosinophils in response to VIP stimulation

Stimulant	Buffer	VIP	VIP+anti-VIP
30 min	100	105,2	96,03
24h	100	140,17	84,41