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Research paper

Characterization and use of a rabbit-anti-mouse VPAC1 antibody by flow cytometry

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ABSTRACT

Vasoactive intestinal peptide receptor-1 signaling in lymphocytes has been shown to regulate chemotaxis, proliferation, apoptosis and differentiation. During T cell activation, VPAC1 mRNA is downregulated, but the effect on its protein levels is less clear. A small number of studies have reported measurement of human VPAC1 by flow cytometry, but murine VPAC1 reagents are unavailable. Therefore, we set out to generate a reliable and highly specific α-mouse VPAC1 polyclonal antibody for use with flow cytometry. After successfully generating a rabbit α-VPAC1 polyclonal antibody (α-mVPAC1 pAb), we characterized its cross-reactivity and showed that it does not recognize other family receptors (mouse VPAC2 and PAC1, and human VPAC1, VPAC2 and PAC1) by flow cytometry. Partial purification of the rabbit α-VPAC1 sera increased the specific-activity of the α-mVPAC1 pAb by 20-fold, and immunofluorescence microscopy (IF) confirmed a plasma membrane subcellular localization for mouse VPAC1 protein. To test the usefulness of this specific α-mVPAC1 pAb, we showed that primary, resting mouse T cells express detectable levels of VPAC1 protein, with little detectable signal from activated T cells, or CD19 B cells. These data support our previously published data showing a downregulation of VPAC1 mRNA during T cell activation. Collectively, we have established a well-characterized, and highly species specific α-mVPAC1 pAb for VPAC1 surface measurement by IF and flow cytometry.

1. Introduction

*V*asoactive intestinal peptide/*p*ituitary *a*denylate *c*yclase activating polypeptide receptor 1 (VPAC1) is the prototypical, group II, G protein coupled receptor nearly ubiquitously expressed in mammals (Ceraudo et al., 2008). This receptor binds the neuropeptide called vasoactive intestinal peptide (VIP) with ≈1 nM K_d (Gaudin et al., 1996), and its neuronal source is derived from neurons in the central nervous system (Lorén et al., 1979), and peripheral neurons that deliver this ligand to numerous organs (Felten et al., 1985; Ottaway et al., 1987). Also, VIP is expressed by certain immune cells, including activated CD4 Th_2 cells (Danek et al., 1983; Delgado and Ganea, 2001). The signal transduction pathways elicited by VIP/VPAC1 are through differential coupling between $G_{\alpha s}$, $G_{\alpha i}$ and $G_{\alpha q}$ that appear to be cell context dependent (Van Rampelbergh et al., 1997; McCulloch et al., 2002). Major cellular consequences for the VIP/VPAC1 signaling axis are to regulate electrolyte balance (Schwartz et al., 1974), secretion of soluble mediators (Delgado et al., 1999), proliferation (Rameshwar et al., 2002a), chemotaxis (Johnston et al., 1994) and apoptosis (Delgado and Ganea, 2000). Several disease states have been implicated with abnormal VPAC1 levels, including inflammatory disorders such as Rheumatoid arthritis (Woessner, 1991; Yuko et al., 1999) and inflammatory bowel disease (Abad et al., 2003), and solid cancers originating from the lung, prostate, breast, and brain (Reubi, 1996; Reubi et al., 2000a).

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The VPAC1 gene was first cloned in 1992 using a rat lung cDNA library, followed by the cloning of the human VPAC1 gene in 1995, which made an immediate impact regarding the tissue expression profile revealing high expression in lung, liver, and intestines (Ishihara et al., 1992; Sreedharan et al., 1995). A decade later, many antibodies had been developed using peptide sequences from the amino- and carboxyl-terminal regions of VPAC1, as well as, the entire full-length human VPAC1 sequence (Goetzl et al., 1994a; Schulz et al., 2004; Freson et al., 2008). These antibodies proved invaluable for detecting VPAC1 protein expression by end-point analyses including, immuno blots, immunoprecipitation, immunohistochemistry and immunofluorescence (Goetzl et al., 1994b; Jiang et al., 1998; Schulz et al., 2004; Zhang et al., 2008). Interestingly, there are exceedingly few examples of flow cytometry analyses using antibodies to the VPAC receptors, and of these studies that we are aware of, only human VPAC protein was measured (Marie et al., 1999; Langlet et al., 2005; Sun et al., 2006; Delgado et al., 2008; Freson et al., 2008). Indeed, we and others have relied primarily on RT-PCR, and in situ hybridization to measure mouse VPAC1 expression at the mRNA level (Barberi et al., 2007; Dorsam et al., 2010). Therefore, the need for a species, specific, mouse VPAC1 antibody suitable for flow cytometry would be a valuable resource for the neuroimmunology field.

Some success has, however, been reported using fluorescently conjugated VIP ligands that measured the presence of binding sites on cells (Lara-Marquez et al., 2001b), but this strategy does not distinguish between VPAC1 and a 50% homologous VPAC2 receptor (Igarashi et al., 2002). Radioactively iodinated VIP/PACAP ligand binding assays have also become a routine method for discerning between the non-selective VPAC1 and VPAC2 receptors *verses* the selective pituitary adenylate cyclase activating polypeptide receptor 1 (PAC1) (Vertongen et al., 1997; Reubi et al., 2000b). However, this strategy is dependent on proper recognition of the VIP/PACAP analogs, which can alter: receptor internalization, ligand/receptor affinity, and receptor half-life, thus further complicating the distinction between the known VIP/PACAP receptors (Langer and Robberecht, 2007). Moreover, splice variants of the selective and non-selective VIP/PACAP receptors may in turn alter affinity for their cognate ligand(s) and present an additional variable in identifying the receptor subtype (Markovic and Challiss, 2009). The continued absence of a murine specific VPAC1 antibody suitable for flow cytometry measurements will make it difficult, for example, for the routine identification of hematopoietic subpopulations expressing VPAC1 protein (Delgado et al., 1996).

We have previously published that mouse VPAC1 steady-state mRNA is downregulated during ex vivo TCR activation (anti-CD3 treatment), and this downregulation was blocked in a concentration dependent manner by small molecule inhibitors against Fyn, Lck, and JNK kinases (Vomhof-DeKrey and Dorsam, 2008a). Unfortunately, this study did not measure whether a parallel downregulation of VPAC1 protein levels also occurred, which will be critical to establish an authentic functional significance to VPAC1 mRNA downregulation during T cell activation (Vomhof-DeKrey et al., 2008). Therefore, we generated a polyclonal rabbit anti-mouse VPAC1 antibody (α-mVPAC1 pAb) by utilizing a genegun strategy to inject the full-length mouse VPAC1 cDNA for expression in rabbit muscle (Genovac, Freiburg, Germany). The α-mVPAC1 pAb showed high specificity toward functionally active, mouse VPAC1 protein by flow cytometry using overexpressed CHO-K1 transfectants, but failed to cross-react with functionally active, human VPAC1 protein, or mouse and human VPAC2, or PAC1 protein. Importantly, we were able to corroborate our mRNA studies of mVPAC1 downregulation by demonstrating that resting primary T cells ($CD44^{low}$) showed readily detectable mVPAC1 expression compared to no detection in activated T cells ($CD44^{high}$) by flow cytometry (Mannering et al., 2002). These studies demonstrate a highly specific, mouse VPAC1 antibody that will provide reproducible flow cytometry data, and therefore, is expected to be an important reagent for the neuroimmunology field.

2. Materials and methods

2.1. Reagents

CHO-K1, HT-29, SupT1, Molt4b, and MC3T3-E1 cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA). F12-Kaighn's modification medium, McCoy's 5a medium, RPMI-1640 medium, α-Minimum essential medium with 0.2 mM ascorbic acid, penicillin/streptomycin, phosphate buffered saline without Ca^{2+} or Mg^{2+}, characterized fetal bovine serum and trypsin were purchased from Cellgro (Manassas, VA). α-Minimum essential medium without ascorbic acid, Opti-MEM1, Lipofectamine 2000 Reagent, and the goat anti-mouse IgG-R Phycoerythrin secondary antibody were purchased from Invitrogen (Carlsbad, CA). The rabbit-anti-mouse VPAC1 antibody (rabbits #133 and #134) was purchased from Genovac (Freiburg, Germany). G418 sulfate and β-glycerophosphate were purchased from EMD Chemicals (Gibbstown, NJ). Anti-mouse or human CD16/CD32 Fc blocker, mouse-anti-CD4 (clone RM4-4), mouse-anti-CD44 (clone IM7), mouse-anti-CD19 (clone 6D5) were purchased from BioLegend (San Diego, CA). Red blood cell lysis buffer and mouse-anti-CD8 (clone 53-6.7) were purchased from eBioscience (San Diego, CA). Melon Gel IgG purification kit and the EZ-link NHS-PEO_4 biotinylation kit were purchased from ThermoScientific (West Palm Beach, FL). Qiashredders, RNeasy Mini Kit, and RNase-free DNase I were purchased from Qiagen (Valencia, CA). The deoxynucleotides, reverse transcriptase were purchased from Promega (Madison, WI). The gene specific primers were purchased from Integrated DNA Technologies (Coralville, IA). Fast SYBR green PCR master mix was purchased from Applied Biosystems (Foster City, CA). VIP was purchased from American Peptide (Sunnyvale, CA) and Cyclic AMP EIA Kit from Cayman Chemical Company (Ann Arbor, MI). The DC protein assay and Silver Stain Plus were obtained from Bio-Rad (Hercules, CA). All other reagents were ordered from Sigma-Aldrich (St. Louis, MO) or VWR (West Chester, PA).

2.2. Antibody production

The anti-mouse VPAC1 polyclonal antibody (α-mVPAC1 pAb) was generated by genetic intradermal immunization of two rabbits using Genegun technology (GENOVAC, Freiburg, Germany). Briefly, an immunization vector (GENOVAC, Freiburg, Germany) encoding for the full length mVPAC1 protein was used to evoke an immune response, and final bleeds