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Positive allosteric modulators of $VPAC_1$ receptor-mediated ERK activation

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University of Vermont Senior Honors Thesis 2018

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Important Abbreviations

VIP: Vasoactive intestinal peptide

PACAP: Pituitary adenylate cyclase activating polypeptide

GPCR: G-protein coupled receptor

ECD: Extracellular domain

ERK: Extracellular signal regulated kinase

GCGR: G-protein coupled glucagon receptor

Abstract

Vasoactive intestinal peptide (VIP) is a neuroendocrine peptide that has been implicated in a myriad of functions. VIP promotes neuronal survival during development and apoptotic challenges. Further, VIP has been shown to regulate hormonal release, circadian rhythms, vasodilation, and T cell proliferation in central and peripheral tissues. VIP binds with equally high affinity to $VPAC_1$ and $VPAC_2$ receptors, members of the Class B family of G protein-coupled receptors (GPCRs) that also include the PAC_1, glucagon (GCGR), and CRH receptors. VIP binding has previously been shown to increase downstream MAPK signaling cascades, especially MEK/ERK. Given the structural similarities and functional overlap between $VPAC_1$ and $VPAC_2$ receptors, we sought to investigate whether there are small molecule modulators that can probe and identify the functional distinctions between the two receptor subtypes. For these studies, we used HEK-293 cells stably expressing the $VPAC_1$-EGFP or the $VPAC_2$-EGFP receptor. The cultures were treated with VIP with and without the addition of putative small molecule antagonists. Protein assays and western blotting analysis for phosphorylated ERK (pERK) were performed to define quantitative changes in ERK activation. Many compounds in the small molecule panel were non-selective antagonists at the $VPAC_1$ and $VPAC_2$ receptors. Interestingly, an acyl hydrazide compound, I48, and a previously described glucagon receptor antagonist, MK-0893, behaved as positive allosteric modulators at the $VPAC_1$ receptor on ERK activation, but not at the $VPAC_2$ receptor. Our results suggest that these compounds can uniquely restrict the conformational microstate of the $VPAC_1$ receptor to amplify VIP-mediated ERK signaling.

Introduction

VIP Synthesis

VIP is a neuroendocrine peptide that was first isolated from porcine small intestines (Said & Mutt, 1970). The gene encoding the precursor protein, preproVIP, is located on chromosome 6 (Gozes et al, 1987), and is comprised of 7 discrete exons spanning approximately 9 kilobase pairs (Linder et al., 1987). PreproVIP is cleaved to synthesize the active form of VIP (Bodner, Fridkin, & Gozes, 1985), a 28 amino acid residue peptide that has been found to function as both a putative neurotransmitter, as well as a neuroendocrine hormone (Said, 1986). Given this, VIP has been found to exert a wide range of effects throughout the different systems of the body, the implications of which remain nascent in the literature, especially in regards to its binding to VIP specific receptors.

VIP Tissue Distribution & Physiology

VIP is expressed broadly throughout the body. It is found in neurons in both the peripheral and central nervous systems, as well as in several non neuronal cells extending throughout the immune system (Fahrenkrug, 1993). In this regard, it is both highly and diversely biologically active, the effects of which arise from its downstream activation of adenylyl cyclase and ERK signaling pathways. Broadly, VIP has been found to promote neuronal survival during development and apoptotic challenges (Brennemen & Eiden, 1986). Within the hypothalamus, particularly the suprachiasmatic nucleus (SCN), VIP expression has been correlated with light-dark cycles, suggesting its functions in circadian rhythm regulation (Gozes, Avidor, Biegon, & Baldino, 1989). Within the hypothalamic-pituitary axis, VIP has also been implicated in facilitating the release of prolactin, oxytocin, and vasopressin (Reichlin, 1988). In the periphery, it is highly implicated in areas of systemic vasodilation (Said & Mutt, 1970); similarly, VIP-mediated smooth muscle relaxation results in gastrointestinal tract relaxation (Rattan, Fan, &

Chakder, 1999). In some of these mechanisms, VIP works synergistically with nitric oxide (NO) (Grider, 1993) to inhibit the rhythmic activity (slow waves) that is exhibited at GI basal tone, leading to hyperpolarization and relaxation of smooth muscle (Sababi, Hallgren, & Nylander, 1996). In the intestines, VIPergic enteric neurons can project to other enteric neurons, as well as muscle and epithelial cells in order to stimulate secretion (Masel, Brennan, Turner, Cullingford, & Cullen, 2000) and absorption (Barada, Saade, Atweh, & Nassar, 1998). In the sympathetic system, VIP can facilitate catecholamine release from the adrenal medulla (Malhotra, Wakade, & Wakade, 1988). In the immune system, VIP plays a role in regulating T cell proliferation (Ottaway, 1987) and can promote Th2-like immune responses (Ganea, Hooper, & Kong, 2015), which manifests in its endogenous anti-inflammatory action.

VPAC Receptor Tissue Distribution

VIP binds with equally high affinity as PACAP to $VPAC_1$ and $VPAC_2$ receptors. Detailed studies have found the $VPAC_1$ receptor to be highly concentrated throughout both the central and peripheral nervous system. In the central nervous system, the receptor has been isolated in the pyrifom & cerebral cortex, lateral amygdaloid nucleus, putamen, supraoptic nucleus, choroid plexus, dentate gyrus, and pineal gland (Usdin, Bonner, & Mezey, 1994; Vertongen, Schiffmann, Gourlet, & Robberecht, 1997; Vaudry et al., 2000). In the peripheral nervous system, the receptor has been found in abundance in both tissues and organs, including the: liver, kidney, prostate, breast, spleen, lung, and GI tract (Reubi, 2000).

Similarly, the $VPAC_2$ receptor has also been localized to both central and peripheral tissue. In the CNS, presence of the $VPAC_2$ receptor has been reported throughout the cerebral cortex, PVN, SCN, thalamus, hypothalamus, and amygdala (Sheward, Lutz, & Harmar, 1995; Vertongen et al., 1997; Vaudry et al., 2000). In peripheral tissue, the receptor has been primarily

associated within the smooth muscle of organs and blood vessels, but has also been demonstrated in pancreatic acinar cells, the adrenal medulla, retina, and lung alveoli (Harmar et al., 2004). Although the receptors are co-expressed in certain areas, they are often delineated to different layers within the same tissue.

G-Protein Coupled Receptor Structure

Both VPAC receptor variants are Type II, or Class B GPCRs, indicative of the secretin receptor family (May & Parsons, 2016). Similar to other classes of GPCRs, Class B GPCRs are structurally characterized by an N-terminus extracellular domain (ECD) and intracellular C-terminus tail with 7 transmembrane intervening alpha helices. These are linked by 3 extracellular and 3 intracellular loops. Specific to the Class II receptors is the presence of a large (approximately 120 amino acid) N-terminal extracellular domain that is comprised of 10 highly conserved amino acids. This entails six cysteine residues to form 3 disulfide bonds, N-terminal leader sequences (Laburthe, Couvineau, & Tan, 2007), and anti-parallel beta-sheets. There are clusters of serine residues in the intracellular tail that serve as a docking site for scaffolding proteins (May & Parsons, 2016).

VPAC Receptor Extracellular Binding Domain

A key feature of the $VPAC_1$ receptor is the presence of the N-terminus ECD region, which is thought to be essential to VIP binding. As for all N-terminus domains of Class B receptors, the human $VPAC_1$ receptor ECD is thought to be comprised of two antiparallel beta sheets. These are stabilized by three disulfide bonds between Cys^{50} and Cys^{72}, Cys^{63} and Cys^{105}, and Cys^{86} and Cys^{122}, and a salt bridge between Asp^{68} and Arg^{103}, which are both constrained between the aromatic rings of Trp^{73} and Trp^{110} (Fig. 1). Likewise, the $VPAC_2$ receptor reflects a highly similar ECD, with the exceptions of a salt bridge between Asp^{56} and Arg^{91}, and both the

cysteine disulfide bridges and salt bridge being confined between Trp^{62} and Trp^{98} (Tan et al., 2006). In both receptors, these residues are thought to constitute a short consensus repeat (SCR), comparable to a Sushi domain motif due to their presence towards the N-terminus of the receptor (Schwarzenbacher et al., 1999). This indicates their role in ligand recognition, apart from stabilization of the receptor.

VPAC Receptor Activation

There are currently two models surrounding VPAC receptor activation. The prototypical two domain model suggests that VIP binds to the VPAC receptors in a manner that allows interactions at two separate receptor sites. These include the aforementioned N-terminal ECD domain and a central binding domain within the juxtamembrane core of the receptor that results in conformational dynamics for receptor activation (Laburthe, Couvineau, & Marie, 2002). Upon further investigation, the carboxyl terminal and central component of VIP is thought to bind the receptor ECD, which orients and positions the N-terminus end of the peptide to bind the receptor core. This model is supported by pharmacological data, which supports the notion that N-terminally truncated VIP analogs are common observable receptor antagonists (Laburthe, Couvineau, & Marie, 2002).

Another model for several Class II GPCRs suggests that a hidden epitope may exist within the ECD domain. In regards to the VPAC receptors, the model suggests that VIP binds to the ECD, and upon binding causes a hidden epitope within the ECD region to become exposed, which subsequently interacts with the receptor core (Dong, Pinon, Asmann, & Miller, 2006). This model is currently disfavored, as it does not allow N-terminally truncated peptides to behave as antagonists. Further, the proposed newly exposed epitopes have consensus asparagine

N-glycosylation sites that would prohibit binding to the transmembrane core (Couvineau, Fabre, Gaudin, Maoret, & Laburthe, 1996; Tan et al., 2006).

VPAC Receptor Signaling Cascades

Upon activation, signaling cascades for both receptor subtypes are analogous. In brief, many Class B GPCRs exhibit similar functionality and mechanisms. Upon agonist binding to the extracellular binding domain on the receptor, the GPCR undergoes a conformational change to an active state, which mobilizes the heterotrimeric G proteins ($G\alpha/G_{\beta\gamma}$). Upon activation, the $G\alpha_s$ subunit exchanges GDP for GTP, dissociates from the $G_{\beta\gamma}$ subunit, and stimulates adenylyl cyclase to catalyze ATP to cAMP (May & Parsons, 2016). In the activated receptor, the $G\alpha_s$ subunit likely docks at the intracellular face of the receptor by interacting with the third intracellular loop (ICL3) and C-terminal domain (Laburthe, Couvineau, & Tan, 2007). It is notable that unlike some class B receptors, the VPAC receptors appear to preferentially activate $G\alpha_s$ and not other G proteins such as $G\alpha_{q/11}$ to transduce PLC as independent second messenger generators (May & Parsons, 2016).

In addition to heterotrimeric mobilization, agonist binding to the extracellular binding domain activates G-protein receptor kinases (GRKs), predominately GRK-2, -3, which phosphorylate the receptor on serine and threonine residues in the third cytoplasmic loop and/or the C-terminal tail region. The phosphorylation at some of these sites signals β-arrestin -1, -2 binding to the phosphorylated sites, which is necessary for receptor internalization as well as sterically hindering G-protein coupling and plasma membrane-delimited signaling. Of note, β-arrestins also exhibit cAMP phosphodiesterase interactions, implying that the arrestins dually desensitize $G\alpha_s$ through subunit uncoupling and accelerated cAMP degradation (Reiter & Lefkowitz, 2006). β-arrestin is essential for protein scaffolding for clathrin, which is integral for

receptor internalization. While it was originally believed GRK-β-arrestin interactions were mechanisms for receptor internalization and desensitization, new evidence appears to suggest that the functions are more nuanced. Following β-arrestin dependent clathrin-mediated endocytosis, it has been shown that the complex has the ability to scaffold MAPK enzymes for long term MEK/ERK signaling (Clason, Girard, May, & Parsons, 2016).

VPAC Receptor Duality

It is apparent that the distinctions between the $VPAC_1$ and $VPAC_2$ receptors are not well understood. Given that they are two highly related receptors that bind the same endogenous VIP ligand, generate similar second messenger signals, and have evolved in parallel to each other, there must exist differences for evolutionary divergence. There has been a small amount of studies that have thus far shown small differences in receptor functioning. For example, it has been shown that $VPAC_1$ receptor activation appears to inhibit GABA release, whereas $VPAC_2$ receptor activation enhances it (Cunha-Reis, Ribeiro, Almeida, & Sebastião, 2017). It has also been shown that $VPAC_2$ receptors are essential to circadian functioning in mice in comparison to $VPAC_1$ receptors (Hamar et al., 2002). Conversely, VIP induced $VPAC_1$ receptors have been shown to attenuate acute pancreatitis in mice, whereas $VPAC_2$ receptors did not (Kojima et al., 2005). However, the quantity of research into these functional differences is scarce, with even fewer studies examining the differences in structural/mechanistic nature of these receptors and implications on downstream signaling. We aimed to begin to address this deficiency using a panel of pharmacological compounds, including a myriad of acyl hydrazides and a previously described glucagon receptor antagonist, MK-0893 (Jazayeri et al., 2016). This was done in an effort to elucidate whether there are structural differences that distinguish their function. The compounds were tested on their abilities to block or alter VIP signaling in HEK-293 cells stably

expressing the $VPAC_1$-EGFP or $VPAC_2$-EGFP receptors. Phosphorylated ERK fold change was measured using Western blotting techniques.

Methods

Samples

Stable HEK-293 cells expressing either the $VPAC_1$-EGFP or $VPAC_2$-EGFP receptor were treated for 15 minutes with a test compound (20 uM) before the addition of 25 nM VIP for an additional 15 minutes. The samples were subsequently lysed in lysis buffer containing protease and phosphatase inhibitors (May et al., 2010). The compounds consisted of a panel of acyl hydrazides, previously described as PAC_1 receptor antagonists, in addition to the glucagon receptor antagonist AB120 (MK-0893).

Bradford Assay

A standard curve was generated through the addition of BSA standards (150 uL) in duplicates to corresponding wells on a ninety-six well plate. The experimental samples were diluted 1:400 with Milli-Q water, followed by the addition of 150 uL of samples that were similarly added in duplicates to adjacent wells. The Coomassie Plus™ Protein Assay Reagent was pipetted into both standard and sample wells for colorimetric detection at 595 nm using a Synergy Biotech plate reader. The sample absorbance was entered into an Excel sheet to calculate sample volume for loading 35 ug of total protein onto the gel lanes.

Western Blotting

Day 1

The samples were diluted and combined with 7.5 uL of 4x LDS and 3 uL of 10x DTT sample reducing agent. Samples were denatured on a heating block at 97 degrees Celsius for 10 minutes and centrifuged down to collect droplets in the tube. Pre-cast 4-12% Bis-Tris, 12 well gels were used in an Invitrogen Novex Mini Cell apparatus. 1x SDS MOPS running buffer (50

mL 20x SDS + 950 mL Milli-Q water) was placed in both the inner and outer cores, followed by 500 uL of antioxidant that was pipetted into the inner core. Gels were pre-run for 5 minutes. 25 uL of sample were added to individual wells, flanked by 2 uL of SeeBlue®Plus2 standard ladder on the end lanes. The gel was run for approximately 60 minutes at 150 V. Upon removal, the gel cassette was opened, and the gel segments containing the wells and dye front were removed. Whatman paper that had been soaking in Transfer buffer (50 mL 20x Transfer buffer + 200 mL methanol + 750 mL Milli-Q water) was placed directly onto the gel; the gel was overlaid with an Immobilon transfer membrane (0.45 um pore) that had been pre-soaked with methanol. A second piece of Whatman paper was placed on top of the membrane, and the complex was gently rolled with a cylinder to remove trapped air bubbles. 3 filter pads soaked in transfer buffer were placed in a separate Novex Mini Cell transfer box holder, followed by the gel sandwich and an additional 3 filter pads. The box was locked into the Mini Cell and filled with transfer buffer in both inner and outer cores. The transfer was run for 75 minutes at 25 V. After protein transfer, the membrane was recovered and placed into a small box with aquablock (1:1 with PBS) on a platform shaker for 60 minutes. The aquablock was removed, and a 1:1000 dilution of a mouse pERK antibody (4 mL aquablock, 40 uL 10% Tween20, 4 uL antibody) was added. The membrane was incubated with gentle agitation in a cold room overnight.

Day 2

The membrane was removed from the cold room. 3x10 minute 1x PBST (900 mL Milli-Q water, 100 mL 10x PBS, 1 mL Tween20) washes were performed. After washing, the blot was incubated in a 1:20000 dilution of a donkey anti-mouse IgG secondary antibody (15 mL 1x PBST, 5 mL aquablock, 1 uL antibody) for 60 minutes. A second 3x10 minute 1x PBST wash was performed. The blot was allowed to rest in a solution of 1x PBS (900 mL Milli-Q water, 100 mL 10x PBS) for 5 minutes, and was analyzed using a LiCor Odyssey imager at 800 nm. Bands

were quantified for pERK expression and exported to an Excel program. The blot was then incubated in a 1:1000 dilution of a rabbit pan-ERK primary antibody (4 mL aquablock, 40 uL Tween20, 4 uL antibody) with gentle agitation in the cold room overnight.

Day 3

The membrane was removed from the cold room. 3x10 minute 1x PBST (900 mL Milli-Q water, 100 mL 10x PBS, 1 mL Tween20) washes were performed. Upon washing, the blot was set in a 1:20000 dilution of a goat anti-rabbit IgG secondary antibody (15 mL 1x PBST, 5 mL aquablock, 1 uL antibody) for 60 minutes. A second 3x10 minute 1x PBST wash was performed. The blot was then rested in a solution of 1x PBS (900 mL Milli-Q water, 100 mL 1x PBS) for 5 minutes, and was permitted to fully dry before being analyzed using the Odyssey imager at 700 nm. Bands were quantified for total ERK expression and exported to an Excel program. Normalized pERK expression was calculated, and the pERK fold change was standardized to the control well. From sample replicates, the mean ± SEM was calculated for each treatment. Statistical analysis was conducted using SigmaPlot/SigmaStat 12.0 software. One-way ANOVA was performed followed by Student-Newman-Keuls posthoc analyses.

Results

Quantitative Western analyses for pERK levels were used to test the abilities of a panel of 13 small molecule compounds (mass ~450 Dal) to antagonize or modulate $VPAC_1$ and $VPAC_2$ receptor activation. All of the compounds, except for compound AB120, were acyl hydrazide analogues, some of which had been described previously as PACAP receptor antagonists. Compound AB120 is the glucagon receptor antagonist MK-0893 used previously to facilitate the determination of the glucagon receptor structure. Preliminary quantitative results

from Western blotting analyses suggest that I48 and AB120 exhibit positive allosteric effects in $VPAC_1$ (Fig. 2) but not $VPAC_2$ receptors (Fig. 3).

VIP alone stimulated $VPAC_2$ receptor pERK levels approximately 8-fold. There are 2 forms of the ERK enzyme, p42 and p44 ERK; both forms were phosphorylated after VIP stimulation. To varying degrees, all 13 compounds demonstrated abilities to block VIP-stimulated $VPAC_2$ receptor ERK activation. Whereas compounds I54, MB216 and AB120 (MK-0893) blocked ERK activation approximately 50%, the acyl hydrazide compounds I12, I42 and MB208 appeared more potent and appeared to block VIP-induced ERK signaling to control levels ($P < 0.05$; Fig. 4).

VIP stimulation of pERK levels at the $VPAC_1$ receptor was approximately 2-fold and hence less robust, which may reflect differences in $VPAC_1$ receptor expression levels or a more transient nature of ERK activation. Even though several of the small molecule acyl hydrazide compounds in the panel, such as MB208 and I42, also appeared to behave as receptor antagonists and returned the VIP-stimulated ERK levels to control levels, the overall variance among samples precluded identification of significant statistical changes. However, acyl hydrazide I48 and GCGR receptor antagonist AB120 (MK-0893) appeared to be strong positive allosteric modulators and significantly increased VIP-mediated ERK activation 4- and 7-fold from control levels respectively i.e. I48 and AB120 (MK-0893) compounds amplified VIP-stimulated ERK activation 2- and 3.5-fold above levels seen for VIP alone ($P < 0.05$; Fig. 4).

Discussion

We expected the preconceived antagonists to blunt ERK signaling. Contrary to this notion, I48 and AB120 were found to function as positive allosteric modulators of the $VPAC_1$

receptor, resulting in an increase in pERK expression (and thus increased pERK fold change) compared to lone VIP treated cells.

Unlike Class A GPCRs, there are currently no active structures for Class B GPCRs. Until recently, there has been only one defined ligand binding site for Class B GPCRs (CRF_1R) that was found to be located deep within the TM7 bundle. However, recently Jazayeri et al. utilized MK-0893 (AB120), a glucagon receptor antagonist, to define a novel second binding site that differed from the canonical helical bundle (2016). MK-0893 was found to bind to an allosteric site on the GCGR in between TM6 and TM7 (Fig. 5). It is theorized that, in comparison to Class A GPCRs, Class B GPCRs may also mobilize upon activation by rotating and outwardly extending the cytoplasmic interface of TM6 to allow for G-protein binding (Deupi & Standfuss, 2011). By binding here, MK-0893 is permitted to function as a clamp, holding TM6 in an inactive state (Jazayeri et al., 2016). However, although the GCGR and $VPAC_1$ receptors are both categorically Class B, MK-0893 appears to have an opposite effect in $VPAC_1$ receptors. MK-0893 serves as a positive allosteric modulator in $VPAC_1$ receptors, but not $VPAC_2$ receptors (Fig. 4). For $VPAC_2$ receptors, it is both logical and likely that MK-0893 functions in a similar way as it does in the GCGR, holding the receptor in an inactive state and preventing VIP activation. Briefly, as exhibited in the GCGR, MK-0893 may connect to TM6 from within the lipid bilayer, causing TM6 to bisect the binding site into a hydrophobic region facing TM5 and a hydrophilic region oriented towards TM7. Of its numerable interactions with both regions, it is notable that the methoxynaphthalene (hydrophobic) and phenylethylpyrazole core (polar) moieties will make contact at the TM5-TM6 and TM6-TM7 interfaces with $Phe345^{6.36b}$ and $Thr353^{6.44b}$ respectively (Fig. 5; Jazayeri et al., 2016). However, MK-0893 binding and modulating effects on $VPAC_1$ receptors is inherently more complex.

Due to the novel discovery of a secondary binding site, it remains unclear how MK-0893 functions as a positive allosteric modulator to the $VPAC_1$ receptor. Our data illustrate that MK-0893 administration results in enhanced pERK expression (Fig. 4), indicating that the ERK pathway is highly active for an extended period of time. Presumably, MK-0893 binds the same TM6-TM7 region as in the GCGR. However, given similar pharmacodynamics, the $VPAC_1$ receptor molecular composition must essentially differ from that of the GCGR and $VPAC_2$ receptors so that the cytoplasmic interface of TM6 is favored in the outwardly extended conformation. This may be associated with the aforementioned $Phe345^{6.36b}$ and $Thr353^{6.44b}$ residues, since they are the only key binding site residues that exhibit weaker conservation across other Class B receptors (Jazayeri et al., 2016). Thus it is likely they are involved, to some degree, in the divergence of MK-0893 activity in glucagon/$VPAC_2$ and $VPAC_1$ receptors. Given this, it is probable the agent works synergistically with VIP binding to the ECD as a positive allosteric modulator. This is further supported by unpublished results suggesting that MK-0893 alone cannot stimulate downstream ERK phosphorylation; that is, it cannot function as an agonist.

I48 is derived from the pharmacophore of all acyl hydrazides. As such, it shares almost identical molecular homology among MB208, I12, I48, I54 and I55. The difference in these structures, from which stems the different magnitudes of $VPAC_1$ receptor activation, is localized to the most distal modifiable aromatic group. In I48, the distal modifiable aromatic group comprises a trifluoromethyl at the ortho position. This is in contrast to MB208 and I12, whose trifluoromethyl substituent rests at the meta and para positions respectively. This is also in contrast to I54, whose modifiable aromatic group is comprised of two trifluoromethyls located at the meta positions, and in further contrast to I55, which forgoes this moiety in exchange for a nitrogen dioxide located at the meta position.

At a glance, given the structure of I48, it would appear unlikely to bind the TM6-TM7 region in comparison to MK-0893. Structurally, these acyl hydrazides are largely apolar and thus do not serve the role of bipartite interactions as well as MK-0893 does. However, unpublished results examining these two agents in concert with each other suggests that they do operate at the same allosteric binding domain. This presupposes a particular binding problem, in that it must be further examined how this agent orients itself within the binding site, and what binding site residues are essential to its docking in comparison to MK-0893. Furthermore, similar to MK-0893, it has also been shown that none of the acyl hydrazides, including I48, have the capacity for agonist like effects. Thus, our results illustrate that I48 serves as solely a positive allosteric modulator in $VPAC_1$ but not $VPAC_2$ receptors, with increasing interest being placed on how the agent interacts with the same helical binding site as MK-0893 does.

Given insight into both pharmacological positive allosteric modulators presented here, it follows that there remains significant research to be investigated. Ideally, this includes isolating a high resolution structure of both $VPAC_1$ and $VPAC_2$ receptors. However, given the longevity of such a project, what can be done immediately is to synthesize analogs of the acyl hydrazides to more closely mimic the structure of MK-0893. Comparing those results against the ones presented here would serve as one way in determining essential binding interactions between I48 and the helical site. This would also permit observance of how the agent orients itself into the site. Additionally, time pulse experiments are essential in defining ERK activation pathways, given that both receptor isoforms have been shown to differentially activate ERK. Addressing these may aid in the discovery of novel therapeutic targets for VIP pathophysiology, such as VIPomas, GI distress, and hormonal regulation.

It may also be prudent to further investigate I12, which exhibited apparent inverse agonist like effects in the $VPAC_2$ but not $VPAC_1$ receptor. Although not statistically significant, there exists a qualitative discrepancy that may lend itself to further characterization of how acyl hydrazides bind to the helical site, given that the agents differ only in ortho or para positioning of the trifluoromethyl substituent.

In addition to these agents' effects on VIP binding to VPAC type receptors, similar experimentation should be performed and compared using a PACAP ligand. Given their close association and equal affinity binding to VPAC receptors, this would be important in classifying the drug-receptor binding interactions presented here as VIP specific, further differentiating the two endogenous ligands from one another.

Limitations from this study result from the variance between samples that precluded statistically significant findings. Due to the magnitude of consistent pERK fold change resulting across both I48 and AB120, we find these results to retain their integrity. However, as mentioned above, I12 may in fact serve as an inverse agonist, and should be thoroughly reexamined. Additionally, $VPAC_1$ ERK activation in lone treated VIP cells was not found to be statistically significant from control levels. While this may be due to a more transient level of receptor activation as compared to $VPAC_2$ receptors, this should also be reexamined for the integrity of results presented here.

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References

Barada, K. A., Saade, N. E., Atweh, S. F., & Nassar, C. F. (1998). Neural mediation of vasoactive intestinal polypeptide inhibitory effect on jejunal alanine absorption. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *275*(4), G822-G828.

Bodner, M., Fridkin, M., & Gozes, I. (1985). Coding sequences for vasoactive intestinal peptide and PHM-27 peptide are located on two adjacent exons in the human genome. *Proceedings of the National Academy of Sciences*, *82*(11), 3548-3551.

Brenneman, D. E., & Eiden, L. E. (1986). Vasoactive intestinal peptide and electrical activity influence neuronal survival. *Proceedings of the National Academy of Sciences*, *83*(4), 1159-1162.

Clason, T. A., Girard, B. M., May, V., & Parsons, R. L. (2016). Activation of MEK/ERK signaling by PACAP in guinea pig cardiac neurons. *Journal of Molecular Neuroscience : MN*, *59*(2), 309–316. http://doi.org/10.1007/s12031-016-0766-z

Couvineau, A., Fabre, C., Gaudin, P., Maoret, J. J., & Laburthe, M. (1996). Mutagenesis of N-glycosylation sites in the human vasoactive intestinal peptide 1 receptor. Evidence that asparagine 58 or 69 is crucial for correct delivery of the receptor to plasma membrane. *Biochemistry*, *35*(6), 1745-1752.

Cunha-Reis, D., Ribeiro, J. A., Almeida, R. F., & Sebastião, A. M. (2017). VPAC1 and VPAC2 receptor activation on GABA release from hippocampal nerve terminals involve several different signalling pathways. *British Journal of Pharmacology*, *174*(24), 4725-4737.

Deupi, X., & Standfuss, J. (2011). Structural insights into agonist-induced activation of G-protein-coupled receptors. *Current opinion in structural biology*, *21*(4), 541-551.

Dong, M., Pinon, D. I., Asmann, Y. W., & Miller, L. J. (2006). Possible endogenous agonist mechanism for the activation of secretin family G protein-coupled receptors. *Molecular pharmacology*, *70*(1), 206-213.

Fahrenkrug, J. (1993). Transmitter role of vasoactive intestinal peptide. *Basic & Clinical Pharmacology & Toxicology*, *72*(6), 354-363.

Ganea, D., Hooper, K. M., & Kong, W. (2015). The neuropeptide vasoactive intestinal peptide: direct effects on immune cells and involvement in inflammatory and autoimmune diseases. *Acta Physiologica*, *213*(2), 442-452.

Gozes, I., Avidor, R., Biegon, N., & Baldino, F. (1989). Lactation elevates vasoactive intestinal peptide messenger ribonucleic acid in rat suprachiasmatic nucleus. *Endocrinology*, *124*(1), 181-186.

Gozes, I., Nakai, H., Byers, M., Avidor, R., Weinstein, Y., Shani, Y., & Shows, T. B. (1987). Sequential expression in the nervous system of C-MYB andVIP genes, located in human chromosomal region 6q24. *Somatic cell and molecular genetics*, *13*(4), 305-313.

Grider, J. R. (1993). Interplay of VIP and nitric oxide in regulation of the descending relaxation phase of peristalsis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *264*(2), G334-G340.

Harmar, A. J., Marston, H. M., Shen, S., Spratt, C., West, K. M., Sheward, W. J., ... & Kelly, J. S. (2002). The VPAC2 receptor is essential for circadian function in the mouse suprachiasmatic nuclei. *Cell*, *109*(4), 497-508.

Harmar, A. J., Sheward, W. J., Morrison, C. F., Waser, B., Gugger, M., & Reubi, J. C. (2004).

Distribution of the VPAC2 receptor in peripheral tissues of the mouse. *Endocrinology*, *145*(3), 1203-1210.

Jazayeri, A., Doré, A. S., Lamb, D., Krishnamurthy, H., Southall, S. M., Baig, A. H., ... & Andrews, S. P. (2016). Extra-helical binding site of a glucagon receptor antagonist. *Nature*, *533*(7602), 274.

Kojima, M., Ito, T., Oono, T., Hisano, T., Igarashi, H., Arita, Y., ... & Nawata, H. (2005). VIP attenuation of the severity of experimental pancreatitis is due to VPAC1 receptor-mediated inhibition of cytokine production. *Pancreas*, *30*(1), 62-70.

Laburthe, M., Couvineau, A., & Marie, J. C. (2002). VPAC receptors for VIP and PACAP. *Receptors and Channels*, *8*(3-4), 137-153.

Laburthe, M., Couvineau, A., & Tan, V. (2007). Class II G protein-coupled receptors for VIP and PACAP: structure, models of activation and pharmacology. *Peptides*, *28*(9), 1631-1639.

Linder, S., Barkhem, T., Norberg, A., Persson, H., Schalling, M., Hökfelt, T., & Magnusson, G. (1987). Structure and expression of the gene encoding the vasoactive intestinal peptide precursor. *Proceedings of the National Academy of Sciences, 84*(2), 605-609.

Malhotra, R. K., Wakade, T. D., & Wakade, A. R. (1988). Vasoactive intestinal polypeptide and muscarine mobilize intracellular Ca2+ through breakdown of phosphoinositides to induce catecholamine secretion. Role of IP3 in exocytosis. *Journal of Biological Chemistry*, *263*(5), 2123-2126.

Masel, S. L., Brennan, B. A., Turner, J. H., Cullingford, G. L., & Cullen, D. J. (2000). Pancreatic vasoactive intestinal polypeptide-oma as a cause of secretory diarrhoea. *Journal of gastroenterology and hepatology*, *15*(4), 457-460.

May, V., Lutz, E., MacKenzie, C., Schutz, K. C., Dozark, K., & Braas, K. M. (2010). Pituitary Adenylate Cyclase-activating Polypeptide (PACAP)/PAC1HOP1 Receptor Activation Coordinates Multiple Neurotrophic Signaling Pathways Akt ACTIVATION THROUGH PHOSPHATIDYLINOSITOL 3-KINASE γ AND VESICLE ENDOCYTOSIS FOR NEURONAL SURVIVAL. *Journal of Biological Chemistry*, *285*(13), 9749-9761.

May, V., & Parsons, R. L. (2016). G Protein-Coupled Receptor Endosomal Signaling and Regulation of Neuronal Excitability and Stress Responses: Signaling Options and Lessons from the PAC1 Receptor. *Journal of Cellular Physiology*.

Ottaway, C. A. (1987). Selective effects of vasoactive intestinal peptide on the mitogenic response of murine T cells. *Immunology*, *62*(2), 291.

Rattan, S., Fan, Y. P., & Chakder, S. (1999). Mechanism of inhibition of VIP-induced LES relaxation by heme oxygenase inhibitor zinc protoporphyrin IX. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *276*(1), G138-G145.

Reiter, E., & Lefkowitz, R. J. (2006). GRKs and β-arrestins: roles in receptor silencing, trafficking and signaling. *Trends in endocrinology & metabolism*, *17*(4), 159-165.

Reubi, J. C. (2000). In vitro evaluation of VIP/PACAP receptors in healthy and diseased human tissues: clinical implications. *Annals of the New York Academy of Sciences*, *921*(1), 1-25.

Sababi, M., Hallgren, A., & Nylander, O. (1996). Interaction between prostanoids, NO, and VIP in modulation of duodenal alkaline secretion and motility. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *271*(4), G582-G590.

Said, S. I. (1986). Vasoactive intestinal peptide. *Journal of endocrinological investigation*, *9*(2), 191-200.

Said, S. I., & Mutt, V. (1970). Polypeptide with broad biological activity: isolation from small

intestine. *Science*, *169*(3951), 1217-1218.

Schwarzenbacher, R., Zeth, K., Diederichs, K., Gries, A., Kostner, G. M., Laggner, P., & Prassl, R. (1999). Crystal structure of human β2-glycoprotein I: implications for phospholipid binding and the antiphospholipid syndrome. *The EMBO Journal*, *18*(22), 6228-6239.

Sheward, W. J., Lutz, E. M., & Harmar, A. J. (1995). The distribution of vasoactive intestinal peptide2 receptor messenger RNA in the rat brain and pituitary gland as assessed by in situ hybridization. *Neuroscience*, *67*(2), 409-418.

Tan, Y. V., Couvineau, A., Murail, S., Ceraudo, E., Neumann, J. M., Lacapère, J. J., & Laburthe, M. (2006). Peptide agonist docking in the N-terminal ectodomain of a Class II G protein-coupled receptor, the VPAC1 receptor photoaffinity, NMR, and molecular modeling. *Journal of Biological Chemistry*, *281*(18), 12792-12798.

Usdin, T. B., Bonner, T. I., & Mezey, E. (1994). Two receptors for vasoactive intestinal polypeptide with similar specificity and complementary distributions. *Endocrinology*, *135*(6), 2662-2680.

Vaudry, D., Gonzalez, B. J., Basille, M., Yon, L., Fournier, A., & Vaudry, H. (2000). Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacological reviews*, *52*(2), 269-324.

Vertongen, P., Schiffmann, S. N., Gourlet, P., & Robberecht, P. (1997). Autoradiographic visualization of the receptor subclasses for vasoactive intestinal polypeptide (VIP) in rat brain. *Peptides*, *18*(10), 1547-1554.

Figures

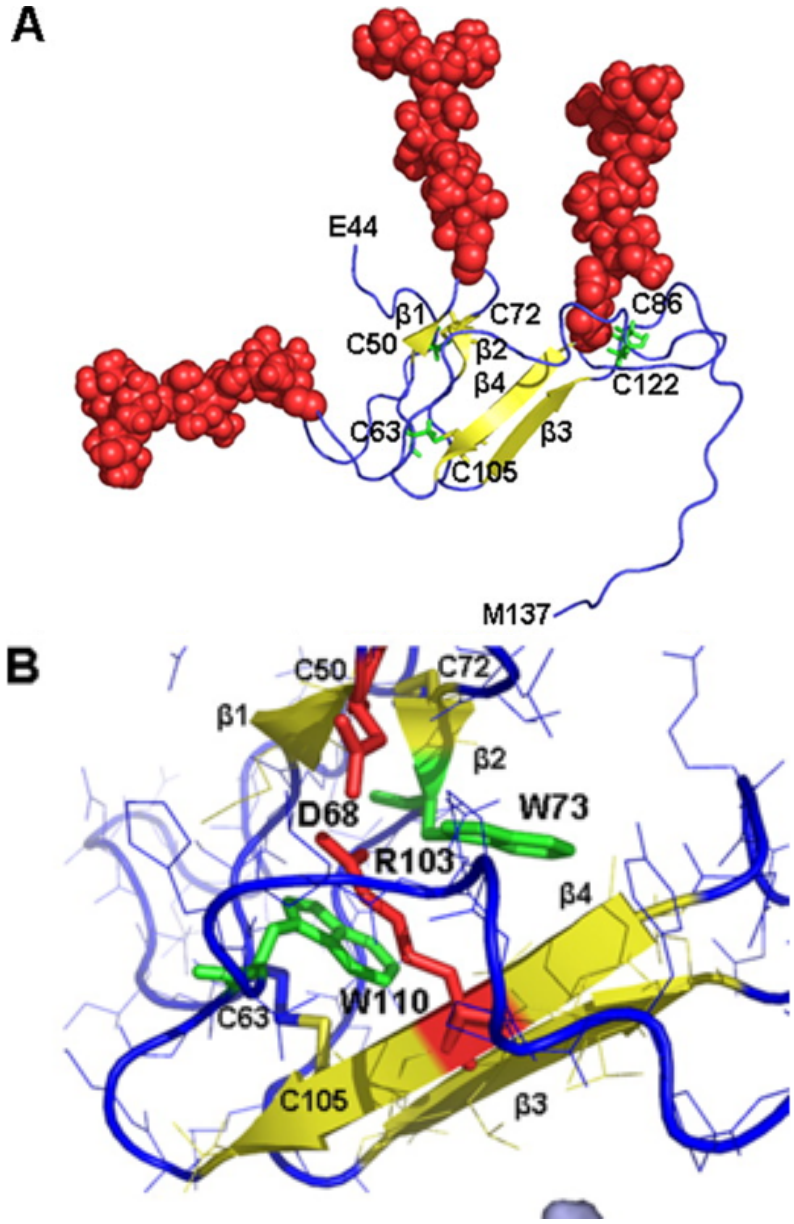

Figure 1. Ribbon representation of the $VPAC_1$ extracellular binding domain. Three essential binding moieties are presented. *A*) Representation of β sheet (yellow) and three essential disulfide bonds (green). *B*) Representation of salt bridge (red) confined between tryptophan aromatic rings (green). Tan et al., 2006.

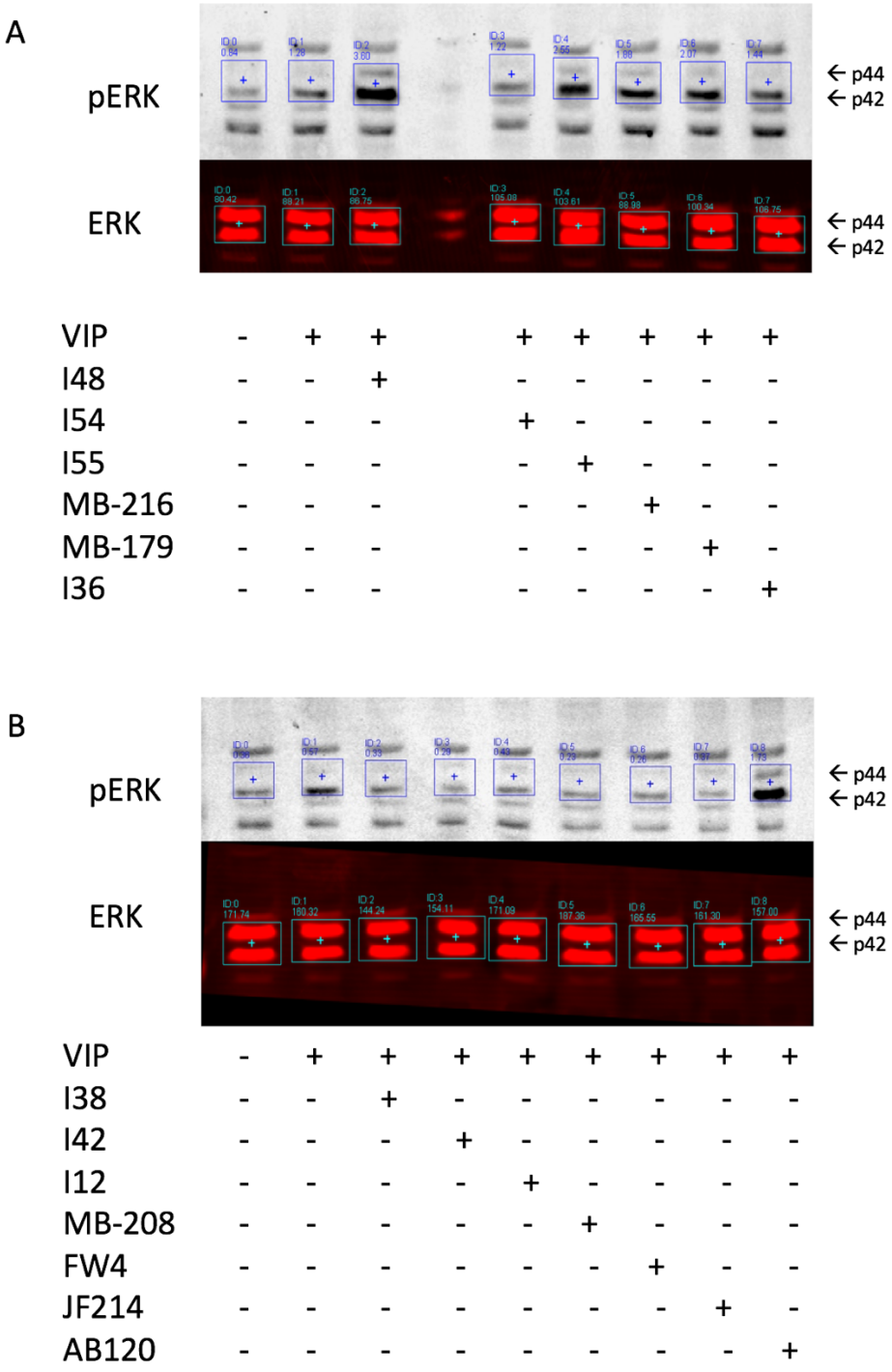

Figure 2. Acyl hydrazide I48 and GCGR antagonist AB-120 (MK-0893) act as positive allosteric modulators of VIP-induced ERK phosphorylation. Representative Western blot analysis of Plate 1 (*A*) and Plate 2 (*B*). VPAC$_1$ receptor cell cultures were treated with VIP (25 nM) for 15 minutes. Other cell cultures were pretreated with a panel of pharmacological agents (20 uM) for 15 minutes before exposure to VIP for an additional 15 min.

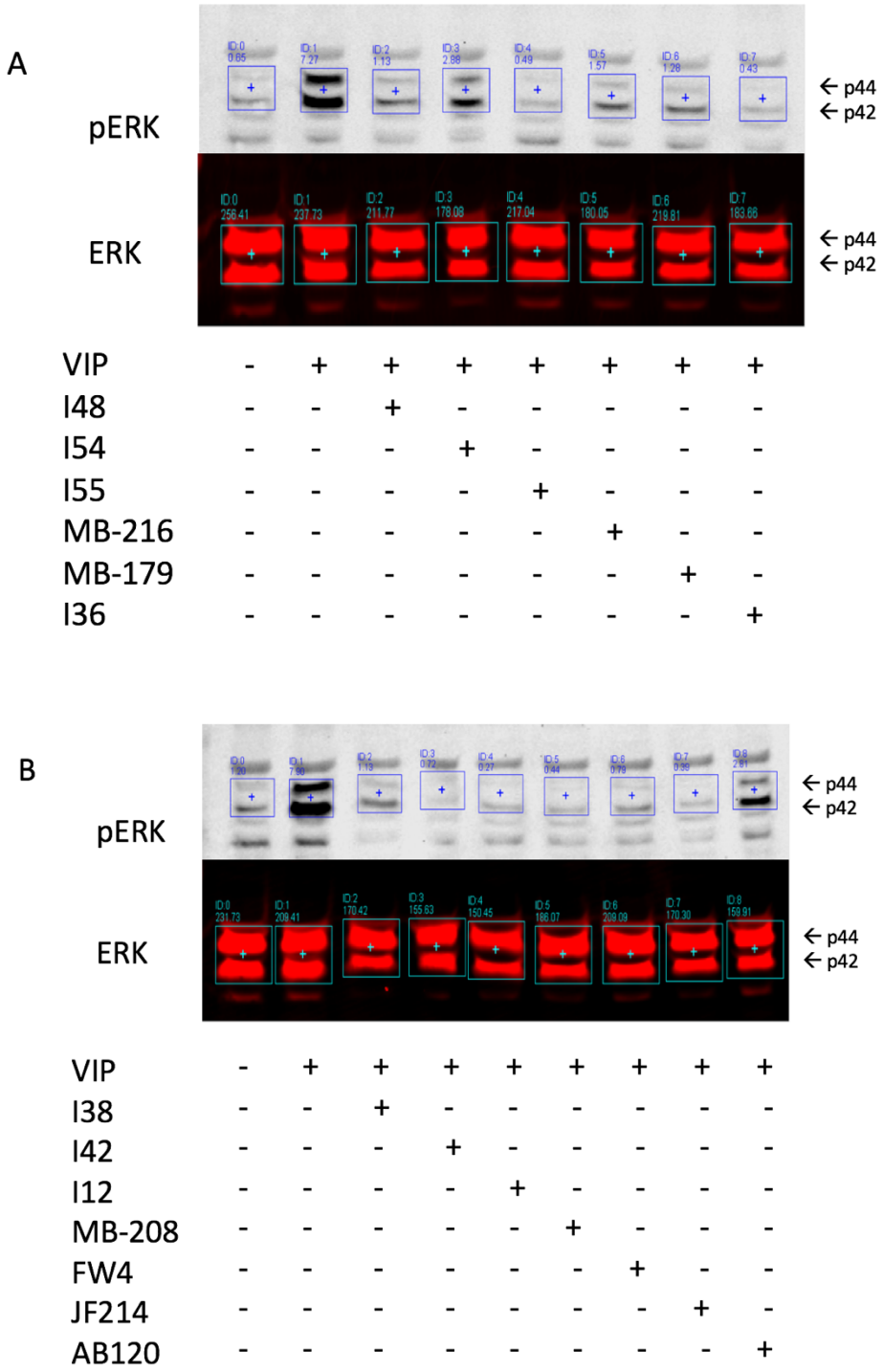

Figure 3. Acyl hydrazide I48 and GCGR antagonist AB-120 (MK-0893) do not act as positive allosteric modulators of VIP-induced ERK phosphorylation. Representative Western blot analysis of Plate 1 (*A*) and Plate 2 (*B*). $VPAC_2$ receptor cell cultures were treated with VIP (25 nM) for 15 minutes. Other cell cultures were pretreated with a panel of pharmacological agents (20 uM) for 15 minutes before exposure to VIP for an additional 15 min.

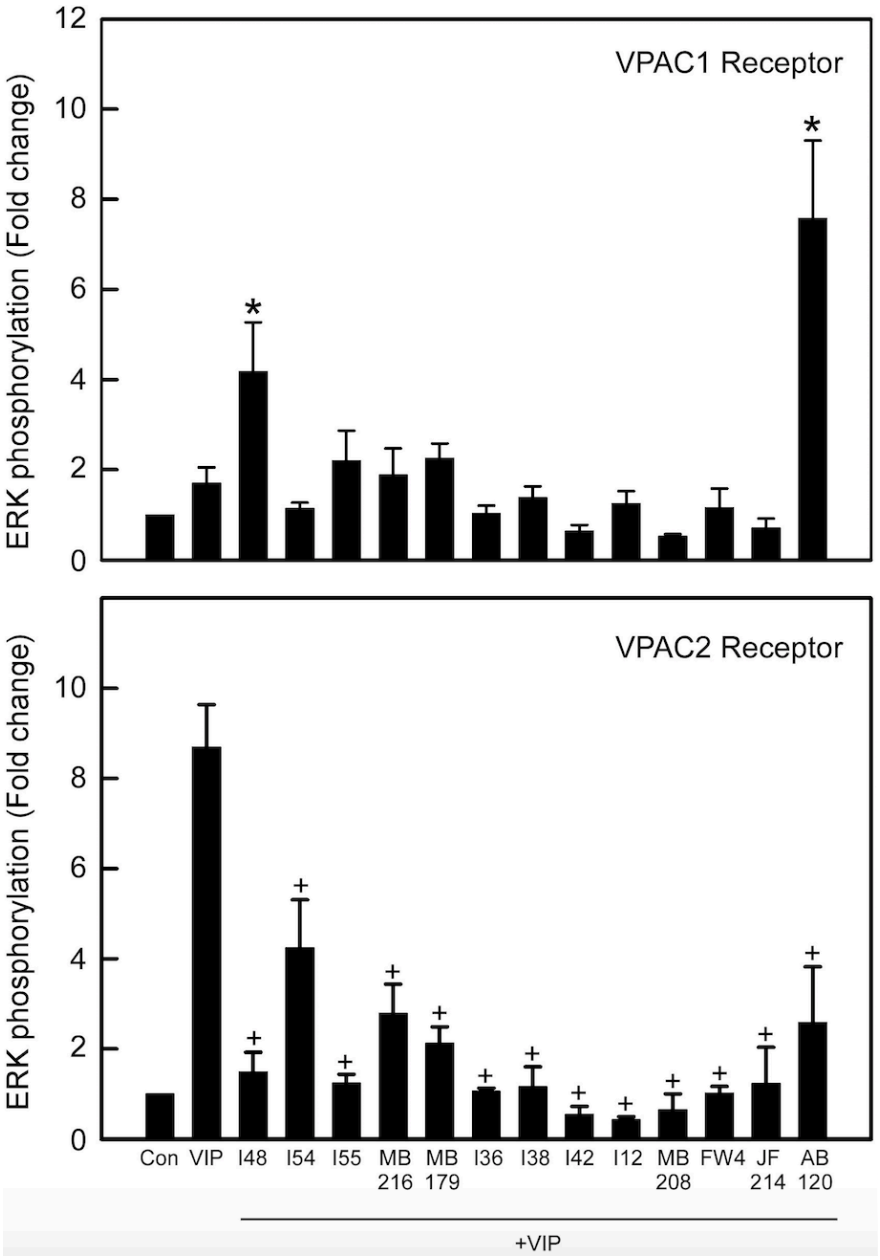

Figure 4. Quantification of phosphorylated ERK in VPAC$_1$ and VPAC$_2$ receptors. pERK was normalized to total ERK in each sample. VIP alone stimulated ERK activation ~2-fold in VPAC$_1$ receptors compared to control. Following pretreatment with I48 and AB-120, the VIP-induced ERK phosphorylation increased significantly ~4 and ~7-fold from control levels respectively. In contrast, in VPAC$_2$ receptors, VIP alone stimulated pERK levels ~8-fold from control levels. Pretreatment with I48 and AB-120 did not significantly increase the VIP-induced ERK phosphorylation. Data represent mean fold change ±SE; n=3 independent experiments different from control and lone VIP treated samples, all at $P < 0.05$.

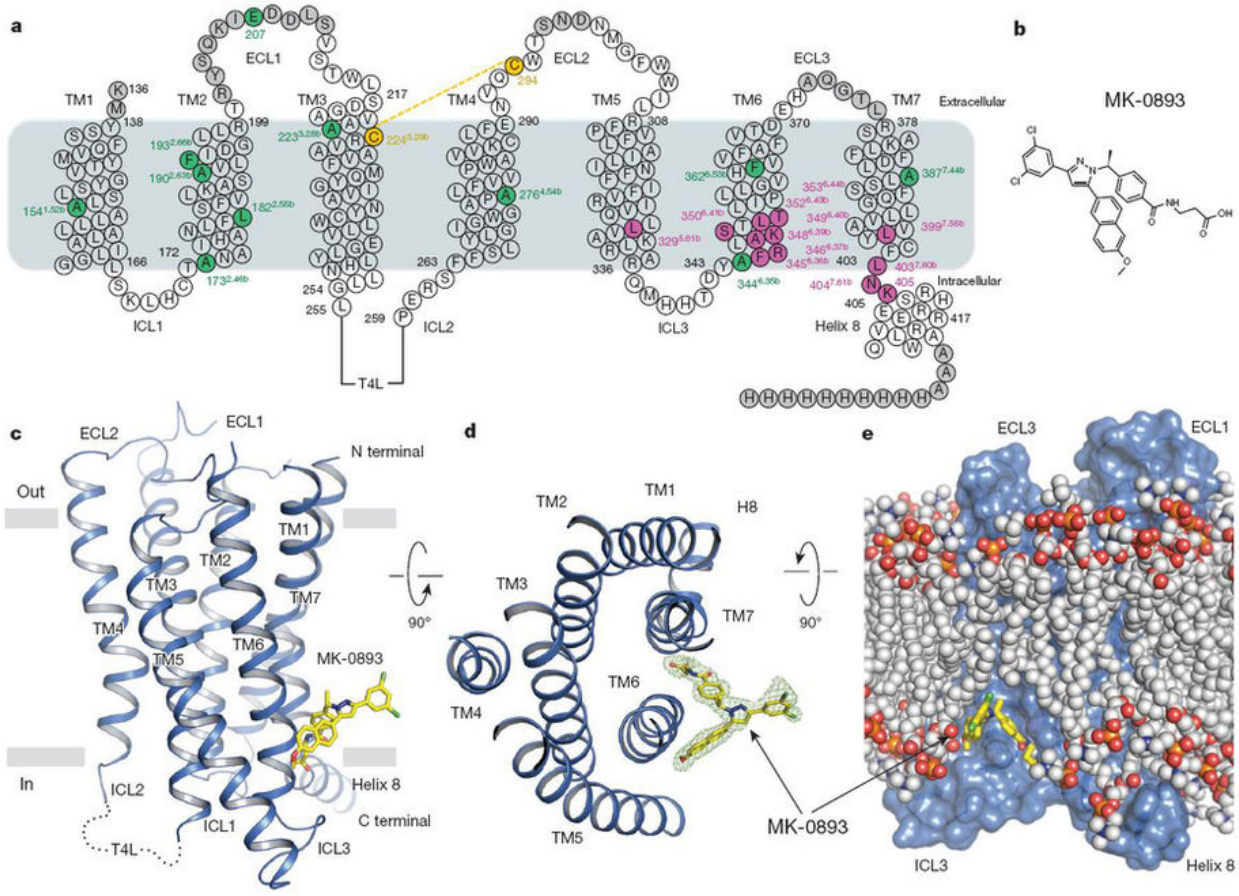

Figure 5. Glucagon receptor sequence and structure. a) Glucagon receptor transmembrane domain amino acid arrangement. b) MK-0893 structure. c) GCGR ribbon representation of MK-0893 binding at the helical site, transmembrane orientation. d) GCGR, top view from extracellular face demonstrating MK-0893 pincher to restrict TM6 dynamics. e) MK-0893 modeled in the lipid bilayer (taken from Jazayeri et al., 2016).