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Article

Novel Fluorescently Labeled PACAP and VIP Highlight Differences between Peptide Internalization and Receptor Pharmacology

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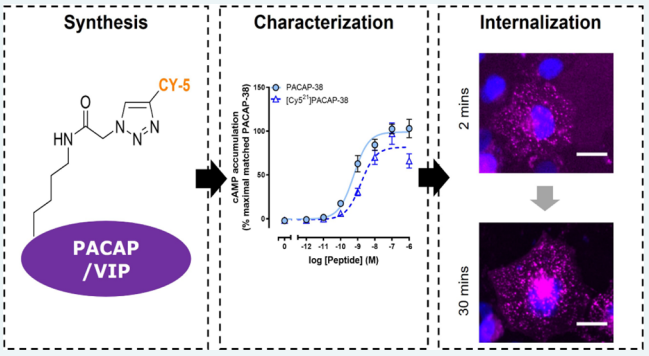
ABSTRACT: The related peptides pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) have diverse biological functions in peripheral tissues and the central nervous system. Therefore, these peptides and their three receptors represent potential drug targets for several conditions, including neurological and pain-related disorders. However, very little is known about how these peptides regulate their receptors through processes such as internalization. Therefore, we developed tools to study receptor regulation through the synthesis of fluorescently labeled analogues of PACAP-38, PACAP-27, and VIP using copper-mediated 1,3-dipolar cycloaddition of the Cy5 fluorophore. The functionality of Cy5-labeled peptides at their receptors was confirmed in cAMP accumulation assays. Internalization of the Cy5-labeled peptides was then examined and quantified at two distinct PAC_1 receptor splice variants, $VPAC_1$ and $VPAC_2$ receptors in transfected cells. All labeled peptides were functional, exhibiting comparable cAMP pharmacology to their unlabeled counterparts and underwent internalization in a time-dependent manner. Temporal differences in the internalization profiles were observed between Cy5-labeled peptides at the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors. Interestingly, the pattern of Cy5-labeled peptide activity differed for cAMP accumulation and internalization, indicating that these peptides differentially stimulate cAMP accumulation and internalization and therefore display biased agonism. This novel insight into PACAP-responsive receptor signaling and internalization may provide a unique avenue for future therapeutic development. The fluorescently labeled PACAP and VIP peptides described herein, which we validated as tools to study receptor internalization, will have utility across a broad range of applications and provide greater insight into this receptor family.



KEYWORDS: *PACAP, VIP, Cy5, internalization, bias*

The pituitary adenylate cyclase-activating polypeptide (PACAP) peptide family includes two biologically active forms of PACAP: the full-length 38 amino acid peptide (PACAP-38) and shorter 27 amino acid peptide (PACAP-27) in addition to the related 28 amino acid vasoactive intestinal peptide (VIP).[1] PACAP and VIP are linked to diverse biological functions in both peripheral tissues and the central nervous system.[2−4] Not surprisingly, these peptides are implicated in numerous disorders, including neurodegenerative conditions and migraine.[1,2,5]

The important role PACAP plays in migraine has led to the development of clinical drug candidates targeting the PACAP peptide and receptor family.[4,6,7] These drug candidates act to block the activity of PACAP and prevent downstream signaling events following receptor activation. An anti-PACAP antibody has been developed and is currently in clinical trials.[4,6] However, an anti-PAC_1 receptor antibody, which blocks receptor activation, did not exhibit clinical efficacy against migraine.[7] Efficacy in other neurological disorders or diseases has yet to be examined. The reason for this lack of efficacy is unclear but emphasizes the need to better understand how these peptides activate and regulate their receptors to develop effective therapeutics.

The PACAP family of peptides produces their effects through three different PACAP-responsive G protein-coupled receptors; PAC_1, $VPAC_1$, and $VPAC_2$. Characteristically, both PACAP-38 and PACAP-27 exhibit higher activity than VIP at

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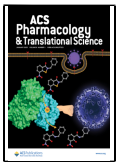

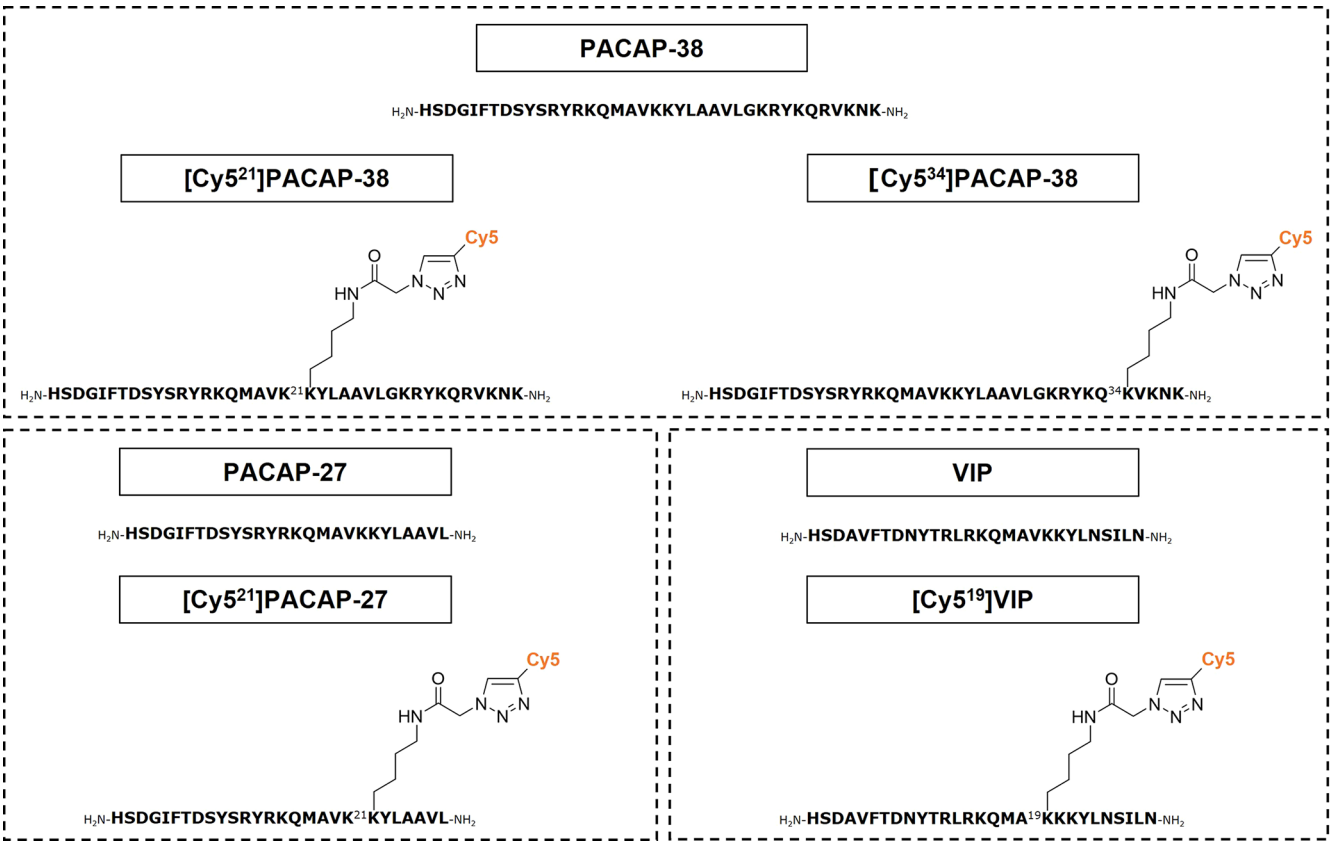

Figure 1. Chemical structures of fluorescently labeled peptides illustrating the position of Cy5 attachment to PACAP-38, PACAP-27, and VIP.

the PAC_1 receptor, whereas PACAP and VIP activate the $VPAC_1$ and $VPAC_2$ receptors equivalently.[1] However, the PAC_1 receptor can undergo alternative splicing to generate variants that may differ in their pharmacology and signaling behaviors.[8−10] PAC_1 receptor splice variants are broadly categorized into two groups based on the location and type of splicing event: either N-terminal deletions or intracellular loop 3 (ICL3) insertions. The receptor with a complete N-terminus and no ICL3 insertion is considered the PAC_{1n} variant. N-terminal variants are known as PAC_{1s} and PAC_{1vs} and ICL3 variants as PAC_{1Hop} and PAC_{1Hip}.[11] Although PACAP is generally reported to have a higher potency than VIP at PAC_1 receptors, this is not the case at the PAC_{1s} receptor splice variant, where PACAP and VIP display similar potency.[8,10,12]

The activation of a PACAP-responsive receptor initiates a series of intracellular signaling events, which canonically involve the accumulation of cAMP.[10] Interestingly, biased agonism, which refers to the activation of different signaling pathways by different ligands, has been reported for the PAC_1 receptor.[13−15] Thus, different peptides could activate the same PACAP-responsive receptor but stimulate different signaling events, yielding diverse pharmacological outcomes.

Receptor internalization is an important regulator of intracellular signaling responses by limiting the number of receptors at the cell surface and preventing further cell surface receptor activation. This occurs through the translocation of activated receptors from the cell surface into endosomal vesicles, which can then be targeted for degradation or recycling.[16,17] However, evidence now suggests that internalized receptors can continue to signal from endosomes, where they can activate similar signaling pathways to those at the cell surface or specific endosomal signaling pathways.[16,18,19] Interestingly, endosomal signaling through the PAC_1 receptor has been linked to chronic pain and anxiety-like responses.[20,21] This is consistent with other reports where endosomal signaling contributes to sustained neural excitation, which results in enhanced pain behaviors.[16,19,22]

Investigation of PACAP-responsive receptor internalization has been undertaken.[23−27] Studies using tagged receptors or fluorescently labeled peptides suggest that these receptors can undergo internalization in a time-dependent manner with most studies focusing on PAC_1.[25−29] However, typically only a single peptide, receptor subtype, or splice variant have been examined in any individual study. Consequently, there is currently an incomplete picture of PACAP-responsive receptor internalization, and potential peptide or receptor-dependent differences may have been missed. Such differences could have significant implications for understanding the role of the PACAP peptide family in physiological functions. Thorough comparisons using multiple peptide ligands at multiple receptor subtypes are required to elucidate these behaviors.

Traditionally, radiolabeled peptide probes have been used to investigate receptor binding and peptide-stimulated receptor internalization.[30,31] More recently, fluorescent imaging techniques have matured and are increasingly used to investigate these aspects of receptor biology.[32,33] These methods involve using either fluorescently labeled peptides or antibodies that target the receptor to allow visualization and quantification of internalization. Characterized fluorescently labeled probes are invaluable pharmacological tools that aid the understanding of agonist-mediated internalization for a wide range of receptors.[32−35] Fluorescently labeled PACAP or VIP has previously been generated and used to investigate receptor

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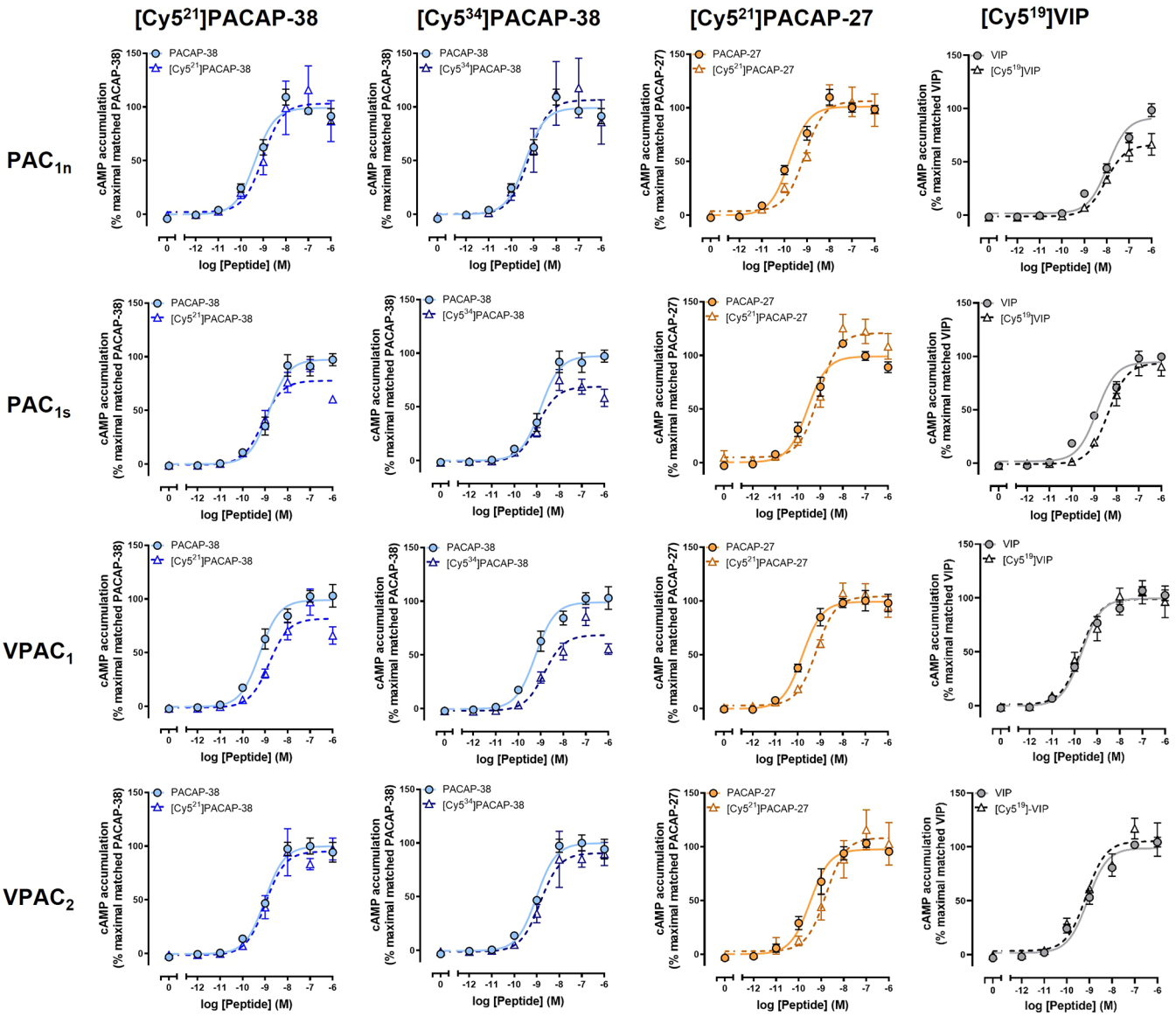

Figure 2. Stimulation of cAMP accumulation by unlabeled and Cy5-labeled PACAP-38, PACAP-27, and VIP in Cos7 cells transfected with the PAC_{1n}, PAC_{1s}, $VPAC_1$, or $VPAC_2$ receptor. Data were normalized to the maximal responses produced by the corresponding unlabeled peptide and expressed as a percentage. Data points are the mean ± SEM of the combined data from 5 independent experiments.

internalization or tissue distribution.[23,24,36−38] However, several of these tools were not site-specifically labeled, as the synthesis approach used could generate a mixture of peptides labeled at different sites, or were not robustly pharmacologically characterized.

This study aimed to develop and validate a series of fluorescently labeled PACAP and VIP peptides that could be used as tools to study cellular aspects of PACAP-responsive receptor behavior, including receptor internalization. We synthesized Cy5-conjugated PACAP-38, PACAP-27, and VIP peptides by click chemistry and pharmacologically characterized their ability to stimulate PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptor-mediated cAMP accumulation in transfected cells. These were then used to investigate internalization by fluorescent imaging at all three receptor subtypes, including the previously unexplored PAC_{1s} receptor splice variant.

RESULTS AND DISCUSSION

Synthesis of Cy5-Labeled PACAP and VIP Peptides.

Our selection of labeling sites was guided by published targeted mutagenesis studies as at the time of initiating these studies, only limited structural data were available.[39−41] These suggested that several positions within PACAP and VIP may tolerate the incorporation of an azido-lysine and the subsequent conjugation of a Cy5 alkyne fluorophore. Position 21 of both PACAP-27 and PACAP-38 was selected as mutagenesis to glutamic acid had a minimal effect on peptide binding at the three receptor subtypes.[39] In addition, position 34 of PACAP-38 was selected to synthesize a second Cy5-labeled PACAP-38 analogue as mutagenesis to lysine did not significantly affect peptide binding.[40] Alanine scanning and screening of VIP binding at the $VPAC_1$ receptor suggested that modification at position 19 would be well tolerated.[41] Therefore, 1,3-dipolar cycloaddition of an in-house Cy5 alkyne fluorophore was employed to synthesize the following site-

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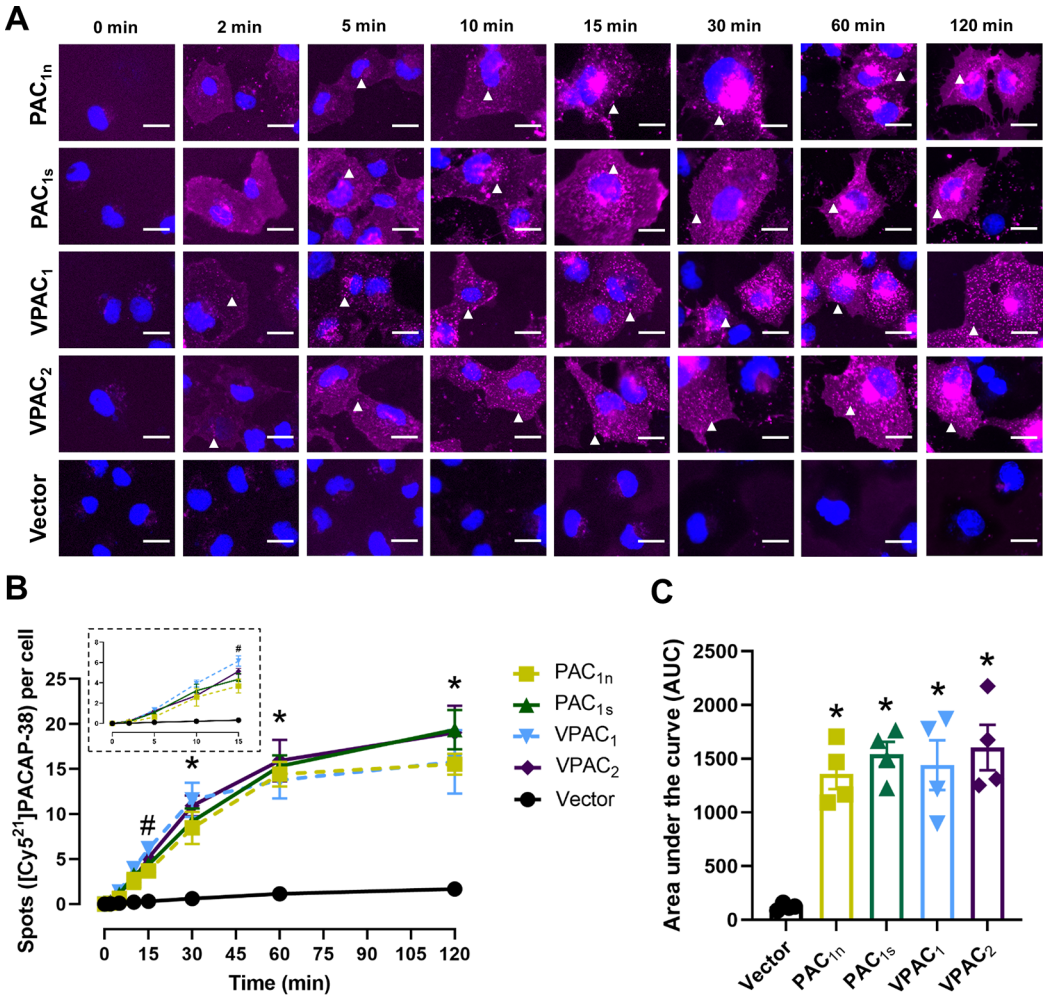

Figure 3. Internalization of [Cy5[21]]PACAP-38 (10 nM) over time in Cos7 cells transfected with the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors. (A) [Cy5[21]]PACAP-38 fluorescence shown in magenta and nuclear 4′,6-diamindino-2-phenylindole (DAPI) in blue; white arrowheads indicate examples of spots. Images are representative of one field of view from 4 independent experiments. Scale bar, 20 μm. (B) Quantification of the number of [Cy5[21]]PACAP-38 spots per cell at each time point. (Inset) Magnified view of the spot number from 0 to 15 min. Statistical significance was determined by repeated measures two-way ANOVA with post hoc Tukey's test comparing all receptors and vector-transfected cells to each other at each time point. * $p < 0.05$ for all receptors compared to vector-transfected cells, and # $p < 0.05$ for $VPAC_1$ and $VPAC_2$ compared to vector-transfected cells. (C) Area under the spot counting curve from B. Statistical significance was determined by one-way ANOVA with post hoc Tukey's test. * $p < 0.05$ compared to vector-transfected cells. Data in B and C are plotted as the mean ± SEM combined from 4 independent experiments.

specifically labeled fluorescent peptides: [Cy5[21]]PACAP-38, [Cy5[34]]PACAP-38, [Cy5[21]]PACAP-27, and [Cy5[19]]VIP, as depicted in Figure 1.

Incorporation of Cy5 into PACAP and VIP Was Tolerated Pharmacologically. Following synthesis, it was essential to characterize the pharmacological activity of the Cy5-labeled peptides at their receptors to determine if the activity had been affected. To achieve this, peptide-induced cAMP accumulation was measured in receptor-transfected Cos7 cells. Cos7 cells were selected as they do not respond to PACAP endogenously.[10,42,43] cAMP accumulation was selected as this pathway was robustly activated in the Cos7 cell model system and displayed similar relative pharmacological profiles to other signaling pathways.[10] Furthermore, cAMP accumulation is a well-described measure of receptor activation, which is relative to receptor binding for the PACAP-responsive receptor family.[8,12,43] We therefore opted to use cAMP accumulation as a measure of receptor activation and an indirect indicator of receptor binding. Multiple peptide concentrations were used to generate concentration−response curves from which the peptide potency (pEC_{50}) and maximal response (E_{max}) at each receptor were calculated and compared to the corresponding unlabeled peptide. [Cy5[21]]PACAP-38 and [Cy5[34]]PACAP-38 were equipotent to unlabeled PACAP-38 at stimulating cAMP accumulation at the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors (Figure 2, Table S1). However, a significantly lower E_{max} was observed for both labeled peptides at the PAC_{1s} and $VPAC_1$ receptors. [Cy5[21]]PACAP-27 was significantly less potent compared to unlabeled PACAP-27 at all receptors; however, this difference was relatively small at less than 5-fold (Figure 2, Table S2). The retention of potency for both Cy5-labeled PACAP-38 and PACAP-27 is not surprising based on cryo-EM structures of the peptide-bound PAC_1 and $VPAC_2$ receptors that are now available.[44−46] These studies identified that position 21 of PACAP did not make crucial receptor interactions, while the C-terminus of PACAP-38 could not be resolved and is likely a highly flexible region that forms weak receptor interactions.

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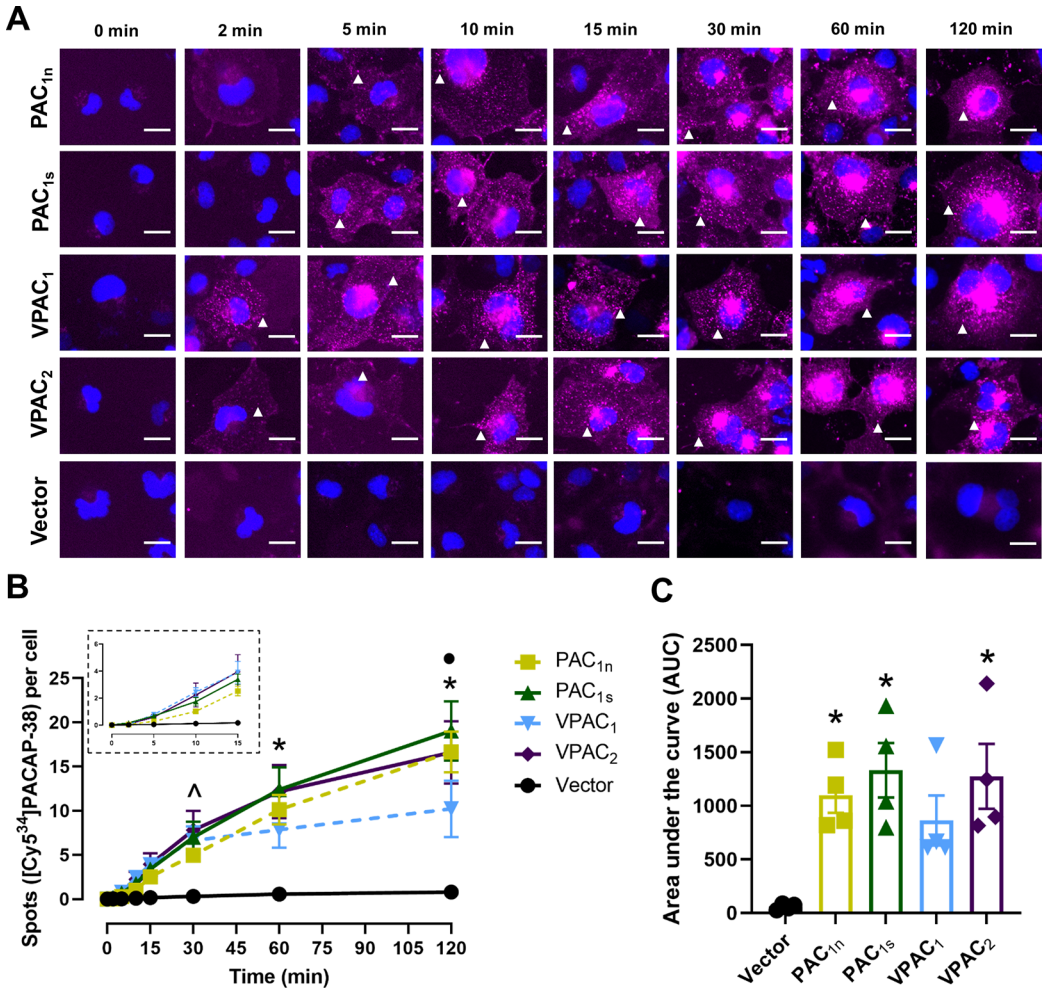

Figure 4. Internalization of [Cy5[34]]PACAP-38 (10 nM) over time in Cos7 cells transfected with the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors. (A) [Cy5[34]]PACAP-38 fluorescence shown in magenta and nuclear DAPI in blue; white arrowheads indicate examples of spots. Images are representative of one field of view from 4 independent experiments. Scale bar, 20 μm. (B) Quantification of the number of [Cy5[34]]PACAP-38 spots per cell at each time point. (Inset) Magnified view of the spot number from 0 to 15 min. Statistical significance was determined by repeated measures two-way ANOVA with post hoc Tukey's test comparing all receptors and vector-transfected cells to each other at each time point. * $p <$ 0.05 for all receptors compared to vector-transfected cells, ● $p < 0.05$ for $VPAC_1$ compared to all receptors. and ^ $p < 0.05$ for PAC_{1s}, $VPAC_1$, and $VPAC_2$ compared to vector-transfected cells. (C) Area under the spot counting curve from B. Statistical significance was determined by one-way ANOVA with post hoc Tukey's test. * $p < 0.05$ compared to vector-transfected cells. Data in B and C are plotted as the mean ± SEM combined from 4 independent experiments.

[Cy5[19]]VIP stimulated cAMP accumulation at all receptors equipotently to its unlabeled peptide but exhibited a significantly lower E_{max} at the PAC_{1n} receptor (Figure 2, Table S3). No VIP-bound receptor structure has been solved, but the findings of the current study suggest the valine at position 19 is not required for high-affinity binding. Overall, Cy5 labeling of all peptides was well tolerated at the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors. Peptide potencies were consistent or within a ~5-fold range to those reported in the literature for unlabeled PACAP and VIP.[8,10,43] The fluorescent peptides were therefore further characterized for their ability to undergo receptor-mediated internalization.

Internalization of Cy5-Labeled PACAP and VIP Peptides. To confirm that PACAP-responsive receptors were capable of internalization in receptor-transfected Cos7 cells, ligand-stimulated internalization of HA-tagged (HA) PAC_1 receptors was measured using cell surface ELISA. PACAP-38 stimulated a time-dependent reduction in HA-PAC_{1n} and HA-PAC_{1s} receptor cell surface expression, indicative of internalization (Figure S1). In contrast, PACAP-27 appeared to stimulate a reduction in HA-PAC_{1s} but not HA-PAC_{1n} receptor surface expression; however, this difference did not reach significance (Figure S1). This suggests that PAC_1 receptors can internalize and that the transfected Cos7 cell model was suitable for investigation of Cy5-labeled peptide-stimulated internalization. For analysis of internalization, a Cy5-labeled peptide concentration of 10 nM was used because this produced robust stimulation of cAMP production at each receptor (Figure 2). Diffuse fluorescent staining would be observed in the absence of internalization, whereas distinct fluorescent spots within the cell cytoplasm are indicative of peptide-bound receptor internalization into endosomes. To differentiate between spots on the cell surface and within the cytoplasm, the cytoplasmic stain CellMask Green was used (Figures S2–S5). The time points were chosen to reflect the rapid nature of receptor internalization which can begin within 5 min of peptide addition.

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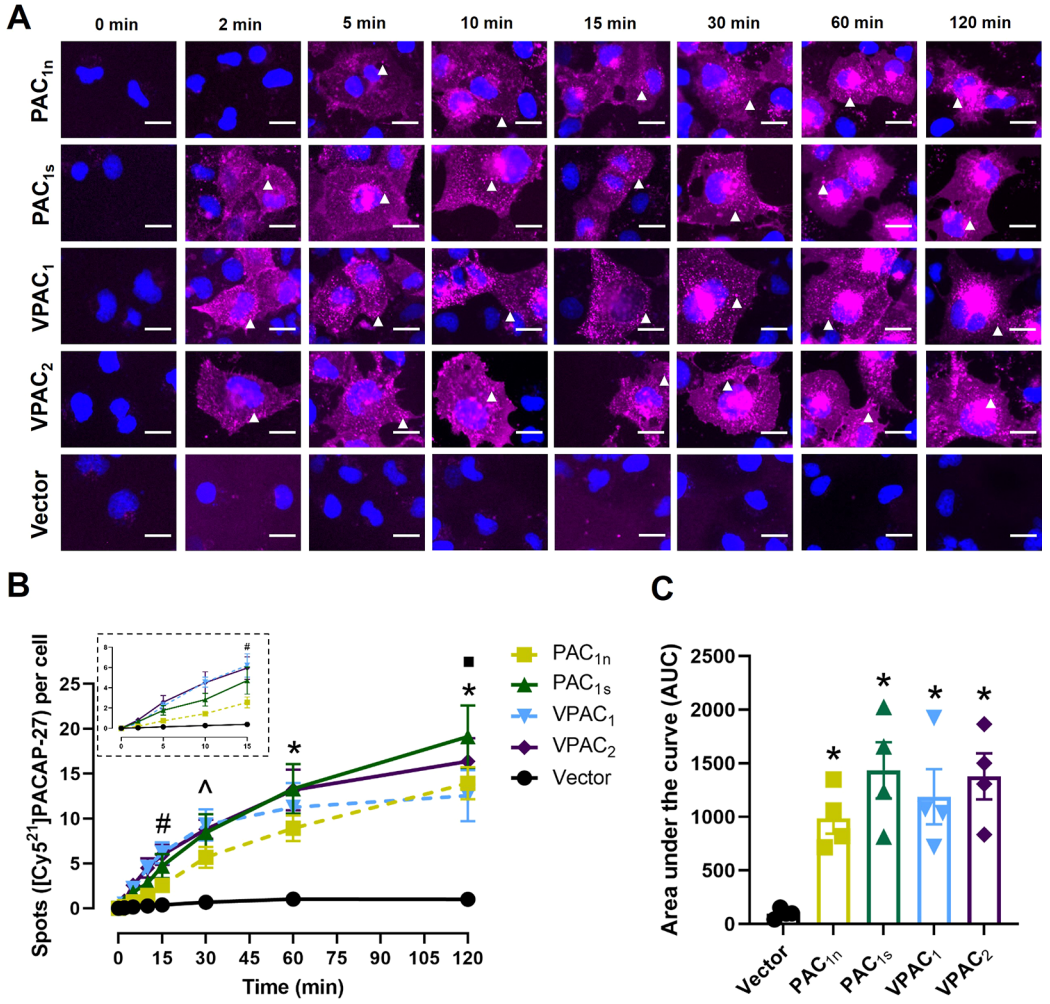

Figure 5. Internalization of [Cy5[21]]PACAP-27 (10 nM) over time in Cos7 cells transfected with the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors. (A) [Cy5[21]]PACAP-27 fluorescence shown in magenta and nuclear DAPI in blue; white arrowheads indicate examples of spots. Images are representative of one field of view from 4 independent experiments. Scale bar, 20 μm. (B) Quantification of the number of [Cy5[21]]PACAP-27 spots per cell at each time point. (Inset) Magnified view of the spot number from 0 to 15 min. Statistical significance was determined by repeated measures two-way ANOVA with post hoc Tukey's test comparing all receptors and vector-transfected cells to each other at each time point. * $p <$ 0.05 for all receptors compared to vector-transfected cells, ■ $p < 0.05$ for $VPAC_1$ compared to PAC_{1s}, ^ $p < 0.05$ for PAC_{1s}, $VPAC_1$, and $VPAC_2$ compared to vector-transfected cells, and # $p < 0.05$ for $VPAC_1$ and $VPAC_2$ compared to vector-transfected cells. (C) Area under the spot counting curve from B. Statistical significance was determined by one-way ANOVA with post hoc Tukey's test. * $p < 0.05$ compared to vector-transfected cells. Data in B and C are plotted as the mean ± SEM combined from 4 independent experiments.

A time-dependent increase in the number of [Cy5[21]]-PACAP-38 spots was observed in cells transfected with the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors but not the vector control (Figure 3A and 3B). Interestingly, the $VPAC_1$ and $VPAC_2$ receptors exhibited a slightly different temporal profile from the PAC_1 receptors, where distinct fluorescent spots were evident at 2 min for $VPAC_1$ and $VPAC_2$ but were comparatively delayed at the PAC_1 receptors (10 min). The temporal difference was reflected in quantification, whereby the number of spots reached statistical significance for the VPAC receptors by 15 min, compared to 30 min for the PAC_1 receptors (Figure 3B). All receptors reached a similar maximum at ∼60 min, and the degree of [Cy5[21]]PACAP-38-stimulated internalization (area under the curve) was comparable between all receptors examined (Figure 3C, Table S4).

[Cy5[34]]PACAP-38 produced a time-dependent increase in the number of spots at all receptors, though the temporal profile between receptors was slightly different (Figure 4A and 4B). The PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors, but not the PAC_{1n} receptor, exhibited significantly more fluorescent spots compared to vector at 30 min (Figure 4B). However, the PAC_{1n} receptor had reached significance from 60 min. The number of spots per cell increased gradually until 120 min for all receptors except $VPAC_1$, which appeared to plateau after 30 min (Figure 4B). Overall, [Cy5[34]]PACAP-38 stimulated a comparable degree of internalization at the PAC_1 and $VPAC_2$ receptors, whereas internalization at the $VPAC_1$ receptor was lower and not significantly different from the vector (Figure 4C, Table S4). [Cy5[34]]PACAP-38 appeared to be less effective at stimulating internalization than [Cy5[21]]PACAP-38; however, this was not significantly different (Figure S6).

A time-dependent increase in the number of [Cy5[21]]-PACAP-27 spots was observed at all receptors except the PAC_{1n} receptor, which appeared to have a delayed response (Figure 5A and 5B). Significant differences from the vector were detected from 15 min for the $VPAC_1$ and $VPAC_2$ receptors, 30 min for the PAC_{1s} receptor, and 60 min for

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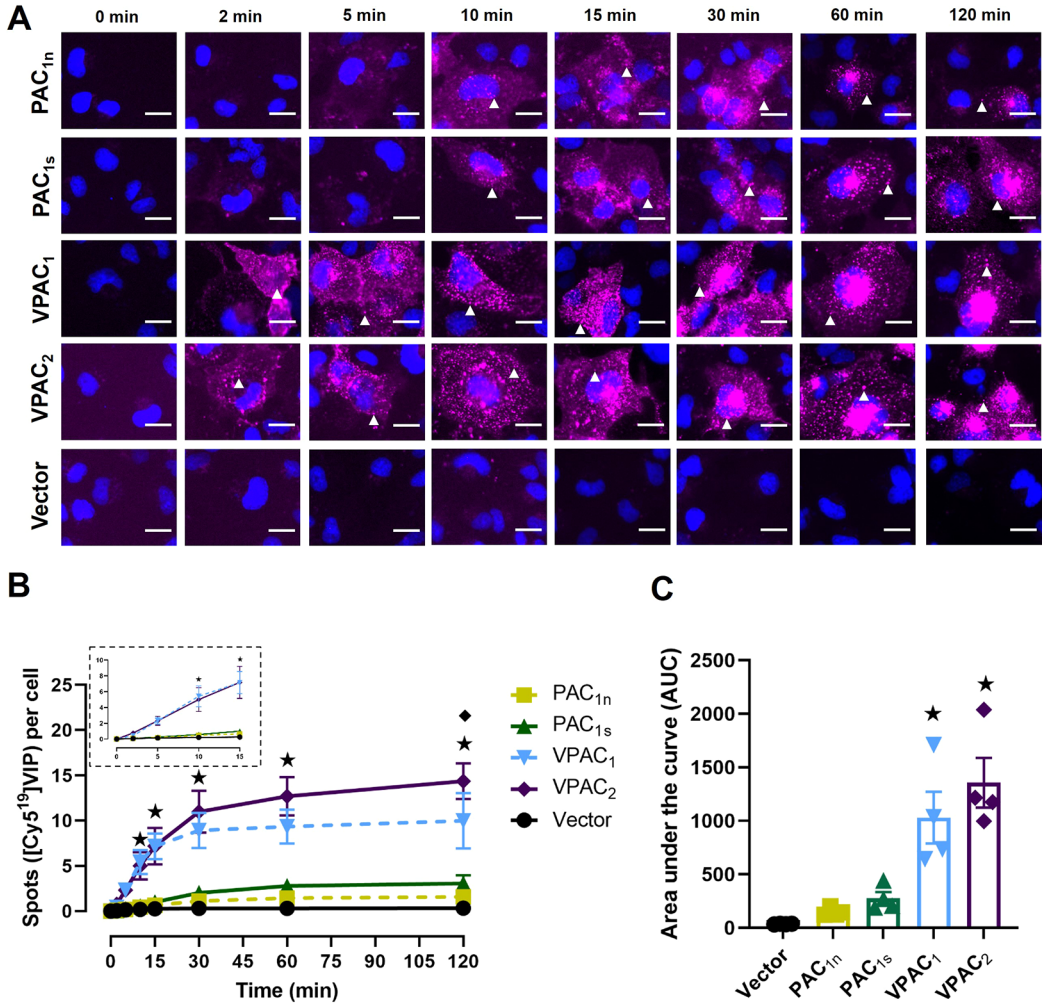

Figure 6. Internalization of [Cy5[19]]VIP (10 nM) over time in Cos7 cells transfected with the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors. (A) [Cy5[19]]VIP fluorescence shown in magenta and nuclear DAPI in blue; white arrowheads indicate examples of spots. Images are representative of one field of view from 4 independent experiments. Scale bar, 20 μm. (B) Quantification of the number of [Cy5[19]]VIP spots per cell at each time point. (Inset) Magnified view of the spot number from 0 to 15 min. Statistical significance was determined by repeated measures two-way ANOVA with post hoc Tukey's test comparing all receptors and vector-transfected cells to each other at each time point. ★ $p < 0.05$ for $VPAC_1$ and $VPAC_2$ compared to PAC_{1n}, PAC_{1s}, and vector-transfected cells, and ◆ $p < 0.05$ for $VPAC_1$ compared to $VPAC_2$. (C) Area under the spot counting curve from B. Statistical significance was determined by one-way ANOVA with post hoc Tukey's test. ★ $p < 0.05$ compared to PAC_{1n}, PAC_{1s}, and vector-transfected cells. Data in B and C are plotted as the mean ± SEM combined from 4 independent experiments.

the PAC_{1n} receptor (Figure 5B). Although the number of spots increased gradually over time, $VPAC_1$ receptor internalization appeared to plateau from 60 min and was significantly lower than the PAC_{1s} receptor at 120 min (Figure 5B). Overall, [Cy5[21]]PACAP-27 stimulated a comparable degree of internalization at the PAC_{1s} and $VPAC_2$ receptors, whereas responses at PAC_{1n} and $VPAC_1$ appeared to be more limited; however, these were not significantly different (Figure 5C, Table S4). Furthermore, the degree of [Cy5[21]]PACAP-27-stimulated internalization was comparable to both Cy5-labeled PACAP-38 peptides (Figure S6).

[Cy5[19]]VIP stimulated a time-dependent increase in fluorescent spots in cells transfected with the PAC_{1n}, PAC_{1s}, $VPAC_1$, and $VPAC_2$ receptors but not the vector control (Figure 6A and 6B). Fluorescent spots were initially observed at 2 min at the $VPAC_1$ and $VPAC_2$ receptors followed by the PAC_{1n} and PAC_{1s} receptors from 10 min, and the VPAC receptors exhibited significantly more fluorescent spots at each time point compared to the PAC_1 receptors (Figure 6B). Interestingly, the number of observed spots at the $VPAC_1$ and $VPAC_2$ receptors appeared to plateau at 30 min with $VPAC_2$ reaching a significantly higher maximum compared to $VPAC_1$ at 120 min (Figure 6B). Overall, [Cy5[19]]VIP stimulated a comparable degree of internalization at the $VPAC_1$ and $VPAC_2$ receptors with very low levels of internalization observed at the PAC_{1n} and PAC_{1s} receptors (Figure 6C, Table S4).

Internalization of PACAP or PACAP-mediated receptor internalization has been investigated, but very few studies characterize the tools used or examined responses at multiple receptor subtypes in the same study. Site-specific FITC-labeled PACAP-38 and PACAP-27 peptides have been reported and were used to show time-dependent internalization of both PACAP-38 and PACAP-27.[36] In this study, the internalization response plateaued within 30 min, whereas only $VPAC_1$ receptor internalization with Cy5-PACAP plateaued in the current study (Figures 4B and 5B). Furthermore, the FITC-PACAP internalization was reported to be receptor independent, which contrasts with our results as internalization was not

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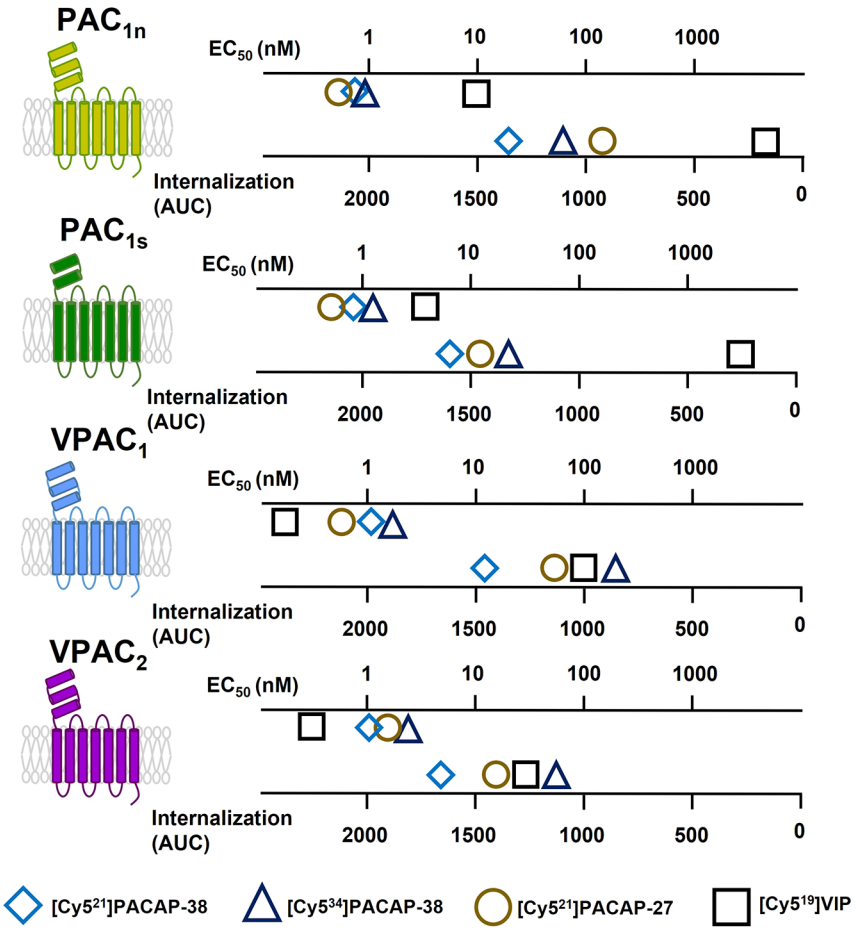

Figure 7. Summary of Cy5-labeled peptide cAMP accumulation potency and internalization capacity. Internalization was measured with 10 nM peptide. AUC = area under the curve.

observed with the Cy5-labeled PACAP-38 or PACAP-27 in vector-transfected cells. This difference in results could reflect distinct properties of the labeled peptide or the cells used. Alternatively, many cells contain endogenous PACAP-responsive receptors.

Another study generated and investigated the internalization of an AlexaFluor488-labeled PACAP-27.[23] Time-dependent internalization of PACAP-27 was reported in cells expressing the PAC_{1Hop1} receptor; however, in contrast to the current study with [$Cy5^{21}$]PACAP-27, a more pronounced plateau was observed from 30 min. PACAP-38-mediated receptor internalization has been observed using the PAC_{1Hop1} receptor tagged with green fluorescent protein (GFP).[25] Following receptor activation, GFP was observed to accumulate in endosome-like structures after 3 min, which was comparable to that observed in the current study. PACAP-27-stimulated internalization has also been investigated using a GFP-tagged PAC_{1Hop1} receptor.[27,47] Consistent with the internalization profile of [$Cy5^{21}$]PACAP-27 in the current study, they reported a disappearance of cell surface fluorescence within 10−20 min following PACAP-27 stimulation and an increase in fluorescence within the cytoplasm (Figure 5A).[27,47] A similar pattern of internalization has been reported for the PAC_{1n} receptor.[30,48,49]

The current study represents the first to explicitly look at the internalization of the PAC_{1s} receptor. The combination of our data using the PAC_{1n} and PAC_{1s} receptors with prior studies suggests that the internalization profiles are similar for different PAC_1 receptor splice variants. However, PACAP-27 appeared to stimulate internalization more rapidly and robustly at the untagged and HA-tagged PAC_{1s} receptor compared to the PAC_{1n} receptor, which may indicate unique temporal differences in responses between variants. Despite PACAP-38 and PACAP-27 being potent agonists of VPAC receptors, no previous studies have investigated PACAP-38-mediated internalization of either the $VPAC_1$ or the $VPAC_2$ receptor. The current study now shows that both forms of PACAP can promote receptor internalization across the PACAP-responsive receptor subtypes.

Compared to the PACAP peptides, internalization of VIP or VIP-stimulated receptor internalization has been more thoroughly investigated. Three previous studies have investigated the internalization of fluorescently labeled VIP peptides.[24,37,38] The internalization and localization of Cy3-VIP within the cytoplasm of Panc1 cells have been reported after 15 min.[38] However, this internalization could not be attributed to a specific receptor subtype as Panc1 cells endogenously express PAC_1, $VPAC_1$, and $VPAC_2$ receptors. Consistent with the profile observed by [$Cy5^{19}$]VIP, a second study using FITC-VIP reported internalization of the peptide from 5 min in cells expressing the $VPAC_1$ receptor.[24] Using antibodies against the $VPAC_1$ and $VPAC_2$ receptors, VIP (100 nM or 1 μM) was reported to stimulate a rapid decrease of cell surface fluorescence and increase in cytosolic fluorescence within 30 min, indicative of receptor internalization.[26,28,29,49] The observation of internalization occurred more rapidly than

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that seen by [Cy5[19]]VIP at the $VPAC_1$ or $VPAC_2$ receptor in the current study; this could be due to the much lower concentration of VIP used.[50]

Receptor activation and intracellular signaling are intimately connected to receptor internalization, whereby the presence of different agonists or intracellular signaling proteins can have dramatic effects on receptor internalization.[15−18] The experimental design of the current study involved testing multiple peptide agonists at multiple PACAP-responsive receptor subtypes over several time points. This allowed peptide-mediated internalization to be quantified and directly compared across different receptor subtypes for the first time. Furthermore, this approach allowed a comparison between peptide-mediated cAMP accumulation and internalization to be performed (Figure 7). Differences between the relative pattern of cAMP signaling and internalization may indicate the presence of biased signaling where specific peptides may display preferential activation of cAMP signaling over internalization at individual PACAP-responsive receptors. However, the relative potency of PACAP-responsive receptor internalization appears to be consistently lower than cAMP accumulation, which may indicate that differential coupling between these processes may be a confounding factor. It is therefore possible that differences in receptor coupling, downstream signaling events, or sensitivity between our ability to detect cAMP and receptor internalization may contribute to the overall pattern. We previously observed greater stimulation of G_s (cAMP accumulation) compared to G_q (IP_1 accumulation), ERK1/2 phosphorylation, and Akt phosphorylation at the PACAP-responsive receptors.[10] The data suggested that these receptors may couple more effectively to G_s than G_q and other downstream signaling events. PACAP-responsive receptor internalization may therefore correlate more closely to other pathways than cAMP accumulation. This possibility is particularly intriguing as PACAP-mediated phosphorylation of ERK1/2 and Akt is reported to require internalized PAC_1 in the endosomal compartment.[27,48] Further exploration of G_q and other downstream signaling events may therefore prove valuable in understanding receptor internalization.

Consistent with the cAMP signaling profile of VIP at the PAC_{1n} receptor, the degree of [Cy5[19]]VIP internalization was significantly less when compared to the Cy5-labeled PACAP-38 and PACAP-27 (Figure 7, Figure S6). Interestingly, although [Cy5[19]]VIP produced a potent cAMP response at the PAC_{1s} receptor, it exhibited less internalization than expected, which could indicate potential biased signaling toward cAMP accumulation over internalization (Figure 7, Figure S6). Alternatively, the relatively low VIP concentration used (10 nM) may have influenced the amount of detectable signal as this concentration does not produce a maximal cAMP response at the PAC_{1s} receptor, and [Cy5[19]]VIP is slightly less potent than unlabeled VIP (Figure 2). However, a study investigating VIP-stimulated internalization of the PAC_1 receptor reported that PAC_1 remained at the cell surface, even when higher concentrations of VIP were used.[49] This along with the lack of observed [Cy5[19]]VIP internalization in the current study at the PAC_1 receptors may suggest VIP is not as readily internalized at this receptor subtype compared to the PACAP peptides. Furthermore, the lack of potent VIP internalization at the PAC_{1s} receptor indicates this receptor variant may exhibit unique agonist profiles for previously unexplored signaling pathways. The relative difference between cAMP and internalization at the PAC_{1s} receptor we observed was greater for VIP than either PACAP-38 or PACAP-27; this suggests that functional selectivity or biased signaling may be involved where VIP is biased away from internalization relative to PACAP-38. However, in a prior study using the Cos7 cell model, although VIP-stimulated IP_1 accumulation displayed a trend toward bias, significant biased signaling was not observed relative to PACAP-38 at the PAC_{1s} receptor.[10] Interestingly, relative to PACAP-38, the VIP-mediated IP_1 response at the PAC_{1s} receptor displayed the most divergence from reported binding affinities and cAMP accumulation responses.[8,10] Formal quantification of this potential bias requires further investigation, including direct comparisons between receptor internalization and additional signaling molecules. Similarly, at the $VPAC_1$ and $VPAC_2$ receptors, [Cy5[19]]VIP yielded the most potent stimulator of cAMP accumulation. However, when internalization was measured, [Cy5[21]]PACAP-38 appeared to exhibit the greatest response (Figure 7, Figure S6). This difference could represent biased signaling of [Cy5[19]]VIP for cAMP accumulation over internalization when compared to [Cy5[21]]PACAP-38. However, formal investigation and quantification of biased signaling is required to confirm this observation.

CONCLUSIONS

To effectively target the PACAP peptide system in neurological conditions, including neurodegenerative and pain-related disorders, a deeper understanding of how these receptors behave is required. Internalized peptides, or their receptors, could present a novel target for therapeutic development, but we currently lack sufficient, well-characterized tools to explore this process. This study reports the synthesis of novel, functional, and characterized Cy5-labeled PACAP and VIP peptide analogues that can be used as tools to examine internalization. The ability of PACAP-38, PACAP-27, and VIP to internalize demonstrates they may all play an important role in mediating the endosomal signaling of PACAP-responsive receptors, including those involved in pain behaviors. Furthermore, this is the first study to consider internalization at the PAC_{1s} receptor splice variant and identify that biased agonism across the PACAP peptide family may involve internalization. This could have significant implications for understanding the biology of these receptors and drug development. The Cy5-labeled analogues developed herein provide insight into the role internalization plays across the PACAP peptide family. These novel tools represent a new resource that could be used to further study internalization and investigate peptide binding sites in tissues important in pain and other physiological functions.

METHODS

General Peptide Synthesis. Native PACAP-38, PACAP-27, and VIP were synthesized in house by a N^{α}-9-fluorenylmethoxycarbonyl (Fmoc) solid-phase synthesis strategy as previously described (Supporting Information).[10] Two analogues of Cy5-labeled PACAP-38 were generated: one labeled at amino acid 21 and the second at amino acid 34. PACAP-27 was labeled at amino acid 21 and VIP at amino acid 19. To facilitate Cy5 labeling, an azido-lysine was substituted into the sequence at these positions in place of the native amino acid. 1,3-Dipolar cycloaddition of an in-house Cy5 alkyne fluorophore was then performed and purified, and fractions were collected and analyzed by ESI-MS and RP-

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RPLC. Fractions with correct m/z were combined and lyophilized to afford the desired product with a purity of >98%. Full details of the Cy5-labeled peptide synthesis can be found in the Supporting Information. All peptides were assumed to have 80% peptide content and were made up as 1 mM stock solutions in sterile water under reduced light and stored at −30 °C.

Cell Culture and Transfection. Cos7 cells were selected for this study as they do not respond endogenously to PACAP and were cultured as previously described.[51] Cos7 cells were plated at 20 000 cells per well into 96-well SpectraPlates (PerkinElmer, MA, USA) for measurement of cAMP and ELISA and into 96-well CellCarrier Ultra plates (PerkinElmer) at a density of 15 000 cells per well for internalization studies. Cos7 cells were transiently transfected using polyethylenimine with 0.25 μg per well of either vector plasmid (pcDNA3.1) or receptor containing plasmid as previously described.[51] Experiments were performed 48 h after transfection. The human PAC_{1n}, $VPAC_1$, and $VPAC_2$ receptors in the pcDNA3.1 vector were purchased from the cDNA Resource Centre (Bloomsburg University, PA, USA). The human PAC_{1s} receptor was originally obtained in the pCMV6-XL6 vector (Origene, MD, USA) and subsequently cloned into pcDNA3.1 as previously described.[10] Empty pcDNA3.1 was used as the vector control.

cAMP Measurement. Peptide-stimulated cAMP accumulation was measured as previously described.[52] Briefly, Cy5-labeled and unlabeled peptides were serially diluted in cAMP assay media (DMEM containing 0.1% (w/v) BSA and 1 mM 3-isobutyl-1-methylxanthine) to give a final concentration range from 1 pM to 1 μM. Cos7 cells transiently transfected with receptor were incubated with peptide, media alone, or the positive control forskolin for 15 min at 37 °C. The reaction was stopped by aspiration of the media and addition of 50 μL of ice-cold absolute ethanol. The ethanol was then evaporated from the wells and cAMP reconstituted in 50 μL of cAMP detection buffer (PerkinElmer). cAMP was then quantified using the LANCE cAMP detection kit (PerkinElmer) as per the manufacturer's instructions.

Fluorescent Imaging. Cells were serum starved in DMEM containing 0.1% (w/v) BSA for 30 min at 37 °C. The cells were then incubated at 37 °C with 10 nM fluorescent peptide for 0−120 min. Following this, the media was aspirated, and cells were fixed with 8% paraformaldehyde (PFA). Cells were then washed in PBS and incubated with Cell Mask Green (1:1000; Life Technologies, CA, USA) and DAPI for 30 min at 37 °C. Cells were washed a further two times with PBS and imaged on an Operetta High-content Imager (PerkinElmer) using a 20× nonconfocal 0.75 high NA lens.

Measurement of HA-PAC_1 Receptor Cell Surface Expression by ELISA. Cos7 cells were transfected with HA-tagged $hPAC_{1n}$ and $hPAC_{1s}$ receptors and assayed for receptor cell surface expression as described previously with minor modifications.[51,53] Briefly, transfected cells were stimulated with 100 nM unlabeled PACAP-38 or PACAP-27 for 0−120 min and fixed using 4% PFA. Cells were washed with PBS and incubated with 0.6% hydrogen peroxide for 20 min. Cells were blocked with 10% goat serum/PBS for 1 h, followed by a 30 min incubation with anti-HA primary antibody (1:1000, Biolegend, CA, USA). Cells were washed in PBS, incubated with antimouse horseradish peroxidase (HRP) secondary antibody (1:500, GE Healthcare, IL, USA) for 1 h, and washed again in PBS. Sigma FAST OPD was added to each well and incubated in the dark for 15 min. Sulfuric acid was added to stop the reaction, and the absorbance was measured.

Image Analysis. Spot counting was performed using the Columbus software package (Figure S7). Cells were identified by nuclear staining of DAPI and CellMask Green staining of the cell cytoplasm. The cytoplasm region was set at 90% of the cell volume to ensure that fluorescent peptide at the cell surface was not counted.[34] Fluorescent peptide spots were quantified within the cytoplasm region of the cells in each field of view. Each condition was performed in duplicate. Data are the average of 8 fields of view for each well outputted as the number of spots per cell. The area under the curve was calculated and plotted as a bar graph.

For presentation, the raw TIFF files were acquired using the Harmony software on the Operetta and merged, pseudocolored, and adjusted for brightness and contrast, and scale bars were added in ImageJ. Image adjustments were applied uniformly for all time points related to a peptide at a particular receptor. The brightness and contrast of vector-transfected cells were matched to each time point from [$Cy5^{21}$]PACAP-38, [$Cy5^{34}$]PACAP-38, and [$Cy5^{21}$]PACAP-27 at the PAC_{1n} receptor or [$Cy5^{19}$]VIP at the $VPAC_1$ receptor.

Data and Statistical Analysis. All graphing, curve fitting, and statistical analysis were performed using GraphPad Prism 7.0. Statistical significance was defined as $p < 0.05$.

cAMP Measurement. Data points are the mean ± SEM from 5 independent experiments performed with 2−3 technical replicates (wells). Data from individual experiments were initially fitted with a four-parameter logistic equation. F tests were then used to determine if the Hill slope was significantly different from 1. In the majority of experiments, the Hill slope was not significantly different from 1, and the curves were then refitted to a three-parameter logistic equation to obtain the peptides pEC_{50}. The mean pEC_{50} from independent experiments was calculated for each peptide, and significant differences were determined using an unpaired two-tailed t-test (PACAP-27 and VIP) or one-way ANOVA with Dunnett's test (PACAP-38). Significant differences in peptide E_{max} were determined from the non-normalized peptide values by a ratio-paired two-tailed t-test (PACAP-27 and VIP) or log transformed followed by a repeated measures one-way ANOVA with Dunnett's test (PACAP-38). For presentation purposes, the concentration−response data from individual experiments were normalized to the curve-fitted maximum (E_{max}) and minimum (E_{min}) cAMP response produced by the matched unlabeled peptide and expressed as a percentage.

ELISA Assays. Data points are the mean ± SEM from 4 or 5 (PACAP-27 at HA-PAC_{1n}) independent experiments performed with 2 technical replicates (wells). In each independent experiment, values were normalized to vector-transfected cells (0%) and the receptor expression in unstimulated cells at 0 min (100%).

The mean normalized receptor surface expression was then combined from individual experiments. Significant differences to vehicle-treated wells at each time point were determined by repeated measures two-way ANOVA with post hoc Bonferroni's test. The area under the curve of PACAP-stimulated receptors was calculated and plotted as a bar graph. Significant differences to vehicle-treated wells were determined using a one-way ANOVA with post hoc Dunnett's test.

Internalization Spot Count Analysis. For spot counting, significant differences for each peptide were determined using

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a two-way ANOVA with post hoc Tukey's test comparing all receptors and vector-transfected cells to each other at each time point. Significant differences in the spot count area under the curve were determined by one-way ANOVA with post hoc Tukey's test.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsptsci.2c00124.

Additional biology methodology (spot count analysis), colocalization of Cy5 peptides with a cytoplasm marker (CellMask green), ELISA results with PACAP-stimulated HA-PAC_1 receptors, table of peptide potencies and degree of internalization, and detailed chemistry methods describing the synthesis of fluorescent peptides (PDF)

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Author Contributions

This study was conceived and designed by D.L.H. and C.S.W. A.S., M.A.B., and P.W.R.H. performed peptide synthesis. T.I.A., Z.T., and T.A.R. performed peptide characterization and internalization experiments. The manuscript was written through contributions from T.I.A., Z.T., A.S., M.A.B., P.W.R.H., D.L.H., and C.S.W.

Notes

The authors declare the following competing financial interest(s): D.L.H. is or has been a consultant or speaker for Lilly, Amgen, Teva, Intarcia, and Merck Sharp and Dohme and has received research funding from Living Cell Technologies and AbbVie in the past 3 years. C.S.W. has received research support from Living Cell Technologies and AbbVie.

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■ ABBREVIATIONS

PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; cAMP, cyclic adenosine monophosphate.

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