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Characterization of the pharmacology, signal transduction and internalization of the fluorescent PACAP ligand, fluor-PACAP, on NIH/3T3 cells expressing PAC1

Patrizia M. Germano, James Stalter, Sang V. Le, Mark Wu, Dean J. Yamaguchi, David Scott, Joseph R. Pisegna*

CURE: VA/UCLA Digestive Diseases Research Center, VA Greater Los Angeles Health Care System and Dept of Medicine, the University of California, Los Angeles, Los Angeles, CA 90073, USA

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Abstract

Fluor-PACAP, a fluorescent derivative of PACAP-27, has been confirmed to share a high affinity for PAC1 receptors transfected into NIH/3T3 cells and to have comparable pharmacological characteristics to the unconjugated, native form. Through competitive binding with ^{125}I-PACAP-27, the two ligands exhibited similar dose- dependent inhibition. Additional examination of the efficacy of activating adenylyl cyclase revealed that both ligands analogously stimulated the production of cyclic AMP. Furthermore, PAC1 internalization visualized by our Fluor-PACAP, is compareable to that performed with the radioligand, ^{125}I-PACAP-27, with maximal internalization achieved within thirty minutes. Thus, Fluor-PACAP exhibits intracellular signaling abilities homologous to the native ligand.

Keywords: PACAP; Confocal microscopy; Fluo-PACAP; PAC1; NIH; 3T3 cells; Pharmacology

1. Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP), isolated in 1989 from ovine hypothalamus, occurs as two carboxyl-amidated peptides, PACAP-38 (P-38) and PACAP-27 (P-27), sharing the same N-terminal 27 amino acids [1,8]. These two are the most recently described biologically active peptides comprised in the Vasoactive Intestinal Peptide (VIP)/secretin/glucagon family [1,8]. PACAP occurs at the highest concentration in the central nervous system, and can be found at moderate concentration in testis and adrenal medulla as well as at lower concentrations, in the ovary, lungs, gastrointestinal tract, and pancreas [1,14]. Specific binding sites for PACAP have been identified in the human hypothalamus, brain stem, cerebellum, and cortex. Although their functional significance is unknown [14], they might be involved in regulating the release of adrenocorticotropin, growth hormone, prolactin and luteinizing hormone. Recently PACAP has been identified in the rat gastric neuroenteric plexus, where it has been described to stimulate ECL growth and the release of histamine in response to central neural stimulation and hence plays an important role in the regulation of gastric acid secretion [10,15].

The gonadotroph-derived αT3–1 cell line has also been found to be PACAP-responsive [14]. In the rat epididymas PACAP may play a regulatory role in epididymal and sperm functions [5] and has been localized in human penile erectile tissue [4].

The cloning of the high affinity receptor for PACAP (PAC1) receptor, specific for PACAP-27 and PACAP-38, has identified this receptor as a member of the seven transmembrane G protein-coupled receptors, which is, in turn, highly homologous to the receptors for VIP and secretin [11,12]. Furthermore, the PAC1 receptor (previously PACAP type I receptor, or PVR1) has been re-classified as PAC1 in the current IUPHAR nomenclature (Table 1). The pharmacological characterization of receptors within this superfamily has demonstrated that PACAP hormone binds with a high affinity to PAC1 and with a lower affinity to the

* Corresponding author. Tel.: +1-310-478-3711 ext 41940; fax: +1-310-268-4096.

E-mail address: Jpisegna@ucla.edu (J.R. Pisegna).

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classic VIP_1 and VIP_2 receptors (VPAC1 and VPAC2, respectively) [3]. Furthermore, PAC1 has been shown to be coupled with both adenylyl cyclase and inositol phosphate turnover [2]. The widespread localization, function, and dual signal transduction pathways of PACAP and PAC1 represents an ideal model to perform receptor pharmacological and physiological studies.

In the past, pharmacological and drug screening assays were typically performed using ligands radiolabeled with isotopes ^{125}I or ^{3}H; however, the use of fluorescent peptides has recently been proposed as an alternative, environmentally safe and convenient modality for carrying out such analysis. Fluorescent peptides also allow real-time, continuous experiments, such as binding activity and internalization studies, as opposed to radioactive peptides, which can only be measured at established time points. The limiting factor for using fluorescent peptides, nonetheless, is the potential difference in affinity for their respective receptor. Therefore, it is critical to characterize as well as verify that the pharmacological properties of these fluorescent compounds are comparable to those of the native ligands, before they can be preferentially used to perform localization and functional assays.

Accordingly, we describe here the pharmacological comparison between PACAP-27, the native ligand for PAC1, and Fluo-PACAP, a new fluorescent derivative of the native. Fluor-PACAP is a fluorescent analog of PACAP-27, conjugated with Alexa-488 in our laboratory. This study describes for the first time its relative potency and efficacy for radioligand binding displacement, adenylyl cyclase stimulation and internalization in cells stably transfected with PAC1.

2. Methods

Materials- [^{125}I]-PACAP-27 was purchased from Dupont NEN (Boston, MA), [^{3}H]adenine was purchased from Amersham Life Technologies (Arlington Heights, IL), and PACAP-27 was purchased from Bachem (Torrance, CA). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, penicillin G, and streptomycin sulfate were purchased from Gibco-BRL (Gaithersburg, MD). Bovine serum albumin (BSA) was purchased from ICN Biomedicals, Inc. (Aurora, OH). Alexa-488 was purchased from Molecular Probes (Eugene, Oregon).

2.1. Establishment of stably transfected NIH/3T3 cells expressing WT

The pCDL-SRα/Neo plasmid containing the human PAC1 insert was linearized at its Aat II restriction site within the Ampicillin-resistance gene and stably transfected into NIH/3T3 fibroblasts using an electroporator (BTX model T820, San Diego, CA) set at 475 V by 1 msec for four pulses on 2×10^7 cells in 0.25 ml containing 20 μg vector, Wt cDNA, and 500 μg/ml salmon sperm. Geneticin® (250 mg/ml, Gibco, Gaithesburg, MD) was used to select clones. Ten clones were subjected to radioligand binding. The clones, that exhibited the highest efficacy for adenylyl cyclase and radioligand binding inhibition were selected for further study.

2.2. [I^{125}]-PACAP-27 radioligand binding inhibition

These studies were performed as described previously [11,12]. Stably transfected cells were seeded overnight on 24 well plates in Dulbecco's Eagle Medium (DMEM) with 10% fetal bovine serum, at a density of 60,000 cells per well. The following day, the cells were washed once with 1% BSA/PBS at 4°C and incubated in DMEM containing 1%BSA for 60 min at 37°C with 50pM of [^{125}I]-PACAP-27 (2200 Ci/mmol) (DuPont, NEN, Boston, MA) with either the indicated concentrations of unlabeled peptides, PACAP-27 (Bachem, California) or Fluor-PACAP. The density of cell surface receptors expressed was determined by measuring the amount of saturable binding (total binding in the presence of [^{125}I]-PACAP-27 alone minus nonspecific binding in the presence of 1 μM PACAP-27). Nonspecific binding was defined as total binding in the presence of 1 μM unlabeled PACAP. The reaction was terminated by washing the cells twice with 1% BSA/PBS at 4°C. The cells were then solubilized with 1 ml of 0.1 N NaOH and radioactivity was detected with a gamma counter. At least three experiments were performed in triplicate.

2.3. Adenylyl cyclase assay

These experiments were performed as described previously [11,12]. Stably transfected NIH/3T3 cells were seeded on 24 well plates in DMEM/10% fetal bovine serum in the presence of [^{3}H]adenine (Amersham, Arlington Heights, IL) at a concentration of 2 mCi/ml. The cells were washed with DMEM, incubated with the indicated concentrations of PACAP-27 or Fluor-PACAP-27 in DMEM containing 1 mg/ml BSA and 2.5 mM 3-isobutyl, 1–1 methyl-xanthine (IBMX) for 30 min and then aspirated. One hundred microliters of 2% SDS, 1 mM cAMP solution was used to lyse the cells. Cyclic AMP was assessed with consecutive Dowex AG-50W-X4 resin (BioRad, Richmond, CA) and aluminum oxide (Sigma, St. Louis, MO) column chromatography, according to a modification of the procedure described by Salomon, et al. [13]. Elutes were collected in vials and ^{3}H-cAMP was counted using a Beckman Liquid Scintillation Counter (Fullerton, CA). At least three experiments were performed in triplicate.

2.4. Immunocytochemical detection of PAC1 expression on NIH/3T3 cells using anti-HA antibody or Fluor-PACAP

Cells were plated overnight at a low density on polylysine-treated coverslips. The following day, the cells were

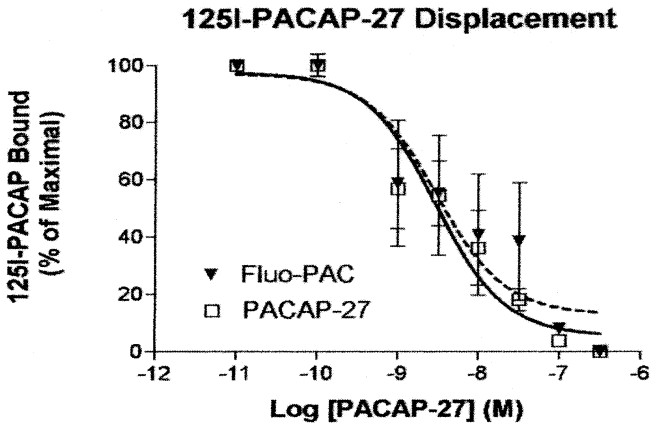

Fig. 1. Radioligand binding inhibition of ^{125}I-PACAP-27. Dose-dependent inhibition of ^{125}I-PACAP-27 binding on NIH 3T3 cells expressing PAC1 was assessed using increasing concentrations of Fluo-PACAP or PACAP-27. The results represent the averages of 3 experiments performed in triplicate.

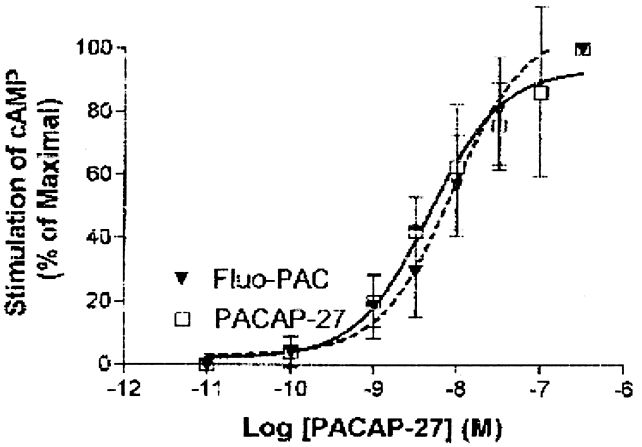

Fig. 2. Dose-dependent stimulation of cAMP with increasing concentrations of PACAP-27 or Fluor-PACAP. The results represent the averages of 3 experiments performed in triplicate.

washed once with Phosphate-buffered saline (PBS) and incubated with Fluor-PACAP for 1 h at 4°C or with a monoclonal anti-HA antibody (Boehringer Mannheim, USA) for 2 h at 37°C, followed by a biotinylated anti-mouse IgG (H+L), used as a secondary antibody, and fluorescein-conjugated avidin for 1 h at room temperature. Immunocytochemical results were visualized using fluorescence microscopy and photomicrographs were made.

2.5. Radioligand stripping studies

These studies were performed as described previously [2]. Transfected NIH/3T3 cells were washed twice with PBS as described above and incubated with 50 pM ^{125}I-PACAP at 37°C for various time intervals between 1 and 90 min. At the indicated times, cells were washed twice with 1% BSA/PBS, and treated with 0.5 M KSCN at room temperature for eight minutes. Following solubilization of the cells with 0.1 N NaOH, radioactivity was measured as described above for the radioligand binding. Parallel incubations were performed in the presence of 1 μM unlabelled PACAP-27 to determine nonsaturable binding for each period. Internalized receptor is described as ^{125}I-PACAP-27 that could not be stripped by KSCN and is expressed as a percent of the total bound ^{125}I-PACAP-27 in control cells processed without KSCN stripping of the ligand from the receptor. At least three experiments were performed in triplicate.

2.6. PACAP-27 conjugation with Alexa-488 and internalization studies

Using the Alexa Fluor™ 488 Protein Labeling Kit from Molecular Probes (Eugene, Oregon USA), a solution of 1 mg PACAP-27, 500 μl PBS, and 50 μl of 1M sodium bicarbonate (pH 8.3) was prepared on ice. The protein solution was then added to a vial containing the active dye and mixed with a magnetic stirrer 4 h at 4°C. BSA1% was used to quench the reaction, neutralizing the unbound dye. 2 μl of Fluor-PACAP per ml of cell culture were used to label the cells on ice in the dark. The cells were then washed with PBS before internalization studies were performed. Dyed cells were visualized via the FITC channel of a Zeiss confocal microscope.

3. Results

3.1. [I^{125}]-PACAP-27 binding inhibition

Competitive radioligand binding was performed via specific binding of ^{125}I-PACAP-27 at 37°C for 60 min in the presence of either Fluor-PACAP or PACAP-27 at the indicated concentrations. The displacement curves were nearly identical for both Fluor-PACAP and PACAP-27 in NIH/3T3 cells stably expressing the PAC1 receptor (Fig. 1). For both ligands dose dependant inhibition is noted at 50 pM and the half-maximal inhibition (IC_{50}) at $\approx$ 5 nM (Fig. 1). Previous studies have shown that binding of PACAP-27 and PACAP-38 are identical and therefore only PACAP-27 was used in these studies [11,12].

3.2. Adenylyl cyclase assay

To determine the efficacy and potency of activating adenylyl cyclase, cAMP was measured in response to increasing concentrations of either Fluor-PACAP or PACAP-27 (Fig. 2). Cyclic AMP formation was measured following a one-half stimulation with either Fluor-PACAP or PACAP-27 at the indicated concentrations. As shown in Fig. 2, there is a similar dose dependent stimulation of cAMP with increasing concentration of either peptide. Detectable stimulation occurred at 0.1 nM while half-maximal (IC_{50}) stimulation occurred at $\approx$ 5 nM for both peptides

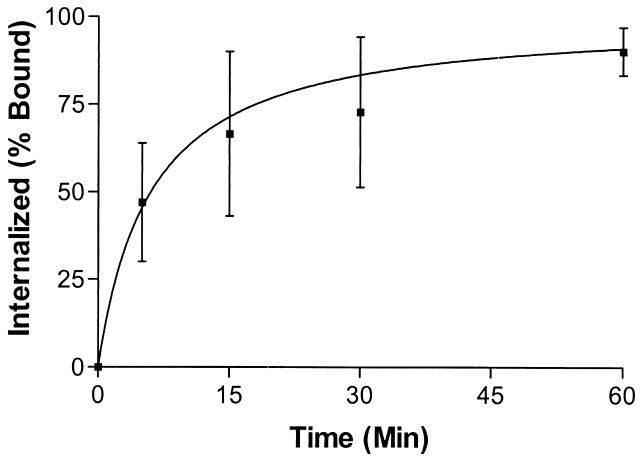

Fig. 3. Internalization of ^{125}I-PACAP-27. Radioligand internalization of NIH/3T3 cells transfected with wild type PAC1 cDNA. Data presented reflect the percentage of receptor internalized following acid washing and represent the average of three experiments performed in triplicate.

(Fig. 2). AC stimulation for both PACAP-27 and Fluor-PACAP were readily superimposable, so statistical analysis was not necessary.

3.3. Radioligand stripping studies

^{125}I-PACAP-27 internalization of PAC1 expressed in NIH/3T3 cells was determined for the Hop splice variant (Fig. 3). Radioligand internalization was rapid with the majority of receptors internalized within the first 5 min. Internalization was maximal at 60 min and reached a plateau after this time period. The half-maximal response as defined as the time period by which 50% of receptors are internalized was 3 min.

3.4. Confocal microscopy

Confocal microscopy was used to visualize PAC1 receptors staining of stably transfected cells by Fluor-PACAP, or anti-HA antibody. Staining of the cells was evident on the cell surface. Internalization of the Fluor-PACAP occurred following warming of the cells to 37°C (Fig. 4). These experiments visually confirmed both the binding and internalization ability of Fluor-PACAP, letting us visualize the internalized receptors. Internalization detected after 1 min. and was completed by 30 min (Fig. 4). The half-maximal internalization occurred at 3–4 min using quantification of the confocal images.

4. Discussion

Given the molecular structure of the PAC1 receptor via the recent cloning of the rat and human PAC1 receptor, we are now able to further define its unique structure, localization and pharmacology with the intent to increase our knowledge of its potential biological actions. Previously, we have demonstrated that PAC1 has a high affinity for only PACAP-27 and PACAP-38 [3]. The recombinant rat and human PAC1 receptors, expressed on NIH/3T3 cell lines, were shown to have half-maximal stimulation of cAMP within 1 nM (IC_{50} ~1 nM), which was much higher in potency compared to VIP (IC_{50} 1000nM) by radioligand binding studies [11]. Like the native ligand, Fluor-PACAP also demonstrates similar high specific binding to the PAC1 receptor.

In addition, internalization of PAC1 following radioligand stimulation was compared to that visualized via confocal microscopy using our fluorescent PACAP derivative. By conventional radioligand techniques, radioligand internalization was rapid with the majority of internalization occurring by 5 min. These results are similar to those obtained using confocal microscopy. Following warming of the perfusing buffer to 37°C, internalization appeared completed by 30 min. Receptors internalized in a perinuclear distribution, possibly influenced by the carboxyl terminus of PAC1 which recently has been shown to aid internalization [9]. The present study indicates that $hPAC_1$ receptor internalization for the Hop splice variant occurs with a $t_{1/2}$ (half-maximal internalization time) of about 5 min. Although the issue of receptor recycling is not addressed, the evidence reveals that Fluor-PACAP could be used to assess the intracellular compartmentalization of internalized receptor, permitting further study of this topic.

Therefore, the data suggests that the addition of the fluorescent dye to PACAP-27 does not alter the peptide's ability to bind to the receptor. These results are in close agreement with the observation by Scatchard analysis from membrane preparations using PACAP-27 and PACAP-38 for the cloned PAC1.

Based on the results of cloning the rat and human PAC1, it was shown that both PACAP-27 or PACAP-38 have identical potencies and efficacies for activating signaling pathways. Therefore, in our current study, only PACAP-27 was studied. Similarly, because it has been well established that PACAP is coupled to the activation of both adenylyl cyclase as well as phospholipase C, only one of these signaling pathways was studied. In previous studies, both PACAP-38 and PACAP-27 were shown to increase cAMP in a dose-dependant manner with a potency of 1 nM. The activation of cAMP by either PACAP-27 or PACAP-38 is 5–10 fold greater than the half-maximal inhibition of binding due to "receptor spareness" [12]. In agreement with our previous observations obtained by using the native peptides, the current study comfirms that "receptor spareness" also occurs with Fluor-PACAP [11]. Although the current study did not specifically investigate the effects of Fluor-PACAP on inositol phosphate turnover, we expect that the results would be similar to those reported with native peptides. Differences in receptor surface expression may have important implications for the efficacy of signaling through the phospholipase C signaling pathway [11]. Through the use of

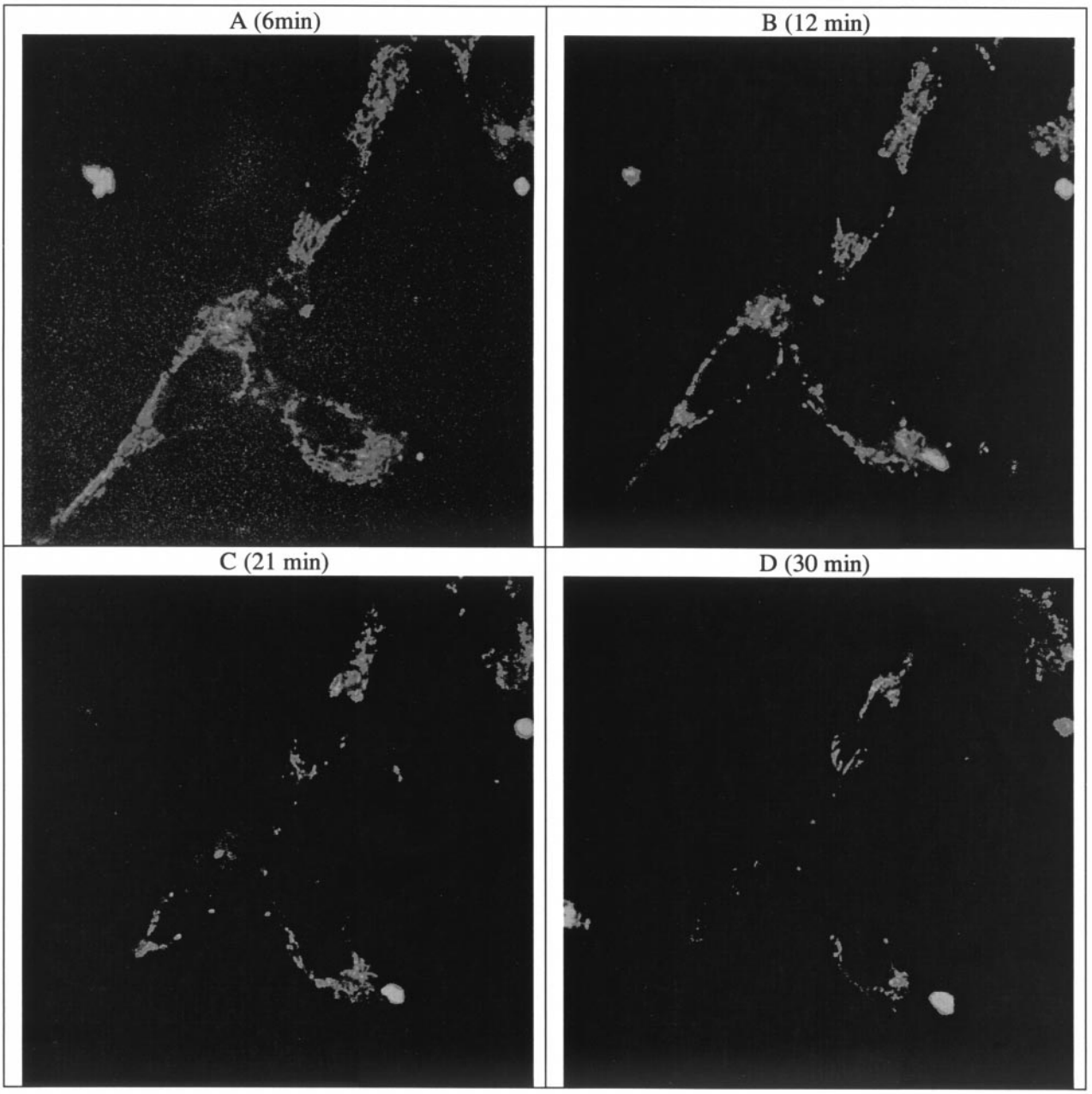

Fig. 4. Internalization of PACAP27 conjugated with Alexa-488 in NIH/3T3 cells transfected with the wild type PAC1 receptor cDNA. Internalization was assessed with confocal microscopy, magnification 100×.

Fluor-PACAP, quantification of receptor expression can be performed using FACS analysis.

NIH/3T3 cells were used in this study because of the preponderance of pharmacological data, which has been accumulated to date in our lab using this cell line. We have shown in preliminary studies that prior to receptor transfection, this cell line does not express native PAC1, VIP1 or VIP2 receptors. Cells were transfected with the Hop splice variant of the human PAC1 using electroporation as described previously [11,12]. Therefore, this cell line expressing PAC1 is a practical model for investigating the pharmacology and ability to activate signaling pathways. As demonstrated in Fig. 1, Fluo-PACAP is shown to have an affinity for PAC1 receptors, which is similar to that of the native ligands, PACAP-27 and PACAP-38, perhaps supporting the hypothesis that this compound is pharmacologically equivalent to the native ligand.

PAC1 receptors are similar to other members of the VIP/secretin/glucagon receptor family. In addition to expanding the pharmacological characterization of these receptors, the cloning of PAC1 demonstrated that this receptor has a long amino acid terminus which is presumably the critical site for ligand recognition, although the current study did not demonstrate specific sites of amino acid in-

Table 1
Nomenclature for PACAP and related VIP receptors (adapted from reference 12)

Receptor type		Selective agonists	Selective antagonists	Fluorescent agonists	
IUPHAR nomenclature	Previous nomenclature				Selective antagonist
$PACAP_1$	PACAP Type I PVR1	maxadilan	PACAP 6-38	Fluo-PACAP	PACAP(6-38)†
VIP_1/PACAP	VIP VIP_1 PACAP Type II PVR2	[Arg^{16}]chicken secretin* [$K^{15}R^{16}L^{27}$]VIP(1-7)GRF(8-27)-NH_2	Ro-	Fluo-VIP	[Ac-His^1, D-Phe^2, Lys^{15}, Arg^{16}] VIP(3-7)GRF(8-27)-NH_2
VIP_2/PACAP	VIP_2 PACAP-3 PVR3	Helodermin Ro 25-1553 Ro 25-1392			

teraction [11,12]. The PAC1 receptor possesses three potential glycosylation sites in the amino terminus similar to the $VPAC_1$, $VPAC_2$, glucagon, GLP-1 and secretin receptors which may be important for receptor processing and targeting of the cell membrane [11]. Mutational analysis of the glycosylation sites and imaging with Fluor-PACAP will permit the elucidation of the importance of these sites. In addition, PAC1 contains five cysteine residues that are highly conserved among PACAP, VIP_1, VIP_2, secretin, GLP-1 and glucagon receptors and are thought to form disulfide bonds necessary for agonist binding conformation as shown for rhodopsin. More recently, we have demonstrated that two amino acids of the carboxyl terminus of PAC1 are required for signal transduction and to a lesser degree, internalization [7].

In conclusion, since PACAP has been shown to stimulate the growth of lung cancer as well as breast cancer cells via PAC1 [6,17], the use of a specific fluorescent PACAP agonist may provide significant insight into the expression pattern and enable the characterization of receptor internalization in tumor cells. This research has revealed that the alternative ligand Fluor-PACAP has comparable affinity for the PAC1 receptor when compared to the native ligand. It also indicates that Fluor-PACAP effectively couples to intracellular signaling molecules. In addition, internalization of PAC1 has been demonstrated to occur rapidly as characterized by both radioligand and Fluor-PACAP binding. Thus, these studies have determined that Fluor-PACAP is an effective ligand that can also be used to perform internalization studies.

Table 2
Relative affinities of the three PACAP and VIP receptors for the ligands PACAP-27, PACAP-38, VIP, and helodermin (adapted from reference 12).

Receptor type	
IUPHAR nomenclature	Relative affinities
$PACAP_1$	PACAP27 = PACAP38 ≫ VIP > Helodermin
VIP_1/PACAP	PACAP27 = PACAP38 = VIP ≫ Helodermin
VIP_2/PACAP	Helodermin > PACAP27 = PACAP38 = VIP

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