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The G protein-coupled receptor PAC1 regulates transactivation of the receptor tyrosine kinase HER3

Terry W. Moody[a], **Lingaku Lee**[b], **Robert T. Jensen**[b]

[a]Department of Health and Human Services, National Institutes of Health, National Cancer Institute, Center for Cancer Training, Bethesda, MD 20892

[b]National Institute of Diabetes, Digestive and Kidney Disease, Digestive Diseases Branch, Bethesda, MD 20892 U.S.A.

Abstract

Peptide G protein-coupled receptors (GPCR) for pituitary adenylate cyclase activating polypeptide (PACAP) regulate the growth of non-small cell lung cancer (NSCLC) cells. PACAP binds with high affinity to PAC1, which causes transactivation of receptor tyrosine kinases (RTK) for the EGFR and HER2 but its effect on HER3 is unknown. Using 3 NSCLC cell lines (NCI-H358, NCI-H441 and Calu-3), proteins for EGFR, HER2, HER3 and PAC1 were detected. Addition of PACAP-27 to Calu-3 cells increased EGFR, HER2 and HER3 tyrosine phosphorylation by 420, 240 and 190%, respectively. The increase in EGFR tyrosine phosphorylation caused by PACAP was blocked by the EGFR tyrosine kinase inhibitor (TKI) gefitinib, or PACAP(6–38), a PAC1 antagonist. The increase in HER2 tyrosine phosphorylation caused by PACAP was inhibited by trastuzumab, a monoclonal antibody (mAb) for HER2, or PACAP(6–38). The increase in HER3 tyrosine phosphorylation caused by PACAP was inhibited by HER3 mAb3481 or PACAP(6–38). Immunoprecipitation experiments indicated the PACAP addition to Calu-3 cells resulted in the formation of EGFR/HER3 and HER2/HER3 heterodimers. Addition of the HER3 agonist neuregulin (NRG)-1 increased HER3 tyrosine phosphorylation in NSCLC cells. PACAP or NRG-1 increased the proliferation of NSCLC cells, whereas PACAP(6–38), gefitinib, trastuzumab or mAb3481 inhibited proliferation. The results indicate that PAC1 regulates the proliferation of NSCLC cells as a result of transactivation of the EGFR, HER2 and HER3.

Keywords

PAC1; transactivation; HER3; lung cancer; proliferation

*Correspondence to: Dr. Terry W. Moody, NCI Office of the Director, CCT, 9609 Medical Center Drive, Room 2W-340, Bethesda, MD 20892 USA, 240-276-7785, moodyt@mail.nih.gov.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

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Introduction

Pituitary adenylate cyclase activating polypeptide (PACAP) is a member of the vasoactive intestinal peptide (VIP) family of peptides (Arimura, 1993; Said and Mutt, 1970). PACAP-27 and PACAP-38 are derived from a 176 amino acid preproPACAP (Sherwood et al., 2000). PACAP-27 and VIP have 67% sequence homology (Miyata et al., 1989). PACAP-27 binds with high affinity to the class B/secretin-like G protein-coupled receptors (GPCR) VPAC1, VPAC2 and PAC1. When PACAP binds with high affinity to PAC1 interaction with Gs, results in increased adenylyl cyclase activity elevating the cAMP (Harmar et al., 2012), whereas interaction with Gq causes phosphatidyl inositol (PI) turnover (Vaudry et al., 2009). The resulting inositol-1,4,5-trisphosphate (IP_3) released causes elevation of cytosolic Ca^{2+} whereas the diacylglycerol (DAG) released increases protein kinase (PK)C activity.

Addition of PACAP to lung cancer cells increases the phosphorylation of ERK, FAK, paxillin and the EGFR (Moody et al., 2002, Moody et al, 2012 a,b). When ERK is phosphorylated increased nuclear oncogene expression occurs. PACAP-27 increased the expression of c-fos which forms heterodimers with c-jun and alters the expression of growth factor genes (Draoui et al., 1996). PACAP increases the proliferation of brain cancer, colon cancer, lung cancer, neuroendocrine tumors and pancreatic cancer (Le et al., 2002; Buscail et al., 1992, Germano PM et al., 2009; Nakamachi et al., 2014; Moody et al., 2016a). PACAP(6–38), which is a PAC1 antagonist, decreased the proliferation of breast, lung and prostate cancer (Zia et al., 1995; Leyton et al., 1999; Moody et al., 2018). PAC1 may play an important role in the regulation of cancer cellular proliferation.

PAC1 regulates the transactivation of receptor tyrosine kinases (RTK) in NSCLC cells. Addition of PACAP to NSCLC cells increases the tyrosine phosphorylation of the EGFR (Moody et al., 2012b). When the EGFR is mutated in NSCLC patients, they respond to gefitinib (Lynch et al., 2004; Paez et al., 2004). Addition of PACAP to NSCLC cells, increases the tyrosine phosphorylation of HER2 (Moody et al., 2019). When HER2 is amplified, cancer patients respond to trastuzumab (Mitri et al., 2017). PACAP induced the formation of EGFR/HER2 heterodimers, which activate the MEK/ERK pathway (Moody et al., 2019). Here the ability of PACAP to tyrosine phosphorylate HER3 was investigated using NSCLC cells.

2. Materials and Methods

2.1 Cell Culture.

NCI-H358, NCI-H441 and Calu-3 cell (American Type Culture Collection, Rockville, MD) lines were adherent and were split 1:10 weekly with trypsin/EDTA after washing in PBS. The cells were cultured in a T175 flask containing Roswell Park Memorial Institute (RPMI)-1640 medium with 10% fetal bovine serum (FBS; Invitrogen, Grand Island, NY). The cells were used when they were in exponential growth phase after incubation at 37°C in 5%CO_2/95% air.

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2.2 Receptor binding

Calu-3 cells were cultured in 24 well plates. When confluent, the cells were washed 3 times in SIT medium (RPMI-1640 with 10 μg/ml apotransferrin, 3×10^{-8} M Se_2O_3 and 5 μg/ml insulin (Sigma-Aldrich, St. Louis, MO)). The cells were incubated in SIT medium containing 2 mg/ml bovine serum albumin and 0.2 mg/ml bacitracin (receptor binding buffer; Sigma-Aldrich, St. Louis, MO). Various concentrations of inhibitor were added along with 100,000 cpm of ^{125}I-PACAP-27 (2200 Ci/mmol). After 30 min at 37°C, the plates were rinsed 3 times with receptor binding buffer at 4°C to remove free ^{125}I-PACAP-27. The radiolabeled ^{125}I-PACAP-27 bound to the cells was dissolved in 0.2 N NaOH and the samples counted in a LKB gamma counter.

2.3 Western Blot.

The ability of PACAP-27 (Bachem Inc., Torrence, CA) or NRG-1 (R&D Systems, Minneapolis, MN) to stimulate phosphorylation of the HER3, EGFR, HER2, ALK or ERK (p42/p44 MAP kinase) was investigated by Western blot. NCI-H358 or Calu-3 cells were placed in 10 cm dishes. After the cells were confluent, they were placed in SIT medium for 3 hr. Routinely, NSCLC cells were treated with mAb3481 (R&D Systems, Minneapolis, MN), gefitinib, PACAP(6–38), DPI, N-acetyl cysteine or Tiron (Sigma-Aldrich, St. Louis, MO) for 30 minutes. Then cells were incubated with 1 μM PACAP-27 or 0.1 μg/ml NRG-1 for 2–30 min, washed twice with PBS and treated with 0.5 ml of lysis buffer. The lysate was sonicated for 5 s at 4°C and centrifuged at 10,000 × g for 15 min. Protein concentration was determined using the BCA reagent (Pierce Chemical Co., Rockford, IL), and 600 μg of protein was incubated with 4 μl of anti-phosphotyrosine (BD Biosciences), 1 μl of anti-EGFR or 1 μl of anti-HER2 (Cell signaling technologies, Danvers, MA) and 15 μl of immobilized protein A/G PLUS agarose (Santa Cruz Biotech, Santa Cruz, CA) overnight at 4°C. The immunoprecipitates were washed 3 times with RIPA buffer and fractionated using 4–20% polyacrylamide gels (Novex, San Diego, CA). Proteins were transferred to nitrocellulose membranes and after washing the blot, it was incubated with Super Signal Dura West enhanced chemiluminescence detection reagent (Thermo-Fischer Scientific, Rockford, IL) for 5 min and exposed to Biomax XAR film (Carestream, Rochester, NY). The band intensity was determined using a densitometer.

Alternatively, 40 μg of cellular extract was loaded onto a 15 well 4–20% polyacrylamide gels. After transfer to nitrocellulose, the blot was probed with anti PY^{1289}-HER3, PY^{1068}-EGFR, PY^{1248}-HER2, HER3, EGFR, HER2, $PT^{202}PY^{204}$ERK, ERK, PT^{308}-AKT, AKT or tubulin (Cell Signaling Technologies, Danvers, MA). In most experiments the Abs were diluted 1:2000, however, in the immunoprecipitation experiments the Abs were diluted 1:500 to obtain a stronger signal.

2.4 Cytosolic Ca^{2+}

NCI-H358 or Calu-3 cells were detached from the flask and 2×10^6 cells/ml incubated with 5 μM FURA-2 AM (Sigma-Aldrich, St. Louis, MO) at 37°C. After 30 min, the cells were centrifuged and resuspended in SIT medium. The cells were treated with 0.1 μM PACAP-27, 0.1 μM VIP, 5 μM PACAP(6–38) or 5 μg/ml ionomycin. Fluorescence measurements were

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taken at the various times using excitation wavelengths of 340 or 380 nm and an emission wavelength of 510 nm.

2.5 Proliferation.

Growth studies in vitro were conducted using the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) and clonogenic assays. In the MTT assay, NCI-H358, Calu-3 or NCI-H441 cells were placed in SIT medium and various concentrations of mAb3481 added. After 1 day, 15 μl of 0.1 % MTT solution added. After 4 h, 150 μl of dimethylsulfoxide was added and the optical density at 570 nm was determined. In the clonogenic assay, the effects of PACAP-27, PACAP(6–38), NRG-1, mAb3481, trastuzumab or gefitinib were investigated on Calu-3 cells. The bottom layer contained 0.5% agarose in SIT medium containing 5% FBS in 6 well plates. The top layer consisted of 3 ml of SIT medium in 0.3% agarose, PACAP-27, PACAP(6–38), NRG-1, mAb3481, trastuzumab and/or gefitinib using 5×10^4 Calu-3 cells. Triplicate wells were plated and after 2 weeks, 1 ml of 0.1% p-iodonitrotetrazolium violet was added and after 16 hours at 37°C, the plates were screened for colony formation; the number of colonies larger than 50 μm in diameter were counted using an Omnicon image analysis system.

2.6 Statistical analysis

The results are expressed as means ± S.D. Statistical significance of differences was performed by one-way or two-way repeated measures analysis of variance (ANOVA).

3. Results

3.1 Receptor binding

The pharmacology of binding was investigated. Table I shows that PACAP-27, PACAP-38 and PACAP (6–38), but not VIP or NRG-1, inhibited specific ^{125}I-PACAP-27 binding to Calu-3 cells (IC_{50} values of 3.0, 5.3, 31.4, >1000 and >1000 nM, respectively). Similar binding data were obtained using NCI-H358 cells (data not shown). Because VIP bound with low affinity, PACAP is binding to PAC1 in these NSCLC cells.

3.2 Cytosolic calcium

The ability of PACAP to increase cytosolic Ca^{2+} in Calu-3 cells was investigated. Fig. 1A shows that PACAP addition to FURA-2 AM loaded Calu-3 cells resulted in a rapid increase in cytosolic Ca^{2+} within seconds followed by a slow decline (minutes). Fig. 1B shows that PACAP(6–38) had no effect on the cytosolic Ca^{2+} but antagonized the ability of PACAP-27 to increase cytosolic Ca^{2+}. Fig. 1C shows that VIP had no effect on the cytosolic Ca^{2+}, however, the positive control, ionomycin (Ca^{2+} ionophore) increased the calcium. The results indicate that addition of PACAP-27 to NSCLC cells increases the metabolism of PI, and the resulting IP_3 elevates the cytosolic Ca^{2+}.

3.3 Western blot

The time course of phosphorylation of HER3, ERK and AKT was investigated. Fig. 2A shows that PACAP weakly stimulates HER-3 and ERK phosphorylation at 2 min, but

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moderately stimulates phosphorylation at 5, 10 and 30 min. Fig. 2C shows that HER3 and ERK phosphorylation is significantly increased at 5, 10 and 30 min relative to the control. In contrast, PACAP increased AKT phosphorylation only after 10–30 min. Fig 2C shows that AKT phosphorylation significantly increased at 10 and 30 min relative to the control. Fig. 2B shows that the HER3 agonist NRG-1 weakly stimulates HER3 and ERK phosphorylation at 2 min, but the phosphorylation is moderate at 5, 10 and 30 min (Fig. 2B). Fig. 2D shows that HER3 and ERK phosphorylation were significantly increased a 2, 5, 10 and 30 min. Equal amounts of tubulin were present at all time points. The results indicate that PACAP and NRG-1 increase the phosphorylation of HER3 and ERK within minutes, however, the time-course for AKT phosphorylation is slower.

PAC1, EGFR, HER2 and HER3 were investigated in three NSCLC cells lines (Calu-3, NCI-H441 and NCI-H358) by Western blot. Fig. 3A shows that low densities of PAC1 but moderate densities of HER3 and EGFR were present in Calu-3, NCI-H441 and H358 cells. In contrast, Calu-3, NCI-H441 and H358 cells had high, moderate and low densities of HER2, respectively. As a control, equal amounts of tubulin were present in each cell line. Fig. 3C shows that the densities of PAC1, EGFR, HER2 and HER3 were higher in cell line Calu-3 than NCI-H441 and H358. Fig. 3B shows that PACAP-27 addition to Calu-3 cells increases P-HER3 but has no effect on total HER3. The increase in HER3 tyrosine phosphorylation caused by PACAP-27 is inhibited significantly by PACAP(6–38) or mAb3481 (Fig. 3D). The results indicate that the ability of PAC1 to regulate HER3 tyrosine phosphorylation is impaired by a PAC1 GPCR antagonist and HER3 mAbs.

The effects of reactive oxygen species (ROS) inhibitors on the PAC1 regulation of HER3 transactivation was investigated. Fig. 4A indicates that diphenylene iodonium (DPI inhibits Nox and Duox enzymes) weakly but N-acetyl cysteine (NAC is an antioxidant) or Tiron (Tir is a superoxide scavenger) strongly inhibited the ability of PACAP to increase HER3 tyrosine phosphorylation. Fig. 4B shows that NAC and Tiron significantly impair the ability of PAC1 to transactivate HER3. The results indicate that PAC1 regulates the transactivation of HER3 in a time-, concentration- and ROS-dependent manner.

3.4 Immunoprecipitation

The formation of heterodimers between the EGFR, HER2 and HER3 was investigated. Fig. 5A shows that addition of 1 or 10 nM PACAP to Calu-3 cells had little effect, however, addition of 100 or 1000 nM PACAP significantly increased HER3 tyrosine phosphorylation. The set of samples immunoprecipitated with HER2 antibody, had a similar PACAP dose-response curve, however, the density of the bands was 31% less (Fig. 5B). The set of samples immunoprecipitated with EGFR Ab had a similar dose- response curve, however, the density of the bands was 73% less. The results indicate that there are more HER3-HER2 heterodimers than HER3-EGFR heterodimers.

3.5 Proliferation

The ability of mAb3481 in a dose-dependent manner to inhibit the proliferation of NSCLC cells was investigated. In the MTT assay, Fig. 6 shows that low doses (0.05 μg/ml) of mAb3481 had little effect on the proliferation of NCI-H358, NCI-H441 and Calu-3

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cells. In contrast, moderate doses (1 μg/ml) of mAb3481 inhibited the growth of NCI-H358, NCI-H441 and Calu-3 cells. In the clonogenic assay, 0.1 μg/ml mAb3481reduced significantly colony number by 31% (Table II). In contrast 0.1 μg/ml NRG-1 stimulated growth significantly by 33% and this increase in colony number caused by NRG-1 was reversed by mAb3481. PACAP-27 (100 nM) increased significantly colony number by 49% whereas PACAP(6–38), gefitinib and trastuzumab reduced basal colony number significantly by 35, 64 and 27%, respectively. The increase in colony number caused by PACAP-27 was impaired by PACAP(6–38), gefitinib, trastuzumab or mAb3481. The results indicate that a PAC1 antagonist, EGFR TKI, and mAbs against HER2, as well as HER3, inhibit the growth of NSCLC cells in the presence or absence of PACAP.

4. Discussion

NSCLC, which kills approximately 130,000 U.S. citizens annually, is treated with platinum chemotherapy but the 5 year survival rate is only 16% (Qin et al 2019). Pembrolizumab, an immune checkpoint inhibitor, has improved the therapy of certain lung cancer patients (Qiu et al., 2019). NSCLC patients with EGFR mutations such as L858R, G719C or deletions in amino acids 747–75 who have failed chemotherapy can be treated with gefitinib or erlotinib (Santoni-Rugiu et al., 2019). The EGFR extracellular amino terminal has 621 amino acids and domains I as well as III participate in binding of ligands such as EGF, TGFα or amphiregulin (Wang, 2017). The EGFR has a 23 amino acid transmembrane domain and a 542 amino acid intracellular domain with tyrosine kinase activity. Domain II the EGFR (170 kDa) can form homodimers and resulting in the phosphorylation of protein substrates such as PI3K and PLCγ (Lemmon et al., 2014). The amino terminal of HER2 has no known ligand, a TM domain and an intracellular domain with tyrosine kinase activity. HER2 (180 kDa) is amplified in many cancer patients (Mitrie et al., 2012). HER3 has an amino terminal which binds NRG-1 or NRG-2 with high affinity. HER3 (190 kDa) has a TM domain but the intracellular domain has little tyrosine kinase activity. HER2 and HER3 can phosphorylate protein substrates, however, if they form heterodimers (Wang, 2017). Also, EGFR-HER3 and EGFR-HER2 heterodimers active.

Numerous studies on the transactivation of EGFR and HER2 by peptide GPCRs have been conducted (Moody et al., 2016b). Bombesin addition to NSCLC cells increases HER3 tyrosine phosphorylation (Lee et al., 2020) and here the effects of PACAP were investigated. EGFR, HER2, HER3 and PAC1 proteins are present in NCI-H358, NCI-H441 and Calu-3 cells (Fig. 3). Calu-3 cells bind PACAP-27, PACAP-38 and PACAP(6–38), but not VIP, with high affinity (Table I). PACAP, but not VIP addition to FURA-2AM loaded NSCLC cells increased the cytosolic Ca^{2+} within seconds (Fig. 1). PACAP(6–38) functioned as an antagonist and blocked the increase in cytosolic Ca^{2+} caused by PACAP-27. The results indicate that PAC1 is present and biologically active in these NSCLC cells.

PAC1 regulation of HER3 transactivation was time dependent. Two minutes after addition of PACAP to NSCLC cells, there was little increase in HER3 tyrosine phosphorylation, however, a 2-fold increase in P-HER3 occurred after 5 min (Fig. 2). Low doses of PACAP-27 (1 or 10 nM) had little effect on HER3 tyrosine phosphorylation, however, 100 nM and 1000 nM PACAP-27 increased P-HER3 moderately and strongly, Previously, we

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found that 100 nM PACAP-27 increased TGFα release from lung cancer cells leading to tyrosine phosphorylation of the EGFR (Moody et al., 2012b). Currently, we are investigating if higher doses of PACAP-27 such as 1000 nM, are required for the release of NRG-1 from lung cancer cells leading to tyrosine phosphorylation of HER3. The increase in P-HER3 caused by PACAP addition to Calu-3 cells was inhibited by PACAP(6–38) or mAb3481. MAb3481 is selective for HER3 and inhibits the ability of NRG-1 to increase the proliferation of cancer cells. MAb3481 inhibits the ability of NRG-1 to tyrosine phosphorylate HER3 in lung cancer cells (Lee at al., 2020). It remains to be determined if mAb3481 blocks the HER3 NRG-1 binding site.

Using anti-PY^{1289}HER3 as a probe, PACAP increased tyrosine phosphorylation 2-fold. Using other cells, phosphorylation of HER3 Y^{1054}, Y^{1197}, Y^{1222}, Y^{1260}, Y^{1276} or Y^{1289} targets P85 resulting in activation of PI-3K and increased phosphorylation of AKT leading to cellular survival (Wang, 2017). Phosphorylation of HER3 Y^{1199}, Y^{1262} or Y^{1328} targets Grb2 or Shc leading to Ras activation and increased phosphorylation of ERK and cellular proliferation. Addition of PACAP-27 to Calu-3 cells increased AKT and ERK phosphorylation after 10 and 5 min, respectively (Fig 2.). Addition of PACAP to neuronal cells stimulated ERK1/2, AKT and ERK5 but abrogated SAPK/JNK and p38MAPK activity (May et al., 2010).

ROS, which can be produced by electron transport, P450 enzymes and/or Nox/Duox enzymes, increased after addition of PACAP to NSCLC cells (Moody et al., 2019). DPI, NAC and Tir each inhibited the ability of PACAP to cause EGFR and HER2 tyrosine phosphorylation. Fig. 4 shows that NAC and Tir but not DPI impaired significantly the ability of PACAP to cause HER3 tyrosine phosphorylation. The ROS in NSCLC cells that alter PAC1 induced HER3 transactivation remain unknown.

PACAP increased tyrosine phosphorylation of the EGFR and HER2 4- and 3-fold respectively (Moody et al., 2019) due to the formation of EGFR homodimers and EGFR-HER2 heterodimers. Due to a weak tyrosine kinase domain, HER3 does not form active homodimers and must form heterodimers with the EGFR or HER2 to be biologically active. Fig. 5 shows that HER3 forms heterodimers with HER2 (69%) and the EGFR (27%). The results indicate that PAC1 regulates the transactivation of the EGFR, HER2 and HER3. PACAP causes transactivation of TrkA in neuroendocrine PC12 cells in a Src-dependent manner (Lee, FS et al. 2002; Shi GX et al., 2010). The results indicate that PAC1 can transactivate several RTK.

PAC1 contains 468 amino acids with an extracellular N-terminal, 7 transmembrane (TM) domains, 3 extracelluar loops (EL), 3 intracellular loops (IL) and an intracellular C-terminal (Pisegna and Wank, 1993). It has a large N-terminal of 125 amino acids which contains antiparallel β-sheets and binds to the C-terminal of PACAP-38 (Sun et al., 2007; Kumar et al., 2011). PAC1 has an open state (G4) and 3 closed transition states (G1–G3) (Liao et al., 2017). PAC1 splice variants (SV) have been detected in the N-terminal and intracellular loop IL3 (Pisegna and Wank, 1996). PAC1 has 18 exons and deletion of exons 5,6 or 4–6 reduces the size of the N-terminal by 7, 21 or 57 amino acids (Lutz et al., 2006). Addition of a 28 amino acid segment to IL3 results in PAC1 hip SV. Addition of a different 28 amino

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acid segment to PAC1null results in the PAC1 hop SV. Addition of both segments results in the PAC1 hip-hop SV (Ushiyama et al, 2007). The signal transduction of PAC1 varies as a function of the SV (Moody et al., 2016a). The role of the PAC1 SV on cancer cellular proliferation in unknown.

Several somatic mutations of HER3 have been identified in cancer cells (Kiavue et al., 2020). In NSCLC cells, HER3 is mutated at D297Y in the EC domain II and at S846I as well as E928G in the TK domain. It remains to be determined if these HER3 mutations increase tumor onset, tumor progression and immune escape.

The role of PAC1 and HER3 on lung cancer cellular proliferation was investigated. In the MTT assay mAb3481 inhibited the proliferation of NCI-H441, NCI-358 and Calu-3 cells in a dose dependent manner with IC_{50} values of 0.7, 2.8 and 1.1 μg/ml, respectively. In the clonogenic assay, mAb3481 moderately inhibited Calu-3 proliferation (basal or that stimulated by PACAP or NRG-1). PACAP(6–38) or trastuzumab inhibited growth (basal or that stimulated by PACAP-27), however, gefitinib strongly inhibited proliferation. NSCLC patients become resistant to gefitinib as a result of additional EGFR mutations, MET amplification or HER3 amplification. In lung cancer cells resistance to gefitinib was associated with increased HER3 expression (Engelman et al., 2007). In prostate cancer cells, erlotinib resistance was associated with increased expression of NRG-1, HER2 and HER3 (Carrion-Salip et al., 2012). Numerous HER3 mAbs have been developed which inactivate HER3 by impairing NRG-1 binding, however, they have not shown meaningful clinical benefit in trials (Jacob et al., 2018). It remains to be determined if PACAP(6–38) potentiates the action of HER3 mAbs and/or TKI.

In summary, PACAP increases the tyrosine phosphorylation of HER3 in NSCLC cells. PAC1 regulates the formation of EGFR/HER3 and HER2/HER3 heterodimers. This results in the phosphorylation of ERK and AKT which increase cancer cellular proliferation and survival, respectively. Also, PAC1 regulates the tyrosine phosphorylation of the EGFR and HER2. The GPCR PAC1 regulates the activation of numerous RTK in NSCLC cells.

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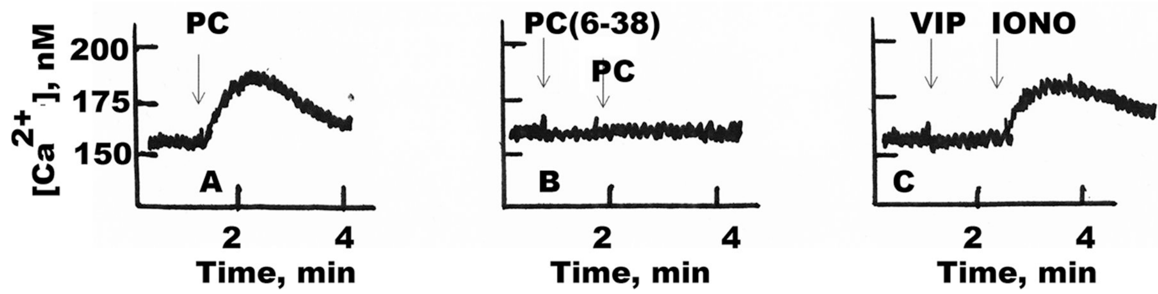

Fig. 1.

Cytosolic Ca^{2+}. The ability of 100 nM PACAP-27 (PC) to increase cytosolic Ca^{2+} in Fura2-AM loaded Calu-3 cells was determine in the presence of (A) no additions and (B) PACAP(6–38), the PAC1 antagonist. (C) VIP had no effect on cytosolic Ca^{2+} but the positive control 5 μM ionomycin increased cytosolic Ca^{2+}. This experiment is representative of 2 others.

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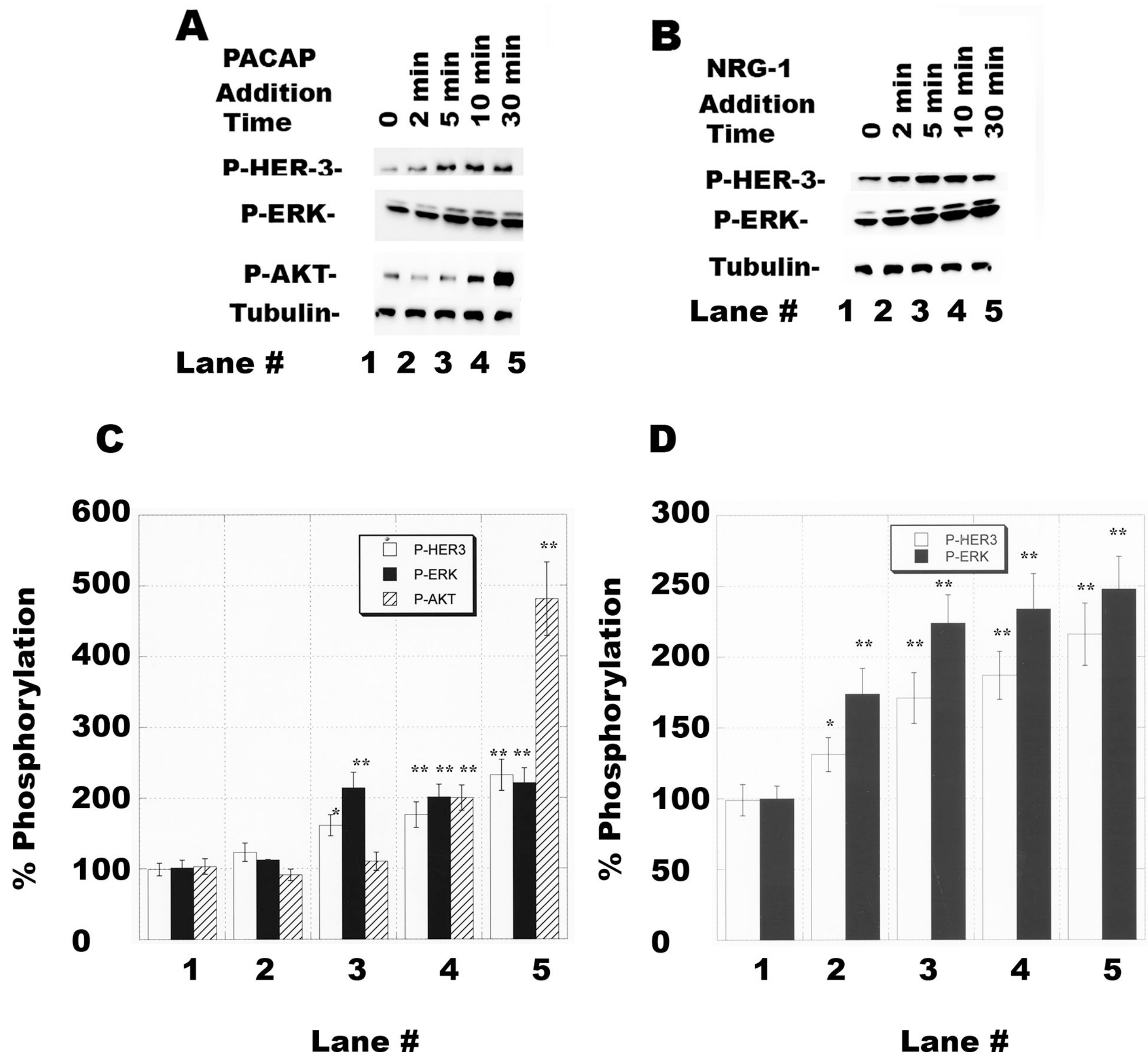

Fig. 2.

HER3 and ERK tyrosine phosphorylation. (A) PACAP-27 (1000 nM) was added to Calu-3 cells as a function of time and the P-HER3, P-ERK, P-AKT and tubulin determined. (B) NRG-1 (1 μg/ml) was added to Calu-3 cells and the P-HER3, P-ERK and tubulin determined as a function of time. (C) Graphical representation of Fig. 2A. (D) Graphical representation of Fig. 2B. This experiment is representative of 3 others; $p < 0.05$, *; $p < 0.01$, **; relative to control by ANOVA..

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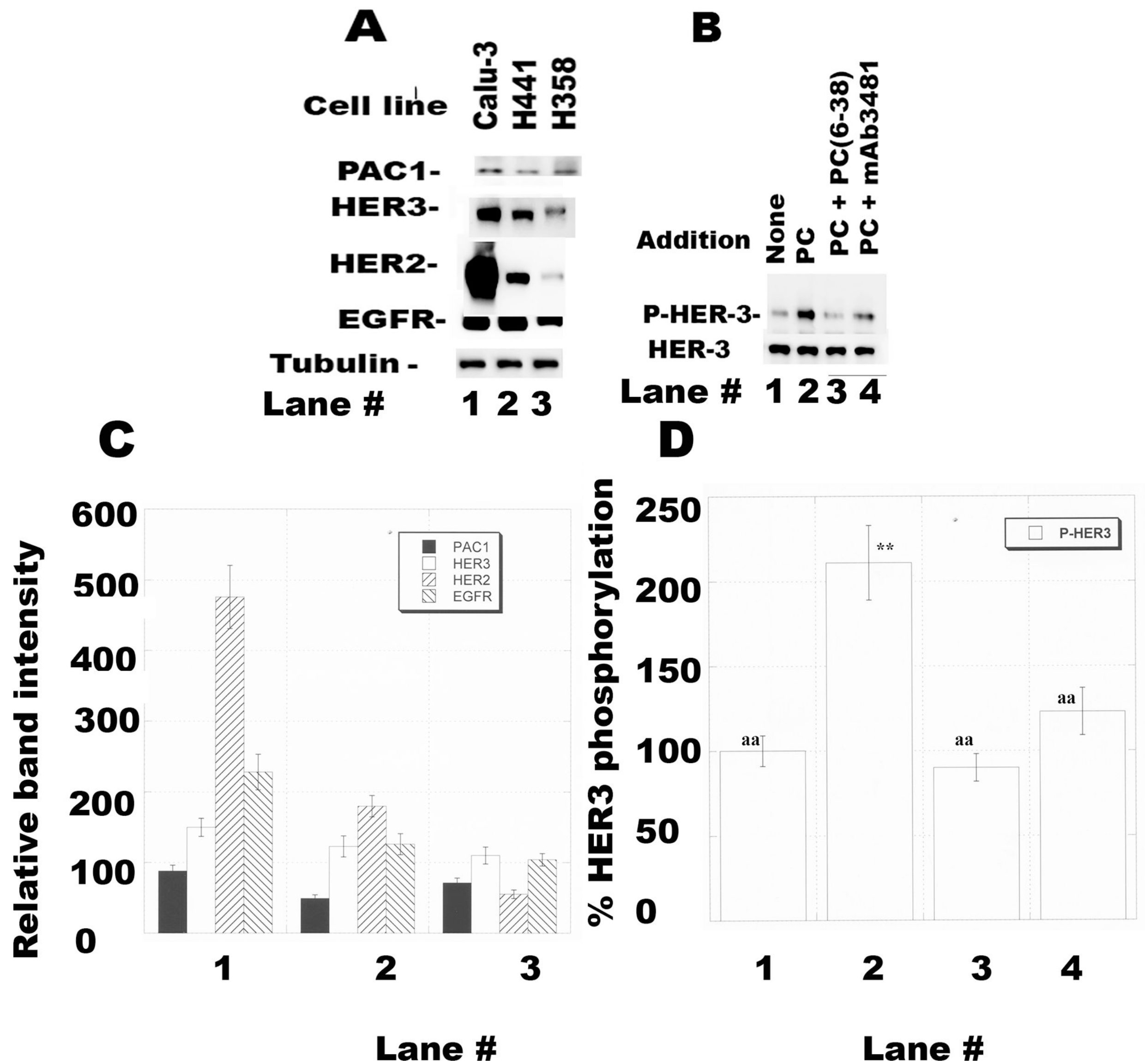

Fig. 3.

PAC1 and RTK in NSCLC cells. (A) PAC1, EGFR, HER2, HER3 and tubulin were determined in Calu-3, NCI-H441 and NCI-358 cells. (B) The ability of PACAP-27 to increase P-HER3 in Calu-3 cells was impaired by PACAP(6–38) or mAb3481. (C) Graphical representation of Fig. 3A. (D) Graphical representation of Fig. 3B. This experiment is representative of 2 others; $p < 0.01$, ** relative to control; $p < 0.01$, ^{aa} relative to PACAP by ANOVA.

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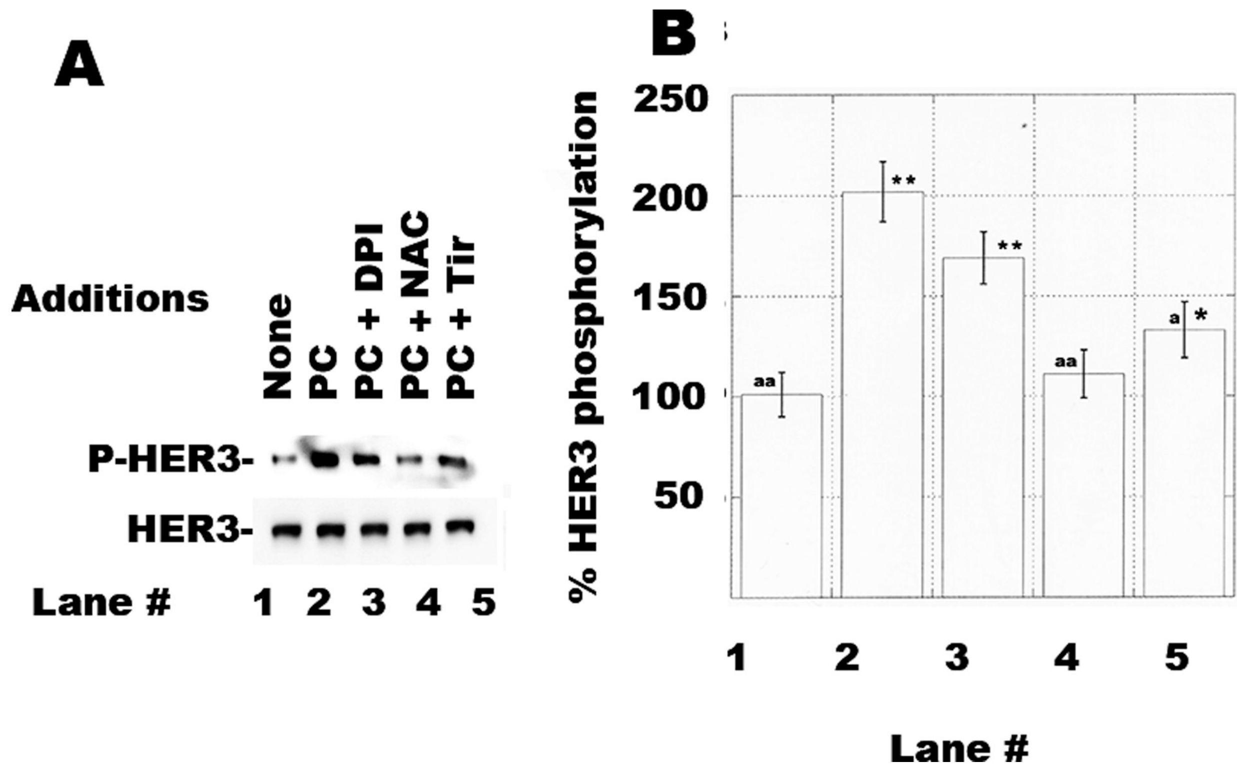

Fig. 4.

Effect of ROS inhibitors on HER3 transactivation. (A) The effects of DPI, NAc or Tir on the ability of PC to increase HER3 phosphorylation was investigated by Western blot. (B) Graphical representation. The mean value ± S.D. of 3 experiments are shown; $p < 0.05$, *; $p < 0.01$, ** relative to control; $p < 0.05$, [a]; $p < 0.01$,[aa]; relative to PACAP by ANOVA.

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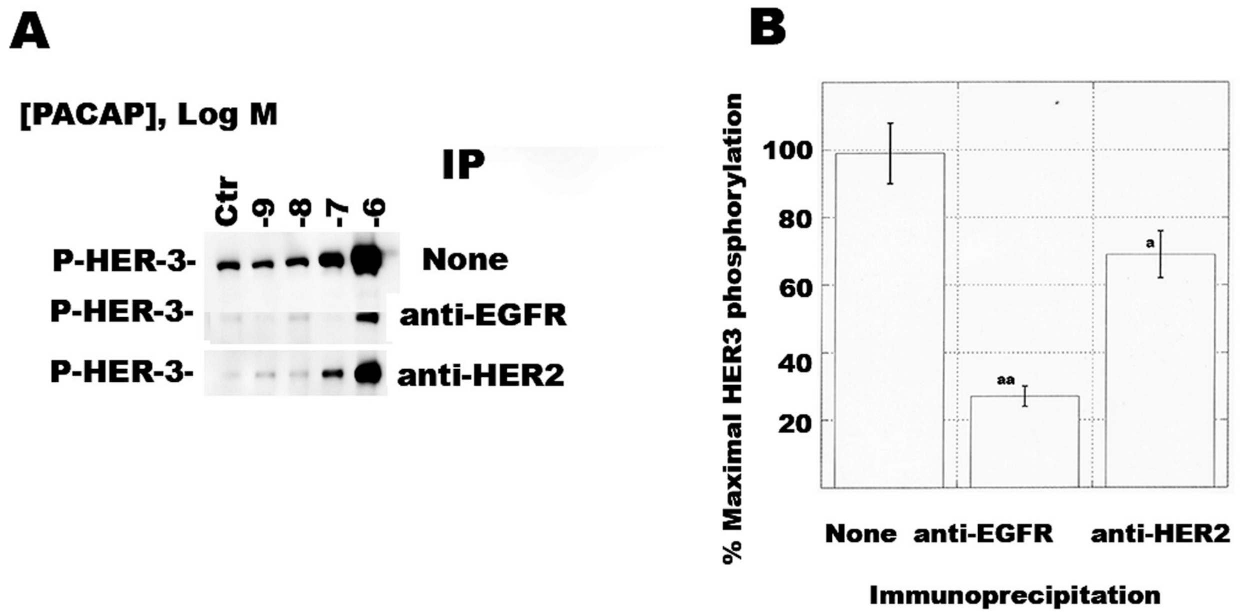

Fig. 5.

Immunoprecipitation experiments. Calu-3 cells were incubated with varying doses of PACAP-27 for 5 min. (A) The total lysate was compared to lysate immunoprecipitated with anti-HER2 or anti-EGFR. The immunoprecipitation samples were then analyzed for PY^{1289}-HER3. (B) Graphical representation. The mean value ± S.D. of 3 experiments are shown; $p < 0.05$, [a]; $p < 0.01$,[aa] ; relative to PACAP by ANOVA.

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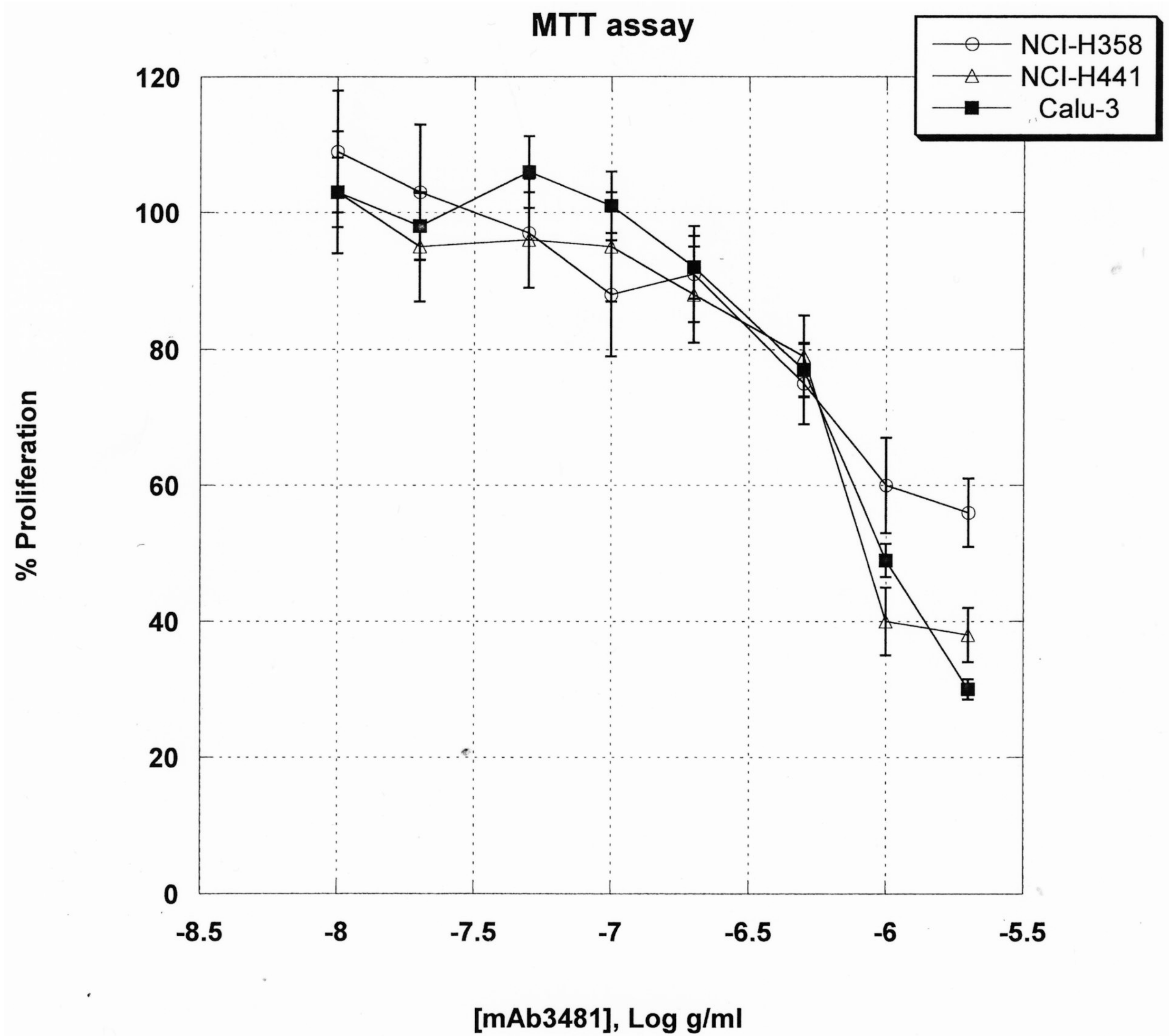

Fig. 6.

NSCLC proliferation. Using the MTT assay the growth of Calu-3 (■), NCI-441 ( ) and NCI-H358 (○) cells is indicated as a function of mAb3481 concentration. The mean value ± S.D. of 8 determinations is indicated. This experiment is representative of 2 others.

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Table I.

Binding to NSCLC cancer cells.

Ligand	IC_{50}, nM
PACAP-27	3.0 ± 0.3
PACAP-38	5.3 ± 0.6
PACAP(6–38)	31.4 ± 4.9
VIP	> 1000
NRG-1	> 1000

The ability of the ligands to inhibit specific ^{125}I-PACAP-27 binding to Calu-3 cells was determined at 37°C. The mean IC_{50} ± S.D. is indicated for 4 determinations. The structures of the peptides are shown below and sequence homologies relative to PACAP-27 are underlined.

PACAP-27 His-Ser-Arg-Gly-Ile-Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-NH_2

PACAP (6–38) Phe-Thr-Asp-Ser-Tyr-Ser-Arg-Tyr-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Ala-Ala-Val-Leu-Gly-Lys-Arg-Tyr-Lys-Gln-Arg-Val-Lys-Asn-Lys-NH_2

VIP His-Ser-Asp-Ala-Val-Phe-Thr-Asp-Asn-Tyr-Thr-Arg-Leu-Arg-Lys-Gln-Met-Ala-Val-Lys-Lys-Tyr-Leu-Asn-Ser-Ile-Leu-Asn-NH_2

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Table II.

Clonogenic assay using Calu-3 cells.

	Colony number	
Addition	**Basal**	**+ PACAP**
None	75 ± 7	112 ± 15*
PACAP(6–38)	49 ± 6*	72 ± 8
Gefitinib	24 ± 3**	43 ± 5*
Trastuzumab	54 ± 6*	67 ± 8
mAb3481	52 ± 5	79 ± 6
NRG-1	99 ± 7*	n.d.
NRG-1+ mAb3481	75 + 7	n.d.

The colony number was determined in the presence or absence on 100 nM PACAP-27 using the following inhibitors; PACAP(6–38) (5 μM); gefitinib (1 μM); trastuzumab (0,1 μg/ml); mAb 3481 (0.1 μg/ml). The mean value ± S.D. of 3 determinations is indicated; $p < 0.05$, *; $p < 0.01$, ** by ANOVA. Not determined, n.d.