**Western Blot Semi-Quantitative Analysis of Non-Canonical cAMP-Dependent Protein Expression Induced by PACAP**

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## ABSTRACT

The neuropeptide pituitary adenylate cyclase activating polypeptide (PACAP) is a slow transmitter and a neuroendocrine hormone in the central and peripheral nervous system. It binds to its cognate G-protein-coupled receptor PAC1, elevating intracellular cAMP and calcium. PACAP protects neurons from excitotoxicity and hypoxic damage. In previous experiments, a target gene was identified in microarray to be upregulated by PACAP treatment in PC12 cells. The present study aimed to identify a novel signal transduction pathway that PACAP uses to stimulate expression of the target gene. Neuronal cells - the cell line NG108-15 and rat cortical neurons - were stimulated with PACAP and various secondary messenger activators and inhibitors. Furthermore, this study aimed to develop a method to calibrate immunodetection protein assay results to quantify protein fold increase. A non-canonical pathway via ERK and not PKA activation was confirmed, as validated by calculated induction fold increase, in both cell types. In addition, a hyperbolic regression curve to approximate volume of protein from standard dilution curves was used to quantify observed Western Blot band intensities, though this method needs further work. This pathway should be further investigated to see if drug treatments could target this in preventing cell death caused by stroke or neurodegenerative disease.

**Acronyms:** ATP, adenosine triphosphate; BSA, Albumin Bovine Fraction V; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; ddAd, 2',5'-dideoxyadenosine; ERK, extracellular signal-regulated protein kinase or p44/p42 MAPK; G-protein, guanine nucleotide binding protein; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride; HRP, horseradish peroxidase; $IP_3$, inositol triphosphate; LDS, lithium dodecyl sulfate; MAPK, mitogen-activated protein kinase; MOPS, 3-(N-morpholino) propanesulfonic acid; NG108-15, neuroblastoma x glioma hybrid cells; NG108-$15_{hop}$, NG108-15 cells that normally express the $PAC1_{hop}$ receptor; PACAP, pituitary adenylate cyclase-activating polypeptide; $PAC1_{hop}$, PACAP receptor; PACAP38, PACAP with 38 residues; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKA, protein kinase A; SDS, sodium dodecyl sulfate; STC-1, stanniocalcin-1; TBS, Tris-Buffered Saline; TBST, TBS with 0.1% Tween-20; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene

## INTRODUCTION

PACAP is a hormone of the secretin superfamily which has two variants: PACAP38 and PACAP27 (Sherwood, Krueckl, & McRory, 2000). It is involved in many functions in the central and peripheral nervous system, including neuroprotection, neuronal development, neurotransmission, cell proliferation and transformation, and neuritogenesis (Sherwood, Krueckl, & McRory, 2000). For this experiment, PACAP38, which is endogenous in neuronal cells, was used. PACAP and its corresponding G-protein-coupled receptor PAC1 are widely expressed in the central nervous system (CNS). The PAC1 receptor occurs in different splice variants of the 3rd intracellular loop, including the PAC1-hop, -hip and -null variant (Spengler et al., 1993). Cortical cells express predominantly the $PAC1_{hop}$ variant expressed in rat cortical cells; $PAC1_{hop}$ is also the most abundant form in the CNS (Eiden et al., 2008).

PACAP performs its roles in the CNS via upregulation of multiple genes. Activation of particular target genes may be due to a unique combination of elevation of cAMP, calcium, and MAPKs, which are major downstream pathways induced by PACAP (Somal et al., 2007). PAC1 preferentially stimulates adenylate cyclase via the G-protein $G_{\alpha s}$ to convert ATP into cAMP (Mustafa, Grimaldi, & Eiden, 2007). Moreover, PACAP activates $G_{\alpha q}$ to generate diacylglycerol and $IP_3$, leading to calcium mobilization from intracellular stores and activation of protein kinase C (Squire et al., 2008), as illustrated in Figure 1. The classical model of cAMP signaling to the nucleus goes through protein kinase A. However, other cAMP effectors have been recently identified. For example, Ravni et al. showed in 2008 that PACAP induced differentiation of PC12 cells depends on cAMP, Rap1 and ERK1/2, not requiring PKA.

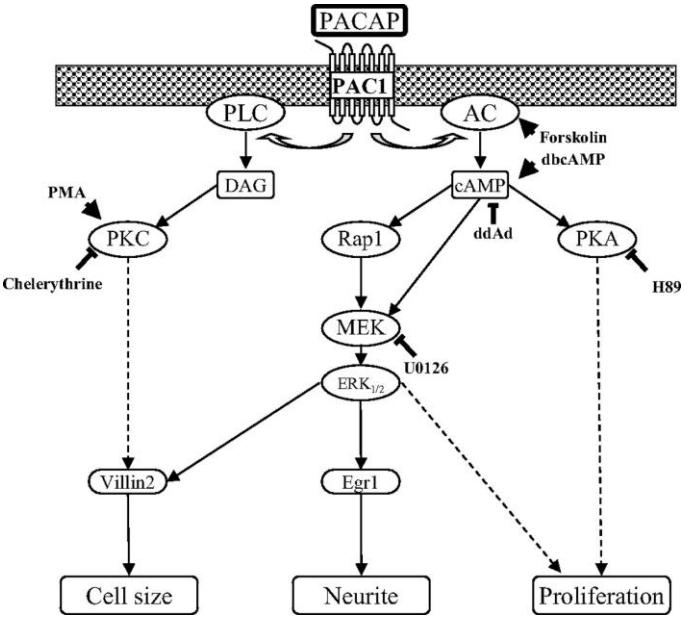


**Figure 1**: PACAP signal transduction pathways (Ravni et al., 2008)

One of the many important roles of PACAP in cell regulation is neuritogenesis and prevention of cell damage and cell death after cerebral ischemia (Stumm, 2007). Protein expression of genes upregulated by PACAP may therefore have neuroprotective properties. Such target genes were determined in a previous unpublished study as identified in a microarray analysis of PC12 cells; among them was STC-1. This gene regulates phosphate and calcium homeostasis in response to elevated environmental calcium levels by lowering the calcium uptake, increasing phosphate reabsorption, and inhibiting calcium transport. It prevents hypoxic damage and hypercalcemia in vitro, the major mediators of hypoxic cell death, suggesting that it too plays a role in maintaining integrity of terminally differentiated neurons following ischemic insult.

Western Blots can be used to quantify protein when analyzed in combination with a standard curve or housekeeping protein. A correlation between measurable results and amount of protein must be established; this correlation is determined by blotting procedure, primary and secondary antibody binding kinetics, chemiluminescence reaction, and image acquisition and processing. Irregularities in chemiluminescent substrate incubation can be corrected for by background deletion, while variations in SDS-PAGE gel loading can be corrected for by division by a loading control; a protein known not to change in this pathway paradigm, total ERK, was used. Furthermore, the band intensities of the resultant exposures can be calibrated via a hyperbolic curve determined to describe the relationship between measured and actual protein concentrations developed by a hyperbolic regression script that uses a standard curve of dilutions as the parameters (Heidebrecht et al., 2009).

This information suggests that PACAP may be involved in the upregulation of a gene with neuroprotective capabilities. Additionally, this gene regulation may be mediated via a novel signal transduction pathway, requiring ERK and cAMP, but not PKA (Eiden et al., 2008).

## MATERIALS AND METHODS

### *2.1 Drugs*

PACAP-38 was purchased from Phoenix Pharmaceuticals (Mannheim, Germany). Forskolin, H89, and U0126 were obtained from Calbiochem (San Diego, CA), and 2',5'-dideoxyadenosine (ddAd) was purchased from Sigma-Aldrich (Louis, Missouri). If inhibitors were used, cells were pretreated with 300μM ddAd, 10μM H89, or 10μM U0126 30 minutes before treatment with 100nM PACAP or 10μM forskolin. Cortical cells were stimulated with PACAP for 5 minutes while NG108-15 cells were stimulated for 2 minutes. These concentrations and treatment times were determined to be effective by prior unpublished studies.

*2.2 Cell Culture*

Embryonic rat cortical cells harvested from 18-day pregnant Wistar rats were used at 8-9 days *in vitro* (div); NG108-15 cells expressing $_{PAC1hop}$ were generated by viral infection. Cells were grown at 37°C in a 5% $CO_2$, 95% humidified air atmosphere in complete medium (Invitrogen, Carlsbad, CA). Pharmacology experiments had eight samples each that were treated thusly: unstimulated control with no drug applied, stimulated control with PACAP treatment, ddAd applied without PACAP, ddAd applied with PACAP, H89 with and without PACAP, and U0126 with and without PACAP. Some blots had forskolin treatment instead of PACAP for all instances of PACAP treatment. Calibration curve blots had eight samples all treated with PACAP or forskolin and diluted before blotting.

*2.3 Western Blot*

Proteins were run on NuPAGE Novex Pre-Cast 4-12% Bis-Tris gels in NuPAGE MOPS SDS Running Buffer using SeeBlue Plus2 Pre-Stained Standard and Magic Mark XP Western Standard, all obtained from Invitrogen. Proteins were transferred onto a 0.45µm pore nitrocellulose membrane on the XCELLII Blot Module using NuPAGE transfer buffer, also from Invitrogen.

Cells were washed in PBS and lysed in 1x LDS sample buffer containing 10mM reducing agent and protease and phosphatease inhibitors. Samples were sonicated for 10-15 seconds and heat-treated at 95°C for 5 minutes, cooled on ice and microcentrifuged for 2 minutes at 12,000 rpm. Samples were then loaded onto a 1.5mm, 10-well gel or 1mm, 12-well gel; 25µL of sample was loaded into each lane for NG108-15 cells and 15µL of each sample was loaded for cortical cells for pharmacology blots. A dilution series of 25µL, 20µL, 15µL, 12.5µL, 6.25µL, 3.13µL, 1.6µL, and 0.8µL sample diluted to 25µL in lysis buffer was loaded for calibration curve blots. The gel was run at 120V for 90 minutes. As a running standard, 9-10µL SeeBlue Standard and 4µL Magic Mark Standard were loaded into one lane each on every gel. After running, proteins were transferred onto a membrane at 30V for 90 minutes in prechilled 50µM transfer buffer and 100µM methanol.

*2.4 Immunodetection*

Phospho-ERK and total ERK primary antibodies were purchased from Cell Signaling (Danvers, Massachusetts). The secondary antibody, HRP-conjugated goat anti-rabbit IgG, was obtained from Pierce (Rockford, Illinois). Chemiluminescent imaging was achieved by incubating blots in Super Signal West Pico Chemiluminescence Substrate from Pierce, exposing the blots using Fluor Chem 8800 Imaging System from Alpha Innotec (San Leandro, CA), and digitizing the results using iVision software. After exposure, blots were stripped with Restore Western Blot Stripping Buffer from Pierce if necessary.

All of the following incubations were carried out with agitation:
After transfer, blots were dried to fix proteins to the membrane, then were blocked in TBST plus 5% skim milk powder for 1 hour at room temperature. The primary antibody phospho-ERK was diluted 1:1000 in blocking buffer plus 5% BSA, and the membrane was incubated overnight in the solution at 4°C. The membrane was washed 3 times for 15 minutes at room temperature in blocking buffer, then incubated in the secondary antibody, diluted 1:3000 in blocking buffer, for 40 minutes at room temperature, then washed 3 times for 15 minutes in blocking buffer. After rinsing the membrane once with TBST and once with TBS, HRP was detected with chemiluminescence substrate, and the membrane was exposed to a camera for 30 seconds, 2 minutes, or 8 minutes and digitized. After phospho-ERK was detected, the blot was stripped with Restore Western Blot Stripping Buffer (Pierce) for 15 minutes at room temperature, washed twice with TBS, blocked, and reprobed with the total ERK primary antibody.

*2.5 Quantification*

The quantification of Western blots was performed using a method detailed in Heidebrecht et al. (2009). Band intensities were quantified using ImageJ (NIH). Pictures were divided by base images of blank membranes with the same exposure length to eliminate background. Pictures were then inverted, and using the gel analysis features band intensities, were measured by selecting the band with the rectangle tool, graphing the intensity, and calculating the area of the intensity plots. The averages band intensities measured from calibration curve blots were inputted into a hyperbolic regression script (provided by A. Heidebrecht) to generate a regression formula, which was then used to calculate protein fold increase from control in pharmacology blots.

**RESULTS**

*3.1 Immunodetection Analysis*

For each SDS-PAGE gel, blots were incubated in phospho-ERK and total ERK antibodies. Changes in ERK activation along the non-canonical cAMP-dependent pathway were evaluated by quantifying band intensities on phospho-ERK blots and comparing to total ERK blot band intensities as a loading control. All blots were carried out for NG108-15 cells (see Figure 2), the model cell type, and cortical cells (see Figure 3), the target cell type investigated for the pathway. Background was eliminated from blot exposures by dividing blots by pictures of blank membranes using the image calculator in ImageJ. Rectangles were drawn around each band, and the area of resulting intensity graphs of each were found as previously described (Heidebrecht, 2009).

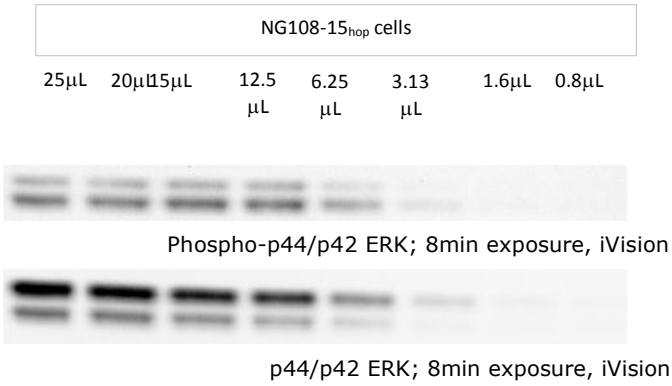


**Figure 2**: Sample dilution series blot using NG108-15 cell samples incubated in phospho-ERK and total ERK antibodies.

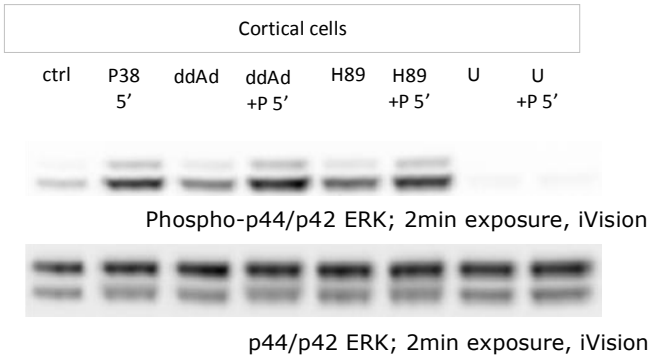


**Figure 3**: Sample pharmacology blot using cortical cell samples incubated in phospho-ERK and total ERK antibodies.

### *3.2 Calculating a Calibration Curve*

The relationship between band intensity and actual protein concentration was established using a hyperbolic regression script using known loading amounts along a calibration curve and measured band intensities. The program ran an iterative optimisation using these paramaters and outputted a standard hyperbola which established a relationship between the parameters for each cell type and each exposure length. However, protein concentrations for pharmacology blots were only evaluated at 2 minutes exposure for cortical cell blots and NG108-15 cell blots incubated in total ERK antibodies and 8 minutes exposure for NG108-15 cell blots incubated in phospho-ERK antibodies, as we established that this was these times produced distinct bands for most samples without oversaturation. Two standard blots were used to calculate the regression for each cell type as illustrated in Figure 4 and Figure 5.

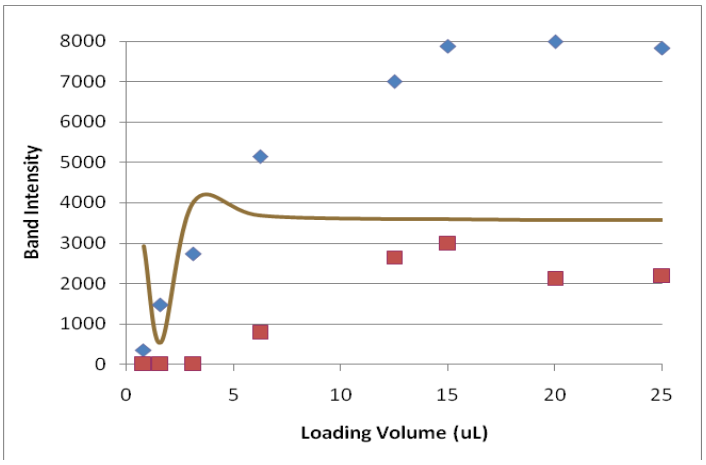


**Figure 4**: Measured band intensity versus actual protein volume in NG108-15 standard blots incubated in phospho-ERK antibodies and the hyperbolic regression line of best fit.

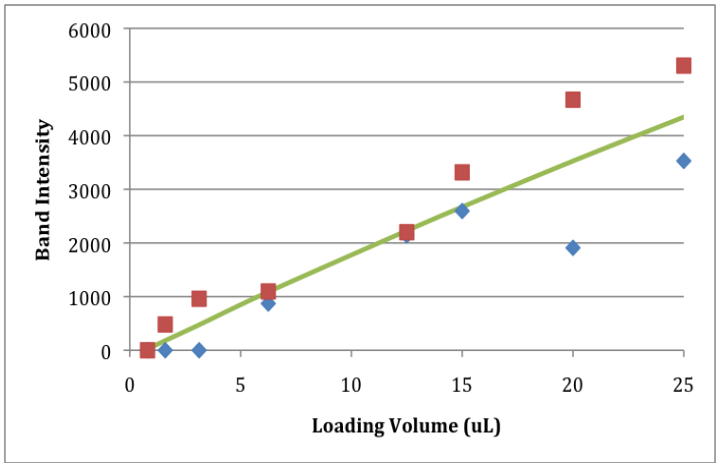


**Figure 5**: Measured band intensity versus actual protein volume in cortical standard blots incubated in phospho-ERK antibodies and the hyperbolic regression line of best fit.

*3.3 Quantifying Protein Fold Increase*

Total band intensities for samples on pharmacology blots were calibrated by plugging in the measured values into the regression formula to find the corresponding loading volume of cells stimulated only with PACAP. These volumes were then divided by the calculated volume for the sample of unstimulated cells on each blot to correct for blot differences and to calculate protein fold increase as shown in Tables 1 and 2.

**Table 1**: Calculated phospho-ERK protein concentrations in NG108-15 cells stimulated with PACAP and various inhibitors.

| Cell Stimulation | Calculated Protein Concentrations | | | | |
|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Average |
| none | 1 | 1 | 1 | 1 | 1 |
| 100nM PACAP38 (2min) | -1.329023 | 1.029632 | 1.806005 | -0.163765 | 0.335712 |
| 300µM ddAd | -0.925865 | 0.482223 | 1.037984 | -0.243008 | 0.087834 |
| 300µM ddAd + PACAP38 2' | -1.434127 | 0.579611 | 1.001293 | -0.240078 | -0.02333 |
| 10µM H89 | -1.372334 | 1.148057 | 1.009056 | -0.182646 | 0.150533 |
| 10µM H89 + PACAP38 2' | -1.283579 | 0.985554 | -0.843777 | -0.340947 | -0.37069 |
| 10µM U0126 | -1.075406 | 0.81951 | 1.037984 | -0.251415 | 0.132668 |
| 10µM U0126 + PACAP38 2' | -1.080926 | 0.820934 | 1.037984 | -0.251415 | 0.131644 |

**Table 2**: Calculated phospho-ERK protein concentrations in cortical cells stimulated with PACAP and various inhibitors.

| Cell Stimulation | Calculated Protein Concentrations | | | | |
|---|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Trial 4 | Average |
| none | 1 | 1 | 1 | 1 | 1 |
| 100nM PACAP38 (5min) | 3.473844 | 1.875222 | 1.644294 | 5.284614 | 3.069493 |
| 300µM ddAd | 2.63921 | 1.64376 | 1.107498 | 1.479957 | 1.717606 |
| 300µM ddAd + PACAP38 5' | 4.377874 | 2.325401 | 1.760065 | 3.562081 | 3.006355 |
| 10µM H89 | 3.322231 | 1.473202 | 1.173373 | 1.687619 | 1.914106 |
| 10µM H89 + PACAP38 5' | 4.229149 | 2.112417 | 1.623408 | 4.193806 | 3.039695 |
| 10µM U0126 | 0.339331 | 0.512304 | 0.586351 | 0.145105 | 0.395773 |
| 10µM U0126 + PACAP38 5' | 0.312778 | 0.621475 | 0.52291 | 0.145105 | 0.400567 |

Due to chemiluminescent substrate difficulties that prevented accurate data from being collected from certain blots, some blots were omitted from analysis. Because of this and regression errors discussed below, total ERK data were used as a qualitative rather than quantitative method for loading control of phospho-ERK data. Additionally, the calibration curve was inaccurate for analysis of NG108-15 samples, so the calibration resulted in negative protein concentration increases from control levels of phospho-ERK protein, as shown in Figure 6.

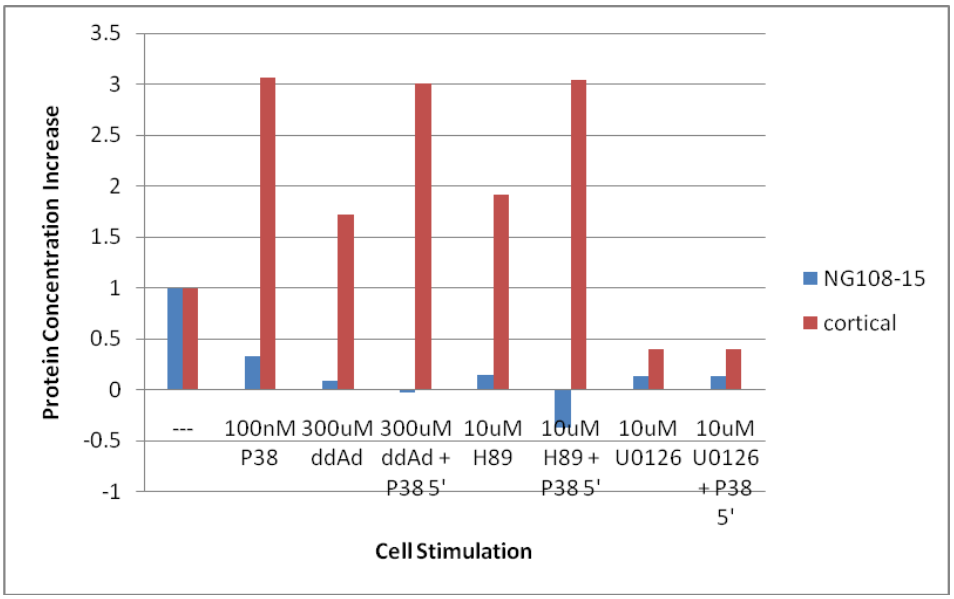


**Figure 6**: Average phospho-ERK protein fold increase in NG108-15 and cortical cells stimulated with various inhibitors.

## DISCUSSION

*4.1 Statistical Analysis of Protein Fold Differences*

We predicted that PACAP would regulate the target gene in neuronal cells in a non-canonical cAMP-dependent pathway, requiring ERK1/2 activation. By using various second messenger activators and inhibitors, the transduction pathway from PAC1 receptor activation to activation of ERK was investigated. These data indicate that this non-canonical cAMP-dependent pathway is used by PACAP in neuronal cells. The patterns of upregulation of cell samples stimulated with inhibitors in NG108-15 cell samples was similar to these outcomes in rat cortical cells, indicating that the NG108-15 cell line is an accurate model for rat cortical cells in this context. In both cell types, the pathway was not inhibited (and possibly promoted) by H89 and was completely blocked by U0126. Although ddAd treatment did not produce a complete block in cortical cells, further studies of ddAd treatment concentrations showed that a higher concentration of ddAd are necessary for full inhibition. However, since H89 did not produce any inhibition when stimulated with PACAP, the non-canonical cAMP-dependent pathway was confirmed because PKA block did not inhibit upregulation. Furthermore, since forskolin activated ERK in the same fashion as PACAP in cortical cells, ERK activation was induced by PACAP via AC.

When phospho-ERK concentrations were compared to total ERK concentrations, the loading was generally consistent. Total ERK band intensities had a mean of 6831 and a standard deviation of 1970 for cortical cells and a mean of 5141 and a standard deviation of 1496 for NG108-15 cells. Blots were omitted from analysis when background deletion failed (i.e. a signal could be measured from the membrane surrounding the bands).

*4.2 Accuracy of Western Blot Quantification Method*

The calibration curve for NG108-15 phospho-ERK blots and cortical total ERK blots were inaccurate because the saturation point for band intensities was 12.5μL, thus making the regression curve very sensitive to fluctuations at smaller dilutions and flatten out above 12.5μL. This caused band intensities of 3300 to 3500 for NG108-15 phospho-ERK and band intensities above 7000 for cortical total ERK to be correlated to a negative, and thus impossible, volume, leading to what appears to be protein fold decreases when in reality all stimulated samples exhibited a protein fold increase from the unstimulated control, as demonstrated in Appendix 1. Furthermore, the models for these two sets of blots are inappropriate due to high variation of actual values from predicted values, as shown in the sum of errors for the regression: 24,287,533 for NG108-15 phospho-ERK, 631,011 for NG108-15 total ERK, 160,739 for cortical phospho-ERK, and 1,233,319 for cortical total ERK.

*4.3 Implications for Neuroprotection and Further Research*

The conclusions of this study have multiple furthur applications. Other genes may be upregulated by PACAP via ERK activation and could be investigated using the inhibitors and methods described. Furthermore, functional assays should be conducted to investigate if this pathway is implicated in neuroprotection. Thus, this pathway could be targeted in drug development if this pathway exists only in neuronal cells, for PACAP treatment could be used to prevent cell death during the progression of neurodegenerative disease and post-ischemic insult. In addition, this method of immunodetection analysis could be developed to make Western Blots accurately quantitative for when other immunodetection methods are not practical. If a calibration curve were produced using more blots that had dilutions within a more viable range (in which all bands are visible but not saturated), the method could be used generate correct results; this method should further by evaluated by using known protein concentrations rather than a dilution series and by comparing results to ELISA studies to verify accuracy.

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**APPENDIX A:** Total measured band intensity and calculated protein fold increase compared to control levels.

| Cortical Cells - pERK | Trial 1 | | Trial 2 | | Trial 3 | | Trial 4 | |
|---|---|---|---|---|---|---|---|---|
| Contents | Total | Fold | Total | Fold | Total | Fold | Total | Fold |
| --- | 2516 | 1.000 | 4223 | 1.000 | 5129 | 1.000 | 796 | 1.000 |
| 100nM P38 | 7855 | 3.474 | 7370 | 1.875 | 7886 | 1.644 | 4376 | 5.285 |
| 300µM ddAd | 6220 | 2.639 | 6589 | 1.644 | 5619 | 1.107 | 1230 | 1.480 |
| 300µM ddAd + P38 5' | 9468 | 4.378 | 8796 | 2.325 | 8339 | 1.760 | 3013 | 3.562 |
| 10µM H89 | 7569 | 3.322 | 5991 | 1.473 | 5913 | 1.173 | 1415 | 1.688 |
| 10µM H89 + P38 5' | 9213 | 4.229 | 8136 | 2.112 | 7803 | 1.623 | 3524 | 4.194 |
| 10µM U0126 | 801 | 0.339 | 2213 | 0.512 | 3116 | 0.586 | 0 | 0.145 |
| 10µM U0126 + P38 5' | 729 | 0.313 | 2681 | 0.621 | 2788 | 0.523 | 0 | 0.145 |

| Cortical Cells - Total ERK | Trial 1 | | Trial 2 | | Trial 3 | | Trial 4 | |
|---|---|---|---|---|---|---|---|---|
| Contents | Total | Fold | Total | Fold | Total | Fold | Total | Fold |
| --- | 6308 | 1.000 | 3395 | 1.000 | 7784 | 1.000 | 6689 | 1.000 |
| 100nM P38 | 7986 | -0.704 | 3394 | 0.999 | 8387 | 0.629 | 8031 | -0.243 |
| 300µM ddAd | 8297 | -0.564 | 3511 | 1.061 | 7539 | 1.357 | 8180 | -0.218 |
| 300µM ddAd + P38 5' | 8976 | -0.405 | 3980 | 1.357 | 7529 | 1.377 | 7669 | -0.346 |
| 10µM H89 | 9137 | -0.382 | 4308 | 1.628 | 6796 | -6.231 | 7873 | -0.278 |
| 10µM H89 + P38 5' | 9330 | -0.358 | 4768 | 2.146 | 6674 | -3.083 | 8098 | -0.231 |
| 10µM U0126 | 9008 | -0.400 | 3166 | 0.890 | 5658 | -0.520 | 7297 | -0.657 |
| 10µM U0126 + P38 5' | 9601 | -0.330 | 4109 | 1.456 | 7435 | 1.610 | 7684 | -0.340 |

| NG108-15 Cells - pERK | Trial 1 | | Trial 2 | | Trial 3 | | Trial 4 | |
|---|---|---|---|---|---|---|---|---|
| Contents | Total | Fold | Total | Fold | Total | Fold | Total | Fold |
| --- | 3356 | 1.000 | 7020 | 1.000 | 897 | 1.000 | 3469 | 1.000 |
| 100nM P38 | 6694 | -1.329 | 6162 | 1.030 | 4150 | 1.806 | 2706 | -0.164 |
| 300µM ddAd | 2022 | -0.926 | 2809 | 0.482 | 0 | 1.038 | 838 | -0.243 |
| 300µM ddAd + P38 5' | 5294 | -1.434 | 2592 | 0.580 | 874 | 1.001 | 1044 | -0.240 |
| 10µM H89 | 5913 | -1.372 | 4862 | 1.148 | 727 | 1.009 | 2541 | -0.183 |
| 10µM H89 + P38 5' | 8360 | -1.284 | 7681 | 0.986 | 3345 | -0.844 | 5087 | -0.341 |
| 10µM U0126 | 160 | -1.075 | 56 | 0.820 | 0 | 1.038 | 0 | -0.251 |
| 10µM U0126 + P38 5' | 0 | -1.081 | 0 | 0.821 | 0 | 1.038 | 0 | -0.251 |

| NG108-15 Cells - Total ERK | Trial 1 | | Trial 2 | | Trial 3 | | Trial 4 | |
|---|---|---|---|---|---|---|---|---|
| Contents | Total | Fold | Total | Fold | Total | Fold | Total | Fold |
| --- | 4519 | 1.000 | 3770 | 1.000 | 5641 | 1.000 | 4638 | 1.000 |
| 100nM P38 | 5443 | 1.349 | 3632 | 0.954 | 6220 | 1.210 | 6851 | 2.077 |
| 300µM ddAd | 5317 | 1.295 | 3005 | 0.764 | 6176 | 1.192 | 6649 | 1.936 |
| 300µM ddAd + P38 5' | 6246 | 1.756 | 3179 | 0.814 | 5738 | 1.032 | 6702 | 1.972 |
| 10µM H89 | 6075 | 1.659 | 2805 | 0.709 | 5878 | 1.081 | 5918 | 1.515 |
| 10µM H89 + P38 5' | 6225 | 1.744 | 2405 | 0.607 | 5930 | 1.099 | 6003 | 1.558 |
| 10µM U0126 | 6137 | 1.693 | 1862 | 0.482 | 5449 | 0.940 | 6552 | 1.873 |
| 10µM U0126 + P38 5' | 5770 | 1.501 | 1814 | 0.472 | 5717 | 1.025 | 6253 | 1.693 |