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

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Abstract

Pituitary adenylate cyclase activating polypeptide (PACAP) is a neuroendocrine hormone that protects neurons from excitotoxicity and hypoxic damage. It binds to its cognate G-protein-coupled receptor PAC1, elevating intracellular cAMP and calcium. The present study aimed to identify a novel cAMP-dependent PACAP signal transduction pathway. 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 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.

Background

PACAP

•PACAP binds to receptor → receptor activates g-protein → G-protein activates AC → AC produces cAMP
Known pathway: cAMP activates PKA
New pathway: cAMP activates MAPKs, which activate ERK1/2
→ Genes are transcribed into proteins
•Strokes trigger hypertoxicity
Elevated calcium and phosphate levels are mediators of glutamatergic death
PACAP homeostasis to prevent cell damage and death

Western Blot

•Method of separating at proteins by size
Intensity of "bands" of sample can be measured
Analyzed with a standard curve or housekeeping protein
•Band Intensity vs. Protein Amount is not a linear relationship:
Correct for substrate and gel variation and calculate calibration equation via hyperbolic regression script

Results

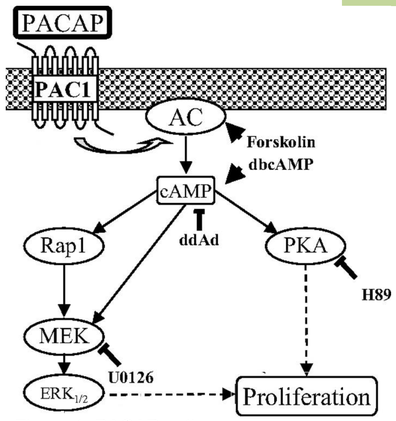

Figure 1: PACAP pathway (adapted from Ravni et al, 2008)

---	ddAd	H89	U0126
PACAP (or forskolin)	PACAP + ddAd	PACAP + H89	PACAP + U0126

Table 1: pharmacology blot samples

25µL PACAP	20µL PACAP + 5µL buffer	15µL PACAP + 10µL buffer	12.5µL PACAP + 12.5µL buffer
6.25µL PACAP + 18.75µL buffer	3.13µL PACAP + 21.87µL buffer	1.6µL PACAP + 23.4µL buffer	0.8µL PACAP + 24.2µL buffer

Table 2: calibration blot samples

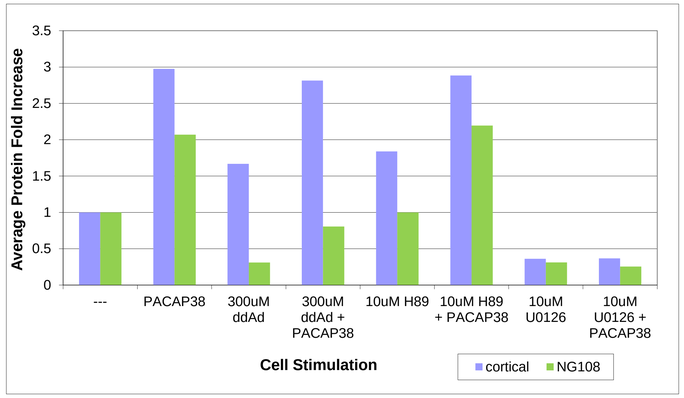

Figure 2: Average protein fold increase

•NG108-15 cells are a good model for cortical cells
•ddAd: did not complete block pathway
→Need to investigate concentrations
•H89: no block, so alternate pathway exists
•U0126: full block, so alternate pathway exists
•Total ERK band intensities were generally consistent, but didn't divide due to background deletion problems
•High blot-to-blot variation lead to high standard deviations, so quantitative conclusions not accurate

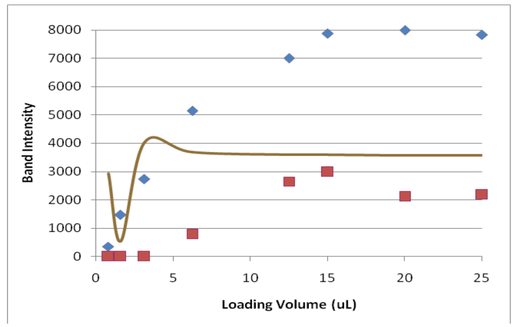

Figure 3: Calibration curve for NG108-15 cells

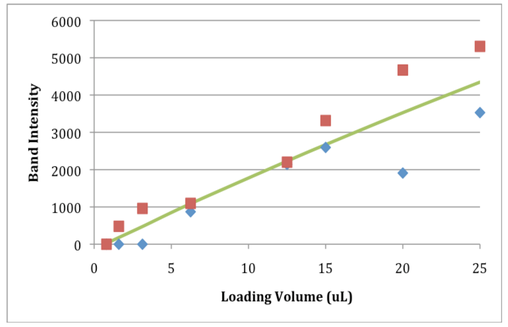

Figure 4: Calibration curve for cortical cells

High sum of errors for NG108-15 phospho-ERK blots and cortical ERK blots because the saturation point for band intensities was at 12.5µL sample, thus curve was very sensitive to fluctuations at smaller dilutions and flattened out above 12.5µL.

Methods

An SDS-PAGE using NG108-15 and cortical cell samples (see Tables 1&2) was run to separate proteins by length. Then, the gel was transferred to a membrane and incubated in phospho-ERK and total ERK antibodies. Finally, membranes were incubated in chemiluminescent substrate and pictures were taken.

The background was deleted from the pictures, then band intensities were measured with the ImageJ gel analysis tool. Calibration blot intensities were inputted into a hyperbolic regression script from A. Heidebrecht to generate a calibration formula.

Conclusions

•Analysis incomplete: did not have enough blots to correct curve due to chemiluminescent substrate difficulties
•Non-canonicalcAMP-dependent pathway via ERK and not PKA activation exists in cortical and NG108-15 cells
•Target gene discovered by microarray also regulates calcium and phosphate concentrations *in vitro*

Future Research

•Test pathway in other cells with PAC1 receptor
•Pathway could be targeted in drug development if only exists in neuronal cells
•PACAP could be used to prevent damage during neurodegenerative disease progression or post ischemic insult
•Create calibration curve with more than two blots
•Evaluate accuracy of method using known protein concentrations or by comparing results to ELISA studies