Phosphorylation of ERK signalling pathway molecules in response to step-stimulation by Epidermal Growth Factor

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The ERK signalling cascade is a key pathway for information transfer in the cell, and has a major role in cell fate determination. The pathway is known to be activated by Epidermal Growth Factor (EGF), and is capable of inducing either cell proliferation or differentiation dependent on whether the activation is transient or sustained, respectively. By modelling this pathway, taking into account experimental data from *in vitro* stimulation of PC12 cells, I predicted that step stimulation of the cell by EGF would elicit a diminishing level of ERK activation with each subsequent addition of the stimulus. I also predicted that the ratio of EGF concentrations between each step, rather than the number of stimulations would be a determining factor in the activation of ERK. By measuring the phosphorylation – and thus activity levels - of two key molecules within the ERK signalling pathway, I was able to test these hypotheses experimentally. The experimental resulting data indicated that the pattern of ERK phosphorylation reflects that predicted by the model; however, the pattern of upstream phosphorylation of EGFR did not appear so strongly conserved, possibly due to limitations in the protein detection technique. There are several possible explanations for the observed pERK response, including ubiquitination and degradation. This response could play an important role in temporal coding and directing cell fate.

Introduction

Epidermal Growth Factor (EGF) is a common component of numerous signalling pathways. It is capable of activating the ERK pathway through binding to and inducing phosphorylation of the Epidermal Growth Factor Receptor (EGFR), which in turn results in the phosphorylation and activation of ERK. Thus, by measuring the abundance of phosphorylated EGFR (pEGFR) and phosphorylated ERK (pERK) in a cell sample it is possible to obtain a clear indication of the level of activity at any given point in time.

By performing PC12 cell stimulation *in vitro* using different concentrations of EGF and measuring the phosphorylation of the two aforementioned key molecules at various time points using Western blotting, I was able to produce a simple model of the pathway. This model was then used to predict the response of the cells to a step stimulation procedure. The model indicated that the ratio of EGF concentrations used in each step would be an important determining factor in the final pattern of ERK activity observed: In a three-step stimulation, when the ratio of concentrations between the first two stimulations was larger than that between latter two, only two peaks were observed in pEGFR and pERK activity. However, when the ratios were reversed, three peaks were clearly observed (Figure 1).

I performed two experiments (Hereafter referred to as ‘Experiment 1’ and ‘ Experiment 2’) involving three step stimulations per cell culture plate using concentrations of EGF derived from my model: The ratios of EGF concentration per stimulation were 1:20:40 and 1:2.5:100 respectively. The cells were then lysed and pEGFR & pERK sampled at 10 time points over a period of 1 hour.

The observed phosphorylation of ERK closely resembled that predicted by the model suggesting that the signalling pathway is well represented by the model.

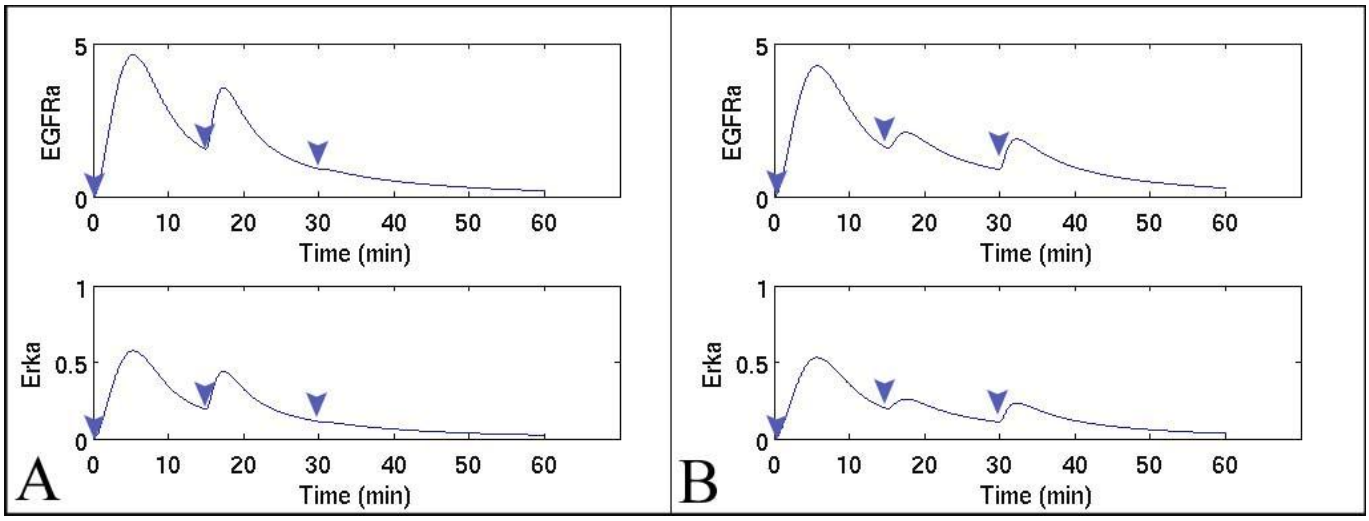


Figure 1: Graphs showing the levels of phosphorylated EGFR (EGFRa) and phosphorylated ERK (Erka) as predicted by the created model. Concentrations of EGF in the first, second and third stimulation are 0.5, 10.0 and 20.0ng/ml in **graph A,** and 0.4, 1.0, and 40.0ng/ml in **graph B** respectively. The first, second and third stimulations occurred at 0, 15, and 30 minutes (blue arrows).

Materials and Methods

Preliminary Experiment:
PC12 cells were seeded on 6cm dishes at a density of $8x10^{-5}$ cells. Serum starvation was performed after 24 hours, and the plates were subsequently incubated for 16 hours at 37°C. The cells in sets of 7 plates were then stimulated using 20µl of Epidermal Growth Factor (EGF) of concentrations 50, 10, 5, 1 or 0.5ng/ml respectively. The EGF preparations had been diluted in 0.1% BSA containing PBS.

The cells were lysed (See Appendix) and harvested at specific time points: 0, 1, 2, 5, 10, 15, 30 & 60 minutes. The resulting lysates were transferred to Eppendorf tubes and centrifuged at 15,000 rpm at 4°C. The tubes were then sonicated for 1 minute to induce DNA fragmentation. Following a further round of centrifugation, the samples were prepared for Western blotting.

The cells underwent heat shock treatment and further centrifugation. A Western blot was then performed following typical protocol. Polyacrylamide gel was prepared (See Appendix), and 10µl of each sample was loaded. Following electrophoresis at 40mA, separated proteins were transferred to a membrane. The membrane was then non-specifically blocked using a milk-TBST buffer.

Separated sections of membrane were incubated at 4°C overnight with specific antibodies targeting either pEGFR or pERK. They were subsequently washed with TBST and the secondary antibody was applied (Anti-Rabbit).

The membranes were finally photographed using LAS-4000 detecting chemiluminescence. The resulting images were subject to digital analysis.

Analysis and Modelling:
The gels were analysed using Phoretix / TotalLab software in order to quantify the level of phosphorylation. These data were then input into MATLAB and a program designed for modelling the EGF-mediated ERK signalling pathway. The resulting ODE model was later used to design my main experiment.

Main Experiment:
The main experiment followed the protocol outlined in the Preliminary Experiment section. However, rather than undergoing a single stimulation, the PC12 cells were subject to step stimulation: three stimulations per culture plate, using 20µl of EGF of

increasing concentrations (Table 1). These values were identified as optimal in the ODE model. Two simultaneous experiments were performed in order to assess the effect of EGF concentration ratios on the subsequent response.

Table 1: Table showing the concentrations of EGF (ng/ml) used for each stage of the step stimulations in Experiment 1 and 2. Stimulations 1, 2 and 3 occurred at 0, 15, and 30 minutes respectively.

	Stimulation (ng/ml)		
	1	2	3
Experiment 1	0.5	10	20
Experiment 2	0.4	1	40

The cells were lysed and harvested at specific time points: 1, 5, 10, 15, 18, 30, 32 & 60 minutes after the initial stimulation. Western blot analysis was performed, as before.

Results

Western blot analysis showed that the level of phosphorylation of ERK in my cell cultures - following EGF stimulation - closely resembled what was predicted by my model (Figure 1). In Experiment 1, two peaks in pERK were clearly observed at 5 and 18 minutes after the initial stimulation (Figure 2A). In Experiment 2, three peaks were observed at 5, 18, and 32 minutes (Figure 2B). In both cases, the level of pERK drops close to the baseline in between stimuli.

The detected levels of pEGFR, however, were not so similar to the model. In Experiment 1, a more prolonged activation of EGFR was observed following the second EGF stimulation. This was followed by a much steeper decline in activity (Figure 3A). In Experiment 2, the maximum level of pEGFR did not decrease with each stimulation as predicted, but instead increased at a steady rate. pEGFR levels in between stimulations also remained higher than expected (Figure 3B).

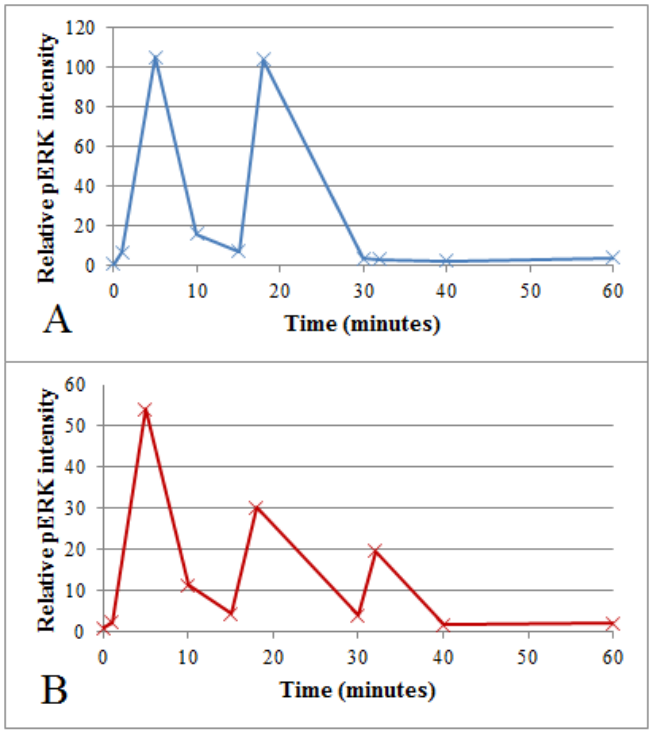


Figure 2: Graphs showing the relative intensity of pERK - relative to time zero - in the cell at various time points following EGF stimulation as determined experimentally. **Graph A** shows Experiment 1, and **Graph B** shows Experiment 2.

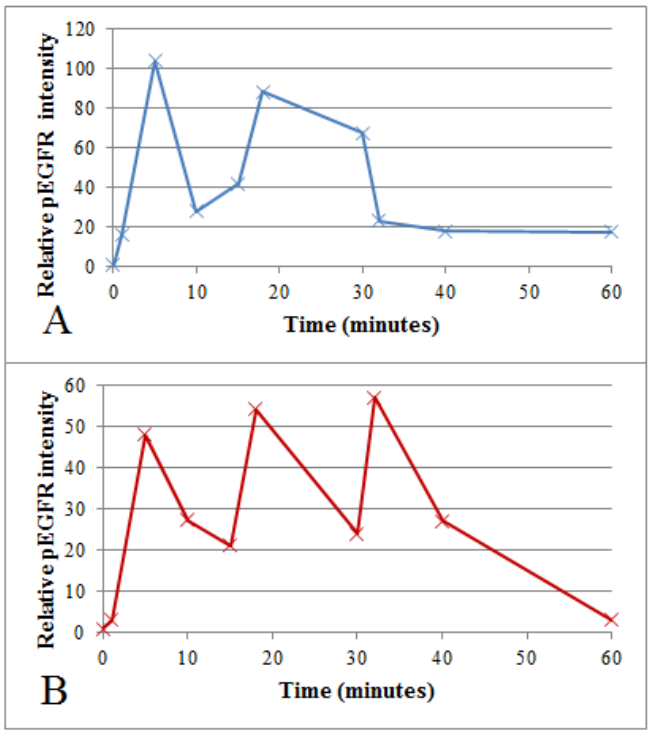
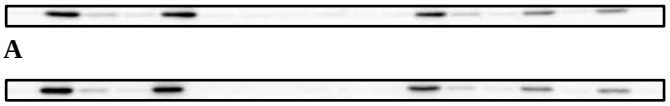


Figure 3: Graphs showing the intensity of pEGFR - relative to time zero - in the cell at various time points following EGF stimulation as determined experimentally. **Graph A** shows Experiment 1, and **Graph B** shows Experiment 2.

In almost all experiments, the level of phosphorylated ERK or EGFR had returned to the baseline by the final measurement taken at 60 minutes after initial stimulation.

Western blotting for pERK was performed twice in order to assess reproducibility and thus reliability of the results. Both replicates produced the same pattern of phosphorylation (Figure 4) and there was no statistically significant difference observed between them (paired sample *t*-test, $t = 1.329$, $df = 9$, $sig = 0.216$).



A

B

Figure 4: Western blot gels showing the phosphorylation pattern of ERK following EGF stimulation. **A** shows the gel from the 1st replicate; **B** shows the gel from the 2nd replicate.

Discussion

The ERK activation maxima were seen to decrease with each sequential stimulus, as predicted by the model. There are several possible reasons why this could occur. Firstly, a second pathway activated by EGF is known to initiate ubiquitination and degradation of the EGF receptor (Sasagawa *et al*, 2005). Thus, activation of this pathway could lead to a reduction in signal transduction along the ERK pathway, and therefore less ERK being phosphorylated. EGF binding to EGFR also causes activation of RasGAP resulting in the inhibition of Ras – a molecule involved in the ERK phosphorylation process. Finally, as EGFR is present in lower abundance in the cell, it is possible that the decrease in ERK activity could be a result of receptor saturation by the rapidly increasing concentrations of EGF in the stimulations.

This diminishing pattern of ERK phosphorylation may have an important function in the cell. Previous studies have shown that the timing and pattern of Nerve Growth Factor (NGF) mediated stimulations determines the way that the signal is decoded by the cell, and its ultimate physiological response (Chung *et al*, 2010). It is possible that a similar mechanism is occurring in the EGF-mediated pathway, and further research could be conducted into how the cell responds to such stimulations. The detection of specific cell markers or a microarray analysis of genes expressed in the cell at various time points following stimulation could be used to determine, for example, whether the stimulation causes cell differentiation. However, the concentration ratios of EGF required to elicit such as response as seen in Experiment 2 are very large. As such, they may not be naturally occurring in the cell.

The observations from the pEGFR analysis did not fit so tightly to the model. The most likely explanation for this is the limitations of the Western blotting technique: EGFR is present in lower abundance in the cell and so its detection is much more difficult. Therefore it is likely that the phosphorylation intensities detected on the gels were not truly representative of actual abundance within the cell. This hypothesis is supported by the fact that ERK, which is downstream of EGFR showed the expected phosphorylation pattern. As a membrane protein - which are notoriously difficult to work with - there are several procedures that can be done to ensure more complete extraction and detection of EGFR (Tan *et al*, 2008).

As the experimental phosphorylation pattern of ERK so strongly resembled the computational model, this implies that we have a good understanding of the EGF-mediated ERK pathway and that few other unknown variables are involved in the process. The inconsistencies regarding pEGFR can be easily explained by the Western blotting technique and as such do not conflict with the predetermined model.

Despite closely resembling the ODE model, the experimental data for pERK in Experiment 1 showed a second peak with slightly higher intensity relative to the first peak than originally predicted. Therefore it would be beneficial to take more samples for analysis around the activity maxima and minima in order to plot the data with greater precision.

It was not possible to take more than 20 measurements due to limitations in time and resources. Therefore an area of potential future study could be to test whether

the cells are able to continue responding to step stimulation when exposed to EGF more than 3 times. The ODE model predicts that with appropriate ratios of EGF concentrations in sequential stimulations, it is possible to observe further phosphorylation peaks (Figure 5). I would like to determine the point at which further peaks are no longer observed due to other limiting factors.

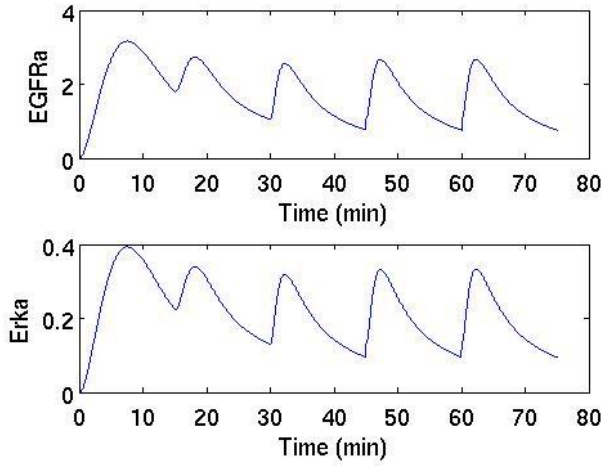


Figure 5: Model simulating continuous step stimulation. The ratios of EGF concentration between each stimulus are 1:4:160:800:1600.

Acknowledgements

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Appendix

Lysis Buffer composition: 200µl 1M Tris-HCl (pH 7.5); 0.4g SDS; 2ml Glycerol; 200 µl 2-mercaptoethanol.

8% Separation Gel composition: 2ml 40% Acrylamide/Bis; 2ml Distilled water; 5.025ml 1.5M Tris-HCl (pH 8.8); 100µl 10% SDS; 16.7µl TEMED; 82.5µl 10% APS.

Stacking Gel composition: 1ml 40% Acrylamide/Bis; 7.62ml DDW; 1.26ml 1M Tris-HCl (pH 6.8); 100µl 10% SDS; 10µ TEMED; 50µl 10% APS.