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Kinase Activity Studied in Living Cells Using an Immunoassay

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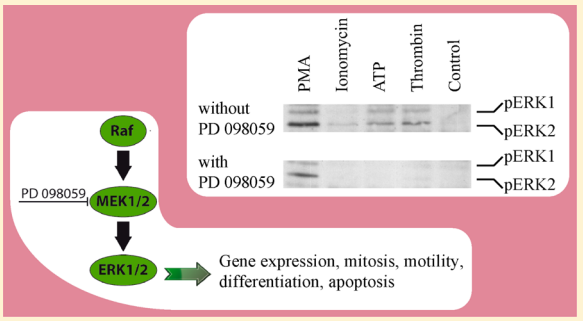
Supporting Information

ABSTRACT: This laboratory exercise demonstrates the use of an immunoassay for studying kinase enzyme activity in living cells. The advantage over the classical method, in which students have to isolate the enzyme from cell material and measure its activity in vitro, is that enzyme activity is modulated and measured in living cells, providing a more realistic picture of cell metabolism. With this experiment, students learn not only how to study protein kinases but also how to study the MAP kinase signaling pathway, how to set up their own experiments, and how to study the effects of pharmacologically important ligands on signaling in a particular metabolic pathway. Signaling with protein kinases, as described here, is a good example of how to study molecular mechanisms of signaling in general.



KEYWORDS: *First-Year Undergraduate/General, Biochemistry, Laboratory Instruction, Biosignaling, Enzymes, Hormones, Metabolism, Receptors*

INTRODUCTION

Biochemistry is a field of science important to students of chemistry and fundamental to those of biology, medicine, and other life sciences. It has become successful in explaining processes in almost all areas of living systems. Its applications are primarily in medicine, nutrition, and agriculture, addressing the improvement of cures, health, and food. Cell signaling is part of the complex pattern of communication that governs basic cellular activities, coordinates cell processes, and is regulated by enzymes, including kinases.

Kinases transfer phosphate groups from high-energy donor molecules, such as ATP, phosphocreatine and phosphoenolpyruvate, to specific substrates, in a process of phosphorylation.[1] If the substrate is a protein, the enzymes are called protein kinases. They are part of the larger family of phosphotransferases (EC number 2.7). Protein kinases modify the activity of specific proteins, their cellular localization, and association with other proteins. They are among the most ancient enzymes involved in the regulation of signal transduction pathways and other physiological processes in cells of bacteria, fungi, plants, and animals.[2] More than 500 different protein kinases have been identified in humans alone. Of these, mitogen activated protein kinase (MAPK) has been studied the most intensively. Like the majority of other protein kinases, it transfers a phosphate group from ATP to serine, threonine, or tyrosine residues on target proteins. MAPK responds to extracellular stimuli (mitogens) and regulates various cell activities, such as gene expression, mitosis, motility, differentiation and apoptosis. MAPK has profound effects on a cell and its activity is thus highly regulated. Activation of MAPK is regulated by phosphorylation of another kinase, MAPK kinase. MAPK is inactivated by a specific phosphatase. In mammals, 14 MAPKs have been characterized and assigned to seven groups.[3] The best characterized are the extracellular signal-regulated kinases (ERKs) (Figure 1). Upstream activation of ERKs is usually initiated at the plasma membrane via receptor tyrosine kinases or G-protein coupled receptors. G-proteins are activated by extracellular ligands, such as ATP and thrombin,

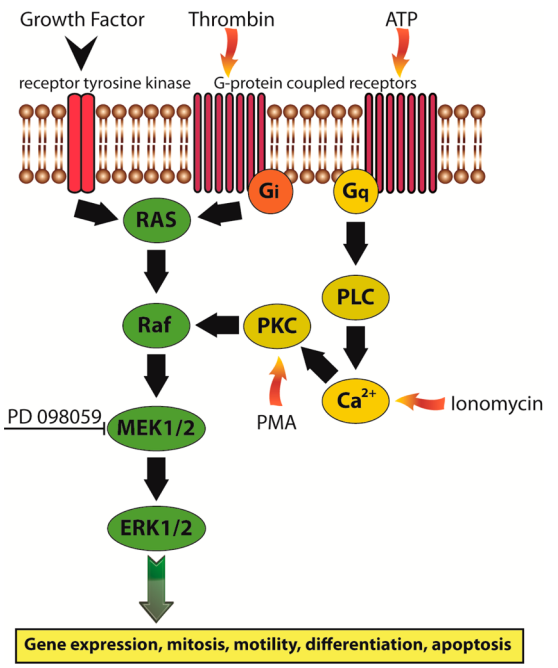


Figure 1. ERK pathway. Signaling pathway activators thrombin, ATP, ionomycin, and PMA are shown. PD 098059 is an inhibitor of MEK1/2.

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which bind to G-protein coupled receptors.[4] Activated G_q protein activates phospholipase Cβ (PLC), thereby triggering phosphoinositide hydrolysis, calcium mobilization and activation of protein kinase C (PKC).[5,6] Signals are then further transmitted via PKC, Raf, MAPK kinase 1/2 (also known as MEK1/2) to ERK1 (p44) or ERK2 (p42).[7] Alternatively, thrombin can activate Ras via G_i proteins, which leads to activation of the Ras-Raf-MEK-ERK signaling pathway.[8] The PKC-Raf-MEK-ERK signaling cascade is regulated by enzymes, small molecules or ions. For example, PKC is especially sensitive to intracellular calcium concentration.[9] Modulation of the PKC activity by calcium affects the ERK activity. Small molecules, such as phorbol-12-myristate-13-acetate (PMA), whose structure is very similar to that of one of the natural activators of PKC, diacylglycerol, can also activate PKC.[10] Activated PKC transmits a signal via Raf-MEK1/2 to both ERKs.

In most cases, it is very difficult to follow enzyme activity in living cells. Normally, students must isolate the enzyme from the cell material and measure its activity separately in vitro. Here, students learn how to study enzyme kinase activity in the living cell using an immunoassay. Moreover, they are introduced to methods of studying complex signaling pathways, following the reactions of various extracellular, pharmacologically important ligands, using inhibitors and other biochemical tools to study signaling cascades in the cell, etc. Finally, the experiment provides students with a basis for discussing the main sustainability concepts, apparent in the experiment, in a more open context. Signaling with protein kinases, as described and studied in the experiment, provides students with a good example of how to study molecular mechanisms of signaling in general.

The experiment has been performed by students with various backgrounds, such as chemistry, biology, biochemistry, and microbiology. Here, an experiment is described for undergraduate student in biochemistry and who have already completed lectures and experimental exercises on general chemistry and cell biology. The experiment has been performed more than 30 times over a five-year period by classes of 20 students of basic biochemistry. Four laboratory periods of 4 h each are needed in order to complete the experiment.

EXPERIMENT

Confluent chinese hamster ovary (CHO) cells were stimulated with thrombin, ATP, ionomycin, or PMA. When inhibition of MAPK kinase was studied, the cells were preincubated with the inhibitor prior to their stimulation by the indicated agonist. Ligand stimulation was stopped by washing the cells with buffer. The cell lysate was then cooled on ice and clarified by centrifugation. Supernatant proteins were separated on SDS-PAGE, then transferred from the gel onto nitrocellulose membranes. The proteins on the blot were stained and subjected to immunoblotting.[11] Immunoreactive proteins ERK1 and ERK2 caught on the blot were exposed to film using a chemiluminescence detection kit. Film was scanned, and MAP kinase activity was quantified. The raw data were obtained by photoimage processing of three Western blots and analyzed with an NIH computer program, software used in most student labs, developed to facilitate manipulation and selection of data from the densitometer trace. The raw data are expressed as the ratio of phosphorylated ERK (pERK1 or pERK2) to total ERK2 (phosphorylated and nonphosphorylated) referred to ERK1 activity and ERK2 activity, respectively. Total ERK1 was not used in the calculation because of the low staining with polyclonal anti-ERK1 antibodies and hence high probability of error. The corresponding ratio using raw results (pERK1/ERK2 or pERK2/ERK2) was converted to the ratio between ligand-induced and control ERK activity expressed in percent represented as ERK1 and ERK2 activity with and without inhibitor PD 098059. An outline of the exercise and details of the experimental procedures are given in the Supporting Information.

HAZARDS

There are many types of polyacrylamide gel electrophoresis, but they all involve handling monomer acrylamide, which is neurotoxic and can be absorbed through the skin. Even after polymerization there may be some free monomer present. Gloves must always be worn when handling the reagents and electrophoresis gels. The following hazardous chemicals are used: acrylamide, *Toxic*; ammonium persulfate – *Harmful*; bis(acrylamide), *Harmful*; mercaptoethanol, *Harmful*; sodium dodecyl sulfate, *Harmful*, TEMED,*Corrosive*. The high voltages used in gel electrophoresis are very dangerous. Electrodes should never be disconnected before turning off the power supply.

RESULTS

Confluent CHO cells are stimulated with various ligands. Thrombin and ATP bind to G-protein coupled receptors, activate Gq-proteins, and transmit a signal via the PKC and Raf-MEK1/2 signaling pathway to ERK1 and ERK2. Alternatively, thrombin activates the Ras-Raf-MEK-ERK signaling pathway. Ionomycin is an ionophore that transports calcium ions across biological membranes, raising their intracellular level. They bind to the calcium binding motif in PKC.[9] Thus, activated PKC transmits a signal via Raf-MEK1/2 to both ERKs. PMA mimics the structure of one of the natural activators of PKC, diacylglycerol. It, thereby, activates PKC and the Raf-MEK1/2 signaling pathway, leading to phosphorylation of ERK1 and ERK2.

Anti-phosphoERK1/2 antibody is used to detect endogenous levels of ERK1 and ERK2 after they are phosphorylated, either individually or dually at Thr202 and Tyr204 of ERK1 and Thr185 and Tyr187 of ERK2. The antibody does not cross-react with nonphosphorylated ERK1/2. The levels of phosphorylation of both ERK1 and ERK 2 by all four ligands (thrombin, ATP, ionomycin and PMA) tested were significantly greater than those of the control (Figure 2A). Moreover, PD 098059, a specific

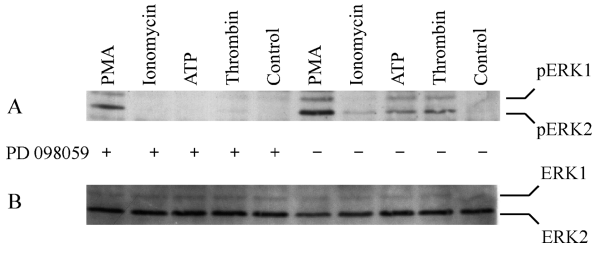

Figure 2. A typical student's Western blot analysis of ERK1 and ERK2 phosphorylation. (A) SDS-PAGE followed by protein transfer from the gel to the nitrocellulose membrane and immunoblotted with primary rabbit polyclonal anti-phosphoERKs 1 and 2. Various ligands were used to stimulate ERK phosphorylation: ATP (100 μM), thrombin (0.5 U/mL thrombin), ionomycin (10 μM), and PMA (200 nM) in the presence (+) or absence (–) of the MEK inhibitor PD 098059 (50 μM). (B) Immunoblot analysis with mouse monoclonal anti-ERK2 on antibody-stripped nitrocellulose membrane from experiment A (see above). Total ERKs (ERK1, ERK2), phosphorylated and non-phosphorylated, are stained on the nitrocellulose membrane.

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inhibitor of MEK that prevents the activation of MEK by Raf,[12] completely blocked phosphorylation of ERK1/2 (Figure 2A). The PKC-Raf-MEK-ERK pathway appears to be only partially blocked by PD 098059, suggesting alternative ways of ERK activation by PMA.[6] The total amounts of ERK1 and ERK2, both phosphorylated and nonphosphorylated, were determined and showed that ERK activities are modulated due to the effect of the ligand on enzyme activity and not on ERK expression. This result was obtained using immunoblot analysis with mouse monoclonal anti-ERK2 on antibody-stripped (antibody-removed) nitrocellulose membrane from experiment Figure 2A. The expression of ERKs during the experiment was unchanged, suggesting that the enzyme activity of ERKs is affected by ligands (Figure 2B). The quantitative analysis of MAP kinase activity is given in Table 1. Detailed description of data analysis is found in the Supporting Information.

Table 1. A Student's Reported Quantitative Analysis of Ligand-Induced MAP Kinase Activity in the Presence or Absence of Inhibitor PD 098059[a]

Ligand	ERK1 activity	ERK2 activity	ERK1 activity + PD 098059	ERK2 activity + PD 098059
Control	100 ± 5%	100 ± 4%	100 ± 3%	100 ± 2%
Thrombin	129 ± 45%	267 ± 88%	101 ± 4%	102 ± 3%
ATP	123 ± 22%	205 ± 19%	105 ± 2%	103 ± 1%
Ionomycin	111 ± 24%	135 ± 35%	104 ± 6%	101 ± 4%
PMA	238 ± 131%	484 ± 128%	136 ± 54%	254 ± 84%

[a]The data are means ± SEM of three independent experiments performed on separate cell cultures on separate occasions. The raw data are expressed in percent as the ratio of phosphorylated ERK (pERK1 or pERK2) to total ERK2 (phosphorylated and nonphosphorylated) referred to ERK1 activity and ERK2 activity, respectively.

DISCUSSION

The procedure outlined was relatively simple and economical, and did not require sophisticated equipment. It can readily be performed with the material and apparatus available in most student laboratories. The demonstration of enzyme kinase activity by immunoblotting has been found to be of particular value as a teaching tool, given the several biochemical concepts it illustrated. First, students learned and used several different techniques of molecular biology in the same experiment. The technical work was very instructive since the student learns the principles of SDS-PAGE, protein transfer, and immunoblotting.

Second, the techniques enable students to study enzyme kinase activity, MAPK signaling, and the principles of molecular mechanisms of signaling in general. Moreover, they designed their own experiments by using different pharmacologically important ligands and studied their effects on signaling in a metabolic pathway of basic interest.

Third, students learned that different ligands affected signaling pathways in different ways. Some ligands potentiated the cellular effect, and some inhibited it. They also learned, by experiment, that different ligands activated different metabolic branches of the same signaling pathway and deduced that different steps in a metabolic pathway are regulated differently. Finally, they learned that inhibitors can be useful biochemical tools for studying signaling mechanisms, in general.

An important feature of the experiment was the visualization of enzyme kinase activity intracellularly, using an immunoblotting technique. In addition, this experiment provided an alternative means of following enzyme kinase activity without the use of common radiolabeled substrates, avoiding the accompanying risk for students.

ASSOCIATED CONTENT

Supporting Information

A student laboratory manual with study questions; instructor notes, including timing, student's pitfalls and possible topics of interest; manufacturers of equipment and materials; hazards; CAS registry numbers of chemicals. This material is available via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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dx.doi.org/10.1021/ed400737t | *J. Chem. Educ.* XXXX, XXX, XXX−XXX