Preparing Posters

A few general notes:

- First read the abstract to remind yourself what you are doing.
- For our posters use Arial 14 pt font for all text and figures. Use Arial 40 pt bold font, all capitals for the headers of each section. Sub sections are underlined but not bold. Titles of figure legends are bolded but not in capitals.
- Each poster has the same basic format. Examples are attached.
    - Abstract: Each poster should have the abstract re-printed in the upper right hand corner.
    - Introduction: First introduce why your project is important. Then introduce each of the proteins or important components of the experiments. End with a few statements of your goals and what you have found.
    - Methods: Using subsections, include the specific methods used, this includes but is not limited to: gel and blotting, NHE assays, cell culture, fixing and staining cells and transfection. In this section you also want to include any unique reagents or materials and where you purchased them. If you are using a clone you did not create, cite who you got it from and where that person works.
    - Results: Each figure should have a figure at least 1/2 of a 8.5 x 11 inch paper. Use the font indicated above. After the figure is made they are copy pasted into either word or power point where figure legends are included. See attached figure for an example.
    - Discussion: Use bullets (closed circles) to describe the bottom line for each experiment or group of experiments. Then using a paragraph or two discuss the significance.
    - Reference: List a few of the key references from the introduction and discussion. Some times methods if it is unique.
    - Acknowledgement: List all funding agencies. This year use "This work was supported in part by the National Science Foundation Award 0080243 and the Minnesota State University Moorhead Faculty Grant and the Rolland Dille Excellence Award. We would also like to thank the ACS Chemistry Club for supporting the travel of the presenters." And for the MAS "Departments of Biology and Chemistry MSUM, President Roland Barden, Vice President Bette Midgarten and Dean of the College of Social and Natural Science, Ron Jeppson".

Do not be afraid to write and re-write. It is far worse to be at the meeting and find mistakes that you can do nothing about. Do not forget what you poster is saying, not just if the pH goes up or down. See the big picture as well as the details… In other words do not lose the forest for the trees.

Here is an example of last year's poster sections. DO NOT USE VERBATUM. The last thing we need is to have all of the posters look the same. However they will be similar due to the nature of what we are doing.

Introduction

Maintenance of intracellular pH (pHi) and regulation of cell volume is the physiological function of several ion transporters, including the Na^+-H^+ Exchanger (NHE) (Orlowski 1997). Extrusion of intracellular H^+ for external Na^+ is the most effective means of eliminating excess acid from actively metabolizing cells. In mammalian cells the increase in pHi critically controls the entry of quiescent cells into the cell cycle. The alkalinization of pHi results almost exclusively from the action of the NHE, and transport activity has been correlated with an increase in mitogenesis, cellular differentiation, cellular migration and neoplastic transformation (Noel 1995). NHE is a member of a multi-protein family that is highly regulated and present in virtually all mammalian tissues and species to date (Hooley 1996). The exchanger is typically inactive at resting pHi and can be activated in two ways: 1) Activity can be increased by an increase in intracellular H^+. This activation returns pHi to resting levels. 2) The exchanger can also

be activated through stimulation from various intracellular signaling pathways. This activation leads to an increase in the resting pHi and a more rapid rate of recovery from an intracellular acid load. The activation of NHE1 is still unclear. However, several protein kinases, G-proteins, and changes in intracellular Ca^{2+} have all been implicated in NHE1 regulation presumably at the carboxy terminus (Orlowski 1997 and Gutkind 1998).

Mitogen activated protein kinase (MAPK) is a group of protein kinases responsible for delivering extracellular signals into the cytoplasm. The three main subfamilies include extra-cellular response kinase (ERK or p44/p42), jun-N terminal kinase (JNK), and p38. The activation of ERK is controlled by a cascade of protein kinases including Raf and MEK. This stimulation is accomplished by the phosphorylation by MAPK kinase (MEK) of both Thr and Tyr residues in the consensus sequence Thr-Glu-Tyr (Vojtek 1998). ERK can also be activated independently of Ras, which entails a sequence involving Raf, Protein Kinase C, and MEK. The proteins involved in the pathway leading to the activation of ERK vary depending upon the cell line under investigation (Della Rocca 1997).

In addition to classic growth factor activation of the MAPK pathway, several G-protein coupled receptors mediate the stimulation of MAPK (Gutkind 1998). Thus, G-protein coupled receptors are potentially responsible for altering the activity of NHE1 (Della Rocca 1997). Receptor activation leads to the subsequent activation of heterotrimeric G-proteins. Activation of the G-protein-linked receptor initiates the exchange of GDP for GTP on the α-subunit. The heterotrimeric G-protein then dissociates into distinct α and $\beta\gamma$ components, each of which can interact with several intracellular effectors (Gutkind 1998).

In Chinese hamster lung fibroblasts (CCL39) MAPK activation is required for the serum stimulation of NHE1. A direct target of MAPK is p90 ribosomal S6 kinase ($p90^{RSK}$), which in turn activates NHE1 through phosphorylation (Takahashi 1999). The lysophosphatidic acid (LPA) receptor has been linked to the activation of several different G-proteins and can also stimulate the activity of both ERK and NHE1. Another G-protein linked receptor, the α_1-adrenergic receptor which is activated by the specific agonist phenylephrine (PE), has been found to be responsible for NHE1 activation in cardiac and smooth muscle cells. Thus there is a potential role for G-protein linked signaling pathways in the regulation of NHE1 in CCL39 cells. This project establishes a link between G-protein coupled receptors, ERK and NHE1.

Methods

Cell culture. CCL39 cells were maintained in attachment culture using high glucose Dulbecco's modified Eagle's medium (DMEM) (Sigma product #D-5648) supplemented with 25 mM sodium bicarbonate, 10% heat-inactivated fetal bovine serum, penicillin (1 unit/mL), and amphotercin B (1 unit/mL) at 37 °C in a 5% CO_2 incubator. Cells were carried in T-25 flasks and split 1:10 when the cells reached 60-70% confluent. A maximum of ten passages in T-25 flasks were allowed in order to maintain consistent cell growth and morphology.

Agonist treatment and cell harvest. Cells were subcultured into 35 mm tissue culture dishes and allowed to grow to approximately 70-80% confluence. Cells were growth-arrested for twelve hours with DMEM high glucose media containing 0.5% serum, 0.5% fatty acid free BSA with antibiotics and antimycotics. One hour prior to agonist treatment, the cells were rinsed with PBS and incubated in serum free DMEM high glucose media containing 0.5% fatty acid free BSA without antibiotics and antimycotics. Cells were then treated with specific agonists, inhibitors, or activators as indicated. Following the incubation periods, the cells were washed twice with chilled PBS and lysed in 200 μL Laemmli sample buffer containing 0.1 mM orthovanadate and 1mM β-glycerophosphate. The samples were boiled and DNA sheered with five passes through a 27-gauge needle.

SDS-PAGE and Western blot analysis. 10-30 μL of lysate was resolved on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane using an electroblotting apparatus. Phosphorylated ERK (p42/p44 MAPK) was detected by immunoblotting the membrane using a 1:1000 dilution of P-ERK mouse monoclonal IgG (Santa Cruz Biotechnology) in TTBS (10 μM Tris and 150 mM NaCl, pH 8.0 with Tween 20) with 5% dry milk overnight at 4 °C. Total ERK was detected by immunoblotting using a 1:1000 dilution of rabbit polyclonal IgG (Santa Cruz Biotechnology). Bound antibodies were detected using a secondary antibody, horseradish peroxidase conjugated IgG (Santa Cruz Biotechnology), diluted at 1:20,000 in TTBS with 5% dry milk. Proteins were detected using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology). Densitometric analysis of Western blots were quantified using NIH Image 1.62.

NHE Assay Changes in intracellular pH (pHi) was determined using the pH-sensitive fluorescent dye 2',7' –bis (2-carboxyethyl)-5(6) carboxyfluorescein (BCECF). Cells were subcultured into T-150 flasks and allowed to grow to 70% confluence. 24 hours prior to experiments the cells were transferred into 50 ml spinner cultures in serum depleted media, DMEM high glucose media containing 0.5% serum, 0.5% fatty acid free BSA with antibiotics and antimycotics. BCECF was loaded into the cells by incubating them for 30 minutes at 37°C in buffer containing 10µM of the acetyoxymethylester form of the dye. pHi was then measured using the fluorescent intensity ratio method where the fluorescent intensity is measured at 525 nm while the dye is excited alternately at the pH sensitive wavelength of 501 nm and the isoexcitation wavelength of 439 nm. The pHi was calculated from the fluorescence intensity ratio using

a calibration plot of the fluorescence intensity ratio vs. pHi, which was obtained by the K-nigericin method. Fluorescence will be measured using a Spex Industries Fluoromax Luminescence Spectrometer.

Figure Legends

Fig. 3. Effect of tyrosine protein kinase inhibitors on LPA mediated phosphorylation of EGFR. 70% confluent, quiescent CCL39 fibroblasts were pretreated with the EGF receptor inhibitor AG1478 (1 μM), the Src family inhibitor PP2, (10 μM) or the platelet derived growth factor receptor inhibitor AG1295 (1 μM) prior to stimulation for 15 minutes. Where indicated, the cells were incubated with LPA (100 μM) for five minutes. The cells were rinsed once with PBS, lysed by five passes through a 27-gauge needle in RIPA buffer. EGFR was immunoprecipitated with an anti-EGFR-protein A/G agarose conjugate overnight at 4°C.. 30 μL of the sample was resolved by 8% SDS-polyacrylamide gel electrophoresis. The phosphorylation state of immunoprecipitated EGFR was determined by Western blot analysis using PY99.

Fig. 5. LPA-induced Activation of NHE1. Serum deprived, quiescent cells were dye-loaded with pH-sensitive fluorescence dye (BCECF) and suspended in Na buffer in a quartz cuvette for 120 seconds. The cells were exposed to LPA (100 μM) and the fluorescence intensity emission at 525 nm was measured for 280 seconds to determine the change in pH_i.

Figure 3. Agonist Stimulation of NHE1 and ERK in PKC Depleted CCL39 Fibroblasts. Steady-state pH_i was measured in cells that had been serum-deprived and treated with 500 nM PMA for 12–18 hours. Following the determination of the resting pH_i the appropriate agonist was added and the change in pH_i was determined. In parallel experiments, the ability of the different agonists to active ERK was also determined.

A. 100 μM LPA was added at t=110 s, after a steady-state pH_i of 7.01 was established. The LPA addition led to an alkalinization of pH_i to 7.22, an increase of 0.21 pH units.
B. 100 μM PE was added at t=100 s, after cells established a steady-state pH_i of 6.98. The PE addition led to an alkalinization of pH_i to 7.02, an increase of 0.04 pH units.
C. 100 nM PMA was added at t=200 s, after cells established a steady-state pH_i of 7.01. At t=1100 s, the pHi was 7.01, showing no increase in pH_i due to PMA addition.
D. Phosphorylation of ERK was determined in 70-80% confluent quiescent fibroblasts. The cells were then incubated for 5 minutes with 100 nM PMA or 100 μM PE. To induce the down regulation of PKC, cells were incubated for 24 hours with 500 nM PMA prior to PE addition. Following agonist treatment, the cells were rinsed with PBS and lysed in Laemmli sample buffer containing phosphatase inhibitors. 30 μL of each sample was resolved on a 12% SDS-PAGE and the phosphorylation state of ERK was determined by immuno-analysis.

Discussion

These data indicate for the first time in CCL39 fibroblasts that two different G-protein linked agonists, PE and LPA signal through ERK to regulate intracellular pH.

We found that the a_1-adrenergic receptor agonist PE can activate ERK in a time and concentration dependent manner.

- Maximal PE stimulation occurs at 100 μM PE
- PE mediates a quick ERK activation quickly and is down-regulated after 20 minutes

Addition of LPA also increased both ERK and NHE in a similar manner.

- Maximal LPA stimulation occurs at 1 μM LPA
- ERK activation is highest after 5 minutes and decreases after 60 minutes.

NHE exchange activity is increased leading to a significant rise in intracellular pH after addition of PE and an increase in pHi by the addition of LPA.

While the activation of both agonists has been established in several cell lines, this is the first demonstrated in CCL39 fibroblasts. Use of this particular cell line is important to study the role of ERK in pH regulation. NHE1 is the primary transporter in these cells leaving little question as to the mechanism of proton extrusion.

The precise role of ERK in activation of NHE by these two agonists is established by pharmacological treatment of the cells. Pre-incubation of the cells with the MEK inhibitor PD98059 as expected blocked the phosphorylation and activation of ERK. While it is not surprising that PE regulation of NHE1 is blocked by the MEK inhibitor it was unexpected that LPA activation of NHE1 was blocked by inhibition of the ERK pathway. Previous studies using both dominant negative MEK and other inhibitors, LPA (Tominaga[a] and Tominaga[b], 1998 and Hooley, 1996) did not seem to involve ERK. To further establish that ERK is involved in this pathway, addition of higher concentrations of anisomycin lead to a transient increase in both ERK and NHE1. While it can be argued that other MAPK isoforms are involved when such high concentrations of anisomycin is used, Pouyssegur showed that lower concentrations of anisomycin activated both p38 and JNK while neither of these kinases were found to be involved in NHE1 regulation

(Bianchini, 1997) In summary, the data presented here indicate that both the MAPK isoform ERK is critical in the regulation of intracellular pH for both G-protein linked agonists PE and LPA.