INVESTIGATION OF PROTEIN KINASE C ACTIVITY IN A POLYCYSTIC KIDNEY DISEASE MODEL

Polycystic kidney disease (PKD) is characterized by cyst formation that eventually leads to renal failure. Recent work has shown that PKD is associated with the loss of the structure/function of the primary cilia in cells. Cilia are long, narrow structures projecting from the apical membrane of polarized epithelial cells. Studies will be performed using duct cells from the Oak Ridge polycystic kidney mouse which lacks primary cilia cells (cilia[−]) vs control cells that possess normal cilia (cilia[+]). In cilia (−) cells, there is increased apical Ca^{2+} entry, resulting in an increased cytosolic Ca^{2+} concentration. It is generally known that elevations in Ca^{2+} concentration activate the enzyme protein kinase C (PKC), and that results in its translocation from the cytosol to the plasma membrane. This investigation tests the hypothesis that there is chronic activation of PKC in cilia (−) cells, and that elevated PKC activity results in a higher rate of cell proliferation in cilia (−) cells. Differences in cytosolic PKC protein levels between cilia (+) and cilia (−) cells will be determined using the protein kinase non-radioactive assay (Calbiochem). Variations in PKC activity will be determined after treatments with PKC activator phorbol 12-myristate 13-acetate (PMA), intracellular calcium chelator Bapta-AM, and protein kinase inhibitor Ro-32-0432. The effects of PKC on the rate of cell proliferation of cilia (+) and cilia (−) cells will be ascertained by cell counting with a hemacytometer during activation or inhibition of PKC activity. Immunofluorescence will be performed with a monoclonal antibody raised against conventional PKC isozymes.

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INTRODUCTION

Polycystic kidney disease (PKD) is the most common genetic disease affecting the kidney, and is characterized by the presence of multiple fluid filled renal cysts. Two primary types of PKD are autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD). ARPKD, a rarer form in which both parents carry the recessive gene, has symptoms that are usually apparent at birth/early infancy, and is often lethal. PKD has been shown to be associated with the loss of the structure/function of primary cilia in cells. Cilia are long, narrow structures projecting from the apical membrane of polarized epithelial cells that act as mechanosensory transducers. In cells with a cilia defect, such as the one noted in PKD, there is increased apical Ca^{2+} entry, via the protein channel polycystin-2, which results in an increased cytosolic Ca^{2+} concentration. Previous studies have shown that cells lacking primary cilia (cilia [−]) proliferate in a Ca^{2+} dependent manner. Cilia (−) cells have been shown to proliferate at a higher rate than normal (cilia [+]) cells, and when both cell types were treated with the Ca^{2+} chelator BAPTA-AM, there was a significant decline in the proliferation rate in cilia (−) cells. It is generally known that elevations in Ca^{2+} concentration activate the enzyme protein kinase C (PKC), and translocate it from the cytosol to the plasma membrane. PKC is responsible for the phosphorylation (chemical addition of a phosphate group) of side chain OH groups of serine and threonine on proteins. PKC consists of approximately 10 isozymes, which are broken up into three subcategories: conventional/classic PKCs, novel PKCs, and atypical PKCs. Our focus was on the conventional/classic type, which includes the isoforms PKCα, PKCβI, PKCβII, and PKCγ. We speculate that there is chronic activation of PKC in cilia (−) cells, and that elevated PKC activity results in a higher rate of cell proliferation in cilia (−) cells. Our studies will be performed using cells isolated from the cortical collecting duct (CCD) of an ARPKD mouse which lack primary cilia versus a control strain of cells that express normal cilia.

METHODS

Determination of PKC activity in the cytosolic fraction of cilia (+) and cilia (−) cells was performed by a non-radioactive protein kinase assay kit (Calbiochem®). The Calbiochem® kit utilized an ELISA based detection method. The kit comes with a 96-well plate coated with a peptide pseudosubstrate that can be phosphorylated by PKC. The protocol called for 10 million cells, so we made lysates of our two cell types and, in order to obtain the cytosolic fraction, we centrifuged our recently isolated cells at 100,000 × g for 60 minutes. We made our reaction mixture from 108 μL of the component mixture and 12 μL of our sample. We then transferred 100 μL of our reaction mixture to the peptide pseudosubstrate-coated plate. The PKC that was present in our sample would phosphorylate the substrate. We then incubated for 20 minutes at 25°C in a water bath, and added 100 μL of stop solution to each well. Then we washed the wells by

completely filling them with wash solution, inverting the plate, and removing the liquid in each well. We repeated this for a total of 5 washes. After completely removing the liquid from each well after the final wash, we added 100 µL of a biotinylated monoclonal antibody that recognizes the phosphorylated form of the peptide pseudosubstrate. We then washed the wells 5 more times as outlined before, and added 100 µL of horseradish peroxidase-conjugated streptavidin to each well and incubated at 25°C for 60 minutes. After the incubation, we washed 5 more times as outlined before and added 100 µL of our substrate solution. We then incubated at 25°C for 5 minutes and added 100 µL stop solution. Next we read the absorbance at 492 nm in a microplate reader, and the absorbance was directly proportional to the PKC activity. Immunofluorescence was performed with a monoclonal antibody raised against conventional PKC isozymes. Our two cell types were grown on permeable supports allowing them to differentiate. We treated our cells with an antibody labeled with a fluorescent dye that would bind to PKC. This was followed by imaging the cells mounted on slides with a confocal laser-scanning microscope. When the antibody was excited by lasers, it showed green. Then we treated each cell type with phorbol 12-myristate 13-acetate (PMA) which is a PKC activator, so that we now had, in addition to our controls, cilia (−) cells treated with PMA and cilia (+) cells treated with PMA. The permeable supports were cut out of the wells that suspended them, and were made into slides. We looked at our slides under the Leica SP5 confocal microscope and took pictures of apical views of our cells. Cell proliferation was assessed by cell counting with a hemacytometer. Our two cell types, cilia (−) and cilia (+), were grown in culture dishes. Both cell types were treated with the PKC inhibitor Ro-32-0432. We now had our cilia (−) control cells, our cilia (+) control cells, our cilia (−) Ro-32-0432 treated cells, and our cilia (+) Ro-32-0432 cells. First we made 3 microcentrifuge tubes of, 50 µL 1× PBS and 30 µL Trypan blue solution, for each cell sample to count. Cells were isolated using trypsin, media was added to each of the dishes, we resuspended everything, and then we transferred it to a conical tube. Then we vortexed our tubes and transferred 20 µL of our cell sample into each microcentrifuge tube. We vortexed each microcentrifuge tube before counting, and loaded both chambers of the hemacytometer with 10 µL of our cell sample. Cells that were not alive appeared blue due to uptake of trypan blue, and were not counted during the counting process. After we finished counting the three tubes of one cell sample, we had to find the average of the three tubes and plug it into the equation to find the number of cells that were in the dish. The equation is: Average # of cells counted from the 3 tubes divided by the # of boxes counted, all multiplied by the dilution factor, and all multiplied by 10,000.

RESULTS

From our Calbiochem® protein kinase assay kit, we knew that the color intensity was directly proportional to PKC activity. Our results showed that there was less color intensity in our cilia (−) cells compared to our cilia (+) cells. This meant that there was less cytosolic PKC in our cilia (−) cells in comparison to our cilia (+) cells. Our immunofluorescence imaging showed that without the treatment of the PKC activator PMA, the cilia (+) cells showed dim labeling at the cytoplasm. It showed that there was little membrane-associated PKC, which is what we are supposed to see in a cell expressing un-activated PKC. In our cilia (−) cells without PMA activation, however, we saw bright labeling which showed that there was more membrane-associated PKC. In both cell types, when we activated PKC with PMA, we saw brighter label. That suggested that the PKC had been activated and had been translocated to the plasma membrane, which is what we expected to see. Our cell counting showed that, in our control groups, the cilia (−) cells proliferate at a faster rate than the cilia (+) cells. When we treated our cell types with the PKC inhibitor Ro-32-0432, we saw a decreased rate of proliferation in our cilia (−) cells.

CONCLUSIONS

Our results using the Calbiochem® protein kinase assay kit and the immunofluorescence imaging suggested that PKC in cilia (−) cells had been activated and had been translocated to the plasma membrane. We therefore confirmed the first part of our hypothesis that there was chronic activation of PKC in cilia (−) cells. On the other hand, inhibition of PKC resulted in attenuation of the enhanced cell proliferation in cilia (−) cells. This provides indirect evidence supporting the second part of our hypothesis that elevated PKC activity results in a higher rate of cell proliferation in cilia (−) cells.

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RESOURCE

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