Research Article

Fluid flow generates delayed calcium current in renal epithelial cells *in vitro*

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ABSTRACT

Background: Primary cilium is the mechanosensor of the cell protruding from the apical side of almost all cells in the body. Primary cilia paly crucial role in regulating various cellular functions, while dysfunctional cilia play an important role in the pathogenesis of many diseases collectively known as ciliopathy. Due it cilium central role, it is vital to study what signal it generate to coordinate these events. **Methods:** In this experiment, renal epithelial cells used to study the signaling events of primary cilia. Genetically encoded calcium indicator (GECI) was used for a precise measurement of calcium ion concentration in real time experiment. In this technique, a single cell live imaging method was utilized with a lateral view of the cell. **Results:** Data revealed that fluid flowing over the cells leads to cilium bending and generates calcium ion peak in the cytoplasm. The calcium ion concentration begin to start after 0.54 second and peak concentration reported in 0.85 seconds after flow start. Depleting the medium and the flowing fluid from calcium by EGTA abolishes calcium peak. **Conclusion:** Renal epithelia cells respond to fluid flow by generating delayed calcium current and is dependent on extracellular calcium entry to cell.

KEY WORDS: Cilia bending, Primary cilia, Renal cyst

INTRODUCTION

Primary cilium is a specialized hair-like cellular organelle projecting from the apical side of the almost all eukaryotic cells. These tubules-based organelles were considered as the cell sensor of flow. When fluid is flowing over the cells, the cilium bends.[1-3] Cilium bending is the primary event that induces intracellular signals for the regulation of various cell functions. Many experimental cellular markers used as a probe for cilia signaling events, however, calcium ion concentration changes is the mostly studies and acceptable record. It is known that primary cilia housed two important proteins in this regard, polycystin 1 (PC1) and PC2. The widely acceptable theory is that cilium bending activates PC1, the mechanosensor compartment of cilium. PC1 interacts with other ciliary membrane proteins essential for flow sensing functions, PC2. Bending of cilium leads to conformational change in PC1 that will induce an activation of PC2.[4-6] The latter is a non-specific cation channel in the ciliary membrane, on activation opens and let calcium ion to enter into cilioplasm. As a summary, fluid flow will induce calcium current that translated into various cell functions. The importance of this signaling pathway appeared from studies documented dysfunctional cilia in many disease collectively known as ciliopathy. Defective ciliary structure/function results in defective ciliary-induced signaling pathway and defective regulation of the corresponding cellular functions. One of the mostly studied disorders is polycystic kidney disease (PKD). As ciliopathy, PKD resulted from dysfunctional primary cilia that lead to many clinical manifestations including cystic kidney, hypertension, cystic liver, and aneurysms.[7-10] Many studies suggest a vital role of primary cilia in the pathogenesis of renal cyst. Many studies measure calcium ion concentration using Fura-2 dye; however, in this study, a more precise way will be used to measure calcium ion concentration utilizing the genetically encoded calcium indicator (GECI) for more clear-cut analysis of calcium current.[11,12] Cell transfected with GECI and calcium ion concentration will be followed up in real-time live imaging method. In addition, we will examine the importance of extracellular calcium ion to the cilium-induced signal.

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MATERIALS AND METHODS

Cell Culture

Renal epithelial LLC-PK cells were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM, HyClone, USA) with 10% fetal bovine serum (HyClone, USA), and penicillin 100 U/ml and streptomycin 100 µg/ml (HyClone, USA) as described previously.[9]

Transfection

Transfection of LLC-PK cells performed with G-CaMP3, a genetically encoded calcium indicator (GECI) that has a green fluorescence. Transfection of cells done by JetPrime transfecting agent (PolyPlus, USA). Transfected cells cloned and selected by G418 (200µg/ml, Ameresco) and examined by fluorescence microscopy.[11,13]

Measurement of Calcium Ion

Cells were let to grow in DMEM on special polymer (Formvar) until the required confluency. Then, cells deprived of serum 24 h before examination to induce ciliation. At the examination time, formvar folded in a special way and cells mounted into examination flow chamber to be ready for fluorescent analysis. Cells in the chamber mounted in a way that allows the lateral view of a single cell. Flow chamber is custom made and specially designed which permits a laminar flow of the fluid over the mounted cells. Calcium ion concentration was measured by fluorescence microscope Nikon TiU incorporated with monochrome digital camera Coolsnap ES2, 12 bit, 20 MHz. The fluorescent signal analyzed by MetaMorph software (Molecular devices, USA). In the experiment of calcium free fluid, ethylene glycol tetraacetic acid (EGTA) (5 nM) added to the DMEM 10 min before examination to chelate all free calcium in the medium. In addition, EGTA is added to the flowing fluid used in this experiment.

Statistics

All experiments repeated at least 3 times. Values were presented as mean ± standard error mean. Calcium ion concentration values normalized by subtraction from their corresponding background values and plotted relative to their baseline values. Data analyzed using ANOVA with post hoc comparisons through Dunnet test. Statistically significant values represent $P < 0.05$. All data analyses were performed using GraphPad Prism v.5.

RESULTS

Calcium ion concentration measured in LLC-PK renal cells transfected with G-CaMP3 to follow up calcium ion changes in real time in response to fluid flow. Transfected cells were examined by fluorescence microscopy to confirm successful transfection [Figure 1a]. Transfected cells examined by fluorescence microscopy to confirm successful transfection [Figure 1a]. Results showed that the transfected cells comprises 94%±0.53 of cell population.

Calcium Ion Concentration in Response to Flow

When the transfected cells ready for the experiment, cells mounted in the specialized flow chamber in a way that viewing the cell from side as illustrated in [Figure 1b]. Precise measurement of calcium ion concentration in real time relative to fluid flow performed by differential interference contrast (DIC) and green fluorescence (GFP) at the same time. Fluid flow generated using pump that produce a shear stress of 1dyne/cm2 on the cell surface. Analysis of the data obtained from following up calcium ion concentration in the cytoplasm of the cell reported a peak concentration immediately after flow [Figure 2]. Once the flow begins, the measured calcium ion showed a peak calculated as 1.64 folds higher compared to baseline concentration significantly ($P < 0.05$). Exactly after the flow start, calcium ion concentration pattern

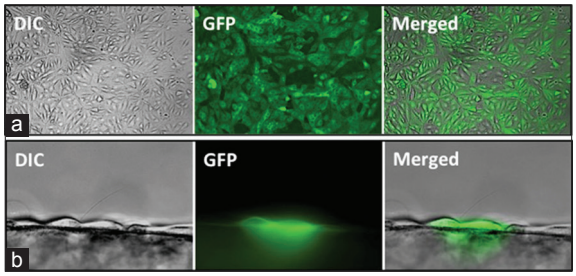

Figure 1: Images of transfected cell. (a) Images of LLC-PK cells transfected with G-CaMP3 (green) to confirm successful transfection; (b) Side view of the cell, green cell is transfected

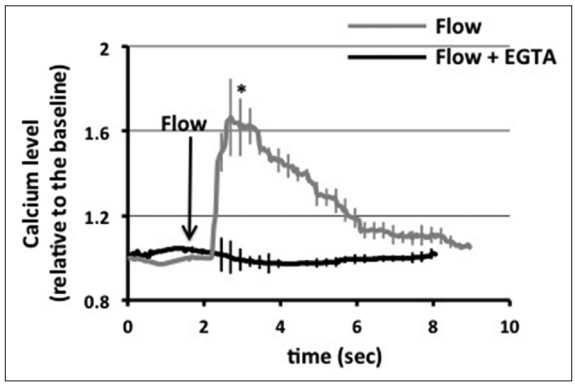

Figure 2: Representative plot of calcium ion concentration in cytoplasm relative baseline in real time in response to flow with shear stress of 1 dyne/cm^2. Cell challenged with flow and calcium ion concentration measured (grey line). Cells incubated with ethylene glycol tetraacetic acid 5 mM 10 min before and challenged with flow (black line). Values are mean ± standard error mean, $n = 3$. *Denotes significant difference ($P < 0.05$) relative to baseline values

begins to increase after 0.45 s from flow start point. The highest calcium ion concentration was reported in 0.85 s after flow start. In spite of continuous fluid flow, calcium ion concentration in the cytoplasm showed gradual decline until it reaches the baseline level. The time required to reach baseline level was measured as 5.1 seconds from the flow start point [Figure 2].

Calcium Ion Concentration in Response to Flow with EGTA

This experiment is done exactly as the above described one, except the Dulbecco's Modified Eagle Medium depleted from calcium by ethylene glycol tetra-acetic acid (EGTA 5 mM). This part was performed to test the role of extracellular calcium ion in ciliary-induced calcium signal in response to flow. After the pump forces the calcium-free fluid over the cells with shear stress of 1 dyne/cm^2 on the surface of cells, calcium ion concentration was measured. Result obtained revealed that the flow could not generate calcium current in cells deprived from extracellular calcium ion [Figure 2]. This is very important report, as it clearly indicated that the initiation of flow-induced calcium ion current is dependent on extracellular calcium ion availability and not on intracellular calcium stores.

According to the above-mentioned experiments, calcium ion peak was recorded in the cytoplasm after flow, and this current is dependent on extracellular calcium ions.

DISCUSSION

In this study, we used an advanced method to measure calcium ion concentration directly and precisely in real time using G-CaMP3 calcium ion probe, which is superior to other chemical dyes like Fura-2 that could disturb some living cell functions.[11,14] Another advances in this method are that we could capture a single cell in the lateral view to track calcium ion concentration. As primary cilia play an essential role in health and disease, thus studying the signaling of this vital organelle is of crucial importance. Primary ciliary function could be recorded using calcium ion concentration. Defective ciliary functions result in defective calcium ion concentration, and thus, it is essential to follow-up this important signal as a record for proper ciliary function.[15]

Reviewing the data obtained from the present experiments, we revealed clearly that fluid flowing over renal epithelial cells generates a specific peak of calcium ion concentration. This calcium signal peak reported with delay of about 0.45 s from the flow start. This result implies that an initial event required before the true calcium signal triggered.[16] This initial event might be initial calcium influx in the cilioplasm immediately after cilium bending.[14,17] The later signal which begins in the cilia may then induce the reported calcium peak in the cytoplasm. This suggestion is further augmented by the second part of the present experiment in which EGTA is used to chelate free extracellular calcium ion. The peak of calcium signal recorded about 1.64 and the baseline calcium level may imply a role of the intracellular stores in this reported rise. However, this will not contradict our suggestion. The result could be explained by the idea that the flow-induced calcium signal initiated in the cilia by extracellular calcium influx and this will trigger cytoplasmic calcium peak folds higher than baseline and delayed by about 0.54 s. This calcium ion pattern recorded in this study is compatible with the suggestion that flow induces cilium bending and triggers calcium ion influx into the cilia.[14,18-20] This mainly occurs through the activation of PC1 and opening of PC2. The entrance of small amount of calcium into the cell from extracellular source will trigger the opening of intracellular calcium stores and generate calcium current eventually.

CONCLUSIONS

In summary, renal epithelial primary ciliary bending with the flow produces a definite pattern of calcium ion current in terms of peak and time. Calcium ion peak in response to flow appeared to be delayed and dependent on extracellular calcium entry.

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