Molecular Physiology

Mechanically induced integrin ligation mediates intracellular calcium signaling with single pulsating cavitation bubbles

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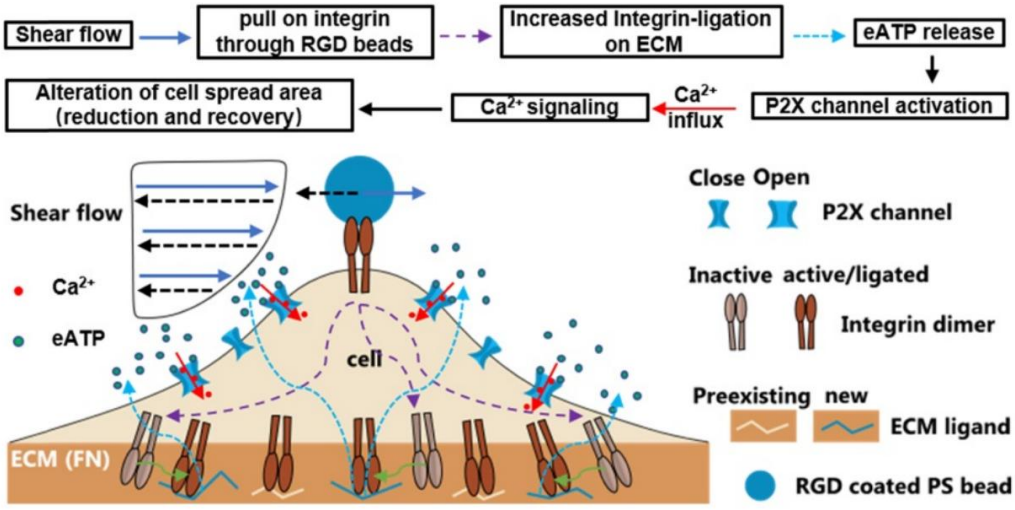
Abstract: Therapeutic ultrasound or shockwave has shown its great potential to stimulate neural and muscle tissue. Accumulating evidence suggest that cavitation microbubble serves as an efficient media transducing the mechanical force to Ca^{2+} influx within neurons. However, the underpinning mechanism is largely unknown, which prohibits the optimization and promotion of its use in non-invasive neuronal regulation. The authors use laser-induced cavitation microbubble to stimulate individual HEK293T cells either genetically knocked out or expressing Piezo1 ion channels with different normalized bubble-cell distance and then identified key molecular players in the bead-enhanced Ca^{2+} response: increased integrin ligation by substrate ECM triggered ATP release and activation of P2X — but not Piezo1 — ion channels. The resultant Ca^{2+} influx caused dynamic changes in cell spread area. This approach to safely eliciting a Ca^{2+} response with cavitation microbubbles and the uncovered mechanism by which increased integrin-ligation mediates ATP release and Ca^{2+} signaling will inform new strategies to stimulate tissues with ultrasound and shockwaves.



Mechanotransduction, the process of converting mechanical forces into biochemical signals, plays a critical role for cells to sense their physical environment and adjust their function accordingly. Intracellular Ca^{2+} is one of the earliest events downstream of mechanotransduction, for example under quasi-static cell loading [1],[2], which regulates myriad cellular processes including exocytosis, contraction, transcription, and proliferation.

Three mechanisms are commonly observed in shear-induced mechanotransduction at single-cell level: 1) activating mechanosensitive ion channels, such as Piezo1 [3]; 2) triggering the release of ATP that activates purinoreceptors (P2X) [4]; and 3) ligating integrins by extracellular matrix (ECM) proteins [5],[6]. The first two mechanisms involve Ca^{2+} influx through ion channels; the third mechanism involves formation of focal adhesions (FAs) on the basal surface of cell and integrin-mediated signaling. Quasi-static loading of integrin-bound beads on the apical surfaces of cells can remotely alter basal focal adhesions via integrin-cytoskeleton interactions [7]. In this work they aim to elucidate the determinants of intracellular Ca^{2+} responses induced by impulsive shear flow from single cavitation microbubbles (SCBs).

First, they want to dissect out which one or combination of aforementioned three mechanisms are recruited to mediate microbubble-induced Ca^{2+} signal. They used HEK293T cells with Piezo1 genetically knocked out (P1KO) or transiently transfected (P1TF) to prove Piezo1 does not contribute to Ca^{2+} responses elicited by SCB-induced impulsive shear flow. They treated the cells with integrin-binding RGD-coated (RGD is widely used to bind integrins) microbeads together with antibodies that block integrin ligation or with a P2X purinoreceptor inhibitor to dissect which of these previously-mentioned molecular players are involved. And found that the RGD beads were required to enhance the mechanical coupling and elicit a Ca^{2+} response without membrane poration. Then they established that the cellular mechanical sensing induced by microbubbles is mediated by increased integrin ligation, which leads to release of extracellular ATP (eATP) and subsequent activation of P2X channels, resulting in Ca^{2+} entry and downstream dynamic cell spreading.

As bubble may cause cell membrane poration, they first studied the bubble-cell system alone without RGD microbeads. They find that for both P1KO and P1TF cells, distinct Ca^{2+} responses were observed depending on the cell injury. Fast Ca^{2+} responses with a short rise time and a large amplitude change were accompanied by Propyl iodide (PI) uptake associated with membrane poration. PI is a small fluorescent molecule that binds to DNA but cannot passively pass through cells with an intact plasma membrane. The relationship between PI uptake and rejection can be used to distinguish whether there is damage to the cell membrane. Slower and milder Ca^{2+} responses were accompanied by negligible PI uptake (little or no membrane poration). Since there was no significant difference in Ca^{2+} response between Piezo1 knockout and Piezo1-expressing cells, Piezo1 does not contribute to Ca^{2+} responses elicited by SCB-induced impulsive shear flow.

Next, they explored the effects of treating the cells with 6-μm diameter RGD-coated polystyrene microbeads. RGD is widely used to bind integrins. In this way, the local resistance enhanced by the microbeads on the cell apical surface can be transmitted into cells through the RGD-integrin link. Again, no significant difference in Ca^{2+} response between Piezo1 knockout and Piezo-expressing cells was observed, indicating Piezo1 is not involved in the Ca^{2+} response.

To discern the processes that initiated the Ca^{2+} response to SCB with RGD beads, they examined spatiotemporal changes in Ca^{2+} signaling within P1KO cells at normalized distances causing a non-injury response. Next, they segmented the cell into three radial regions of interest (ROI) and measured the Ca^{2+} response. The outermost ROI reached 50% of its maximum Ca^{2+} response earlier than the center ROI (~ 7.2 s versus ~ 12.5 s), with the Ca^{2+} wave propagating from the edge to the center of the cell at ~ 2.3 μm/s . This speed is faster than the diffusive rate of Ca^{2+} in cell , indicating the Ca^{2+} wave propagated in the cytosol via a reaction-diffusion process from the endoplasmic reticulum. They examined whether integrin binding was required on the cell apical surface by comparing Ca^{2+} responses of P1KO cells exposed to beads coated with either RGD or BSA. BSA-coated beads non-specifically bind cell membranes but experience the same drag as RGD-coated beads. The Ca^{2+} response was significantly reduced in cells when using BSA beads, indicating that integrin-specific bead attachment was vital to the Ca^{2+} response. No Ca^{2+} response was observed when using Ca^{2+}-free medium with RGD beads, indicating that the increased cytosolic Ca^{2+} was dependent upon extracellular Ca^{2+} entry.

Finally, they investigated whether new ligation of integrins at the cell-ECM substrate interface is necessary to initiate the Ca^{2+} response. After P1KO cells had spread on fibronectin-coated dishes, the fibronectin-specific antibody 16G3 was added to block any new integrin αvβ3 and α5β1 binding sites. In this way, the cells could only maintain previously established integrin-ECM connections during SCB treatment. A non-blocking fibronectin-specific antibody, 13G12, was used as a control. 42% of P1KO cells showed a non-injury Ca^{2+} response with RGD beads. Extracellular Ca^{2+} influx is essential in these responses, as shown by using Ca^{2+}-free medium. Suggesting that not only integrin ligation but also extracellular Ca^{2+} entry is required to get Ca^{2+} response. They hypothesized that the P2X channel was vital for the extracellular Ca^{2+} influx. P2X channels were responsible for shear stress-generated Ca^{2+} waves that propagated from the edge to the center of rat atrial myocytes. So they add the P2X channel-specific blocker pyridoxalphosphate-6-azophenyl-2',5'-disulfonic acid , which eliminated the Ca^{2+} response.P2X is an ATP-gated channel. They depleted eATP with apyrase (an ATP-diphosphatase) and found that the Ca^{2+} response was completely suppressed, reinforcing the hypothesis that P2X activation mediates Ca^{2+} influx following SCB treatment. They next established the causality between integrin ligation and eATP release, as both are required for the non-injury Ca^{2+} response. If integrin ligation is downstream of eATP release, the Ca^{2+} response following eATP stimulation should be affected by blocking integrin-ECM binding sites with 16G3 antibody. They observed a dose-dependent increase in the Ca^{2+} response with ATP-γ-S treatment. However, there was no significant difference in the Ca^{2+} response between 16G3- and 13G12-treated cells stimulated with ATP-γ-S, suggesting that integrin ligation is upstream of eATP release. And they suggest the following model: SCB-induced shear force is exerted on integrins on the cell apical surface by pulling on RGD-coated beads, causing new ligation of integrins by ECM proteins at the cell basal surface, which in turn triggers cellular release of eATP, which opens P2X ion channels, allowing Ca^{2+} influx that regulates a dynamic change in cell spreading.

To summarize, there results establish that applying forces to integrins and activating ATP-gated P2X ion channels is an efficient approach to elicit non-injury Ca^{2+} response in cavitation bubble-generated impulsive shear flow. These results also provide insight into both the mechanism of cavitation bubble-mediated cell stimulation and strategies for improving therapeutic ultrasound applications in tissue stimulation.

References

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