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Cellular biophysical dynamics and ion channel activities detected by AFM-based nanorobotic manipulator in insulinoma β -cells

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Abstract

Distinct biochemical, electrochemical and electromechanical coupling processes of pancreatic β -cells may well underlie different response patterns of insulin release from glucose and capsaicin stimulation. Intracellular Ca^{2+} levels increased rapidly and dose-dependently upon glucose stimulation, accompanied with about 3-fold rapid increases in cellular stiffness. Subsequently, cellular stiffness diminished rapidly and settled at a value about 2-fold of the baseline. Capsaicin caused a similar transient increase in intracellular Ca^{2+} changes. However, cellular stiffness increased gradually to about 2-fold until leveling off. The current study characterizes for the first time the biophysical properties underlying glucose-induced biphasic responses of insulin secretion, distinctive from the slow and single-phased stiffness response to capsaicin despite similar changes in intracellular Ca^{2+} levels. The integrated AFM nanorobotics and optical investigation enables the fine dissection of mechano-property from ion channel activities in response to specific and non-specific agonist stimulation, providing novel biomechanical markers for the insulin secretion process.

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Keywords

Ion channel activities; AFM nanorobotics; cellular stiffness; insulinoma β -cells

BACKGROUND

The molecular mechanism of insulin secretion from pancreatic β -cells in times of elevated glucose levels has been well documented over the past decade. Insulin is produced by β -cells to maintain glucose homeostasis. When the extracellular glucose level rises, glucose will be transported to the cytoplasm through the glucose transporter GLUT2 (Fig. 1A) [1]. The intracellular glucose level rise will increase the pace of glucose metabolism resulting in increased ATP/ADP ratio inside the β -cell [2] followed by the closure of the ATP-sensitive K^+ channel (K_{ATP}) [3]. The closure of K_{ATP} channels causes the increase in plasma membrane (PM) potential [4] and cellular depolarization, a process leading to activation of voltage-dependent calcium channels (VDCC). Activation of VDCC allows Ca^{2+} to flow into the cytoplasm and gives rise to the intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) [5]. In response to the elevated $[Ca^{2+}]_i$, insulin-containing vesicles will fuse with plasma membrane to release the insulin [6]. The whole process is referred to as the stimulation-secretion coupling, a biphasic process in nature. The initial 5–10 minutes with the rapid insulin exocytosis is referred to as the first phase [7], while the subsequent second phase is characterized by a sustained insulin exocytosis, an ATP-dependent process which releases insulin-containing vesicles in reserve pools further away from the releasing site [8].

Capsaicin, the pungent component of chili peppers, is a specific agonist of the transient receptor potential vanilloid type 1 (TRPV1) channel or the vanilloid receptor [9]. TRPV1 receptor protein is expressed in nerve fibers innervating the pancreatic islets of rats and mice [10]. It has also been reported that TRPV1 expression is detected in rat insulinoma cell lines, RIN and INS-1 [11, 12]. Capsaicin may modulate insulin secretion through activation of capsaicin-sensitive afferent nerves [13]. Additionally, capsaicin may stimulate islet β -cells by binding to the intracellular domain of TRPV1 and thereby activating TRPV1 channels [14, 15] as illustrated in Fig. 1A. Thus the regulatory role of capsaicin on insulin secretion may be direct or indirect, a complex effect which remains to be defined. In comparison with glucose stimulation, capsaicin stimulated insulin secretion from islet β -cells is reported to be a sustained process [12] without the biphasic characteristics.

Atomic Force Microscopy (AFM) was invented as a high resolution imaging instrument [16], using a cantilever with a sharp tip at its free end to probe the surface topography of various materials. It operates the cantilever to move across the sample surface in raster scan motion. The interaction force, mainly van der Waals forces, between the scanning AFM tip and the sample surface would bend the cantilever. The bending can be measured by a laser beam reflected back from the cantilever by readings of the laser spot position. AFM works perfectly well in liquid environments where physiological conditions can be maintained for living cells, holding an unparalleled advantage over other high resolution imaging techniques including electron microscopy. Obtaining nanometer resolution images of cell membranes, DNA molecules and other biological structures have become possible with recent development of AFM systems [17]. In addition to imaging, AFM is a natural nanoindenter, a feature that can be employed to probe material mechanical properties. In nanoindentation mode, AFM drives the tip to move in contact with the cell membrane and consequently to deform the membrane. By evaluating the indentation force and the indentation depth, mechanical property of the cell membrane may be obtained. The process will generate force-displacement curves. A Hertzian model is often used to fit the curve and to generate the Young's modulus value [18, 19].

Utilizing the AFM tip as a robotic arm, the capability of AFM has been extended greatly, enabling a new field of nanorobotic investigation. AFM based nanorobotics has had great success in nanomanufacturing, including nanolithography and nanoassembly [20]. More recently, AFM nanorobotics has been applied for investigation of cellular and molecular properties and processes, including using a functionalized tip for biochemical force sensing and for surface receptor mapping [21], twisting DNA molecules [22]; deforming or poking single cells [23], and stretching isolated intermediate filaments [24]. We were able to use the AFM based nanorobotic system to position the AFM tip to the desired locations with a joystick to facilitate the nanoindentation of cells (Fig. 1B) [20].

Moreover, AFM has been applied to study biophysical transformations for a number of biological events, including cellular adhesion loss [17], cell apoptosis [25] and cell metastasis [26]. In these events, cells exhibited distinct mechanical properties as a result of reorganization of membrane structure and cytoskeleton under altered physiological and pathological conditions. Leveraging on the high resolution imaging and high sensitivity force measurement capability, AFM serves as an ideal instrument for investigations involving nanoscale structural and force changes. We reported previously about AFM nanomechanical analysis of islet β -cells in response to glucose and capsaicin stimulation, which may serve as a unique biomarker for insulin secretion process [27]. With the unique capability of AFM based nanorobotic manipulator, the current study went a step further and combines optical microscopy and AFM to unravel the ion channel dynamics in insulin release upon glucose and capsaicin stimulation. The ion channel activity was monitored by capturing fluorescence images of intracellular Ca^{2+} levels upon stimulation. Simultaneously, cellular stiffness was measured in real time with AFM nanoindentation to reveal the reorganization of the cell membrane and cytoskeleton resulting from ion channel reconfiguration.

METHODS

AFM based Nanorobotics for force displacement

The AFM based nanorobotics was developed in our lab as a series of hardware and software upgrade was performed on the original AFM. The Bioscope AFM (Bruker Nano, inc., Santa Barbara, CA) was equipped with Signal Access Module (SAM) which provides all the signals required for external control of the AFM tip position. A Linux RATI framework based closed-loop controller was developed to realize the real-time high frequency manipulation operation with DAQ cards as interface. The joystick would serve as command input where all motion starts so that the AFM tip follows the operators' lead instead of predefined trajectory. Moreover, the force feedback from the joystick makes it an ideal augmented reality environment. With the AFM based nanorobotics, the force measurements could be carried out with ease, especially when statistical analysis requires a large number of measurements. More importantly, it will guarantee a higher degree of spatial and temporal accuracy and sensitivity.

In the AFM nanoindentation experiment, a conically-shaped AFM tip with a silicon nitride cantilever (Bruker Nano, inc., Santa Barbara, CA) was used, the spring constant of which is 0.06 N/m, calibrated using thermal tune method. The applied force can be calculated by Hooke's law as $F = kd$, where d is the deflection of the cantilever and k is the spring constant of the cantilever. According to Hertzian model [18, 19], for a conically-shaped tip,

the relation between indentation and deformation defines: $F = \frac{2}{\pi} \frac{E}{1 - \nu^2} \delta^2 \tan \alpha$, where α is the half open angle of the cone-shaped tip, ν is the Poisson ratio, F is the applied force, δ is the indentation depth and E is the Young's modulus value. Young's moduli can be generated

by fitting the force displacement curves. The half open angle of the tip is 17.5° and we use 0.5 as Poisson ratio for incompressible material.

Cell culture

The insulinoma β -cell line RINm5f cells (ATCC, Manassas, VA) were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum and 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere containing 5% CO_2 . The RINm5f cells used were between passages 20 and 30.

TRPV1 immunofluorescence

For immunoreaction, RINm5f cells were seeded on glass-bottom petri dishes (MatTek Co., Ashland, MA) coated with poly-L-ornithine and incubated under standard conditions until they were 90% confluent. Cultured cells were then processed for immunofluorescence. After three washes with phosphate buffered saline (PBS), cells were fixed by incubation in 4% (v/v) paraformaldehyde in PBS for 15 min at room temperature, rinsed with PBS and incubated overnight at room temperature with anti-TRPV1 antibody, goat polyclonal IgG diluted 1:250 with 1% (w/v) bovine serum albumin (BSA) in PBS (Santa Cruz Biotechnology, Santa Cruz, CA). Control samples were incubated with 1% BSA in PBS. After three washes in PBS, fluorescence was revealed by incubation for 2 h in AlexaFluor[®] 488 labeled secondary antibody, donkey anti-goat IgG diluted 1:100 (Invitrogen, Carlsbad, CA) in PBS. Imaging of immunofluorescence was performed with an epifluorescence microscope equipped with the Nikon B-2A filter (excitation wavelength 450–490 nm). Images were acquired using a digital camera (Photometrics, Tucson, AZ) connected to the microscope and image was captured using software QCapture Pro 6.0.

Loading of Fura-2 AM to RINm5f cells

Fura-2 AM (Invitrogen, Carlsbad, CA) was diluted in the low glucose base medium (2 mM glucose + 1mM CaCl_2 + 140 mM NaCl + 2.8 mM KCl + 2mM MgCl_2 + 10 mM Hepes-NaOH) to a final concentration of 0.5 μM . The RINm5f cells were rinsed with PBS three times. One ml of diluted Fura-2 AM ester solution was added to the dish and incubated for 30 minutes at 37°C . The cells were then washed with PBS three times and then incubated in the low glucose base medium for additional 30 minutes to allow complete de-esterification of intracellular AM esters.

Glucose and capsaicin stimulation and dosage-dependent study

All the experiments were performed at room temperature. Before each glucose stimulation experiment, the cells in the glass-bottom petri dishes loaded with Fura-2 AM were incubated with 5 ml of low glucose base medium (2 mM glucose, 1mM CaCl_2 + 140 mM NaCl + 2.8 mM KCl + 2mM MgCl_2 + 10 mM Hepes-NaOH) for additional 30 minutes until equilibrium. For glucose stimulation, a volume of 40 μl of high glucose medium (2M) was added into the dish to make the final glucose concentrations at 16 mM; a 40 μl of low glucose base medium was added to the control plates. Similar with glucose stimulation, the cells in the glass-bottom petri dishes loaded with Ca^{2+} binding agents were incubated with 5 ml of low glucose base medium for additional 30 minutes until equilibrium before capsaicin was applied. One ml of low glucose base medium with capsaicin (Sigma-Aldrich, St. Louis, MO) concentrations of 100 nM was added to the dish to obtain the final concentrations of 16.7 nM; One ml of low glucose base medium was added to the control plates.

Dosage-dependent experiments for glucose and capsaicin stimulation were carried out in the similar method described above. Dosages of 40 mM, 20 mM and 16 mM for glucose and 1.67 nM, 16.7 nM, 167 nM and 1.67 μM for capsaicin were examined with AFM analysis.

One ml of low glucose based medium was used as control. For glucose stimulation, the stiffness calculated from the first 20 force curves before switching to high glucose were averaged as the baseline. The moduli from the first 20 minutes of measurements after addition of high glucose medium were averaged as the positive results. For capsaicin, 100 cells were randomly selected before the stimulation for base line measurement and another 100 cells 20 minutes after the stimulation on the same cell population, stiffness was measured and compared statistically. To test the specificity of capsaicin stimulation, the TRPV1 was blocked with 1.0 μ M capsazepine (Capz) 30 minutes prior to the capsaicin stimulation. Each data represents a minimum of three independent experiments.

Simultaneous fluorescence imaging of $[Ca^{2+}]_i$ and AFM measurements on RINm5f cells

Before adding glucose or capsaicin, 10 force displacement curves were taken as the baseline on the center of the same cell. After stimulation, force displacement curves were taken at a frequency of 1 Hz to dynamically monitor the stiffness change in real time for an hour. To obtain higher sensitivity and to prevent potential damage of the sharp AFM tip to the delicate cell membrane, force applied to indent the cell was kept minimal. The maximum force applied is controlled at below 10 nN with the resulted deformation less than 100 nm, and all the force curves are taken at a loading rate of 1 Hz, corresponding to a tip velocity of 5.98 μ m/s. At the same time, the cells loaded with Fura-2 AM was observed under the inverted microscope with excitation wavelength 340–380 nm (UV-2E/C Nikon filter). Images were captured in a similar fashion as in the immunofluorescence for TRPV1 except at a much shorter exposure time (0.3 sec). The time-lapse images taken at 1 frame/s were then processed with ImageJ software (NIH) to obtain the mean grayscale values. The images were displayed in 8 bit grayscale (0–255) and each cell in each image was outlined and from which the grayscale value was obtained, averaged and normalized. Each data represents a minimum of three independent experiments

Actin filament staining and imaging

Cells grown on glass coverslips were allowed to pre-incubate in 1 mL of the PBS at 37 °C for 1 h. After pre-incubation, the buffer was removed and replaced with either 16 mM of glucose or 16.7 nM of capsaicin; the low glucose base medium was used for the control samples. After various reaction times (0 min, 1 min, 5 min and 10 min) at 37 °C, cells on glass coverslips were fixed/permeabilized in a solution containing 0.1% Triton X-100 and 3% paraformaldehyde in PBS buffer for 20 min. They were then washed three times by PBS buffer with 1% BSA. The resulting cells were stained with CF488A phalloidin conjugate (Biotium, Inc., Hayward, CA) in PBS with 1% BSA at room temperature for 20 min. Then the excess phalloidin solution was removed by rinsing with 1% BSA in PBS. Coverslips with stained cells were imaged with an inverted fluorescence microscope (Photometrics, Tucson, AZ). Images were processed with the use of QCapture Pro 6.0 software.

RESULTS

Immunofluorescence of TRPV1 on RINm5F cells

Immunofluorescence staining of RINm5f cells with antibody selective for TRPV1 receptors revealed the positive fluorescence signals, primarily distributed peripherally of the cells (Fig. 2A), consistent with the notion that TRPV1 receptors are localized in the cell membrane of RINm5f cells [12]. The control samples incubated with 1% BSA in PBS show negative immunoreactivity (Fig. 2B). The result confirms that RINm5f cells express TRPV1 receptors on their cell membrane.

AFM stiffness observation of dosage responses to glucose and capsaicin stimulation

The normal Young's modulus value for insulinoma RINm5f cells in the low glucose medium was around 7.0 kPa, a similar value as previous reports by AFM based studies [28]. High glucose increased the stiffness, and the increase displayed a dose-dependent pattern (Fig. 2C). The modulus increase for 40 mM glucose stimulation was more than one fold, reaching 14.8 kPa. Stiffness increased to 13.8 kPa and 12.0 kPa for 20 mM and 16 mM, respectively. The control experiment with an equal volume of low glucose medium showed no significant changes in stiffness. Thus cellular stiffness in response to various doses of glucose stimulation is dose-dependent. The dose studies for capsaicin stimulation showed a different pattern compared with glucose. Statistical analysis showed that stiffness increased for lower concentrations of capsaicin at 1.67 nM and 16.7 nM but not at higher concentrations of 167 nM and 1.67 μ M (Fig. 2D). These data support the finding [11] that the high dose of capsaicin had insignificant effects on insulin secretion, possibly due to desensitization of TRPV1 induced by high concentrations of capsaicin [29]. The control samples and the samples treated with Capz, an antagonist of TRPV1, showed no significant change of mechanical property (Fig. 2D). Taken together, these results indicate that increases in cell stiffness are linked to insulin secretion, and the nanomechanical properties may well serve as biomarkers for insulin secretion.

$[Ca^{2+}]_i$ and stiffness observation of glucose stimulation

To look at the activities of ion channel and cellular mechanical property under stimulation, real time observation was performed on a single RINm5f cell. The normal Young's modulus value for the individual RINm5f cell was around 7.0 kPa for the 10 minutes baseline measurement before the addition of high glucose medium to make the final glucose concentration 16 mM in the dish. Immediately following the glucose stimulation at 16 mM, altered ion channel activity was indicated by the sharp increase by about 20% in light intensity of $[Ca^{2+}]_i$ imaging (shown in Fig. 3A). The light intensity remained at a relatively higher level for 3–4 minutes before it dropped to the baseline. It is worth mentioning that the initial slight drop of light intensity was probably due to the photo-bleaching effect, and for the same reason, the imaging capture stopped at minute 20 when the light intensity was bleached to the level below the baseline. The fluorescence images of RINm5f cells at minute 10 (before stimulation), 12 (plateau was reached) and 20 (returns to baseline) were shown in Figs. 3C, D and E. At the same time, the cellular stiffness increased sharply as well, and it took about 5 minutes for the stiffness to reach a plateau at around 18.0 kPa. This was followed by a rapid decline at a similar rate as the climbing. About 10 minutes post stimulation, the stiffness dropped to a value around 10.5 kPa, about 1.5 times the baseline value and leveled off through the rest of the observation. Note that in Fig. 3B, a close up look of the stiffness and light intensity measurement 10 minutes prior and 10 minute post stimulation showed a 2-minute lagging of mechanical property changes when compared with the ion channel activities. The nanomechanical analysis results were echoed by the changes of cytoskeleton structures. The staining of actin showed a disorganized filament structure one minute after glucose stimulation while the control displayed no visible changes shown in Fig. 6.

$[Ca^{2+}]_i$ and stiffness observation of capsaicin stimulation

Upon capsaicin stimulation at 16.7 nM, the light intensity increased immediately (Fig. 4A). Lack of altered light intensity was observed for samples treated with Capz 30 minutes prior to capsaicin stimulation (Fig. 4B). A pulse-like response lasted approximately 3–5 minutes before it dropped to the baseline. The images corresponding to minute 10, 12 and 20 were shown in Fig. 4C, D and E. The Ca^{2+} influx response after capsaicin treatment experienced a similar pattern as that of glucose. The baseline value of stiffness for the cell population under capsaicin stimulation is similar to the one in glucose experiment. However, no rapid

increase of stiffness was observed following the administration of capsaicin. Instead, the stiffness increased gradually, reached the peak at approximately 11.0 kPa about 20 minutes after the stimulation. This sustained stiffness response stopped after the plateau was reached and remained at that value for the rest of the observation. Similar change of actin filament structures was observed but with a distinctive temporal pattern; the structural disorganization didn't start to surface until after 10 minutes of capsaicin stimulation as shown in Fig. 6.

DISCUSSION

It is well documented that increased glucose concentration in the extracellular environment would lead to increases in plasma membrane potential, elevated $[Ca^{2+}]_i$ and ultimately insulin secretion, a process commonly referred to as stimulus-secretion coupling [30–33]. Studies have also shown that capsaicin is an effective compound stimulating insulin secretion from insulinoma β -cells including RINm5f and INS-1 cells [11, 12]. Although glucose and capsaicin have been shown to induce distinctive patterns of insulin release, i.e., a biphasic secretion response upon glucose but not capsaicin stimulation [7, 8, 34, 35], the biophysical characteristics of cells in response to these stimuli have not been well defined. The present study aimed to study the temporal relation of ion channel activity, cell membrane biophysical dynamics, and cytoskeleton reorganization of β cells in real time with AFM nanoindentation to reveal potential mechanisms underlying distinct insulin-secretion processes in response to glucose and capsaicin.

The results from the present study using AFM based nanomechanical analysis show for the first time that the β -cells biomechanically respond to glucose stimulation in a biphasic manner, consistent with the pattern of insulin release. The cellular stiffness increases rapidly and then decreases sharply within 10 minutes of glucose stimulation, followed by a sustained second phase to maintain the cellular stiffness at a relative higher level than the baseline. The biphasic pattern of cell stiffness in response to glucose stimulation is distinct from that induced by capsaicin (fitted curve showing the overall trend in Fig. 5). Capsaicin induces a sustained and slow increase of cellular stiffness without a rapid increase or decrease pattern, a change consistent with that of insulin secretion in response to capsaicin. Although it is known that biophysical changes may result from reorganization of cytoskeleton structure to facilitate the transport and fusion of insulin-containing vesicles with the plasma membrane [34–36], the nature of fine changes in cellular structure and mechanical property presents a daunting challenge for the detection and measurement techniques. The present study shows that biophysical markers captured by AFM based nanomechanical analysis may be used as indicators for altered ion channel activities and thereby insulin secretion.

The initial phase of sharp increases in stiffness in response to glucose stimulation overlaps temporally with altered ion channel activities that result in elevated intracellular Ca^{2+} concentrations, with a few minutes of delay in stiffness to reach the peak. These results indicate that structural changes in the cell membrane or cytoskeleton follow the rapid responses of ion channels activated by stimuli. In contrast, the increases in stiffness in response to capsaicin stimulation do not coincide temporally with increased intracellular Ca^{2+} concentrations, with a long delay in stiffness to reach the peak. These results indicate that structural changes in the cell membrane or cytoskeleton in response to TRPV1 activation induced by capsaicin or to glucose are dissimilar and agonist-specific albeit the changes in $[Ca^{2+}]_i$ concentrations showed similar pulse-response patterns for capsaicin and glucose. The results of immunocytochemistry study of actin filament concur with the biophysical measurements obtained with AFM examination. As seen in Fig. 6, the treatment of glucose and capsaicin causes time-lapse changes of cytoskeleton especially actin filament

structures with distinct patterns. A delayed disorganization of actin around 10 minutes after stimulation was observed in capsaicin treatment, whereas glucose triggered the disorganization 1 minute after treatment, similarly with the biophysical data obtained by AFM nanomechanical analysis.

The biphasic model of insulin secretion from pancreatic β -cells is well established and can be elicited by several compounds including glucose, arginine, and protein kinase C activators[37, 38]; under certain conditions, the first phase of insulin secretion may appear alone, without the second phase[38]. Until recently, there was no compound or treatment condition that elicited the second phase only. Our data shows that the initial calcium influx induced by glucose or capsaicin is similar with a sharp increase in $[Ca^{2+}]_i$, but it diverges hereafter. While a signaling cascade situated between altered $[Ca^{2+}]_i$ and insulin secretion induced by capsaicin has yet to be fully elucidated, our data from cell stiffness changes and actin reorganization support the notion that capsaicin causes only a delayed-phase insulin secretion. It has been indicated that the delayed phase of insulin secretion involves the Rho family of kinases that interacts with and causes the rearrangement of actin cytoskeleton in β -cells [39, 40]. Interestingly, TRPV1 activation with capsaicin may trigger the Rho kinase pathway [41] albeit this has yet to be shown in pancreatic β -cells. Taken together, our data show that in contrast to well described glucose-stimulated dual phase secretion of insulin, capsaicin causes a delayed phase of insulin secretion through TRPV1 activation resulting in actin reorganization and cell stiffness changes.

The islet β -cell insulin secretion process is highly regulated by electrical and ion channel activities [42]. The electrophysiological properties of ion channels play a crucial role in the secretion process, the defect of which may lead to metabolic disorders [43]. These ion channel properties have been investigated to elucidate the underlying secretion mechanism through membrane capacitance measurements. Patch-clamp technique has been employed to measure the capacitance increase resulting from the membrane expansion due to insulin exocytosis [44]. While effective, patch-clamp studies are normally time consuming and demand highly skilled workers. More importantly, electrically-regulated insulin secretion is coupled with biophysical changes of the membrane [35], and the electromechanical coupling can be revealed fully by integrated studies of electrical activity and biophysical monitoring [45].

All the evidence indicates that islet β -cells exhibit a different physical state during the insulin secretion process, on which the biophysically biphasic glucose stimulation and single-phased capsaicin stimulation depend. These biophysical features may be addressed by identifying and characterizing signatures of ion channel activities. Many have reported that β -cells showed similar patterns of Ca^{2+} levels and insulin release *in vitro* and *in vivo* in responses to glucose stimulation [46–48]. Taking into consideration the fact that islet cells tend to cluster *in vivo* [46], changes in biophysical property are expected with three dimensional environments as compared with a cell monolayer. A continued study with cell encapsulation would be better positioned to address these speculations. The current study aimed to develop and apply novel techniques such as this AFM nanorobotic system to examine physical properties of pancreatic insulin secreting cells by characterizing nanomechanical properties of a single cell or a cell population so that unique biomarker(s) in response to general or specific channel activators may be identified. Among many biophysical characterization techniques used for living cells, AFM stands out for a better fit for the task. In recent years, AFM has become an increasingly versatile tool in studies of biological processes for *in situ* nanoscale measurements [49]. With the use of the AFM nanoindentation, nano-even pico-Newton forces may be applied for the biophysics dynamic measurement, rendering the capture of tiny changes of cellular structure and property possible. The integrated approach with AFM and optical microscopy, aimed at unraveling

the dynamic responses to ion channel stimulation, may provide a new perspective in the study of pancreatic β -cell stimulation and secretion.

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Figure 1. The stimulus-secretion coupling model of β -cells

(A) Increased plasma glucose levels will increase the intracellular ATP/ADP ratio after glucose being uptaken by cells, which leads to the closure of ATP-dependent potassium channels, membrane depolarization, and the increase in intracellular Ca^{2+} concentrations through the activation of voltage dependent calcium channels (VDCC) and ultimately insulin secretion. Capsaicin binds to the intracellular domain of the TRPV1 receptor and causes the influx of Ca^{2+} , which triggers the release of Ca^{2+} into the cytoplasm from the endoplasmic reticulum (ER) and increases $[\text{Ca}^{2+}]_i$ concentrations. Increased $[\text{Ca}^{2+}]_i$ will lead to the insulin exocytosis through the fusion of insulin-containing vesicles with the plasma membrane. (B) The AFM based nanorobotic manipulator uses a joystick as position

input to facilitate the movement of the AFM tip in the planar area during the force measurement process.

Figure 2. Immunofluorescence imaging of TRPV1 receptors in RINm5f insulinoma cells
 (A) Cell plasma membrane localization of TRPV1 receptors in RINm5f cells, positive staining indicated by arrows. (B) Control RINm5f cells in which primary antibody was replaced with 1% BSA in PBS show negative staining. Immunofluorescence was performed using goat polyclonal anti-TRPV1 antibody (1: 250) and AlexaFluor® 488-conjugated secondary antibody (1:100). Magnification: 1000×. Scale bar: 20 μm. Biophysical characterization of dose-dependent glucose and capsaicin stimulation in RINm5f cells: (C) The stiffness changes before and after administration of glucose at three concentrations, 16 mM, 20 mM, and 40 mM, plus the control. * $p < 0.05$ vs. before stimulation; + $p < 0.05$ vs. control; $\Delta p < 0.05$ vs. glucose 16 mM; + $p < 0.05$ vs. glucose 16 mM and 20 mM ($n = 20$). (D) The stiffness changes before and after administration of Cap (capsaicin) at four concentrations (1.67 nM, 16.7 nM, 167 nM and 1.67 μM), with * $p < 0.05$ vs. before stimulation; + $p < 0.05$ vs. control; $\Delta p < 0.05$ vs. Cap 1.67 nM; + $p < 0.05$ vs. Cap 1.67 nM and 16.7 nM. Each datum is expressed as mean $\pm$ SEM ($n = 100$).

Figure 3. Dynamic characterization of cellular stiffness simultaneously with $[Ca^{2+}]_i$ fluorescence imaging after glucose (16 mM) stimulation

(A) The ion channel activities overlaps with the overall cellular stiffness change in the first phase when the onset of stimulation induced rapid structural rearrangement. A second sustained phase follows to maintain the structural configuration and stiffness at a relatively higher level than the baseline. (B) A close-look at the lagging of stiffness response compared with that of intracellular Ca^{2+} concentrations, a 2 minute-delay was observed. (C) (D) (E): The fluorescence imaging of $[Ca^{2+}]_i$ before stimulation (C), 12 minutes after stimulation when the light intensity reaches the peak (D), and 20 minutes after stimulation (E) when the light intensity falls to the baseline. The observation stops at minute 20 due to the photo-bleaching effect rendering the light intensity even lower. The normalized grayscale was obtained by ImageJ software analysis. The individual cells were circled and the grayscale values were measured, averaged and normalized.

Figure 4. Dynamic characterization of cellular stiffness simultaneously with $[Ca^{2+}]_i$ fluorescence imaging after capsaicin (16.7 nM) stimulation

(A) The ion channel shows similar pulse-shape response pattern as that of glucose albeit with smaller peak value (B) The cells were also pretreated with Capz 30 minutes before capsaicin stimulation to block TRPV1 receptors. No increase in light intensity was observed for TRPV1-blockade samples shown in the normalized grayscale before and after capsaicin stimulation. * $p < 0.05$ vs. before stimulation; + $p < 0.05$ vs. Cap 16.7 nM. Data was expressed as mean $\pm$ S.D. of five independent experiments. (C)(D)(E): The fluorescence imaging of $[Ca^{2+}]_i$ before (C), 12 minutes after stimulation (D) when the light intensity reaches the plateau, and 20 minutes after stimulation (E) when the light intensity falls to the base line. The normalized grayscale value was obtained the same way as the Fig. 4.

Figure 5. The overall trend of the stiffness responses

(A) Glucose stimulation resulted in a two-phase response: a rapid increase phase in stiffness followed by a sustained elevation phase; (B) Capsaicin stimulation induced a slow and sustained increase in stiffness.

Figure 6. The actin filament reorganization after treatment of low glucose based medium as control, 16 mM glucose and 16.7 nM capsaicin for 0 min, 1 min, 5 min and 10 min
F-29 actin was labeled with CF488A-conjugated phalloidin. The treatment of glucose and capsaicin causes time-lapse change of cytoskeleton especially actin filament structures with distinct patterns. A delayed disorganization of actin around 10 minutes after stimulation was observed in capsaicin treatment, whereas glucose triggered the disorganization 1 minute after treatment, similar with the biophysical data obtained by AFM nanomechanical analysis. No distinctive change was observed for the control sample.