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Characterization of the Mechanodynamic Response of Cardiomyocytes with Atomic Force Microscopy

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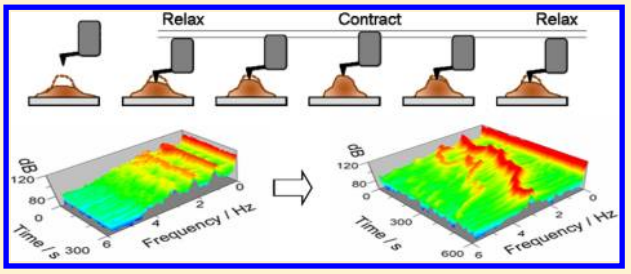
ABSTRACT: Coordinated and synchronous contraction of cardiomyocytes ensures a normal cardiac function while deranged contraction of cardiomyocytes can lead to heart failure and circulatory dysfunction. Detailed assessment of the contractile property of cardiomyocytes not only helps elucidate the pathophysiology of heart failure but also facilitates development of novel therapies. Herein, we report application of atomic force microscopy to determine essential mechanodynamic characteristics of self-beating cardiomyocytes including the contractile amplitude, force, and frequency. The contraction was continuously measured on the same point of the cell surface; the result assessed postintervention was then compared with the baseline, and the fractional change was obtained. We employed short-time Fourier transform to analyze the time-varying contractile properties and calculate the spectrogram, based on which subtle dynamic changes in the contractile rhythmicity were delicately illustrated. To demonstrate potential applications of this approach, we examined the inotropic and chronotropic responses of cardiomyocyte contraction induced by various pharmacological interventions. The administration of epinephrine significantly increased the contractile amplitude, force, and frequency whereas esmolol markedly decreased these contractile properties. As uniquely illustrated in the spectrogram, doxorubicin not only impaired the contractility of cardiomyocytes but also drastically compromised the rhythmicity. We envision that our approach should be useful in research fields that require detailed evaluation of the mechanodynamic response of cardiomyocytes, for example, to screen drugs that possess cardiac activity or cardiotoxicity, or to assess chemicals that could direct differentiation of stem cells into functioning cardiomyocytes.



Cardiomyocytes constitute the fundamental structural and functional unit of a heart. Coordinated and synchronous contraction and relaxation of cardiomyocytes ensure a normal cardiac function and adequate systemic perfusion of vital organs. The deranged contraction of cardiomyocytes can cause a dysfunction of cardiac pumping and circulatory failure, which may in turn lead to malperfusion of vital organs and eventually deteriorate clinical prognosis.[1−3]

Detailed assessment of cardiac function not only helps elucidate the pathophysiological mechanisms of heart failure but also facilitates development of novel therapeutic interventions. Although cardiac function is commonly evaluated in an intact heart with methods such as echocardiography[4] or ventriculography,[5] assessing the contractile characteristics at the cellular level offers several indispensable and unique advantages: it allows a direct examination of the contractile performance of cardiomyocytes in the absence of confounding factors such as preload, afterload, and neurohormonal activities.[6,7] An ability to characterize the contraction of cardiomyocytes also enables one to examine the differentiation of stem cells to functioning cardiomyocytes,[8,9] to help advance stem-cell therapies,[10,11] and to assess novel pharmacological or genomic interventions targeting heart diseases;[12] for example, the inotropic/chronotropic effect or possible cardiotoxicity of new drugs under development can be screened before conducting expensive and tedious preclinical tests on animals.

Video microscopy combined with algorithms of motion analysis is commonly employed to determine the rhythmic characteristics of contracting cardiomyocytes, but the accuracy of the determination of the contraction amplitude is also inevitably limited by the micrometer-sized spatial resolution of the optical system. Fluorescence imaging and Raman spectroscopy have also been employed to investigate the contraction of cardiomyocytes.[13−15] However, essential mechanodynamic information about cardiomyocyte contraction, such as the contractile force, remains lacking. Much effort has recently been made to explore the capability of atomic force microscopy (AFM) to investigate different aspects of living cells, such as release of intracellular ions or morphological change induced by external stress.[16,17] In particular, AFM has also been utilized to study the mechanical property of cardiomyocytes and the dynamic aspect of their contraction. For example, AFM has been employed to map the local elasticity[18−20] and contractile behavior[21] at different regions of single cardiomyocytes; it has

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also been combined with confocal fluorescence microscopy to reveal an intimate relation between the cardiomyocyte contraction and the intracellular concentration of calcium ions.[22]

Herein, we report employment of microcantilevers of AFM to characterize the mechanodynamic aspects of the contraction of self-beating primary cardiomyocytes under various interventions. In comparison with relevant work that reported application of AFM to assess cardiomyocytes, our approach possesses several unique features. As the contraction was measured on the same point of the cell surface, we were able to compare results obtained before and after intervention on the same cell and to assess the dynamic change of the contractile property in real time. Moreover, we employed short-time Fourier transform (STFT) to analyze the temporal displacement of single cells and show that the spectrogram obtained with STFT facilitates intuitive identification of the time-varying contractile property. This approach further allows revelation of subtle dynamic change that might be obscured in results that are obtained before and after intervention and are averaged among multiple cells. We demonstrate our approach using agonists and antagonists that target the alpha and beta adrenergic receptors and a chemotherapeutic drug that is known to cause oxidative injury of cardiomyocytes.[23] We anticipate that our approach should be very useful in various research fields that require a detailed evaluation of the mechanodynamic aspects of cardiomyocytes, for example, to screen drugs that possess cardiac activity or cardiotoxicity or to assess chemicals that could direct differentiation of stem cells into functioning cardiomyocytes.

MATERIALS AND METHODS

These experiments were approved by the Animal Investigation Committee of National Chiao Tung University.

Reagents. Epinephrine, doxazosin mesylate, esmolol hydrochloride, vitamin C, doxorubicin, dimethyl sulfoxide, sodium chloride (NaCl), potassium chloride (KCl), sodium bicarbonate ($NaHCO_3$), magnesium sulfate ($MgSO_4$), sodium dihydrogen phosphate (NaH_2PO_4), calcium chloride ($CaCl_2$), glucose (Sigma Aldrich), bovine albumin, thermally inactivated fetal bovine serum (FBS), calcium- and magnesium-free Hanks' balanced salt solution (HBSS), penicillin−streptomycin solution (PS), trypsin, trypsin inhibitor, and M199 (Invitrogen) were obtained from the indicated sources.

Preparation of Primary Culture of Ventricular Cardiomyocytes. Primary ventricular cardiomyocytes were prepared with a protocol modified from that reported in the literature.[19,24] All solutions used in this work were sterilized using 0.22 μm filters, and the procedures were performed in a sterile hood when applicable.

Hearts from 11-day-old chick embryos were removed aseptically and placed in HBSS. The ventricular tissue was minced into fragments (size about 0.5 mm) and dispersed with trypsin (0.025%, v/v) at 38 °C for 8 min with five repeated cycles. The supernatant that contained dissociated cells was placed in a trypsin stopping solution comprising cold trypsin inhibitor (0.005%, wt/v), bovine albumin (0.83%, wt/v), M199 (42%, v/v), and Barry's salt solution (BSS; 58%, v/v; NaCl 116 mM, KCl 2.5 mM, $MgSO_4$ 0.8 mM, NaH_2PO_4 1.0 mM, $NaHCO_3$ 22 mM, glucose 5.6 mM, $CaCl_2$ 0.87 mM). The solution was centrifuged (1200 rpm) at 4 °C for 5 min, and the supernatant was discarded. The pellet was resuspended in a "chick medium" (CM) that comprised BSS (54%), M199 (36%), FBS (6%), and PS (4%). This suspension was preplated in a cell incubator (5% CO_2, 98% relative humidity, 37 °C) for 45 min. The medium was then collected and centrifuged (1200 rpm) at 4 °C for 5 min to isolate the cardiomyocytes from the fibroblasts. The pellet was resuspended and diluted to a final density of 10^5 cells/mL. A solution that contained about 2×10^4 cells was transferred to a Petri dish (the final density about 500 cells per mm^2); the Petri dish was kept in an incubator. The medium was changed every other day. Cells typically began to contract spontaneously 48 h after culture. Throughout this work, all experiments were performed on cells that were incubated for 5 days.

Characterization of Cardiomyocyte Contraction. A commercial AFM (Bioscope SZ, Veeco, USA) mounted on an inverted optical microscope (TE-2000, Nikon, Japan) and silicon nitride microcantilevers (nominal spring coefficient 0.016 N/m; MLCT, Veeco, USA) were used throughout this work. To control the environment, the entire setup was enclosed in a custom-made chamber maintained at 37 °C and 5% CO_2. The cardiomyocytes typically retained stable contraction for 2 to 4 h after removal from the incubator and placement in the chamber. All data presented here were obtained within 30 min after removal of the cells from the incubator.

As the cells adhered on the substrate, the lateral contraction of the cells resulted in a vertical displacement of the membrane. To determine the amplitude of this vertical displacement while the cardiomyocyte contracted, a microcantilever was brought into gentle contact with the cell. The force exerted on the cell by the microcantilever was kept constant with a feedback loop and a piezo tube that moved the cantilever vertically while the cell was beating. The temporal movement of the cell membrane was thereby tracked precisely with the displacement of the microcantilever (Figure 1a). The amplitude of the contraction was extracted directly from the trajectory (or the temporal trace) of the displacement of the microcantilever.

As described, the load exerted on the cardiomyocyte was maintained constant while the cell contracted. The contractile motion of the cardiomyocytes characterized according to this constant-force mode hence resembles an "isotonic contraction", a muscle contraction of a representative type in which the tension remains constant while the muscle stretches. The amplitude determined under this condition accordingly represents the contractile strength or the contractility of cardiomyocytes that are undergoing isotonic contraction.

To determine the dynamic aspect of the cardiomyocyte contraction, we performed a short-time Fourier transform (STFT) on the temporal trace of the displacement. The temporally varying frequency of the self-contracting cardiomyocyte was then displayed in the resulting spectrogram.

To examine how the external load influenced the contractile property of cardiomyocytes, we systematically characterized the amplitude and the frequency of cardiomyocyte contraction under varied loads. The amplitude remained virtually constant for an applied force less than 10 nN but decreased gradually with a further increased load; the contraction was almost completely suppressed when the load was greater than 35 nN (Figure 1b). In contrast, the contraction frequency possessed a large tolerance of the external load; the frequency exhibited no significant variation within the range of load that we examined (data not shown). The force exerted on the cells was accordingly maintained about 5 nN throughout this work to

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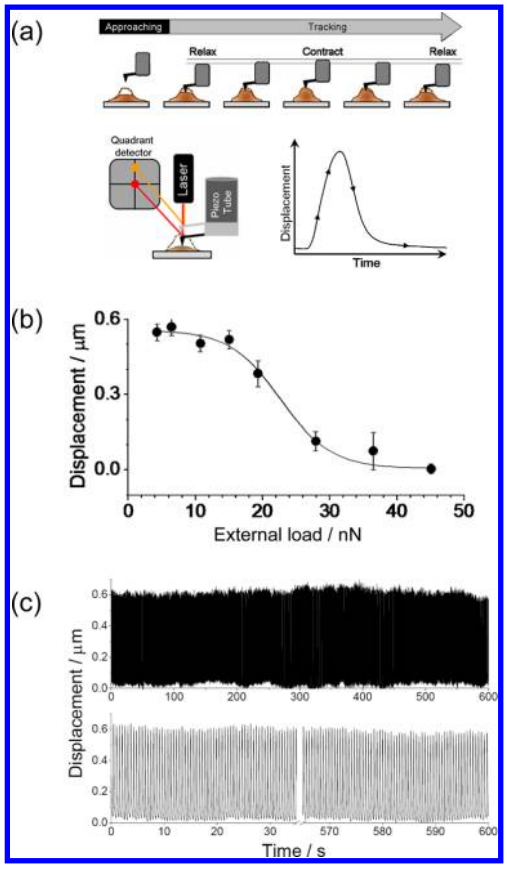

Figure 1. Operation principle for the determination of cardiomyocyte contraction with atomic force microscopy. (a) Cartoon illustration showing a microcantilever is tracking the movement of the cell membrane at a constant load while the cardiomyocyte beats (upper panel). Schematic diagram of the experimental setup (lower left). Hypothetical data illustrating the displacement of a cardiomyocyte while it undergoes one contraction cycle (lower right). (b) Amplitude of the displacement of cardiomyocyte contraction measured at varied external loads. (c) A representative result of the sham control (without administration of chemicals) obtained by a continuous measurement for 10 min. Enlarged sections of the data is displayed in the lower panel to show individual beats and the stability of the contraction.

minimize undesirable perturbation of the contraction due to the excessive load.

To explore the mechanical response of cardiomyocytes to the force applied by the cantilever, we measured force curves on various positions of nonbeating cells and extracted the elastic modulus using the Hertz model.[19] The Young's moduli extracted from these measurements ranged from 10 to 40 kPa. The result is in good agreement with that reported in the literature.[19] From the elastic moduli, we estimated that the indentation was about a few hundred nanometers up to nearly 1 μm under our experimental condition.

Our approach also enabled quantitative determination of the contractile force produced by the cardiomyocyte. To measure this force, we terminated the feedback loop after the cantilever was in contact with the cell. As the cell contracted, the cell exerted a force on the microcantilever and caused the microcantilever to deform. This deformation of the microcantilever increased until the force produced by the cell equaled the counterforce produced by the deflected microcantilever. The maximal deflection of the microcantilever was then recorded to represent the maximal contractile force produced by the cardiomyocyte.

Chemicals of individual interventions were prepared in a prewarmed medium (1 mL) of indicated concentration. Before each experiment, the cardiomyocytes were screened; only batches of cells that exhibited stable and synchronous contraction for a duration greater than 300 s were used for further experiments. To introduce intervention, we gently added the solution to the cells without lifting the microcantilever and waited 180 s. This waiting duration was to ensure complete mixing and cessation of fluctuation due to the mixing. Data were then recorded for a further 300 s or more. As a sham control, we measured also the contraction of cells without addition of chemicals or pharmacological agents for at least 10 min (Figure 1c).

The data measured before the administration of chemicals (the baseline) served as an internal control. The data measured after introduction of intervention was then compared with the internal control obtained on the same cell and reported as a fractional change relative to the internal control ($100 \times (B - A)/A$ %; A: baseline, B: postintervention). Each measurement was repeated at least four times in the same manner. The result of statistical analysis was expressed as mean ± STD.

RESULTS AND DISCUSSION

Dependence of Cardiomyocyte Contraction on the Confluency of Cells. We first examined how the confluency of cells affected the contraction of cardiomyocytes. As observed under an optical microscope, cells cultured to a confluent layer (approximately 500 cells per mm^2) beat regularly with a stable rhythm. The temporal trace of the contraction consistently comprised stable pulses in a series with comparable amplitude (C.V. = 2.16%) (Figure 2a). The spectrogram obtained from a

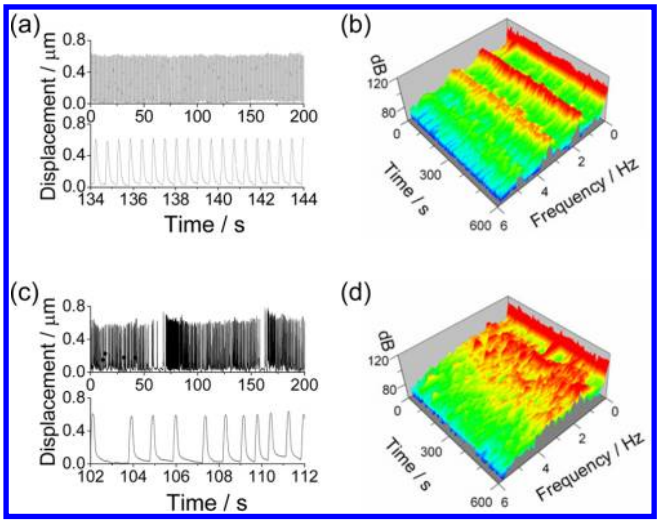

Figure 2. Cardiomyocyte contraction characterized at varied confluency of the cell. (a) Representative displacement data recorded for 600 s on a confluent layer of cardiomyocytes. For clarity, only data recorded during the first 200 s (upper panel) and an enlarged section of the data (lower panel) are displayed. (b) Spectrogram obtained with a short-time Fourier transform (STFT) of the preceding displacement data recorded on a confluent layer of cardiomyocytes. (c) Representative displacement data recorded on a subconfluent layer of cardiomyocytes. (d) Spectrogram obtained from a STFT of the preceding displacement data recorded on a subconfluent layer of cardiomyocytes.

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STFT of the temporal trace shows clearly that the confluent layer of cardiomyocytes possessed a distinct contraction frequency that remained nearly constant during the temporal course of our measurement (Figure 2b).

In contrast, the temporal trace of the contraction measured on a subconfluent layer of cardiomyocytes (approximately 250 cells per mm^2) was distinct from that measured from the confluent layer of cells; it exhibited an abnormal pattern resembling the characteristics of cardiac dysrhythmia. More specifically, the cells stopped contraction occasionally and remained in a quiescent state for varied durations (Figure 2c). Consistent with our observations on this disordered temporal trace, the contraction frequency of the subconfluent cardiomyocytes varied greatly from time to time, as clearly shown in the spectrogram (Figure 2d). Notably, despite the rapid and pronounced alteration of the contraction frequency, the amplitude of the contraction did not change significantly (C.V. = 5.09%). Similar observations were consistently obtained from different batches of cells, indicating that the cardiomyocytes tested in these experiments were stable.

These results demonstrate a unique capability of our approach to characterize intricately cardiomyocyte contraction in a highly quantitative manner. Specifically, the amplitude of the contraction was determined down to a nanometer scale; the temporally varying frequency was directly revealed from the spectrogram. Moreover, the sensitive dependence of the rhythmic property on the confluency of the cells was demonstrated. As the maintenance of a proximal interaction between cells is essential for a group of cardiomyocytes to exhibit synchronous and rhythmic contraction,[25] only confluent cardiomyocytes were used throughout the following experiments.

Agonistic Response of Cardiomyocytes. Epinephrine is both an endogenous hormone and a commonly used drug with potent positive inotropic and chronotropic effects; it is known to augment not only the contractility but also the contraction frequency of cardiomyocytes. According to our results, an administration of epinephrine (10 μM) to the cardiomyocytes resulted in a markedly increase of the contraction amplitude, indicating that epinephrine caused a significant increase in the contractility of cardiomyocytes (23 ± 4%, n = 4; Figure 3a). The spectrogram obtained from a STFT of the temporal trace shows that the contraction frequency increased gradually with time, resulting in a dramatic increase at the end of the measurement (187 ± 21%, n = 4; Figure 3b).

As described in the Materials and Methods, our approach enabled also a quantitative determination of the maximal contractile force of the cardiomyocytes. The result obtained in a separate experiment shows that an administration of epinephrine (10 μM) boosted the maximal deflection of the cantilever significantly, indicating that the administration of epinephrine caused a greatly increased maximal contractile force of the cardiomyocytes (253 ± 39%, n = 4; Figure 3c).

The observation of significantly increased contractility, frequency, and contractile force after administration of epinephrine is consistent with the known agonistic action of epinephrine on both alpha- and beta-adrenergic receptors of cardiomyocytes. According to these collective observations, we have demonstrated a unique capability of our approach to characterize the inotropic and chronotropic properties of cardiomyocytes with or without pharmacological intervention. In particular, the ability to determine a variation of the maximal contractile force shows clearly the superiority of our approach

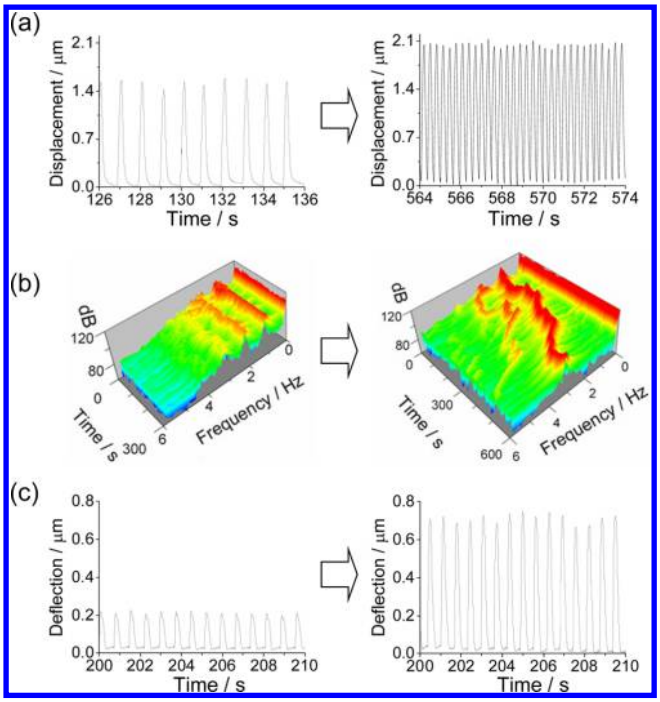

Figure 3. Cardiomyocyte contraction intervened with a potent agonist of the beta-adrenergic receptor of cardiomyocytes. (a) Representative displacement data recorded before (control, left panel) and after (right panel) administration of epinephrine. For clarity, only enlarged sections of the data are displayed. (b) Spectrograms showing the temporal change of the contraction frequency before (left) and after (right) the treatment. (c) Representative deflection data recorded before (left) and after (right) the treatment.

to traditional video-based assessment, especially in revealing the mechanodynamic characteristics of cardiomyocyte contraction. Through a STFT-based analysis of the temporal trace, the spectrogram reveals directly the detailed temporal variation of the chronotropic property of cardiomyocyte contraction upon treatment with epinephrine, which yields further insight into the dynamic aspect of cardiomyocyte contraction altered by pharmacological interventions. We return to this point in a subsequent section.

Antagonistic Responses of Cardiomyocytes. We characterized also the response of cardiomyocytes under treatment with various antagonists of the adrenergic receptor. Esmolol hydrochloride, an antagonist of the beta-adrenergic receptor, is known to possess a potent negative effect on both the inotropic and chronotropic properties of cardiomyocytes. After administration of esmolol hydrochloride (60 μM), the contraction amplitude of the cardiomyocytes decreased significantly (31 ± 2%, n = 5; Figure 4a). The contraction frequency decreased gradually, exhibiting a 14 ± 6% decrease at the end of the measurement (n = 5; Figure 4b). This result conforms to the pharmacological effect of esmolol, which acts as an antagonist of the beta-adrenergic receptor and is expected to decrease both the contractility and the frequency of cardiomyocyte contraction.

To examine the selective antagonistic effect, we tested also doxazosin mesylate, an antagonist that blocks effectively the alpha-adrenergic receptor of cardiomyocytes and that is known to diminish selectively the contractility of cardiomyocytes while having little effect on the chronotropy of cardiomyocytes. As expected, the contraction amplitude exhibited a 29 ± 2%

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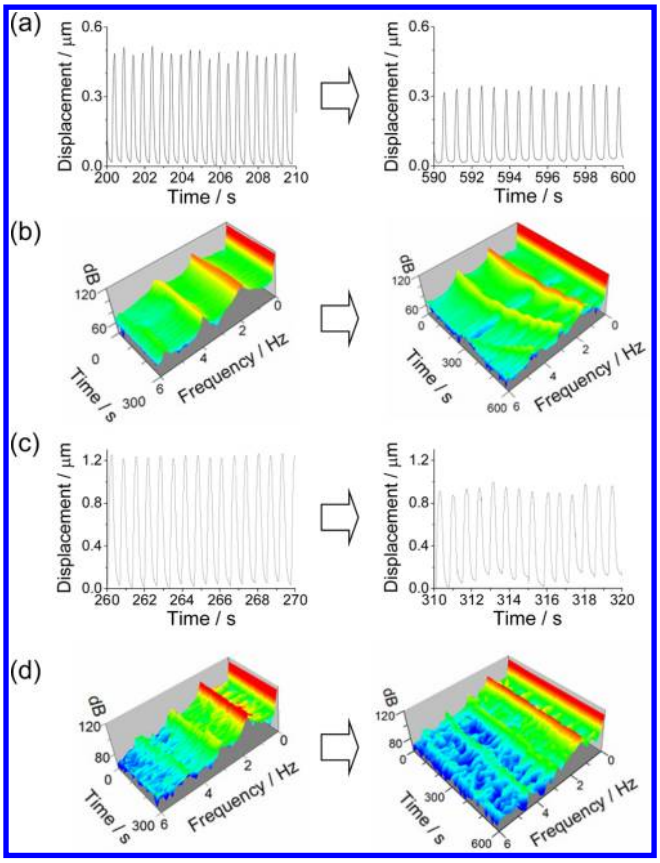

Figure 4. Cardiomyocyte contraction intervened with alpha- and beta-antagonists of the adrenergic receptors of cardiomyocytes. (a) Representative displacement data recorded before (left) and after (right) administration of esmolol hydrochloride. (b) Spectrograms obtained before (left) and after (right) treatment with esmolol hydrochloride. (c) Representative displacement data recorded before (left) and after (right) administration of doxazosin mesylate. (d) Spectrograms obtained before (left) and after (right) treatment with doxazosin mesylate.

decrease after the administration of doxazosin mesylate (0.01 μM), indicating a significant decrease of the contractility (n = 5; Figure 4c). In marked contrast with the appreciably decreased contractility, the treatment resulted in only a minor variation (5 ± 2%) of the contraction frequency at the end of the measurement (n = 5; Figure 4d). This result conforms to the selective alpha antagonistic effect of doxazosin mesylate and contrasts notably with that observed after the administration of esmolol, an antagonist of the beta-adrenergic receptor that suppresses both the contractility and the contraction frequency of cardiomyocytes.

As noted above, the spectrogram uniquely reveals the temporal variation of the chronotropy of cardiomyocytes: the treatments with epinephrine and esmolol hydrochloride resulted in an increasing and a decreasing chronotropy of cardiomyocytes, respectively (Figures 3b and 4b). Comparison of the two spectrograms shows more subtle details of the pharmacological effects of the drugs on cardiomyocytes, which might not be readily intelligible with other approaches. Specifically, the augmented chronotropy of cardiomyocytes caused by epinephrine is not simply a monotonic increase; the contraction frequency fluctuated significantly with time despite its generally increasing trend. In contrast, the variation of the chronotropy of cardiomyocytes due to the treatment of esmolol hydrochloride seemed more moderate, as the contraction frequency of the cardiomyocytes exhibited only a slow and monotonic temporal decrease. This feature uniquely shown with our spectrogram conforms to the known pharmacological effect of esmolol that is commonly used as a potent beta blocker to restore and to stabilize the ventricular rate of patients with atrial fibrillation, despite its depressive effect on the contractility. This result again demonstrates a unique capability and the great potential of our approach to examine subtle but important details of the chronotropic and inotropic effects of drugs on cardiomyocyte contractions.

Cardiotoxicity of Doxorubicin and Its Suppression by Antioxidants. Doxorubicin is a chemotherapeutic agent commonly employed to treat hematological malignancies and solid tumors such as breast cancer, but it is documented that doxorubicin might possess cardiotoxicity and cause cardiomyocyte dysfunction or even cardiomyopathy through the accompanying production of excessive reactive oxygen species (ROS). To explore the potential of our approach to evaluate cardiomyocyte dysfunction of clinical relevance, we attempted to characterize cardiomyocyte contraction before and after a treatment with doxorubicin.

Our results show that the administration of doxorubicin (4 μM) altered greatly the contraction of cardiomyocytes in several respects. There was a significantly decreased contractility of the cardiomyocytes, shown from the decreased amplitude (21 ± 8%, n = 5; Figure 5a). Moreover, the temporal trace of the contraction became highly irregular; the cells ceased contraction occasionally but recovered from a quiescent state of duration ranging from 30 to 100 s (Figure 5a). In addition to these occasional pauses, the spectrogram clearly shows that the contraction frequency appeared to decrease gradually with time (Figure 5b). Most significantly, we observed a greatly depressed contractile force, as shown from the approximate halving of the maximal deflection at the end of the measurement (56 ± 3%, n = 5; Figure 5c).

As oxidative damage is responsible for the cardiotoxicity of doxorubicin, it is hence plausible that a pretreatment with vitamin C, a potent ROS scavenger, might serve to protect cardiomyocytes from the oxidative injury induced by doxorubicin. Our results show that a cotreatment of vitamin C (10 μM) with doxorubicin (4 μM) resulted in only a moderately decreased maximal contractile force (13 ± 3%, n = 5; Figure 5d); this observation is notably in contrast with the approximate halving of the maximal contractile force caused with a treatment with doxorubicin (4 μM) alone (56 ± 3% vs 13 ± 3%). In addition to preventing the cardiomyocyte from a depression in its contractile force, the cotreatment with vitamin C preserved the regular rhythm of the cardiomyocyte contraction to a large extent, resulting in only a slightly varied contraction frequency (Figure 5d).

As shown above, our approach clearly reveals the degraded contractile characteristics of cardiomyocytes caused by a cardiotoxic agent and the suppression of that deterioration with an antioxidative intervention, respectively. These results collectively demonstrate convincingly a prospective application of our approach as an effective and economic means to screen drugs of possible cardiotoxicity and of antioxidative potency.

■ CONCLUSIONS

We have developed a novel application of AFM to characterize the inotropy and chronotropy of self-beating primary cardiomyocytes. Using agonists/antagonists of the alpha- and

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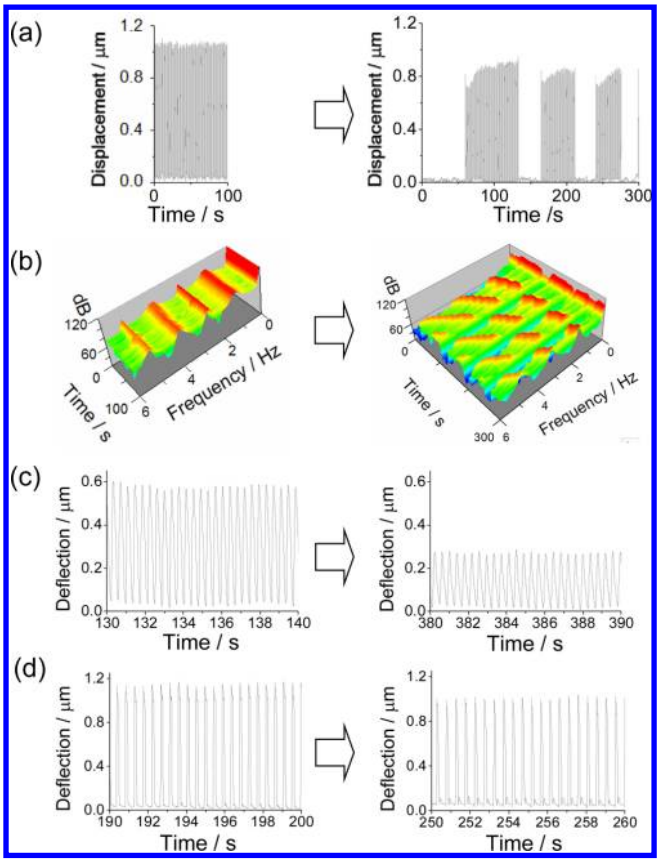

Figure 5. Deterioration of cardiomyocyte contraction with a cardiotoxic agent and its protection with an antioxidant. (a) Representative displacement data recorded before (left) and after (right) administration of doxorubicin. (b) Spectrograms obtained before (left) and after (right) treatment with doxorubicin. (c) Representative deflection data recorded before (left) and after (right) the treatment of doxorubicin. (d) Representative deflection data recorded before (left) and after (right) the cotreatment of doxorubicin and vitamin C.

beta-adrenergic receptors, we demonstrate that our approach uniquely reveals the temporal alteration of the contractile properties of cardiomyocytes under pharmacological interventions. We expect that this approach could open a new route to evaluate the mechanodynamic details of cardiomyocyte contraction that are intimately related to the cardiac function of a heart. This approach might be beneficial in various research fields that require a detailed evaluation of the mechanodynamic aspects of cardiomyocytes such as electromechanical coupling, cardiopharmacology, cardiotoxicity, and stem-cell therapy.

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Notes

The authors declare no competing financial interest.

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