Simultaneous Measurement of Ca^{2+} and Cellular Dynamics: Combined Scanning Ion Conductance and Optical Microscopy to Study Contracting Cardiac Myocytes

Andrew I. Shevchuk,* Julia Gorelik,* Sian E. Harding,† Max J. Lab,† David Klenerman,‡ and Yuri E. Korchev*
*MRC Clinical Sciences Centre, Division of Medicine, †Division of National Heart and Lung Institute, Imperial College School of Medicine, London, United Kingdom, and ‡Department of Chemistry, Cambridge University, Cambridge, United Kingdom

ABSTRACT We have developed a distance modulated protocol for scanning ion conductance microscopy to provide a robust and reliable distance control mechanism for imaging contracting cells. The technique can measure rapid changes in cell height from 10 nm to several micrometers, with millisecond time resolution. This has been demonstrated on the extreme case of a contracting cardiac myocyte. By combining this method with laser confocal microscopy, it was possible to simultaneously measure the nanometric motion of the cardiac myocyte, and the local calcium concentration just under the cell membrane. Despite large cellular movement, simultaneous tracking of the changes in cell height and measurement of the intracellular Ca^{2+} near the cell surface is possible while retaining the cell functionality.

INTRODUCTION

One characteristic of many living cells is their ability to change shape as a result of external or internal stimuli. There are a limited number of methods available to study these processes. Optical methods of motion detection have been used to follow the contraction of cardiac myocytes and the simultaneous changes in calcium by fluorescence (O'Rourke et al., 1990; Cross et al., 2000; Ozaki et al., 1999; Schneider et al., 1994). These methods are nonperturbative and capable of following fast dynamics, but, in general, the minimum size of motion detected is restricted to the diffraction limit for light. In principle, scanning probe microscopy (SPM) offers a means of rapid recording of local motion with nanometer precision. For example, atomic force microscopy has been used to measure the dynamics of cells (Henderson et al., 1992; Schneider et al., 1997, 2000; Schoenenberger and Hoh, 1994; Ushiki et al., 1996). Scanning ion conductance microscopy (SICM) has been used to also follow cellular dynamics and measure volume changes in cells. However, to date, no SPM method has been able to deal with the rapid motion of contracting cells. We report a significant improvement in SICM that extends its capabilities to enable recording of rapid cellular motions.

SICM is a form of SPM based on scanning an electrolyte-filled micropipette over the sample surface, and using the ion current flowing into the pipette to maintain a constant distance from the sample (Hansma et al., 1989). The ion current varies with the distance from the sample, and, by setting the control distance greater than the micropipette diameter, it is possible to image the surface of living cells (Korchev et al., 1997a,b). It has previously been shown that this method is capable of imaging the topography of living cardiac myocytes with a resolution of 50 nm (Korchev et al., 1997a). This method has also been used to measure the changes in cell volume (Korchev et al., 2000a), and the micropipette has been used as a near field light source for scanning near field microscopy (Korchev et al., 2000b). More recently, the ability of the micropipette to locally deliver reagents has been exploited to map the adenosine 5′-triphosphate–dependent potassium channels in a cardiac myocyte (Korchev et al., 2000c). This method was based on local application of potassium ions via the micropipette and detection of the resulting ion flow, via a channel, using patch clamp method. To obtain more reliable control to respond rapidly to the change in distance, we have introduced a modulation method. In this case, we modulate the distance between the pipette and sample, and use a lock-in amplifier to provide the feedback signal. We use this feedback signal to move the sample stage up or down to keep it at the same distance under the pipette. It has been shown that this method of control has several advantages because it makes the measurement insensitive to changes in ionic strength or DC drift.

To demonstrate our method, we have chosen to study cardiac myocytes that undergo regular contractions as this represents an extreme case of rapid cellular motion. We have also combined SICM with simultaneous optical measurements (SNOM) to validate the method. SICM has been combined with SNOM to demonstrate that it is possible to image a contracting cardiac myocyte. The SNOM images can be compared with images previously obtained of a quiescent cardiac myocyte. SICM has also been combined with laser confocal microscopy to measure the relationship between the size of the local motion and the local calcium concentration. The relationship between the intracellular calcium concentration and whole cell contraction has pre-

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Address reprint requests to Dr. Yuri E. Korchev, Imperial College School of Medicine, Division of Medicine, Hammersmith Campus, 5th Floor MRC Cli. Sci. Centre, DuCane Road, London W12 0NN, United Kingdom. Tel.: 44-208-383-2362; Fax: 44-208-383-8306; E-mail: y.korchev@ic.ac.uk.

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viously been studied by optical methods. Hence, the results from the two methods can be compared.

METHODS

Principle of the distance modulation protocol

We have developed a distance modulation scanning protocol that allows us to modulate the ion current flowing through the microscope micropipette and to use this ion current for feed back control of our microscope. The principle of the method is shown in Fig. 1. In our previous microscope, we used nonmodulated (I_{DC}) ion current to control the probe position over the sample (Korchev et al., 1997a,b). In that case, ion current flowing from the micropipette decays rapidly, as soon as the micropipette approaches the sample. In the distance-modulated mode of SICM operation, the movement of the microscope tip (Δd) generates a modulated current (I_{MOD}) that is present in the overall current (Fig. 1 *B*). The modulated signal provides the signal for the feedback loop. Two approach characteristics shown in Fig. 2 illustrate the dependence of both (I_{DC} and I_{MOD}) current signals with the distance of the micropipette from the sample surface. The I_{DC} is at maximum when the pipette is a long way from the surface (Fig. 2 *A*). In contrast, I_{MOD} is only generated when the probe senses the sample, and increases dramatically as the probe approaches the surface (Fig. 2 *B*). I_{MOD} reaches a maximum value at the point that the pipette touches the surface.

Most successful scanning in nonmodulated SICM can be achieved at the distance of approximately one micropipette tip radius from the sample surface, which gives a range of setpoint current within the 97%–99.8% of I_{MAX} (micropipette tip current far from the surface) (Korchev et al., 1997a). This means that any changes in the value of the nonmodulated current that exceed 0.2–3% of I_{DC} will affect the feedback control, even making control impossible. In the distance-modulated mode of SICM operation, the optimal scanning distance is also approximately one micropipette tip radius from the surface which gives a modulated current (I_{MOD}) that stays in the same optimal range of 0.2–3% of I_{DC}. In this case, significant changes in the value of I_{DC} (for example, a 100% increase, i.e., a doubling of the current) will induce a proportional change in I_{MOD} (from 1% to 2% of I_{DC}), still keeping I_{MOD} in the optimal range for the feedback control. This provides a robust feedback control that can tolerate significant changes in ion current because of DC drift or blockage of the pipette, changes in the ionic strength of the solution, or changes in the applied voltage. Distance modulation therefore makes the approach of the micropipette to the surface straightforward and allows one to perform complicated physiological experiments that require alteration of ionic strength of the media. With our modulated protocol we have been able to continuously scan living cells during more than 24 h, and to change ionic strength of the media by up to 4 times during the scanning without loss of the feedback control (these data will be presented elsewhere).

Typically, for an elaborated sample, we select a control point close to the radius of the pipette tip aperture, and the modulation distance is ~20% of the radius.

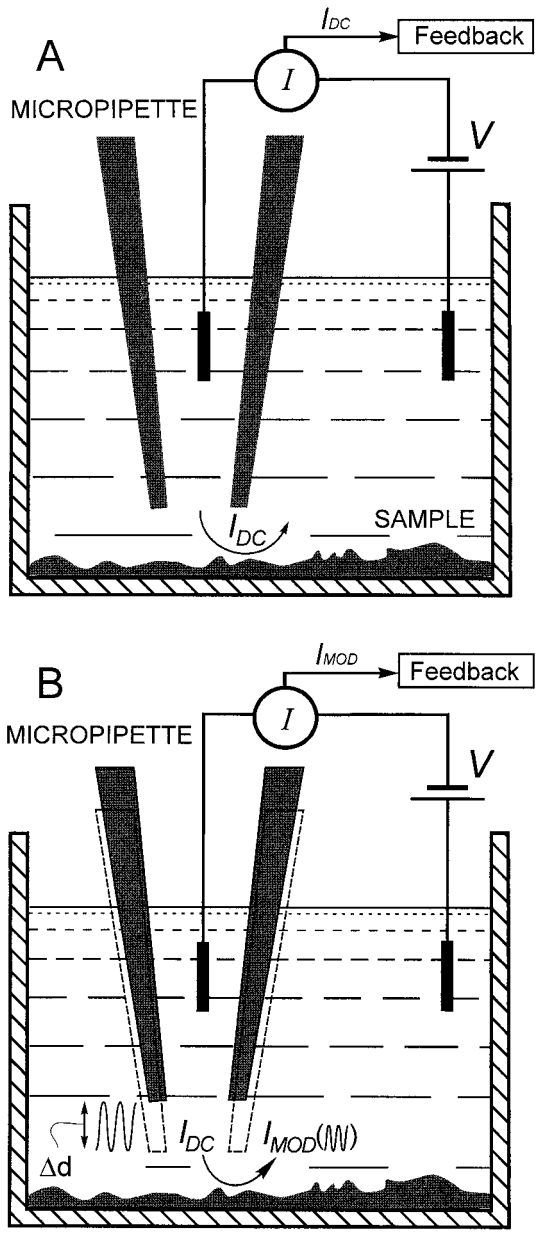

FIGURE 1 Schematics of the two methods in SICM for controlling the micropipette-sample distance. (*A*) A DC current control method. In this method, the position of the micropipette tip relative to the sample surface strongly influences the ion current (*I*) flowing through the pipette and, consequently, its DC component, I_{DC}. These changes of I_{DC} are used for feedback control of vertical position of the tip (Hansma et al., 1989; Korchev et al., 1997a). (*B*) A modulated DC current control method. In this method, the modulation of the micropipette-sample distance by Δd results in an additional component in micropipette current-modulated current (I_{MOD}), which is used for feedback control.

Instrument

The basic arrangement of the SICM for topographical imaging of living cells has previously been described (Korchev et al., 1997a,b). Briefly, the sensitive probe of the SICM is a glass micropipette filled with electrolyte which is connected to a high impedance and head stage current amplifier, and mounted on a piezo-driven three-axis translation stage. The sample was also mounted on a piezo translation stage. The control electronics drive the translation stage to scan the specimen under the micropipette probe. The control/data acquisition hardware and software are produced by East Coast Scientific (Cambridge, England). The electronics comprise a decoder, four digital-to-analog converters and two analog-to-digital converters. The digital signal processor card (DSP32C PC, Loughborough Sound Images PLC, Loughborough, England) of a PC functions as a "front-end" controller and provides digital feedback and scanning control. White light illumination and a camera on one port of the microscope are used to approach the pipette to the sample.

To perform distance modulation, an AC voltage was applied to the piezo on which the sample or pipette was mounted. This led to a modu-

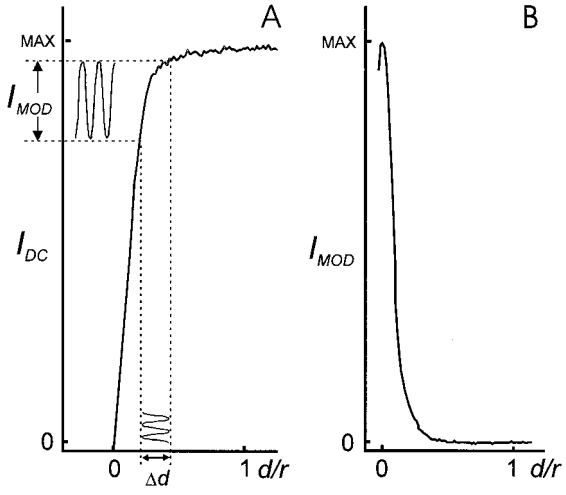

FIGURE 2 Experimental approach characteristics for I_{DC} and I_{MOD} showing the DC current modulation. Both approach characteristics (I_{MOD} and I_{DC} vs. d) were obtained on a flat plastic surface (bottom of a 35 mm petri dish) in phosphate-buffered saline buffer, pH 7.4. I_{MAX} was 3.1 nA and 100 nm pipette tip radius (r) was used. (A) The modulation of DC current that is used for feedback control. The actual approach illustrates characteristically how the overall current, I_{DC}, varies with the distance between the sample and the micropipette, d. The DC current reaches the MAX point when the distance between the micropipette and a sample become greater or equal than the micropipette tip radius, r. Modulation of the sample-micropipette distance by Δd results in a modulated current I_{MOD}. Note the size of I_{MOD} depends strongly on d. (B) Actual approach characteristics for I_{MOD}. Note the signal increases rapidly as the distance between the sample and micropipette becomes small.

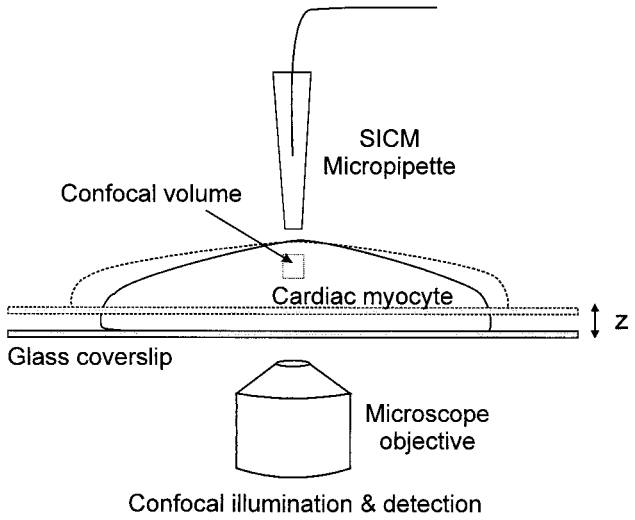

FIGURE 3 Schematic for simultaneous SICM and laser confocal microscopy on a contracting cardiac myocyte. When the cardiac myocyte contracts the feedback control moves the sample stage to maintain a constant distance between the micropipette and the cell surface. The confocal volume probed, therefore, remains at the same point below the cell surface.

lation of the distance between the sample and pipette. The frequency of modulation was from 100 to 10,000 Hz depending on the piezo loading. Piezo loading lowers the resonance frequency of the piezo and, hence, lowers the maximum modulation frequency possible. We operated close to this limit but also selected modulation frequencies away from noise in our system, such as harmonics of mains frequency. The modulated ion current is fed into a lock-in amplifier (SR830 DSP, Stanford Research Systems, Sunnyvale, CA), which provides a signal for the feedback loop, which controls the sample position.

The micropipettes were made from 1.00 mm outer diameter, 0.58 mm inner diameter glass microcapillaries (Intracel, Herts, UK) on a laser-based Brown-Flaming puller (model P-2000, Sutter Instrument Company, San Rafael, CA). The micropipettes and the bath were filled with the same solutions, usually physiological or growth media. The measured micropipette resistance was usually ~250 MΩ. The samples were usually placed on petri dishes, glass coverslips, or membrane filters and imaged in the appropriate medium.

To perform simultaneous SICM and SNOM, the same method was used as previously described (Korchev et al., 2000b). The only difference was that distance modulation was used in these experiments.

To perform simultaneous laser confocal microscopy and SICM, LCS-DTL-364 laser diode (473 nm wavelength, Laser Compact, Moscow, Russia) provided the excitation light source. The optical recording system consisted of a Nikon Diaphot inverted microscope (Diaphot 200, Nikon Corporation, Tokyo, Japan) equipped with oil-immersion objective lOOX 1.3 NA, an epifluorescent filter block and a photomultiplier with a pinhole (D-104–814, Photon Technology International, Surbiton, England). A schematic of this set-up is shown in Fig. 3. In these experiments the sample was moved up and down during scanning to maintain a constant distance between the pipette and the cell surface. The pipette, objective, and confocal volume stayed fixed. This means that, even during contraction, the fluorescence was recorded at the same distance below the cell membrane. The distance the stage was moved and the intensity of the fluo-3 (fluo-3 acetoxymethyl, Molecular Probes, Eugene, OR) fluorescence were simultaneously recorded. The confocal volume was centered at the tip of the micropipette by focusing the laser onto a top surface of the coverslip and bringing the pipette into control above the surface. It was centered in the xy plane by filling the pipette with laser dye and adjusting the pipette position in the xy direction for maximum fluorescence. The position of the pipette was noted on the camera monitor and the pipette was then positioned so as to appear at this point on the monitor before any experiment. This was found to be a reproducible way to position the pipette, in the xy direction, in the laser focus. We estimate that we probed a confocal volume ~500 nm in diameter and 2 μm in length centered at the tip of the pipette. This means that we probed the intracellular calcium within 1 μm of the cell membrane.

Cell preparation

Ventricular myocytes were isolated from the hearts of 1- to 2-day-old rats (Iwaki et al., 1990). Cells were kept in Dulbecco's modified Eagle medium (Gibco, Rockville, MD) with 15% fetal calf serum (Gibco), 1% streptomycin, 1% penicillin (Gibco), and 1% nonessential amino acids (Gibco). One hundred μg/ml G418 geniticin (Gibco) was added for inhibiting fibroblast growth. Cells were maintained at 37°C, in an atmosphere of humidified air plus 5% CO_2. Cells were used 1–3 days after plating. Myocytes were cultured on glass coverslips.

Cardiac myocytes from adult rats were isolated by digestion of intact perfused ventricle as previously described (Harding et al., 1988).

The cardiomyocytes were loaded with the visible wavelength fluo-3 Ca^{2+} indicator by cell incubation with the esterified derivative of 5 μM fluo-3 in a medium containing a mixture of part Leibovitz's L-15 (Gibco) and part Hanks' balanced salt solution buffer (Gibco) at room temperature for 15 min. Cells were rewashed five times with the medium, followed by a postincubation period of 20 min to allow for complete intracellular dye cleavage (Williams et al., 1992; López et al., 1995).

RESULTS

Imaging over contracting cell

The topographic image of a living cardiac myocyte taken using the distance modulation control is shown in Fig. 4 *A*. This image was taken under conditions of low calcium to obtain a clear topographic image of the cardiomyocyte surface. This is similar to the image of the cardiac myocyte previously published using DC control with comparable resolution (Korchev et al., 1997a). It clearly illustrates the *Z*-grooves, the regions where the cardiomyocyte plasmalemma is anchored to an intracellular cytoskeleton to form grooves and give cardiac myocytes a characteristic scalloped surface. The position of *Z*-grooves on topographical picture (Fig. 4 *A*) closely matches the position of *Z*-lines that are shown on the SNOM optical image of the same region of the cardiomyocyte (Fig. 4 *C*).

At higher calcium concentration, the cell undergoes contraction. Fig. 4, *B* and *C*, shows a simultaneous SICM topographic image and SNOM image taken of a contracting cardiac myocyte using distance modulation control. As shown by the SNOM image, the cell seems to return to the same position after contraction. Thus, the SNOM image looks similar to that published previously over a quiescent cell (Korchev et al., 2000b). The lines in the SNOM image are attributable to the change in the structure imaged under the pipette during the contraction. The large motions observed during contraction make the interpretation of the topographic image more complicated (Fig. 4 *B*). However, the rhythmical contractions of the cardiac myocyte are clearly visible. The extent of the vertical cell motion is up to 4 μm and most contractions are similar. The increase of the cell height of 4 μm during contraction occurs in ~200 ms, which is at a rate of ~20 nm/ms. The pipette is 75 nm away from the cell surface and the feedback control needs to operate within 4 ms to prevent the pipette touching the cell surface during contraction. Because we can reliably control over a contracting cell, this means that the feedback control works on a millisecond time scale and we can determine height changes with this time resolution.

This experiment shows that, with using distance modulation, it is possible to scan in control over a contracting cardiac myocyte and obtain a simultaneous optical image.

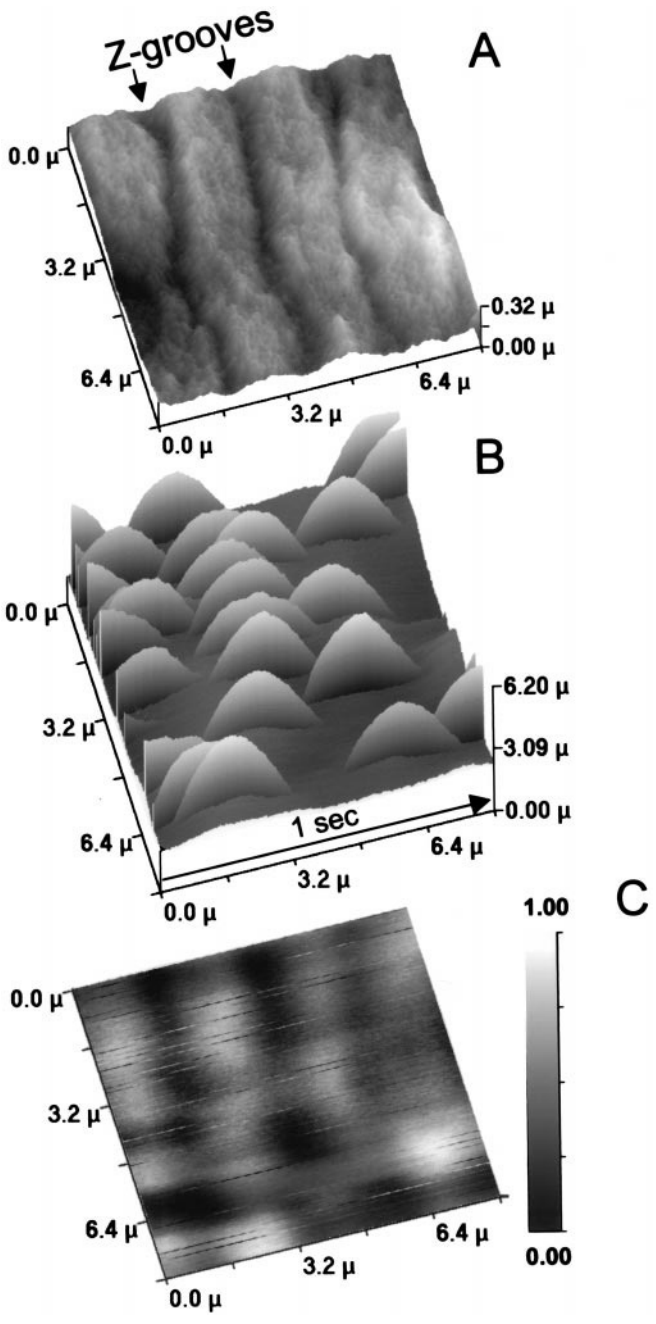

FIGURE 4 Images of an adult cardiomyocyte. (*A*) An image of a quiescent cardiac myocyte obtained using distance-modulation control. The modulation frequency was 198 Hz in this case. The *Z*-grooves are marked on the image with the *arrows*. (*B*) An image of a similar cell under conditions of high calcium where the cell undergoes spontaneous contractions. Each peak on the cardiomyocyte surface was formed by the membrane rising. This was the result of a contraction during the line scan. (*C*) The SNOM image obtained simultaneously with the topographic image in (*B*) using distance-modulation control. The *Z*-lines which correspond to *Z*-grooves (Fig. 4 *A*) are marked with *arrows*. The *gray scale bar* represents the optical signal in arbitrary units.

Ca^{2+} and local motion

The results of the simultaneous measurement of the local motion and relative calcium concentration at the center of a contracting cultured cardiac myocyte just below the cell membrane are shown in Fig. 5 *A–C*. Note the regular changes in calcium level and resulting height changes. There are 35 beats per minute. Fig. 5 *B* is a phase plot and illustrates the variation in cell height with calcium level (Fig. 5 *B* is an overlay of all the contractions in Fig. 5 *A*). All the curves are reproducible. There is an initial period where there is a rapid change in calcium but no contraction. This is followed by contraction of the cell and consequent change

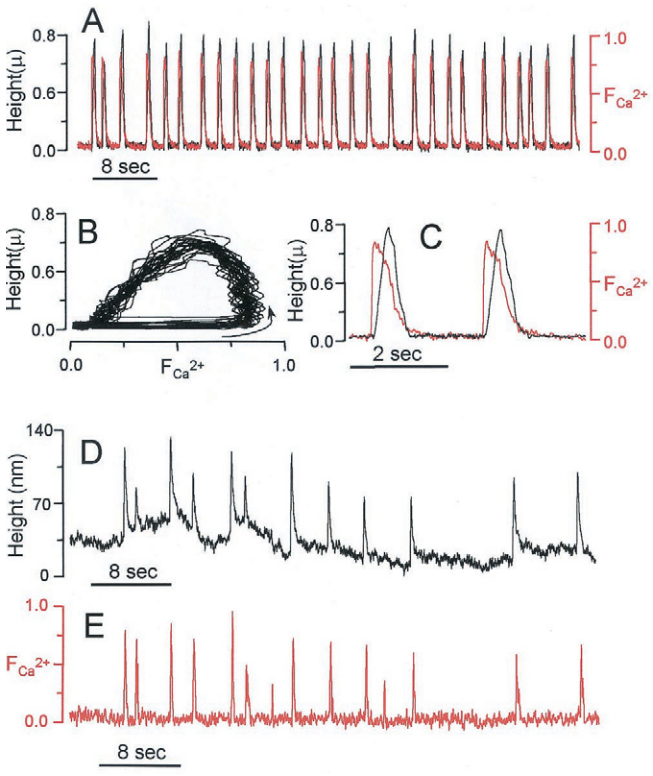

FIGURE 5 A simultaneous measurement of membrane dynamic and the local relative calcium concentration. (*A*) A simultaneous measurement of the position of the surface of 3-day-old cultured rat cardiac myocyte and the local relative calcium concentration just below the cell surface. (*B*) A plot of cell height versus relative calcium concentration for all the contraction in (*A*). (*C*) A blow-up of two of the contractions in (*A*). (*D*) The simultaneous measurement of the cell height and relative calcium concentration of 1-day-old cultured cardiac myocyte

in cell height to reach the maximum height. The calcium is then pumped out the cell and the cell returns to its original height. Fig. 5 *C* is a blow-up of two contractions in time showing the rapid rise in intracellular calcium followed by changes in cell height. As expected, the calcium increases first, and is followed by the local motion. There is a delay of ~250 ms between the two peaks. The cell height changes show a symmetric shape indicating that the contraction and relaxation processes take approximately similar times. In contrast, the calcium increase is much more rapid than the calcium decrease resulting in an asymmetric peak shape. This is similar to what has been observed on whole-cell studies (O'Rourke et al., 1990). Fig. 5 also shows the simultaneous height (*D*) and calcium (*E*) changes over 1-day-old cardiac myocytes. There are irregular and much smaller contractions compared to the mature adult cardiac myocytes, but these nanometric motions are still detectable. There are also undulations of the cell height that do not correspond to any calcium changes. A control experiment over a glass surface with the same feedback parameters used for cell height measurements showed a constant value with $<$10 nm noise. This indicates that these undulations are real motions of the cell surface.

DISCUSSION

In principle, it would have been possible to perform these experiments using the nonmodulated current for feedback control. However, in practice, this is not a robust control mechanism over rapidly contracting cells caused by DC drift. In contrast, this work shows that using the modulated current for feedback control offers several advantages. First, the approach of the pipette to the sample is far easier because the control signal is only generated close to the surface. Second, the control is much more reliable because the control signal is not strongly affected by changes in the value of the ion current because of DC drift, or partial pipette blockage by contaminants in biological media. This has been demonstrated by simultaneous topographic and SNOM imaging over a contracting cardiac myocyte resulting in images similar to those previously published using nonmodulated current control. This greatly increases the range of experiments that can be performed on living cells.

In this work, SICM has been combined with confocal laser spectroscopy to simultaneously record local calcium transients just below the cell membrane and the local motion of the cell at one point during cell contraction. This should be contrasted with normal confocal microscopy where cell contraction would result in the fluorescence being recorded from a focal plane in the cell. The data obtained were in good agreement with the previous whole-cell studies of cardiomyocyte contraction and cytosolic Ca^{2+} dynamics (O'Rourke et al., 1990). However, our measurement is at one point with better time and distance resolution. This has enabled us to detect motions as small as 10 nm and also observe low-frequency small motions of the membrane. These low-frequency motions may be undulations of the membrane, and further work is needed to understand this observation. The combination of SICM with confocal microscopy allows the direct measurement of the relationship between local calcium concentration just under the cell surface and the change in cell height. This allows the size of the calcium transients to be directly correlated to the height changes of the cell. The relationship between the local calcium change and the extent of motion has been measured and this, in future, can be used as a calibration curve to relate fluorescence measurements on cultured cells to extent of contraction. It should also be possible to directly measure the relationship between membrane potential and cellular motion by using a voltage-sensitive dye.

This new form of SICM combined with laser confocal microscopy offers both high time resolution and nanometer sensitivity combined with reliable control over living cells. Whereas such measurements are in principle possible with an atomic force microscope, the mechanical properties of most cells have made this difficult to realize in practice. The

method opens the possibility of performing new types of experiments on living cells because it provides a means to perform high-resolution topographic imaging and simultaneous measurement of the local concentration of many important cellular properties such as calcium, pH, and voltage.

SUMMARY

We have successfully combined SICM and confocal microscopy to simultaneously measure local motion and the local concentration of calcium. The results agree well with whole cell studies validating the method. This method can now be extended to study other chemical species or physical parameters important in signal transduction and the initiation of cell motion such as adenosine 5′-triphosphate, pH, and voltage in combination with measurement of local motion.

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