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Activation and Inactivation of Mechanosensitive Currents in the Chick Heart

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Abstract. The behavior of MS channels in embryonic chick ventricular myocytes activated by direct mechanical stimulation is strongly affected by inactivation. The amplitude of the current is dependent not only on the amplitude of the stimulus, but also the history of stimulation. The MS current inactivation appears to be composed of at least two contributions: (i) rearrangement of the cortical tension transducing elements and (ii) blocking action of an autocrine agent released from the cell.

With discrete mechanical stimuli, the MS current amplitude in the second press of a double press protocol was always smaller than the amplitude of the first MS current. Occasionally, a large MS current occurred when the cell was first stimulated, but subsequently the cell became unresponsive. For a series of stimuli of varying amplitudes, the order in which they were applied to the cell affected the size of the observed MS current for a given stimulus magnitude.

When continuous sinusoidal stimulation was applied to the cells, the MS current envelope either reached a steady state, or inactivated. With sinusoidal stimulation, the MS response could be enhanced or restored by simple perfusion of fluid across the cell. This suggests that mechanical stimulation of the cells produces an autocrine inhibitor of MS channels as well as resulting in cortical rearrangement.

Key words: Stretch — Voltage clamp — Mechanotransduction — Patch clamp — Modeling

Introduction

Mechanosensitivity is a ubiquitous, yet elusive property of cells. Almost every known cell type, from plants to animals, has some type of mechanosensing ability, but very little is known about the transduction pathway, or structure of the mechanosensitive (MS) channels, even in cells where their existence has been confirmed electrophysiologically. Significant progress has been made on determining the structure and behavior of bacterial MS channels (Sukharev et al., 1997*a*; Chang et al., 1998), but little progress has been made with eukaryotic mechanosensitive channels. There is no sequence homology between the components of the known bacterial channels and eukaryotic DNA (Sukharev et al., 1997*b*), which indicates the channels will have different structures and function. Cloned eukaryotic MS channels (although they were not cloned for their mechanosensitivity) include: an MS channel in osteoblasts (Kizer, Guo & Hruska, 1997), NMDA channels (which cannot be activated by stretch alone) (Paoletti & Ascher, 1994), the G protein regulated potassium permeable inward rectifier, GIRK (Krapivinsky et al., 1995), smooth muscle calcium-activated potassium channels (Dopico et al., 1994), and the potassium channels TREK, (Patel et al., 1998) and TRAAK (Maingret et al., 1999).

The continual and repetitive mechanical movement of the heart makes it a strong candidate for influence by MS channels. There are many postulated roles for mechanosensitive channels in the heart including: commotio cordis, resulting from a fatal (though noninjuring) blow to the chest (Link et al., 1998); arrhythmias (Dean & Lab, 1989; Hansen, Craig & Hondeghem, 1990*a*; Franz, 1995), fibrillation, and sudden death following abnormal stretch due to myocardial infarction; a change in beating rate as a function of filling (Bainbridge, 1915; Blinks, 1956); and recovery of a heart from fibrillation by direct heart massage. There is circumstantial evidence that mechanosensitive channels may be involved in these phenomena as gadolinium (Gd^{3+}), a nonspecific blocker of MS channels, blocks stretch-induced arrhythmias in dogs (Hansen et al., 1991) and venom from the spider *Grammostola spatulata*, which has an MS-channel-

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blocking peptide (Chen et al., 1996; Niggel et al., 1996; T.M. Suchyna et al., *submitted*), has a similar effect on atria from both the rabbit (M. Franz, *unpublished data*) and the guinea pig (Nazir et al., 1995), but the lack of data on MS channels at the single-channel and whole-cell level inhibits our understanding of these events. Almost all of the suggested roles for MS channels in the heart concern in pathological situations. However, with the rhythmic and repetitive contraction of the heart, the MS channels probably have a continuous modulatory influence on the electrical activity of the heart.

We need to understand more about the detailed behavior of the cardiac MS channels, and the mechanotransduction pathways, so we can determine, and ultimately manipulate, their contribution to both the diseased and healthy heart. This may be of particular importance in breaking the link between myocardial infarction and its associated ventricular fibrillation (about 80% of sudden death patients, the majority of whom probably die as a result of ventricular fibrillation, have signs of myocardial infarction (Bayes & Guindo, 1990)).

Cardiac MS channels have been identified in a diverse variety of species, including the chick (Ruknudin, Sachs & Bustamante, 1993); frog (Kohl et al., 1992); guinea pig (Bustamante, Ruknudin & Sachs, 1991); rat (Bustamante et al., 1991; Kim, 1992; Craelius, 1993); ferret (White et al., 1993); rabbit (Hagiwara et al., 1992; Suleymanian et al., 1995); mollusc (Sigurdson et al., 1987); and human (Naruse & Sokabe, 1993). Even with the large numbers of cell types exhibiting mechanosensitivity, few detailed electrophysiological studies on cardiac MS channels have been published.

Mechanotransduction research has been hampered by the technical difficulties associated with applying repetitive mechanical stimulation to a myocyte while simultaneously holding the cell under voltage clamp, and keeping the cell physiologically healthy. The lack of biochemical markers for MS channels only compounds the experimental difficulties. Most of the published data on cardiac MS currents are single channel recordings from neonatal or cultured cells, or nonvoltage-clamped whole-cell experiments.

This paper describes the detailed characteristics of the whole cell and single-channel mechanosensitive (MS) current activated by direct stimulation in ventricular myocytes isolated from chick embryos. We show that the mechanotransduction pathway is complex, and in a constant state of flux, from both internal realignment and the effect of an autocrine inhibitor of MS currents.

Materials and Methods

Cell Preparation

The heart cells used in this study were acutely isolated from the ventricle of embryonic White Leghorn chicks at the 16th or 17th day of egg incubation. The chick embryo was removed from the cleaned eggshell and immediately decapitated. The heart was rapidly excised, and washed in calcium-free PBS. The ventricle was separated, placed in fresh calcium-free PBS, and minced with a razor blade. The tissue was placed in 9 ml of preheated (40°C) enzyme solution (9 mg collagenase (SIGMA) and 53 mg trypsin (SIGMA) in 35 ml calcium-free PBS) which was magnetically stirred in an incubator (40°C) for 10 min. The enzyme solution was then refreshed, and the tissue returned to the incubator for further periods of 8, 8 and 6 min. The excess fluid was then removed, and the preparation was washed in 9 ml of minimum essential chick media (MEM, GIBCO) supplemented with 10% calf serum and 2% home-made chick embryo extract. The tissue was triturated carefully before being filtered to remove large debris, then gently centrifuged for 45 sec. The cells were resuspended in 6 ml of media, and distributed onto 12 poly-L-lysine coated coverslips in 35 mm diameter culture dishes. The cells were incubated for 30 min, then a further 2 ml of chick media was added to each dish. The cells were kept under constant temperature (37°C), humidity (88%), and carbon dioxide (5%) until used in the experiments. All cells were used within 12–36 hr of preparation, and all experiments were performed at room temperature.

Equipment

Whole cell Nystatin-perforated patch-clamp techniques were used in these experiments (Hamill et al., 1981; Horn & Korn, 1992). The experimental chamber was placed on the stage of an inverted Olympus microscope located on a vibration isolation table (Newport Research). The patch pipettes were made from capillary glass (Drummond Microcaps, Thomas Scientific, NJ) pulled to form tips with a resistance of a few mΩ on a Sachs-Flaming PC-84 programmable pipette puller (Sutter Instruments, San Rafael, CA). The ground reference was provided by a chlorided silver wire separated from the bath by an agar bridge. Solutions were exchanged rapidly with solenoid valve control system (ALA Scientific Instruments) which allowed the flow of up to 8 different solutions to be controlled remotely and perfused over the cell.

Electrical recordings were made using a CV-203BU headstage (Axon Instruments, Burlingame, CA) connected to an Axopatch 200B Integrating Patch Clamp Amplifier (Axon). The patch electrode was positioned on a quartz rod separate from headstage, and connected by flexible tubing, to isolate as much mechanical movement as possible. Data were recorded by the LABVIEW data acquisition software (National Instruments) or recorded (with audio commentary), after passing through a 2-channel digital data recorder (VR-10 Instrutech, NY) onto videotape (SONY SLV-675HF) for later analysis.

The patch electrode was controlled by hand via a piezoelectric micromanipulator (PCS-PS60, Burleigh Instruments, NY). A second piezoelectric manipulator (PCS-250 Burleigh Instruments, NY) was used to control the mechanical stimulation electrode. Data acquisition and analysis were performed on a DELL Pro200n via a data acquisition board.

Solutions

All in mM unless otherwise noted: *High Sodium:* 150 NaCl; 5 KCl; 2 $CaCl_2$; 1 $MgCl_2$; 10 HEPES, pH 7.4 (titrated with NaOH). *High Potassium:* 150 KCl; 5 NaCl; 0.5 EGTA, 10 HEPES; pH 7.4 (titrated with KOH). *Nystatin:* as for High Potassium, with 200 μg/ml supersaturated solution of nystatin (dissolved in DMSO). In the perforated patch experiments, the tip of the patch electrode was dipped in a nystatin-free electrode solution before being back-filled with nystatin solution. This

ensured a good seal would be achieved before nystatin began to perforate the membrane.

Experimental Protocols

The isolated embryonic ventricular chick myocytes were spherical (diameter 10–20 μm) immediately following dissociation from the heart, and quiescent in normal Tyrode's solution at room temperature (20–24°C). Once a nystatin-perforated patch (Horn & Korn, 1992) was made on the cell and voltage-clamp established, each cell was briefly tested for a normal electrophysiological response to either a series of voltage-clamp steps or a slow voltage ramp before its mechanical responses were investigated.

Whole cell mechanical currents were elicited either by direct pressure on the cell with a mechanical probe, or by suction through the mechanical probe (*not* the patch electrode). Two types of mechanical probe were used in these experiments. The first was made from 1 mm I.D. capillary glass, one end of which was fire polished to form a bulbous tip (diameter 5–10 μm). A fresh probe was used for each cell to reduce the possibility of contaminants from the previous cell sticking to the glass. The position of the probe was controlled by a triaxial piezoelectric micromanipulator (Burleigh Instruments), which was driven by computer using Labview Instruments and/or a sine wave signal generator (Hewlett Packard 8116a Pulse/Function Generator). The piezoelectric manipulator had a manual override feature, which enabled the height of the probe above the cell to be altered while the probe continued to oscillate in a sinusoidal fashion.

The second type of mechanical probe was the cantilever from an Atomic Force Microscope (AFM), positioned so that the sharp tip of the sensing element pointed away from the cell (therefore presenting a solid bar of silicon to the cell) (Fig. 1). The resistance of the cantilever was 50 N/m. The piezoelectric manipulator controlling the mechanical probe had a maximum excursion of 60 μm, and the relative position was monitored by the calibrated driving voltage.

The mechanical probe was used to deform the cell directly. The mechanosensitive response was independent of the particular probe used. The cells were stimulated by bringing the mechanical probe into contact with the cell, which was resting on the bottom of the experimental bath. In some experiments the probe was driven in a series of trapezoidal displacements. These were approximations to square waves that suppressed parasitic oscillation of the probe. In these experiments, the probe was brought down onto the cell by manual override of the piezoelectric manipulator until the cell was visually observed to deform and a mechanosensitive current was registered electrically. The degree of deformation needed to elicit the MS current was noted on the digital readout of the piezoelectric controller when the probe was controlled by hand, and then Labview was programmed to give a reproducible stimulus large enough to initiate an MS response in the computer controlled phase of the experiment. This usually corresponded to 1–2 μm of Z direction probe displacement.

In the sinusoidal stimulus experiments, the probe was set in sinusoidal motion normal to the cell membrane (i.e., the Z direction), then was lowered gently until the pipette was visually observed to be making contact with the myocyte and generated an electrical response. The amplitude and frequency of the sinusoidal motion was held constant throughout the experiment.

The degree of mechanical deformation of the cell (and so the postulated forces in the components connected to the MS channels) was assumed to be directly related to the distance of the probe excursion, i.e., the strain. The cells showed no visible signs of a maintained alteration in shape or position during repeated mechanical stimulation, but it was impossible to track small changes in shape (particularly changes normal to the focal plane of the microscope), or changes in cytoskeletal or membrane bilayer structure during stimulation. The cells were placed on poly-L-lysine coated coverslips (which were anchored to the base of the experimental chamber) to reduce the possibility of cells moving laterally during or after stimulation.

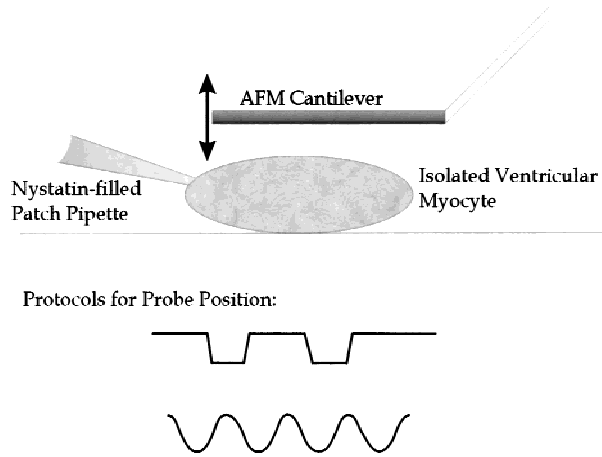


Fig. 1. The experimental setup. The mechanical probe was attached to a piezoelectric manipulator, and moved (under computer control) in the Z direction (with either discrete trapezoidal or continuous sinusoidal movements) to deform the myocyte. The cell was resting on a poly-L-lysine-coated coverslip to provide some adhesion and minimize the lateral movement when being pressed from above. The cell was voltage clamped, and the whole-cell mechanosensitive current was recorded via a nystatin-perforated patch. All experiments were performed at room temperature.

Results

Whole Cell MS Currents in Response to Suction on the Membrane

Prior to the start of the computer-controlled phase of the experiment, the mechanical probe was moved under manual control (via the piezoelectric manipulator) to ascertain where the top edge of the cell was, and to confirm that the cells had a mechanosensitive current. Nearly all cells exhibited mechanosensitivity on initial contact between the probe and the cell.

On a few occasions, a particularly large inward current was seen when the probe was hand controlled and initially made contact with the cell, but later, when control was transferred to Labview, no subsequent MS current was seen, even though the magnitude of the probe excursion was as large, and sometimes larger (as indicated by the piezoelectric manipulator digital meter), than the movement of the probe had been under hand control. This was the first indication that the mechanosensitive response was dependent on stimulation history.

In almost all cases, an MS current of a few was seen when stimulating the cell manually, and this current reappeared when the probe was under computer control.

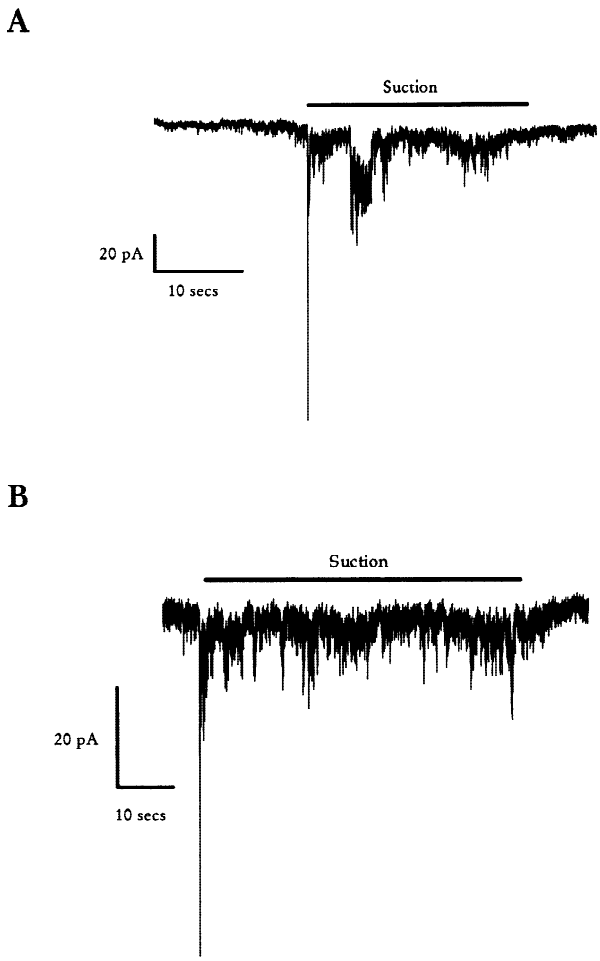


Fig. 2. Whole cell MS currents evoked by membrane suction. An open-tipped glass pipette was gently placed on the cell membrane, on the opposite side of the cell to the recording electrode. Suction applied through the pipette evoked an inward whole cell current, which was preceded by a large and brief inward spike of current.

A few cells exhibited no mechanosensitivity at first, but for the majority of these, changing the position of the probe on the cell resulted in an MS current. For the few cells that showed no response, even after repositioning the probe, an MS current could be elicited by exchanging the mechanical probe for an open tipped glass pipette and applying suction to the cell membrane.

Figure 2 shows that the whole cell current initiated in this way was inward (at negative holding potentials), and was often accompanied by an initial large inward spike of current. The MS current lasted the length of time the suction was applied. Similar inward current spikes are seen at the start of the mechanosensitive response from acutely isolated rat ventricular myocytes activated by direct stimulation with a mechanical probe (Bett & Sachs, 1999).

During the application of suction, the noise level increased dramatically, indicating that channels were being opened. On termination of the suction, the whole cell current and the associated noise levels returned to the control levels. Interestingly, if the open pipette was used as mechanical probe after initiating a suction-activated current, an MS current was then seen with direct strain, even though the cell had been completely inert prior to applying suction to the membrane. This implies that the strain produced by suction disrupted some structures protecting the channels from strain.

Whole Cell MS Currents in Response to Pressing Directly on Isolated Cells with a Mechanical Probe

General Characteristics of the Whole Cell MS Current

The characteristic response of an isolated chick ventricular cell to direct mechanical deformation is shown in Fig. 3. As the probe pressed down onto the cell, the MS current activated rapidly, though not until after the cell was visually observed to deform. When the probe reached its maximum excursion the current peaked and then inactivated even though the mechanical probe maintained its position. There was no visible evidence of the cell moving laterally under the probe. As the probe was lifted off the cell, the MS current decayed quickly (if it had not inactivated completely during the plateau), returning to the original resting current. There was no long-term effect of mechanical intervention on the background current of the cell, which always returned to the prestimulus value.

As the probe began its upward movement, a time at which the deformation of the cell (and the tension in the load bearing elements of the membrane) should be decreasing, a slight increase in the MS current was seen in about 30% of the responses (noted by an asterisk in Fig. 3). This may have been due to the extracellular matrix bonding to the probe when the two were in contact, so that there was a slight increase in tension as the probe began its movement up from the cell.

In a double press protocol with a 1.5 sec interval between stimuli, as shown in Fig. 3, the peak amplitude of the first MS current was always greater than the peak of the second press. The relationship between the first and second press current peaks was independent of the initial (press 1) peak amplitude. The peak-to-peak data were fit linearly with a slope of 0.778 ± 0.063, as is shown in Fig. 4.

The double press protocol was repeated on each cell in a sequence with a progressive variation in the excur-

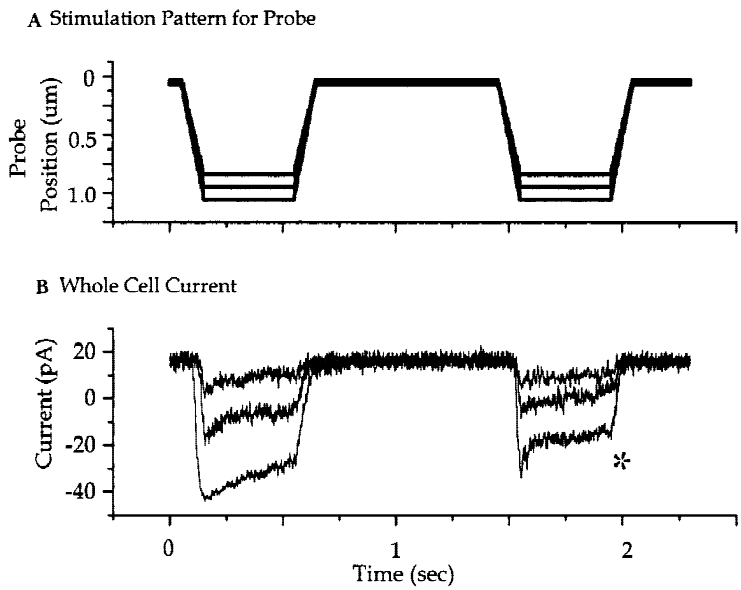


Fig. 3. A typical whole cell MS current. The mechanical probe was moved in a trapezoidal manner (of varying magnitude), starting from just above the top of the cell (as judged by eye). (*A*) Shows the trapezoidal stimulation pattern followed by the probe. (*B*) Shows the whole cell current. The amplitude of the MS whole cell current was proportional to the probe excursion. The current activated quickly, inactivated during the plateau of the trapezoidal stimulus, and decayed quickly once the stimulus was removed. Note the occasional increase in current just as the probe was being lifted off the cell (marked with an asterisk), which was probably caused by adhesion of the probe to the cell. Holding potential was −50 mV.

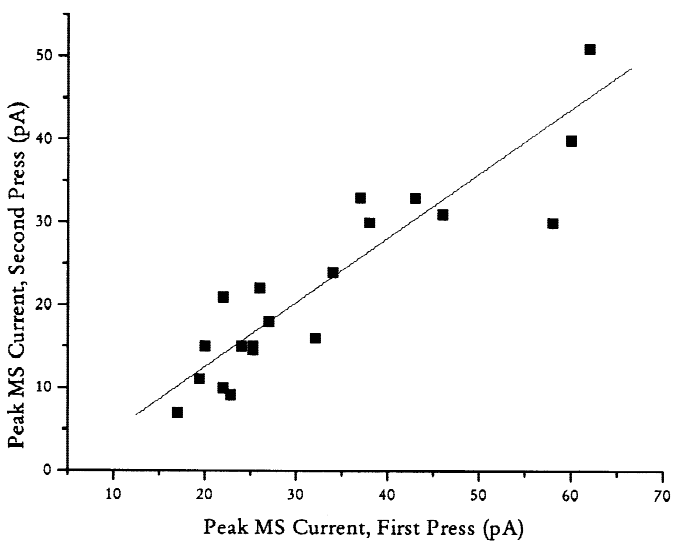


Fig. 4. Relationship between peak MS currents in a double press protocol. The peak MS current evoked by the second press in a double press series is plotted *vs.* the peak current in the first stimulus. There is a linear relationship: the red line represents a fit to: $y = a + bx$ where $a = -3.03 \pm 2.38$ and $b = 0.778 \pm 0.063$.

sion amplitude of the mechanical probe. The relationship between sequential peak MS currents depended on whether the protocol was designed with a sequence of increasing or decreasing magnitude of probe movements, again indicating that the mechanosensitive response was history-dependent. Figure 5 shows the results from a double press protocol. The peak magnitude of the MS current (i.e., the current when the probe first reaches its maximum excursion subtracted from the baseline current) is plotted against the size of the probe excursion. Figure 5 shows the peak MS currents obtained at different probe displacements for seven progressive series.

In Fig. 5*A*, the manipulator was programmed to press twice (at 1.5 sec intervals, with the double press repeated after 2 sec rest interval) onto the cell a distance of 2.0 μm, then 1.8, 1.6, 1.4, 1.2 and 1.0 μm. The peak currents obtained from three repetitions of such a sequence are shown (the smaller excursions elicited a small MS current, if any). The results were fairly reproducible, with a reduction in probe excursion resulting in a corresponding reduction in the magnitude of the peak MS current. The peak of the MS current initiated by the second stimulus of the double pulse protocol (joined by dotted lines in Fig. 5*A*) followed the same pattern as the peak current initiated by the first press, apart from a slight decrease in amplitude. The absolute values of the peak MS current was little changed over the three repetitions of the double press protocol, i.e., the time dependency of repeating this stimulation sequence was minimal.

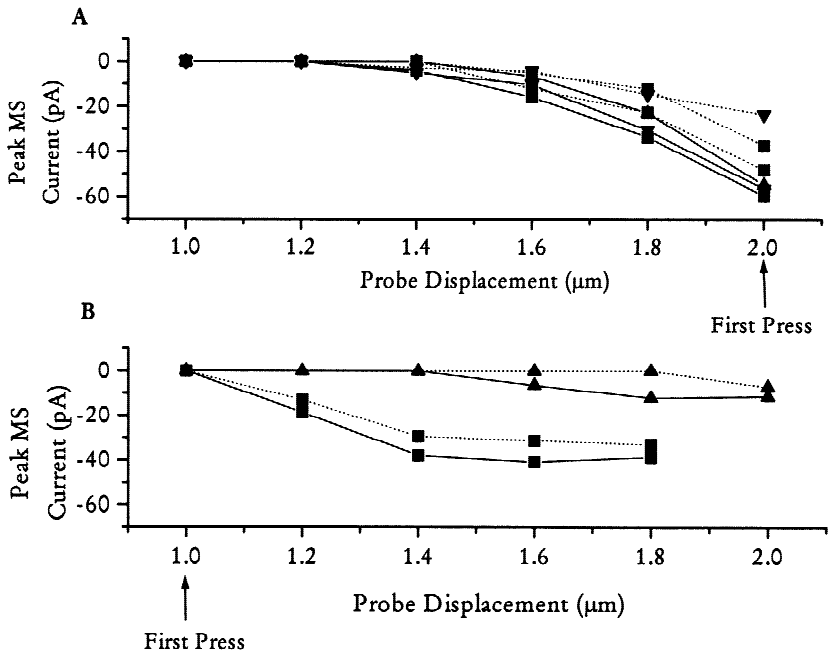


Fig. 5. Pressing on the cell with a graduated sequence of probe movements. The peak whole cell MS current activated by stimulating the cell with: (*A*) Decreasing (2.0 μm to 1.0 μm in 0.2 μm decrements) and (*B*) increasing (1.0 μm to 1.8 μm in 0.2 μm increments) excursion of the probe. (*A*) Shows three repetitions of decreasing magnitude double press protocol on the same cell. (*B*) Shows two sets of data from repetitions of increasing magnitude on the same cell. Solid lines join the peak MS current from the first press and dotted lines join the peaks from the second stimulus in a given double press pair.

Figure 5*B* shows the converse experiment, when the double press series was applied with probe excursions increasing from 1.0 to 1.8 μm in steps of 0.2 μm. Two such sequences from the same cell are shown. The peak MS current generated with an increasing trapezoidal step size was much less reproducible than the ones obtained with decreasing step sequences. The peak current initially increased monotonically with increasing mechanical stimulation, but the magnitude of the increase tapered off as the series progressed. In both the increasing stimulus sequences shown, the peak current generated by the largest deformation was actually smaller than that obtained with the penultimate stimulus. The peak of the MS current evoked by the second press of the double press protocol (joined with dotted lines in Fig. 5*B*) exhibited similar characteristics to the peak MS current stimulated by the first press.

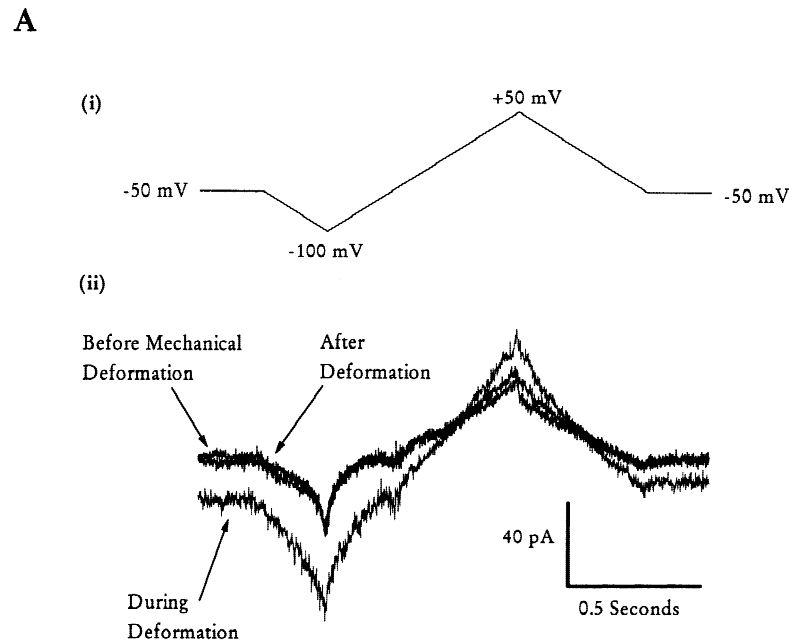
The probe was not moved laterally in the interval between recording the two series of data plotted in Fig. 5*B*, nor was there any visual change in the appearance of the cell, but the reduction in the magnitude of the peak MS current between the two sequences was about 80%. Comparing the peak current magnitudes of Fig. 5*A* and *B*, the MS current activated by pressing on the cell with a large deformation following a period of rest (as in Fig. 5*A*) results in a larger peak MS current than pressing on the cell with a series of deformations and gradually arriving at a stimulus of the same magnitude (as in Fig. 5*B*). Clearly, there is some time or history dependency in the mechanosensitive response of the cells.

Current Voltage Curves with Constant Applied Deformation

Ramp *I-V* curves were recorded from the isolated quiescent chick ventricular myocytes. These were then repeated when cell was undergoing continuous and steady direct mechanical stimulation, as a means of determining the voltage dependency of the steady-state component of the response to mechanical intervention.

Figure 6*A* confirms that there is no long-term component to the mechanosensitive current evoked by pressing on the cell gently with a probe, for the pre- and poststimulus traces are identical. The baseline response to the ramp voltage was subtracted from the current observed in the presence of mechanical stimulation, and an *I-V* curve for the mechanosensitive current was constructed. Figure 6*B* shows four difference traces (the current in the presence of mechanical stimulation minus an average of the before and after currents) indicating that the steady state MS current has a linear relationship to the voltage, and a reversal potential in the range −10 to 0 mV.

The four traces were obtained at different degrees of cell deformation. All traces passed through a single reversal potential, although the slope of the linear MS current varied according to the magnitude of the mechanical stimulation (the larger the probe excursion, the larger the current for a given voltage). It was not possible to plot *I-V* curves obtained by plotting the peak MS current activated by a short-term mechanical stimuli at various



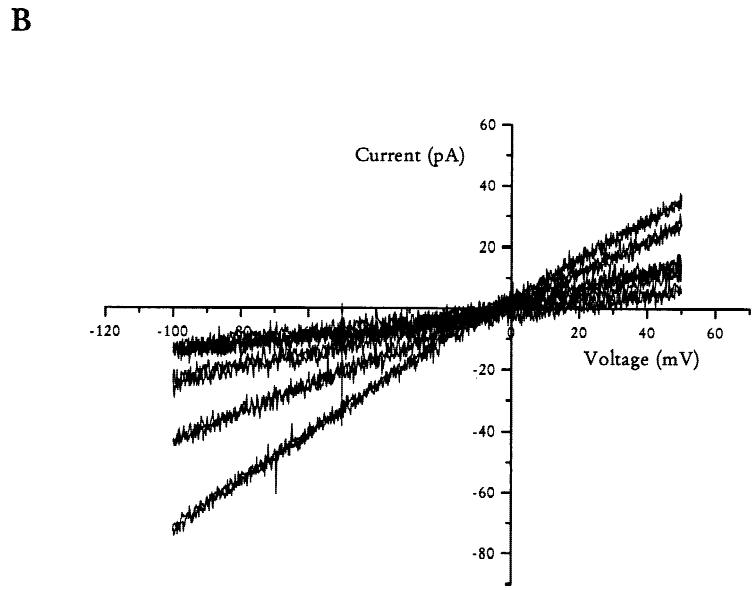


Fig. 6. *I-V* curves of the steady-state MS current. (*A*) (i) The applied ramp voltage. (ii) Whole cell current traces before, during, and after steady direct mechanical stimulation (*B*) Difference currents (MS current—control) showing the voltage dependency of the mechanically activated portion of the whole cell current. Four difference currents from the same cell (recorded at different magnitudes of deformation) cross the $I = 0$ line at the same point, though have different current amplitudes corresponding to the stimulation size. The larger the deformation, the larger the current for a given voltage.

holding potentials, for the magnitudes were too dependent on the history of stimulation.

Activation Characteristics of the MS Current

Although the amplitude of the MS response was highly dependent on the stimulation history of the cell, some elements of the activation of the MS current were consistent. The probe produced a visible deformation of the cell for at least 75% of the stimulus excursion, but the MS current was only ever activated in the last third of the excursion. The link between cell deformation and activation of the MS current is clearly not simple, even discounting the complication of history dependency. There may be a delay in activation somewhere in the transduction mechanism, some slack in the tension-bearing ele-

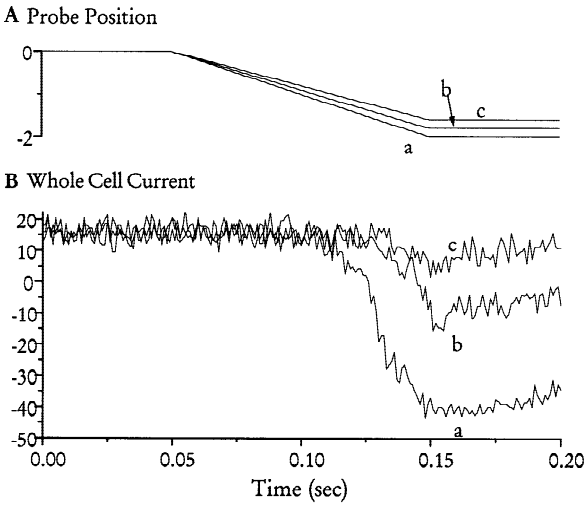


Fig. 7. Activation of the MS currents. (*A*) Probe position. Three probe protocols are shown with excursions to a − 2.0 μm, b − 1.8 μm and c − 1.6 μm. (*B*) Whole cell current. The three current traces evoked by the three probe positions (a, b, and c) are shown. The currents b and c activate rapidly, and reach their peak when the probe reaches its maximum level. Current a has an exceptionally large peak (~60 pA) and the rate of inactivation slows down before the peak current is reached.

ments, or perhaps a certain energy barrier to be overcome before the channel can open.

Once the channels began to open, the rate of activation (*dI/dt*) of the MS current (for a given cell) was independent of the magnitude of the stimulus. The magnitude of the peak MS current appears to be determined solely by the time at which activation starts, with larger currents activating earlier than smaller ones. There was no specific delay associated with a given stimulus size, nor was there a constant relationship between the probe position and start of the activation. The MS current would sometimes continue to increase for a few msec after the peak of the stimulus deformation was reached, but this was rare, and in any case never exceeded 20 msec. The magnitude of the peak MS current was not correlated with the time at which the peak was reached.

The activation rate did vary moderately from cell to cell. This could be due to general cell to cell variation or changes in the area of the cell affected by the mechanical probe, the detailed shape of the probe and perhaps even the position and attitude of the probe relative to the cell. The probe was under micromanipulator control in the Z direction, but a fresh probe was used for each cell (to minimize the effect of cells bonding to the glass), resulting in slight differences in probe shape and attitude with respect to the cell surface for each experiment.

In a double press protocol the kinetics of activation of the current initiated by the second press were identical to the first press, though the onset was delayed by a few msec, thus producing a smaller current. There was no visible alteration in the cell between the first and second presses, so this may relate to some internal restructuring.

The only deviation from the homogeneity of the activation time for a given time was when an unusually large (around 60 pA) current was initiated. In these cases, the activation rate tailed off before the trapezoidal stimulus reached its peak. This may be due to saturating the whole cell conductance or perhaps the unusually large size of the MS current lead to early onset of the inactivation process. There was a clear reduction in noise when the current slowed in activation this way, which suggests the probability of the single channels being open is near maximal. As the current decayed, the noise level increased once more, which suggests that the current inactivation is a result of a decrease in the probability of the channels being open, rather than a decrease in the number of channels available to be opened.

Figure 7 shows an example of MS current activation rates. The three current traces were obtained from three different stimulus magnitudes on the same cell. The largest deformation, trace a, resulted in a 60 pA current, and the rate of activation decreased before the peak current was reached. Traces b and c were obtained with smaller stimuli, and they began activation later than trace a. The "on ramp" of the trapezoidal stimulation was always 100 msec in duration, regardless of the final amplitude reached, so there was a slight difference in the time at which the probe reached a given distance (and presumably similar strain) for these three traces. However, the time of initial activation was not related to the actual position of the mechanical probe.

Inactivation of the MS Current During the Stimulation Plateau

The whole cell MS current always inactivated during the steady plateau of the trapezoidal mechanical stimulation even though the probe remained steady at the maximum excursion. Occasionally, the current would decay completely, with the whole cell current returning to the prestimulus value before the end of the stimulation period, but in most cases the current reached a nonzero steady-state level. In a few cases the current was still declining when the mechanical stimulus was removed.

For those currents which exhibited a steady decrease in MS current without major perturbations, the rate of inactivation of the current during the stimulus plateau was fit (using the least squares method) to a single exponential.

Figure 8 shows that except for the largest peak currents, the rate of inactivation of the MS current during the stimulus plateau was independent of the size of the peak current. The very smallest currents (with peak current less than 10 pA) were difficult to fit, with the small-

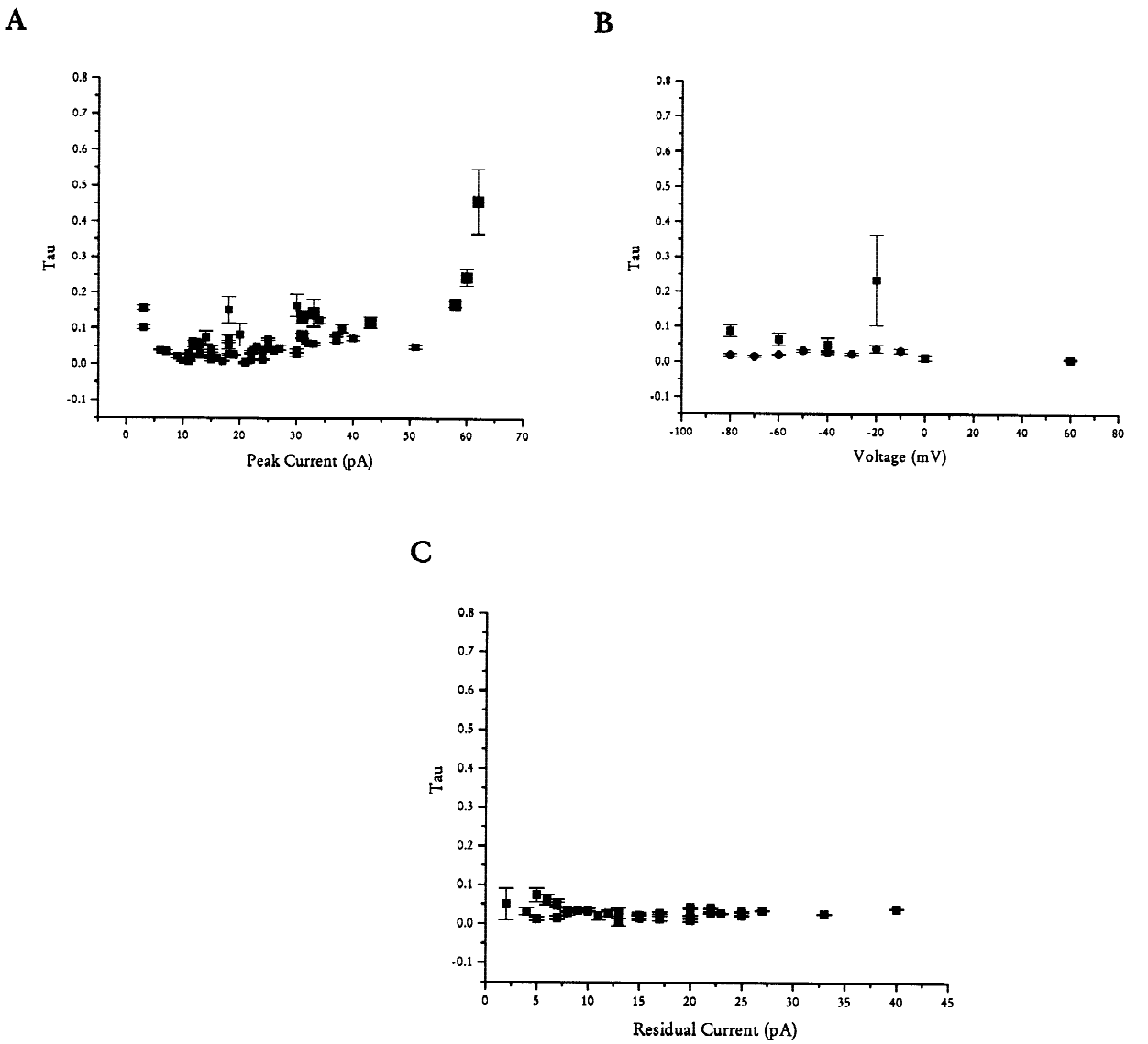


Fig. 8. Inactivation of the current. (*A*) Fitting the inactivation of the MS current during the constant stimulus plateau to a single exponential gave values of tau which were independent of the magnitude of the peak MS current. (*B*) The inactivation rate during the steady mechanical stimulus of the plateau region was independent of voltage, even if the voltage was positive enough (+60 mV) to generate an outward MS current. (*C*) The rate of decay of the residual plateau current when the mechanical probe was lifted away from the cell was independent of the steady-state plateau current, and was the same magnitude as the rate of inactivation of the MS current during the constant stimulation plateau.

est MS current peaks being distinguishable from a large brief peak in the background noise only by their temporal correlation to the mechanical stimulus.

Some of the larger currents (around 60 pA) had relatively slow rates of decay, with the suggestion of an inverse relationship between the size of the initial peak and the subsequent rate of inactivation of the current. However, these were currents whose initial activation had been reduced before the probe reached maximum deformation, and may, as discussed above, have reached the limit of available MS channels.

Figure 8*B* shows the inactivation rate for MS currents as a function of voltage (on the same scale as Fig. 8*A*). The inactivation rate was independent of voltage, and was not dependent on the direction of the current: the decay of the outward MS current at positive holding potentials was similar to that seen in inward currents. This is quite different from the strong voltage sensitivity seen by MS channels *Xenopus* oocytes (Hamill & McBride, Jr., 1992) and in C6 glioma cells (Bowman & Lohr, 1996).

The constancy of the inactivation rate is further emphasized by the fact that the relationship between the peak MS current and the plateau current remaining at the

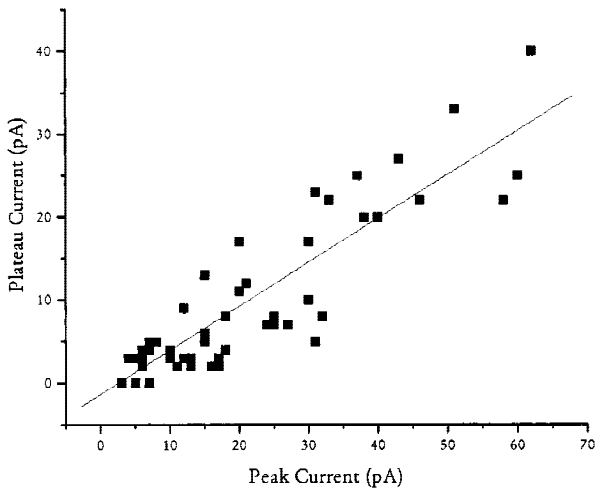


Fig. 9. Relationship between the MS current at the beginning and end of the period of constant stimulus. By the end of the plateau the MS current always fell to about 50% of the peak current. Line is a linear fit to $Y = A + B \cdot X$ where $A = -1.36 \pm 0.96$ pA and $B = 0.529 \pm 0.038$ pA^{-1}.

end of the maximal deformation was linear. This is shown in Fig. 9.

Post-press Deactivation

In a few cases, the MS current decayed away completely before the mechanical probe was lifted from the cell. Whenever there was a significant current remaining at the end of the stimulus plateau, the current decay as the probe was lifted could be fit by a single exponential. The decay rate was, for the range of plateau currents observed, independent of the current, and similar to the rate of inactivation seen during the stimulus plateau (Fig. 8*C*). There was no relationship between the post-press deactivation rate and the initial MS current peak. The mean τ for post-press deactivation was: 0.037 ± 0.004 sec (SE, $n = 41$).

Continuous Stimulation Studies

In an effort to reduce the hysteresis of response, and eliminate the confusion of the history dependence to discrete mechanical stimuli, we used a continuously modulated strain. The mechanical probe was driven by a sinusoidal signal generator so that the probe moved in the Z direction with a constant frequency and amplitude. Once the probe was set in a sinusoidal motion with peak-to-peak movement of 1–2 μm, it was slowly lowered onto the cell under manual control via the piezoelectric manipulator. Once an electrical response was observed, the Z position of the probe was held steady.

An MS current was initiated when the oscillating probe was brought into contact with the cell, but this stimulus resulted in two distinct types of response, as is shown in Fig. 10. If the initial current was small (around 10–15 pA) the peak current decayed over the course of a couple of cycles to a nonzero steady state, as is shown in Fig. 10*A*. The residual peak current was on the order of 5–10 pA. If the initial contact of probe and cell resulted in a large MS current (greater than 20 pA) the current decayed completely with 10 cycles, and no further MS current was seen, even in the presence of continued mechanical stimulation (Fig. 10*B*). In both cases, the MS current that did appear was in phase with the oscillations of the probe with minimal phase delay.

A localized perfusion system which enabled the rapid change of fluid around the cell was placed near the cells undergoing sinusoidal stimulation. We used the system to deliver high sodium solution which was identical to that already in the bath. Most surprisingly, on cells that responded to the sinusoidal stimulus with a nonzero steady state MS current envelope localized perfusion of normal saline solution resulted in an augmentation of the MS current (Fig. 11*A*).

To eliminate the possibility that the enhanced MS response was due to soluble components in the perfusion tubing, a slight temperature difference between the fluid in the tube and the bath, or any other external influence, the localized application system was converted to a localized draining system to remove fluid from around the cell and draw fluid from the rest of the experimental bath across the cell. Figure 11*B* shows that this also increased the mechanosensitive current.

The two techniques of rapid localized fluid movement used to obtained the traces in Fig. 11*A* and *B* apply very different shear stresses to the cells, so it is unlikely that the changes the MS response is a result of the stress of fluid forces per se.

The result of using the localized fluid delivery system with a cell in which the MS current inactivated completely is shown in Fig. 11*C*. Once the initial MS current had decayed completely, high sodium solution was applied to the cell. This resulted in the reappearance of the MS current. When the fluid exchange ceased, the MS current again decayed to zero. These data suggest there is some constituent excreted by the cells that inhibits the MS response.

Modeling

For a channel to be mechanosensitive, there must be a relationship between mechanical stimulation of the membrane-protein-cytoskeleton complex and activation of the channel. The energy imparted to the channel from the mechanical deformation must result in the conduc-

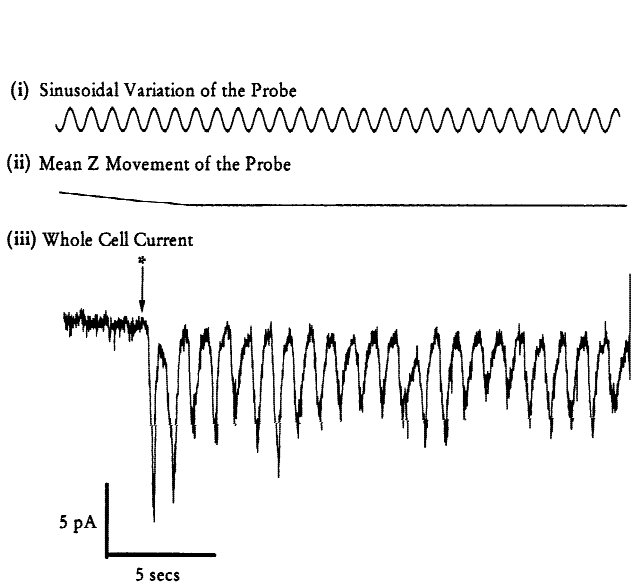
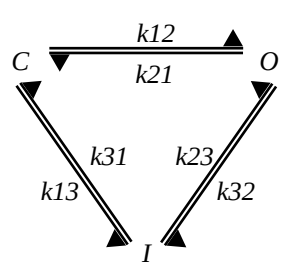


Fig. 10. Sinusoidal stimulation produces two types of response. (i) Sinusoidal variation of the probe in the Z direction. (ii) Mean movement of the probe in the Z direction (controlled by manual override of the piezoelectric micromanipulator). (iii) Whole cell current. The probe was set in sinusoidal motion, then gently lowered onto the cell. The asterisk marks the place at which the cell and probe first came in contact with each other. In *A*, the initial MS current elicited is less than 15 pA, and the current envelope quickly decays to a nonzero steady state level. In *B*, the initial current is large (over 50 pA) but the current envelope quickly decays away to zero. There was no obvious physical or electrical differences between the cells which produced the two responses, apart from the size of the initial MS current.

tance of the channel being altered (some MS channels are closed by mechanical stimulation, whereas others are opened). The MS current in the chick ventricular myocyte is activated by stretch, inactivates under steady-state mechanical deformation, and recovers rapidly when the mechanical stimulus is removed. There is also a dependency on the history of stimulation, possibly due to some combination of cytoskeletal/membrane rearrangement, and the autocrine inhibitor.

The program Scientist was used in attempt to fit the data (using least squares) to a three-state model, with closed (*C*), open (*O*) and inactivated (*I*) states, linked by the rate constants as shown below:



The whole cell MS current was given by:

$$I = I_o + GP_o \quad (1)$$

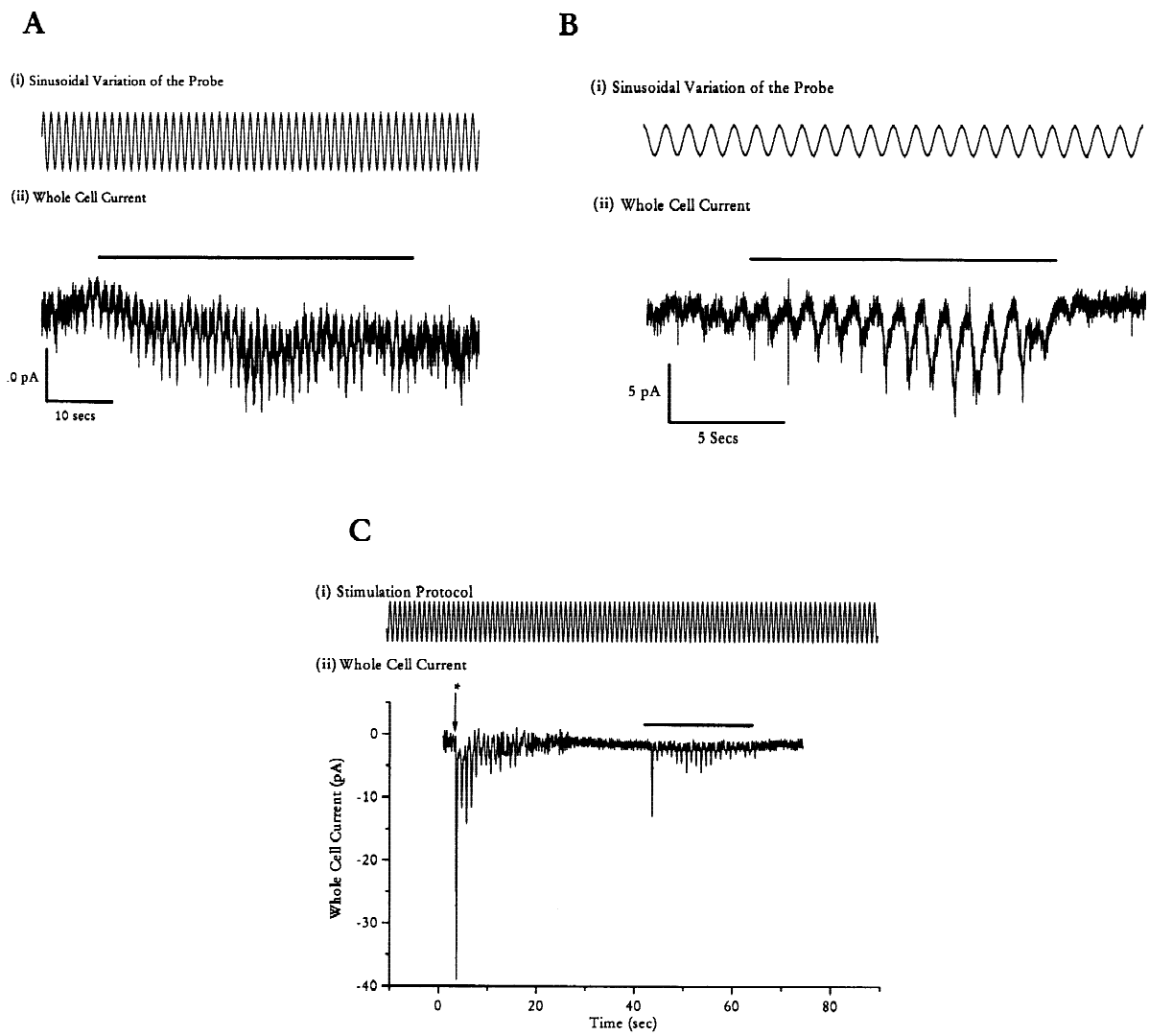


Fig. 11. Rapid exchange of fluid near the cell alters the magnitude of the mechanical response. (i) Sinusoidal stimulation protocol of the mechanical probe in the Z direction. (ii) Whole cell current. In cells that had a nonzero steady-state response to a sinusoidally varying mechanical stimulus: (*A*) local application of regular bath solution resulted in an increase in the MS response and (*B*) withdrawal of bath fluid in a sink localized near the cell had a similar effect. (*C*) In cells which lost mechanosensitive response completely, even when the mechanical stimulation persisted, the MS current would reappear when the fluid surrounding the cell was exchanged for fresh bath solution. The solid bar represents the time at which the solutions were changed.

Where P_o is the probability of the channel being in the open state, G is the whole cell conductance of the mechanosensitive channels and I_o is the background whole cell current in the absence of mechanical stimulation.

The mechanosensitivity of the cell was modeled as being equivalent to two springs and a velocity-dependent sensor coupled to a channel sensor which is deformed by mechanical strain from length x to $(x + dx)$. (The model represents a summation of forces acting on the channel, and should not be interpreted as being equivalent to any specific components of the cell cortex.) Given the arrangement of these components as in Fig. 12*A*, the force across the channel sensor is equal to

$$K1x1 + b(dx1/dt) \tag{2}$$

Where:

$$dx1/dt = (K2(x\text{-}x1) - K1x1)/b \tag{3}$$

and b is a constant related to the dash pot, $K1$ and $K2$ are spring constants and $x1$ is a variable distance. The only rate constant that was made mechanosensitive was $k12$, the transition rate from closed to open states. This rate constant was determined by the following relationship:

$$k12 = k_{120}\exp\{a[K1x1 + b(dx1/dt)]\} \tag{4}$$

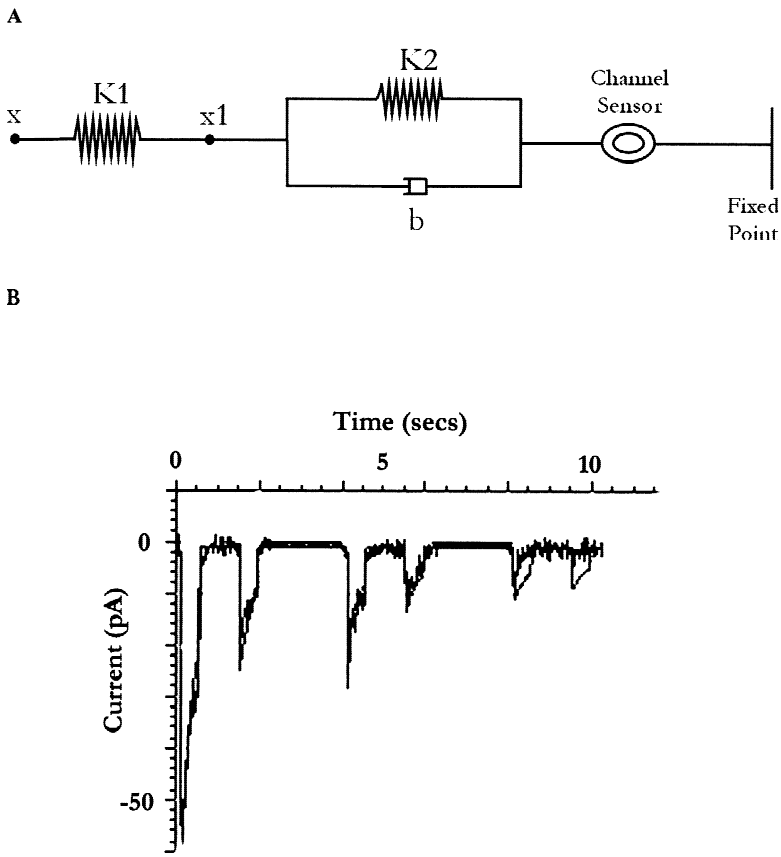


Fig. 12. Modeling the MS current. (*A*) The transduction pathway was modeled as having two springs (representing elasticity in the cell cortex), with constants K_1 and K_2, and a dashpot (representing the viscosity), which relaxes and reduces the force on the channel, producing adaptation. This system was used to describe the activation rate constant from closed to open channels *k12*. (*B*) Modeled data (Solid line) and experimental data (dashed line) from a series of decreasing double press stimulations. The model could fit one series of stimulations of either increasing or decreasing amplitude, but not both.

where k_{120} and a are constants. The probability of being in the open state was calculated from the rate constants linking the model states.

This model was fitted to data obtained from five separate cells. Any single episode or double press activation of the MS current was reasonably fit with the three-state model detailed above. A single series of mechanical excursions of either increasing or decreasing probe amplitude could also be fit by the model, as shown in Fig. 12*B*. However, the parameters used to fit a sequence of decreasing steps would not fit a series of increasing steps from the same cell. Changing the connections of the three-state model, increasing the number of states to four, and making another rate constant force dependent still did not produce a model which could fit the data from both increasing and decreasing series. Typical rate constants for fitting the three-state model to a decreasing series of presses, as shown in Fig. 12*B* are shown in the Table.

Table. Values of parameters in a three-state model that fits the data from a series of mechanical depressions of decreasing amplitude

Parameter	Value
a	25.20
b	5.214
k120	430.16
k230	3.156
k21	108964
k23	10
k32	0
K13	0
K31	0.160
k1	−2.06
k2	0.090

Model and original data are shown in Fig. 12*B*

Discussion

General Comments

MS channels were first discovered in 1984 (Guharay & Sachs, 1984), and identified in the heart in 1988 (Craelius, Chen & El-Sherif, 1988). Although their activity has been recorded in various parts of the hearts from different species, little concrete data exist on their behavior. The data that do exist are sometimes contradictory, although advances have been made in identifying and cloning bacterial (Sukharev et al., 1997*b*), and some eukaryotic mechanosensitive channels (Krapivinsky et al., 1995; Patel et al., 1998; Maingret et al., 1999). Little headway has been made in determining the components

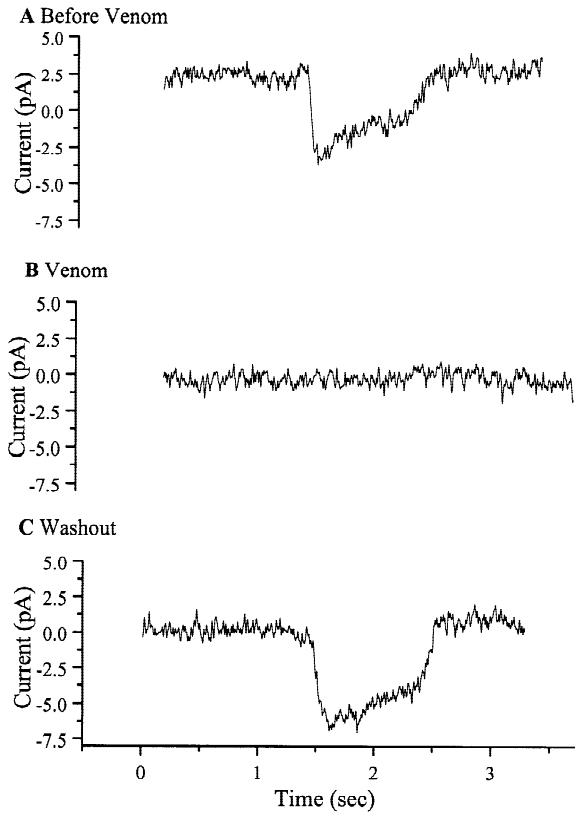


Fig. 13. Block of the MS current by venom from the spider *Grammostola spatulata* (1:100,000). Using a single trapezoidal deformation of the cell at 1-min intervals, the MS response was eliminated in the presence of the venom, within 1 minute of application; 2 min after washout of the venom began, the mechanosensitive response reappeared.

and structure of the eukaryotic mechanotransduction system.

Part of the difficulty in interpreting MS data is that a wide variety of experimental approaches have been taken to activate MS channels. Methods used to stress isolated heart cells include: suction or pressure applied through a pipette (Yang & Sachs, 1989); pulling the cell with adherent carbon fibres (White et al., 1993) or pipettes (Palmer, Brady & Roos, 1996; Zeng, Bett & Sachs, 1999), prodding a cell with a glass pipette (Hu & Sachs, 1997; Bett & Sachs, 1999); and growing cells on flexible culture dishes undergoing cyclic stretch (Naruse & Sokabe, 1996). (Osmotically challenging cells with anisotonic media (*see* Vandenberg et al., 1996) is a distinct mechanism, resulting in activation of chloride currents.)

These diverse techniques probably deliver dissimilar stress to the mechanosensing elements of the cell, so extrapolation from one type of experiment to the other is difficult. Quantification of force in elements of the mechanotransduction system is impossible, no matter how well defined the stimulus may be. The relationship between these various mechanical interventions and the stress and strains undergone by a heart cell in situ is unclear.

Mechanosensitive channels have been identified and well-documented in chick ventricular myocytes (Ruknudin, Sachs & Bustamante, 1993; Hu & Sachs, 1997). They are reversibly blocked by gadolinium (Hu & Sachs, 1997) and venom from the spider *Grammostola spatulata* (*see* Fig. 13). In this paper, we presented detailed behavior of the MS current in isolated chick ventricular myocytes, and in particular detail a feature of MS channels which may have contributed to the difficulty in studying them reproducibly: their inactivation characteristics.

Amount of Force Exerted by This Technique

The cells were approximately 15 μm in diameter, and the probe deformed them by up to 2 μm. Assuming that the cell was originally completely spherical and pressing with the probe deformed the cell into an oblate spheroid, the percentage change in surface area (assumed to be distributed over the entire cell) is given by:

$$\Delta\% = \frac{100\Delta A}{A} = 100\left(\frac{1}{2\varepsilon} + \frac{\varepsilon^2}{4x}\,ln\left\{\frac{1+x}{1-x}\right\} - 1\right) \qquad (5)$$

Where $\varepsilon = c/r$ and $x = \sqrt{1 = \varepsilon^3}$ with r being the radius of the cell, and c the polar axis of the oblate spheroid.

With an average value of $r = 15$ μm and $c = 13$ μm, the percentage change in area would be ~1.0%. Sokabe, Sachs and Jing (1991) and Sigurdson and Sachs (1994) measured the upper limit of the elasticity of the cell cortex in a membrane patch to be in the range 20–60 dynes cm^{-1}. If averaged over the whole cell, this would mean that there was a change in membrane tension of about 0.2 to 0.6 dynes cm^{-1}. The local stresses are probably higher than this, and the ability to evoke an MS current by changing the position of the probe on the cell suggests that the stress of direct stimulation is not spread evenly throughout the cell. Gustin et al. (1988), looking at a mechanosensitive ion channel in the yeast plasma membrane, determined that MS channels have a mean tension for half activation of 1 dyne cm^{-1}.

The Components of the Mechanotransduction Pathway

Clearly, pressing directly on a cell results in the opening of a transmembrane pathway. The rapidity of the basic response rules out the involvement of most second messengers in the primary pathway.

The "membrane" or cell cortex consists of a phos-

pholipid bilayer, cytoskeleton, and an assortment of proteins and sugars attached to the phospholipids. Deforming this aggregation with a mechanical probe will do work on the system and put force on the load-bearing elements. Activation of the MS channel must, by its very definition, be related to the force, or energy, put into the system by the mechanical intervention.

The position of the probe in the experiments described here was known precisely, as it was under control of the piezoelectric manipulator. However, the relationship between the motion of the probe and the resulting deformation of the cell, and the factor which is of prime importance, the force generated in the load bearing elements, is unknown.

Although the MS current was very reproducible in broad terms, there was no constant relationship between the movement of the probe and the magnitude of the current elicited. This was particularly noticeable in the double press protocol, when the second pulse was always smaller than the first one. The variable response suggests that some components of the mechanosensitive pathway are easily altered. The variable includes both structural alterations and modulation by second messengers including the autocrine inhibition we have observed.

The MS Channel Has An Activation Energy Barrier

The MS channel did not activate on immediate contact with the cell although the cell could be seen visibly deformed. In the computer-controlled experiments, the starting position of the probe was just above the cell (determined as a position just above where deformation became visible). The current however, did not appear until the last two thirds of the depression.

The threshold for activation of the channel was not related directly to the position of the probe. There is a delay in activation somewhere in the transduction mechanism. This may be some slack in some tension-bearing elements, or perhaps multiple energy barriers to be overcome before the channel can open.

Movements of the mechanical probe to various amplitudes were all programmed to take 100 msec, so the velocity of the probe will be slightly different for the different stimulation amplitudes. In the intact heart there appears to be a small velocity-sensitive component to arrhythmia generation (Hansen, Craig & Hondeghem, 1990*b*; Franz et al., 1992), but it is doubtful that such a small change in velocity as seen with the different probe velocities would affect the MS activation rate. We observed changes in the delay of activation in response to stimuli with identical velocity of probe movement.

Changes in the time course of activation for a given stimulus size were apparent within a time interval of 1 sec, for the second MS current in a double press protocol was always delayed compared to the time of activation of the first MS current. The position of the probe was tracked digitally, so the change was not in the movement of the probe and therefore must reflect some change in the cell. Some characteristic of the mechanotransduction pathway must have changed within 1 sec.

Even though these experiments were designed to be quantitative, the relationship between probe position and the initiation of the current is not linear, but clearly it is of fundamental importance in understanding the transduction pathway. Whatever controls this delay will also play a part in the hysteresis.

Inactivation of the MS Current During the Stimulus Plateau

The rate of inactivation of the MS current while the mechanical deformation was at its maximum appears to be independent of voltage, the size of the peak current or the type of probe used. The inactivation rate was also constant over many cells, although the specific details of the deformation (e.g., the percentage of surface in contact with the surface; the angle at which the probe contacted the surface, etc.) will vary considerably from cell to cell. This suggests that inactivation is something internal, and common to all cells. The position of the probe was controlled precisely by the piezoelectric manipulator, which was commanded by Labview, and was checked with a digital micrometer to determine its stability, and to rule out any contribution from fluctuations in the position of the probe.

The rate of decay of the current once the probe was lifted off the cell was similar to the rate of inactivation during the constant stimulation phase, which is consistent with some part of the mechanosensing element relaxing following mechanical stimulation.

The increase in the noise level observed in some of the largest MS currents during plateau inactivation suggests that the decay of the current is due to channels closing rather than the number of channels available being reduced at constant probability of activation.

Inactivation of the MS current is complex. There is probably a contribution from changes in the cytoskeleton which results in the channel experiencing an altered force, possibly inactivated kinetic states and inhibition by autocrine agents.

Post Stimulus Recovery

Once the stimulus was removed, the remaining current decayed at a similar rate to the plateau decay, suggesting the same mechanism may underlie both forms of inactivation. The MS current always died away completely after release of the stimulus, indicating that no long-term

MS current was activated. The rapidity of onset also suggests all currents seen were in response to some directly activated channel, and not the result of some complicated second messenger system. Although the conductance always returned to the initial state within a few milliseconds of removing the mechanical stimulus, it is likely that the tension-sensing elements attached to the channel are not back in their original condition at that time.

Sinusoidal Stimulation

The sinusoidal stimulation, which was designed to reduce some of the time dependency of the response, raised further issues concerning inactivation of the MS response. The magnitude of the MS current was increased by simply exchanging the fluid surrounding the cell. This increase in response is unlikely to be due to the shear forces of the fluid flow around the cell, as the effect is seen when the fluid is both drawn into or ejected from a localized application tube, and the flow in these two cases will be different.

These results, and the recovery of an MS current which had previously decayed to zero even though the stimulus was constant suggests that something generated in the solution surrounding the stimulated cell blocks the MS current.

Modeling

A simple three-state model could reproduce any one series of either increasing or decreasing stimulus steps, but not both. Even a four-state model, with two force-dependent rate constants could not reproduce the experimental results from both increasing and decreasing probe amplitudes. This suggests that model was not complex enough to represent all the features of the mechanotransduction pathway. Adding more complexity to the model would not be of physiological value, as relating quantities back to attributes of the cell would be difficult.

Inactivation Has Several Components

The MS current inactivates, sometimes completely, even under constant stimulation. Understanding the inactivation process is therefore important in resolving the mechanotransduction pathway.

There are several pieces of data which suggest that MS current inactivation may be composed of at least two contributions: (a) rearrangement of the tension-transducing elements and (b) blocking action of some solution borne agent, which may be released from the cell.

(i) The large MS current seen when first pressing on the cell with the manipulator under manual control, and the subsequent lack of response to computer controlled stimulation of the same magnitude.

(ii) The amplitude of the peak MS current in the second press of a double press protocol is always smaller than the peak amplitude of the first current.

(iii) The MS current activated by pressing on the cell with a large deformation following a period of rest results in a larger peak MS current than pressing on the cell with a series of deformations and gradually arriving at the a stimulus of the same magnitude.

(iv) The hysteresis of response to increasing and decreasing magnitude of the trapezoidal stimulation.

(v) The initial large MS current with sinusoidal stimulation leading to complete decay of the MS current, and the small initial current leading to a nonzero steady-state response.

(vi) Restoration of the sinusoidal stimulus response by rapid exchange of fluid around a cell.

Conclusions

The behavior of MS channels in embryonic chick ventricular myocytes activated by direct mechanical stimulation is strongly affected by inactivation. The inactivation process is complex, and has contributions from both cortical rearrangement and an autocrine agent released from the cell following stimulation. Both of these factors are dependent on the level of mechanical stimulation of the cell.

The volatility of the transduction system, and the possible presence of MS blockers released from cells other than the embryonic chick ventricular myocytes may underlie some of the confusion surrounding mechanosensitive experiments. Identification of the agent which blocks the channels will not only aid our understanding of MS current inactivation, but may provide a biochemical marker of the MS channels.

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