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Mechanically Activated Currents in Chick Heart Cells

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Abstract. As predicted from stretch-induced changes of rate and rhythm in the heart, acutely isolated embryonic chick heart cells exhibit whole-cell mechanosensitive currents. These currents were evoked by pressing on cells with a fire polished micropipette and measured through a perforated patch using a second pipette. The currents were carried by Na^+ and K^+ but not Cl^-, and were independent of external Ca^{2+}. The currents had linear *I/V* curves reversing at −16 mV and were completely blocked by $Gd^{3+} \geqslant 30$ μM and *Grammostola spatulata* venom at a dilution of 1:1000. Approximately 20% of cells showed time dependent inactivation. In contrast to direct mechanical stimulation, hypotonic volume stress produced an increase in conductance for anions rather than cations—the two stimuli are not equivalent. The cells had two types of stretch-activated ion channels (SACs): a 21 pS nonspecific cation-selective reversing at −2 mV and a 90 pS K^+ selective reversing at −70 mV in normal saline. The activity of SACs was strongly correlated with the presence of whole-cell currents. Both the whole-cell currents and SACs were blocked by Gd^{3+} and by *Grammostola spatulata* spider venom. Mechanical stimulation of spontaneously active cells increased the beating rate and this effect was blocked by Gd^{3+}. We conclude that physiologically active mechanosensitive currents arise from stretch activated ion channels.

Key words: Stretch — Volume — Voltage clamp — Patch clamp — Transduction

Introduction

Mechanical stimulation has long been known to increase automaticity and alter the action potential in whole hearts and isolated tissues (Bainbridge, 1915; Rajala, Kalbfleisch & Kaplan, 1976; Lab, 1980). The simplest explanation for these effects would appear to be stretch-activated channels. Such channels have been observed in a variety of isolated cardiac cells including frog (Kohl et al., 1992), chick (Ruknudin, Sachs & Bustamante, 1993), rat (Kim, 1992; Craelius, 1993), rabbit (Hagiwara et al., 1992), guinea-pig (Bustamante Ruknudin & Sachs, 1991), and human (Naruse & Sokabe, 1993). Despite the wide variety of the data on effects of stretch on cardiac tissues, only a single study has been made of the corresponding whole-cell currents (Sasaki, Mitsuiye & Noma, 1992), and because of the difficulty of applying repeatable stimuli, that study provided limited data. Many researchers have used hypotonic swelling or inflation through the recording pipette as alternative ''mechanical'' stimuli although it is not clear that such stimuli act via a common mechanism (Hagiwara et al., 1992; Van Wagoner, 1993; Kim, 1993; Zhang et al., 1993; Kim & Fu, 1993; Ackerman, Wickman & Clapham, 1994).

Stretching the heart may lead to arrhythmias of clinical significance (Dean & Lab, 1989; Franz et al., 1989; Hansen, Craig & Hondeghem, 1990). Such stretch-induced arrhythmias in dog hearts can be blocked by Gd^{3+} (Yang & Sachs, 1989) but not by organic blockers of Ca^{2+} channels (Hansen et al., 1991) suggesting that mechanosensitive (MS) ion channels may be involved. In this paper, we demonstrate stable whole-cell currents activated by direct mechanical strain and show that these currents are not the same as those activated by hypotonic swelling. The strain sensitive current is capable of altering the beating rate of the cells and this effect can be accounted for by the presence of two types of stretch-activated channels (SACs) found in the cells. We used chick heart cells—an established preparation whose mechanical sensitivity has been demonstrated by pressure-induced changes in rate, strain-induced Ca^{2+} fluxes and the presence of SACs (Rajala et al., 1976; Sigurdson et al., 1992; Ruknudin et al., 1993).

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Materials and Methods

Chick Ventricular Cell Preparation

The cell preparation method was modified from Sada et al. (1988). Briefly, hearts of 16–17 day old White Leghorn chick embryos were dissected, ventricles were removed, washed with divalent-ion free saline (D-PBS, GIBCO, Grand Island, NY), minced, then stirred in a 37°C incubator with an enzyme solution containing 0.02% collagenase (Sigma, St. Louis, MO) and 0.2% trypsin (Sigma). The isolation proceeded stepwise. The supernatant obtained by the first stirring of 15 min contained few cells and was discarded. Steps two through four used fresh aliquots of enzyme solution for 5 min, and the supernatants of these steps were saved. The cell suspensions were mixed with minimum essential medium (MEM, GIBCO, supplemented with 10% horse serum, GIBCO, plus 2% chick embryo extract), and gently centrifuged. The cells were gently resuspended, distributed into culture dishes and maintained in a tissue culture incubator at 37°C. We conducted our experiments 1–3 days after cell preparation when the cells had stuck to the coverslip. We used rounded rather than elongated cells because it was simpler to apply the mechanical stimulation. Using a single pipette to press on the top of the cells, we could expect relatively uniform stimulation (*see below*) compared to the same stimulation applied to part of an elongated cell. The cells could be electrically or mechanically stimulated to beat indicating that they were healthy. All experiments were conducted at a room temperature of 22–24°C.

Electrophysiology and Mechanical Stimulation

We conducted studies using whole-cell, single-channel, and loose-seal recording. Patch-clamp techniques followed standard procedures (Hamill et al., 1981). Micropipettes were made from 100λ capillaries (Drummond Microcaps, Thomas Scientific, Swedesboro, NJ), fire polished, and having a resistance of 2–3 MΩ when the pipette solution was high K^+ saline and the bath solution was normal saline (*see* Solutions below). Currents and voltages were recorded with a CV-3 headstage (Axon Instruments, Foster City, CA), using an Axopatch-1B patch clamp (Axon), a VR-10 CRC digital data recorder (Instrutech, Elmont, NY) and a VCR (JVC, HR-S7000U, Japan). A 902LPF low-pass filter (Frequency Devices, Haverhill, MA) was applied before data acquisition. Bath solutions with a perfusion rate of 1.5 ml/min and the volume of the chamber was 0.5 ml.

In the whole-cell studies, we used one micropipette for perforated-patch voltage-clamp (Horn & Marty, 1988) and another for mechanical stimulation. These are noted respectively as the recording and stimulating pipettes. The whole-cell configuration was considered as formed (noted time 0 for stability tests) when the series resistance decreased to 15 MΩ or lower and was stable for at least 2 min. The tip of the stimulation pipette was a few μm in diameter, fire-polished and mounted on a piezoelectric manipulator with the axis of the pipette tip nearly parallel to the stage. We pressed the side of the pipette against the cell using the vertical displacement of the pipette as a measure of the cell strain. Maximal displacements were less than 4 μm and were expected to produce mean cortical tensions of <1–3 dyne/cm (*c.f.* Discussion). The manipulator (Burleigh PCS-1000, Fishers, NY) was controlled either manually or by a computer running Labview™. In the latter case we used trapezoidal stimulus pulses to avoid mechanical resonances of the probe and manipulator. Unless otherwise noted, the results below refer to noninactivating currents. Single-channel experiments used standard techniques (Hamill et al., 1981), and only seals with a resistance of >10 *G*Ω were used. In the *I/V* study of the single channels, the cell resting potential was measured by the end of the experiment by rupturing the patch and immediately recording the potential in current clamp mode.

In our loose-seal studies (Stühmer et al., 1983), the seal resistance was 20–40 MΩ. In both single-channel and loose-seal studies, suction was applied to the pipette for mechanical stimulation (Guharay & Sachs, 1984).

In the whole-cell experiments, we found that after mechanical stimulation the cells became sensitive to changes in perfusion conditions often causing loss of stable recordings, especially when the replacing solution was nonphysiological. Because of this high failure rate, we were often unable to compare recordings from the same cell in different solutions, and had to resort to population studies. The reason that mechanical stimulation caused cells to become sensitive to perfusion is not clear, although it is possible that strain may disrupt the cells' attachments to the substrate.

In the whole-cell experiments, we monitored the cell under a phase-contrast microscope (magnification 200×) during mechanical stimulation. Cell diameter was measured using a micropipette as a pointer. We moved the pipette from one side of the cell to the other by changing the voltage applied to the stimulating manipulator, and converted the voltage difference to the displacement. In some experiments, particularly when we measured the lateral strain, we videotaped the image of the cell using a CCD camera (GBC, CCD-500, USA) and a VCR (JVC, HR-S7000U, Japan). These images were digitized for off-line analysis using a frame grabber (Data Translation, Model-2861).

Solutions (in mM)

Normal saline (*NS*): 150 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4 titrated with 1N NaOH, 329 mOsm; *High* K^+ *saline* (*HK*): 5 NaCl, 150 KCl, 0.5 K-EGTA, 10 HEPES, pH 7.4 titrated with 1N KOH, 323 mOsm; *Na-isethionate:* 160 Na-isethionate, 10 HEPES, pH 7.4 titrated with 1N NaOH, 330 mOsm; *Mannitol solution:* 330 d-mannitol, 2 $CaCl_2$; 336 mOsm. *Hypotonic mannitol solution:* 250 D-mannitol, 2 $CaCl_2$, 256 mOsm. For perforated patches, the pipette was filled with HK containing 200 μg/ml nystatin (nystatin first dissolved in DMSO at 20 mg/ml) or 100–200 μg/ml amphotericin B and sonicated before filling the pipette, noted as *nystatin* solution and *amphotericin B* solution respectively. For loose-seal experiments, we used both NS and HK, with and without Ca^{2+}, as the pipette solution. The bath solution was always NS unless otherwise specified. *Grammostola spatulata* venom was obtained from Spider Pharm, Seasterville, PA.

Data Acquisition and Processing

Analog signals (real-time or replayed tape recordings) were sampled through a computer interface BNC-2080 connected to an I/O board AT-MIO-16X using LabVIEW software (National Instruments, Austin, TX) into a computer (Gateway2000 Model 486DX2/50). LabVIEW was also used to generate command protocols for whole-cell voltage clamping, to drive the piezoelectrics for mechanical stimulation, and for preliminary data processing as well. AXUM (TriMetrix, Seattle, WA), SCIENTIST™ (Micromath, Salt Lake City, UT), IPROC (Axon), and other software were used for data processing and graphing. The sampling rate ranged from 500 Hz to 10 kHz depending on the speed and the time length of the event of interest, and was bandlimited at the Nyquist limit or lower. When plotting, the less important data were often decimated to simplify graphing while maintaining the visual effect. Unless otherwise specified, all the statistical results given are in the form of mean ± SD.

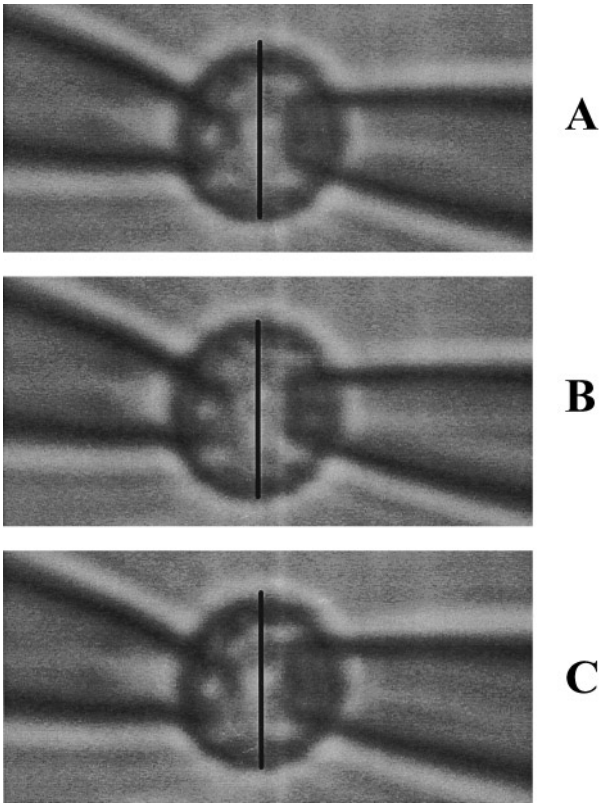

Fig. 1. Phase contrast photomicrographs of a cell and the stimulation (right) and recording (left) pipettes showing the relatively small change in shape associated with stimulation. Panels *A* and *C* were taken, respectively, before and after stimulation, and panel *B* was taken while the cell was pressed by the stimulation pipette. The diameter of the maximal cross-section of the cell in panel *B* is used as the scale bar, and is 16 μm. The diameters of the cell in *A* and *C* are similar, 15 μm, indicating good recovery. The downward displacement of the stimulation pipette was 3.8 μm.

Abbreviations

MS (mechanosensitive), SAC (Stretch-Activated Channel), MSC (Whole-Cell Mechanosensitive Current)

Results

General Description of the Cell

The cells we studied had resting potentials of −69.9 ± 3.6 mV (n = 25, measured from single cells) at room temperature at 22–24°C. They could be electrically or mechanically stimulated to beat and produced action potentials. They displayed typical voltage-sensitive Na^+, Ca^{2+}, and K^+ currents when step-voltage clamped. The cells were generally spherical with a diameter of 15.7 ± 2.1 μm (n = 9), and a capacitance of 7.0 ± 1.97 pF (n = 25). Figure 1 shows photomicrographs of a cell with and without mechanical stimulation. When this cell was pressed against the coverslip by 3.8 μm, the maximal diameter increased from 15.4 μm to 16.1 μm.

The MSC in Normal Saline

An example of whole-cell currents recorded with NS in the bath are shown in Fig. 2*A*. The MSC was inward at holding potential of −71 mV, reversible, and its amplitude was monotonic with the displacement of the mechanical stimulation pipette. The currents were depressed by 83 ± 6% (n = 4) in 20 μM Gd^{3+}, unmeasurable in 30 μM Gd^{3+}, and displayed only partial recovery during washouts lasting 15–30 min (n = 3). Higher concentrations of Gd^{3+} (100 μM) also blocked MSCs, but recovery during washout was small (Fig. 2*B*, n = 3).

Spider venom from *Grammastola spatulata* that has been shown to block SAC activity in other cells (Chen et al., 1996; Niggel et al., 1996), also completely blocked the MSC (Fig. 2*C*, n = 4) and SACs in cell attached patches (Hu, 1996). Washout was generally irreversible within 15–30 min, although the cells could still generate normal voltage dependent currents (Hu, 1996).

To measure the *I/V* curve of the MSC, we applied a ramp voltage in the presence and absence of mechanical stimulation (Fig. 3). These total whole-cell *I/V* curves are representative (Fig. 3*A*) and show the expected strong inward K^+ rectification (Josephson & Sperelakis, 1990). In Fig. 3*B*, the difference current had a linear regression of $I = 1.01 + 0.06V$, indicating a reversal potential of −17 mV and a conductance of 60 pS/pF. The mean overall reversal potential was −16.4 ± 1.3 mV (n = 5), almost identical to the value of −15 mV reported for guinea-pig ventricular cells (Sasaki et al., 1992). The conductance was modulated by the strain, but it was not possible to make quantitative comparisons between cells because of the lack of control of local strain. The maximal observed conductance in NS (but not necessarily saturating) was 600 pS/pF.

Since earlier experiments showed a wide range of responses to mechanical stimulation (Sasaki et al., 1992), it was important to test the reproducibility of our stimulation. For repeated stimuli applied to the same cell, the response was stable over time provided the stimulus was not repeated very often. The peak MSC varied by less than 10% over 2–4 seconds (n = 15 stimuli applied to 4 cells, e.g. trace a of Fig. 2*B*), and less than 15% over 5–20 min (n = 3). For the short term study, we applied sets of stimuli to each cell with patterns as shown in trace d of Fig. 2*B*. The peak displacements ranged from 1.7 μm to 3.3 μm, but were the same in each set. We averaged the 3 peak MS currents within each set, calculated the ratio of each current to the mean current, and pooled these ratios to characterize the stability as a standard deviation. For the long-term study (Fig. 4), we applied

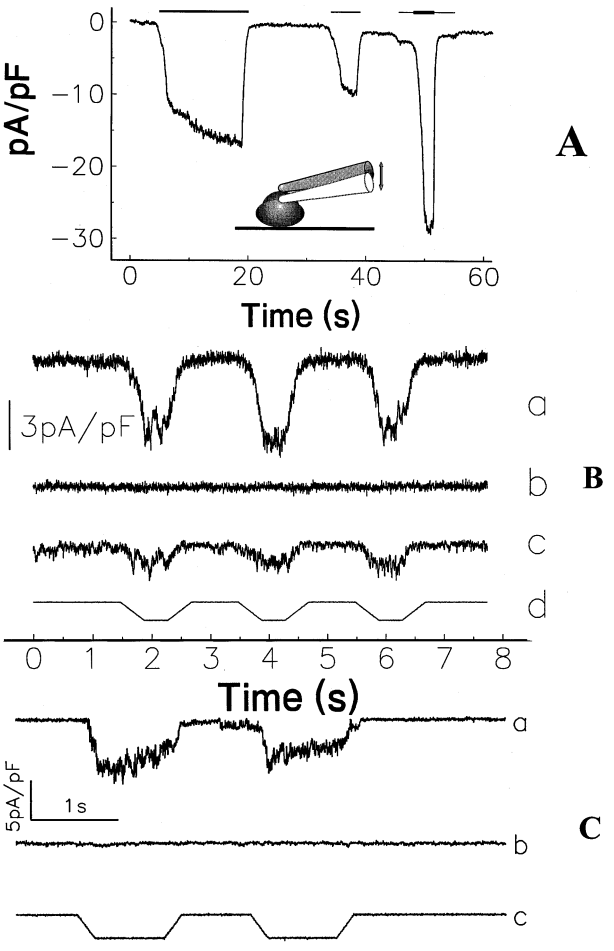

Fig. 2. MSCs in normal saline and blockage by Gd^{3+} and *Grammostola spatulata* venom. (*A*) Stimulus-response relationship and reversibility of MSCs. Inward currents are downward. The three horizontal bars from left to right represent stimuli (manually controlled) of ~2.5 μm, ~1.5 μm and ~3.5 μm, with the line thickness representing the magnitude of the stimuli. Cell clamped at the resting potential of −71 mV, capacitance 4.5 pF. Bath solution: NS; pipette solution: nystatin. The MSC was substantially noisier than the resting current suggesting that the current arose from channels. (*B*) Block of MSCs by high levels of Gd^{3+} (100 μM). Trace *a:* current before Gd^{3+}; trace *b:* with Gd^{3+}; trace *c:* washout; trace *d:* displacement of the stimulation pipette (1.7 μm peak). Cell capacitance 9.1 pF, membrane potential −50 mV. (*C*) Block of MSCs by *Grammostola spatulata* spider venom at a dilution of 1:2,000. Trace *a:* control, the MSC in NS; trace *b:* response in the venom solution; trace *c:* displacement of the stimulation pipette, 1.7 μm peak. Cell capacitance 3.4 pF, membrane potential −45 mV.

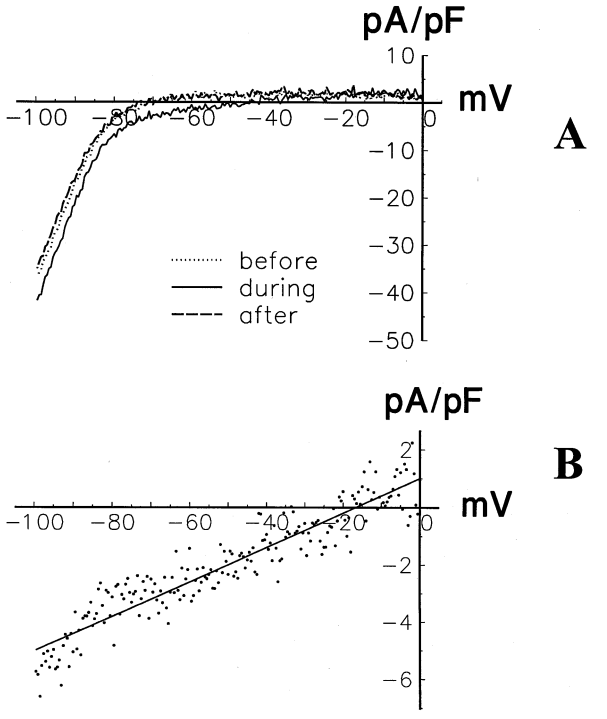

Fig. 3. Measurement of the *I/V* curve of the MSC in normal saline. (*A*) *I/V* curves of the total whole-cell currents. The membrane potential was driven by a ramp voltage, and the currents were recorded before, during and after the mechanical stimulus (dotted, solid and dashed lines, respectively). The currents before and after stimulation were essentially identical. Mechanical stimulation generated increased inward current at negative potentials and outward current at positive potentials. The total currents (average of 3–5 responses) showed strong inward rectification. Bath: NS; pipette: amphotericin B. Cell capacitance 4.8 pF, resting potential −70 mV. (*B*) *I/V* relationship of the MSC (dots) derived by subtraction of the *I/V* curves obtained in the presence and absence of stimulation shown in *A.* Linear regression of the data gives $I = 1.0 + 0.06V$, indicating a conductance of 60 pS (0.06 nS) and a reversal potential of −17 mV.

the mechanical stimulus soon after the whole-cell condition was stable, and then again 10–15 min later. We calculated the ratio of the absolute difference current to the initial current, and averaged these to characterize the stability. Unfortunately, repeated stimulation did cause a loss in responsiveness for reasons unknown and this ''use dependent'' rundown prevented us from using extensive experimental protocols.

The same stimulus displacement produced different currents in different cells. For example, using four different cells, a displacement of 1.7 μm produced a conductance of 146 ± 72 pS/pF. This variability was caused, at least in part, by the uncertainty of judging when the stimulation pipette touched the cell. Such variability discouraged us from attempting quantitative dose-response studies of the MSCs.

Ionic Basis of the MSC

The reversal potential of the MSC, −16 mV, suggested that it might be carried by nonselective cation channels (Craelius et al., 1988; Ruknudin et al., 1993), Cl^- channels (Hagiwara et al., 1992; Zhang et al., 1993), or a mixture of channels. To examine these possibilities, we substituted Na^+, K^+, Ca^{2+}, and Cl^-.

If Cl^- were a major carrier of the MSC, then by

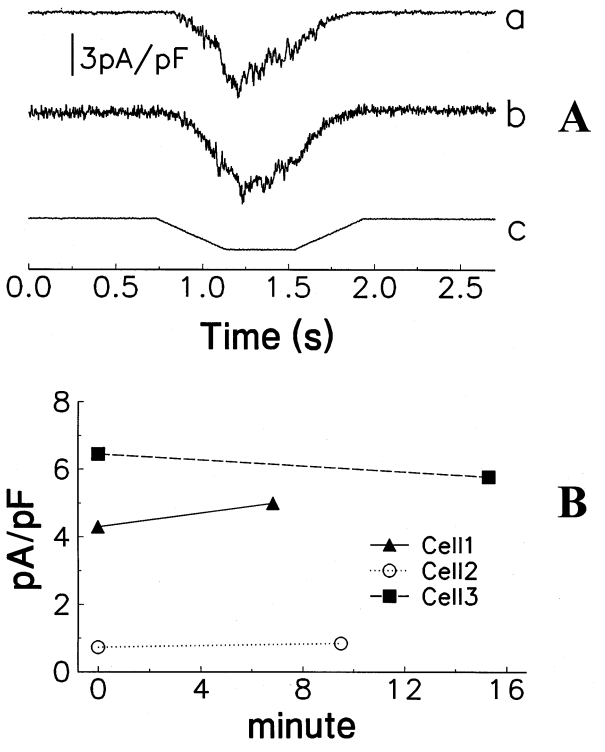

Fig. 4. The MSC of a cell is reproducible over time. (*A*) Currents recorded at different times from a single cell. Trace *a:* current seen when the whole-cell condition was initially formed; *b:* current 6′50″ after trace *a* was recorded; *c:* peak displacement 3.3 μm of the stimulation pipette. The response pattern of *b* was similar to that of *a*, indicating that the MSC was basically unchanged. Bath: NS; pipette: nystatin. Cell capacitance 9.1 pF, held at −50 mV. (*B*) Peak MSC measured at different time intervals from three different cells. The raw data for cell 1 are shown in *A*. Bath: NS; pipette: nystatin.

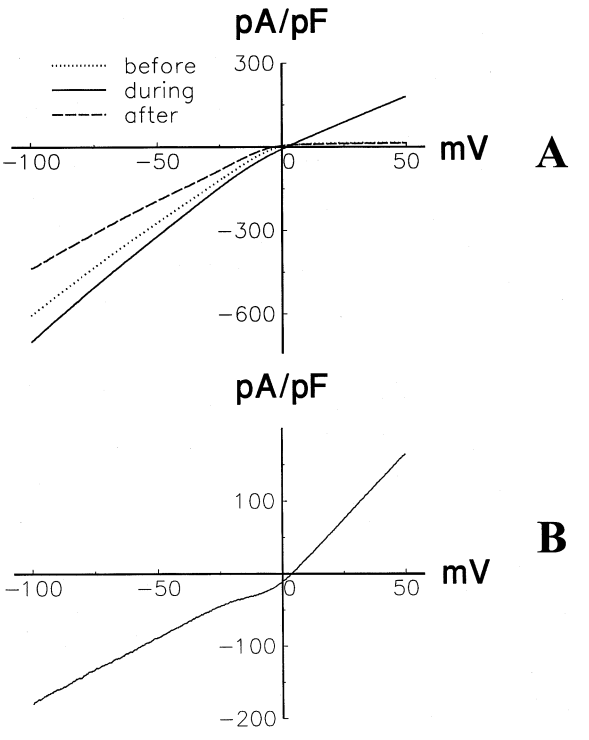

Fig. 5. The MSC in HK bath solution. (*A*) *I/V* curves of total currents before (dotted line), during (solid line) and after (dashed line) a mechanical stimulus. The curves before and after the stimulus showed strong inward rectification, which was greatly reduced in the curve during the stimulation. The current was larger with stimulation indicating that additional conductance was activated. (*B*) The *I/V* curve of the MSC obtained by subtracting the average of the currents before and after mechanical stimulation from the one during the stimulation. The net MSC was not inwardly rectifying.

removing Cl^- from the bath there should be no significant outward current since there would be no Cl^- influx. However, with Cl^- replaced by isethionate we observed distinct outward MSCs, as well as inward MSCs, with conductances similar to those observed in normal Cl^-. The reversal potential was essentially unchanged at −14.0 ± 3.3 mV (n = 3). These results suggest that Cl^- does not carry the MSC. Since Na^+ was the only other external ion in significant concentration, it must have been the charge carrier for the inward current.

MSCs in HK were usually larger than those observed in NS (Fig. 5). For example, with a stimulus displacement of 1.7 μm, the conductance was 690 ± 360 pS/pF (n = 3), compared to 150 ± 70 pS/pF (n = 4) observed in NS. The ranges do not overlap and a t test with unequal variances found the difference significant at 5.8%. In one experiment, the conductance of a cell was first measured in NS and then in HK, and for a 2.5 μm displacement the conductance increased from 280 pS/pF to 1520 pS/pF. This is a ratio comparable to the population means above. These data suggest that the MSC is more permeable to K^+ than Na^+. The reversal potential in HK was 0.7 ± 1.9 mV (n = 5), as expected from the nearly symmetric solutions. Total whole-cell currents in the absence of mechanical stimulation showed strong inward rectification, while during stimulation, the rectification was reduced because of domination by the linear MSC.

MSC is Observed in Nominally Ca^{2+}-Free Solutions

Removing external Ca^{2+} ions did not significantly affect MSCs. For example, in Na-isethionate solution with no added Ca^{2+}, the evoked MSC was similar to that obtained in NS bath solution. Using HK as the bath solution, which was also nominally Ca^{2+} free, we observed even larger MSCs (Fig. 5). When we excluded Ca^{2+} from the NS we still observed MSCs similar to those in NS bath solution, with a mean conductance of 130 ± 50 pS/pF (n = 4) for a displacement of 1.7 μm. These results sug-

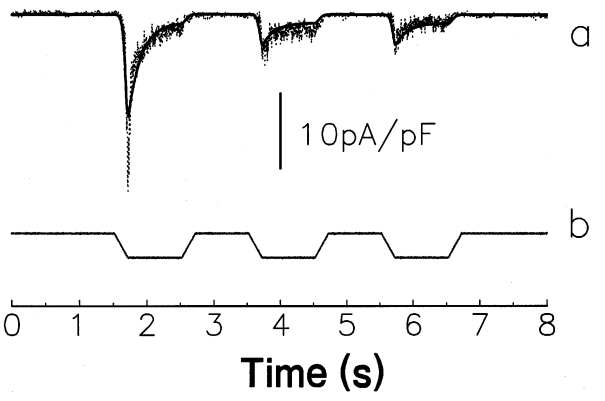

Fig. 6. Inactivation of the MSC. Trace *a:* recorded current (dots) with the model-simulated current (smooth trace) superimposed; trace *b:* displacement, 3.3 μm peak; membrane potential −35 mV.

gest that extracellular Ca^{2+} is not needed to generate MSCs.

Inactivation of the MSC

With constant strain, the MSC rose monotonically to a steady state in most experiments. However, out of 84 cells stimulated under computer control, 15 showed inactivation (Fig. 6), and such inactivation was observed with both inward and outward MSCs. During inactivation, the current decayed with time while a constant stimulus was maintained, reaching a steady-state amplitude. When the stimulation was removed, the MSC recovered, usually within 15–20 sec. By examining the cells with direct imaging and video analysis, the inactivation did not appear to originate from the cell slipping from beneath the stimulation pipette. In contrast to the behavior of SACs in *Xenopus* oocytes (Hamill & McBride, 1992), inactivation properties were not strongly dependent upon the membrane potential.

To summarize the time response of the inactivation process, we fit the currents to a three-state linear model consisting of closed (*C*), open (*O*) and inactivated (*I*) states:

$$C_1 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} O_2 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} I_3$$

with $k_{12} = k_{12}^0 \exp(\alpha_{12} d)$, and $k_{23} = k_{23}^0 \exp(\alpha_{23} d)$, where d is the time-dependent displacement of the stimulation pipette (the directly recorded trapezoidal trace, e.g., Fig. 6*b*), α_{ij}s are the sensitivity parameters and the k_{ij}^0s are the rates in the absence of stimulation. The rates k_{21} and k_{32} could be left independent of stimulation. The model was specified by the first order differential equations to allow the use of the trapezoidal driving function. Using the familiar variables m for activation and h for inactivation, the current was,

$$I = I_{\text{peak}} mh - I_b, \tag{1}$$

where I_{peak} and I_b are peak and background currents respectively,

$$\frac{dm}{dt} = -m(k_{21} + k_{12}) + k_{12}, \tag{2}$$

and

$$\frac{dh}{dt} = -h(k_{23} + k_{32}) + k_{32}. \tag{3}$$

Using the program Scientist™, we fit the data from ten cells exhibiting large currents and the results are shown in the Table (excluding the results from two cells whose values of k_{32} were outliers).

To simplify fitting k_{12}^0 and k_{23}^0 were fixed based on the results of previous trials. The means and standard deviations were calculated from the optimal parameters obtained from the different records, ignoring the covariance obtained from each individual fitting.

The MSC is Distinct from the Hypotonically-induced Current

Because many papers on heart cells have reported a Cl^--selective 'mechanically-induced current' following hypotonic stress (or inflation of the cell) (Tseng, 1992; Hagiwara et al., 1992; Zhang et al., 1993) and our MSCs were cation selective, we compared the result of hypotonic stress with that from direct mechanical strain (Fig. 7). The clearest demonstration of the differences came from using a bath solution almost free of ions (containing only 2 mM $CaCl_2$, as Zhang, Hall & Lieberman, 1994 have reported that Ca^{2+} was needed to observe the hypotonically-induced current). In this distinctly nonphysiological solution, the ionic strength of the bath was much smaller than that of the intracellular phase and the whole-cell current was dominated by efflux. K^+ currents should have had a negative reversal potential, while Cl^- currents should have had a positive reversal potential.

With the low ionic strength bath solution, direct mechanical stimulation produced currents with a negative reversal potential ranging from −10 to −30 mV ($n = 3$). One example is shown as trace a of Fig. 7. The current was primarily outward, and showed complete and rapid recovery upon removal of the stimulus. At more negative voltages there was a tiny inward current, which was likely carried by the 2 mM extracellular Ca^{2+}. Clearly, the current induced by direct mechanical stimulation is cation-selective.

Table. Parameters of the three-state MSC inactivation model

	k_{21} (sec^{-1})	k_{32} (sec^{-1})	α_{12} (μm^{-1})	α_{23} (μm^{-1})	k^0_{12} (sec^{-1})	k^0_{23} (sec^{-1})
Mean ± SD	31 ± 15	0.12 ± 0.06	2.7 ± 0.8	0.6 ± 0.2	0.1	0.5

Means calculated using 10 records from 8 cells.

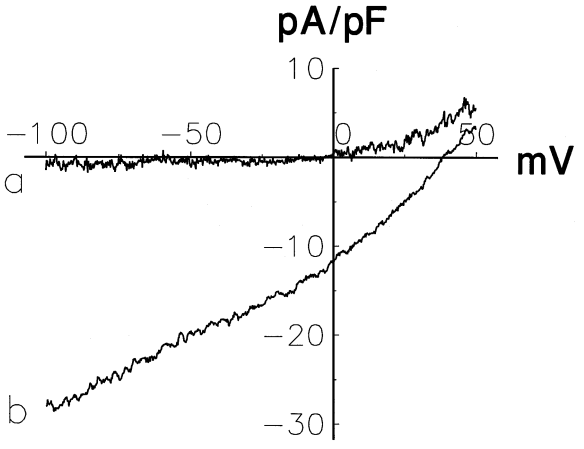

Fig. 7. The MSC is distinct from the hypotonically-induced current. Trace *a:* the net MSC in isotonic mannitol solution, obtained by subtracting the average of the currents before and after mechanical stimulation from the current during stimulation. The MSC is outward-going at positive potentials, indicating a K^+ efflux. Trace *b:* the net hypotonically-induced current obtained in a similar way, by subtracting the current in the isotonic mannitol solution from that in the hypotonic mannitol solution. The current is primarily inward-going, indicating a Cl^- efflux.

Hypotonically-induced currents were generated by perfusing the bath with the same solution, but with lowered mannitol concentrations (hypotonic mannitol solution). A net hypotonically-induced current is shown as trace *b* of Fig. 7. In contrast to the mechanically induced currents, hypotonically induced currents were mainly inward-going with a reversal potential of +45 ± 10 mV ($n = 4$, corrected for junction potentials and interpolated to zero current when necessary). The hypotonically induced conductance recovered by 60–80% after returning to the normal osmolarity solution.

From the composition of the intracellular and extracellular solutions, the only possible carriers for the hypotonically induced inward current were extracellular Ca^{2+} and intracellular Cl^-. The concentration of extracellular Ca^{2+} was too low to generate such a large current and the Nernst potential of Ca^{2+}, ≈125 mV, is far more positive than the observed reversal potential. If the current were carried purely by Cl^-, the observed reversal potential of +45 mV would predict an intracellular Cl^- concentration of 24 mM. This is 11 mM lower than the reported value of 35 mM obtained with an extracellular Cl^- concentration of 85 mM (Zhang et al., 1993), but is reasonable given the low Cl^- bath solution. Regardless of any peculiarities induced by the nonphysiological solutions, it is clear from these experiments that hypotonic stress can induce currents of different ionic selectivity from those induced by direct mechanical stimulation.

Why should the results of direct strain and osmotically induced strain be so different when there is no reason to expect osmotic swelling not to activate MSCs? As opposed to direct mechanical strain in a patch or whole-cell recording, osmotic challenge dilutes the intracellular medium and stresses internal membranous organelles. These latter effects may lead to second messenger activation of Cl^- current. Some researchers have found that swelling-induced Cl^- currents are dependent upon intracellular Ca^{2+} (Zhang et al., 1994) or ATP (Oike, Droogmans & Nilius, 1994). Osmotic swelling probably does cause some activation of MSCs, but the net current is clearly dominated by the anionic Cl^- current. The disparity between the response to osmotic strain and direct mechanical strain has been noted previously (Sasaki et al., 1992; Ackerman et al., 1994).

Single SAC Study

Can SACs account for the MSC? We performed cell-attached single-channel studies using NS in both the bath and the pipette. Out of 13 patches providing sufficient *I/V* data, we observed 5 patches containing a 90 pS SAC whose currents reversed at −70 ± 5 mV ($n = 3$ cells), and 11 patches containing a 21 pS SAC whose currents reversed at −2 ± 4 mV ($n = 6$ cells). Typical results are shown in Fig. 8. (The number of conductance and reversal potential measurements cited above are not equal since we did not obtain the resting membrane potential of all 13 cells.) Several patches exhibited activity of more than one channel, sometimes of both types. The 90 pS channel had a conductance and reversal potential similar to the 100 pS K^+-selective SAC found in tissue cultured chick heart cells (Ruknudin et al., 1993), but the 21 pS channel seemed quite distinct. This difference in channel populations may be due to a number of differences in the preparations including the difference in age of the embryos and the environment-attached *vs.* unattached, rounded *vs.* confluent, electrically uncoupled *vs.* coupled. The activity of both SACs in this preparation was completely blocked by 30 μM Gd^{3+} ($n = 6$ for the 21 pS channel and $n = 4$ for the 90 pS channel), and

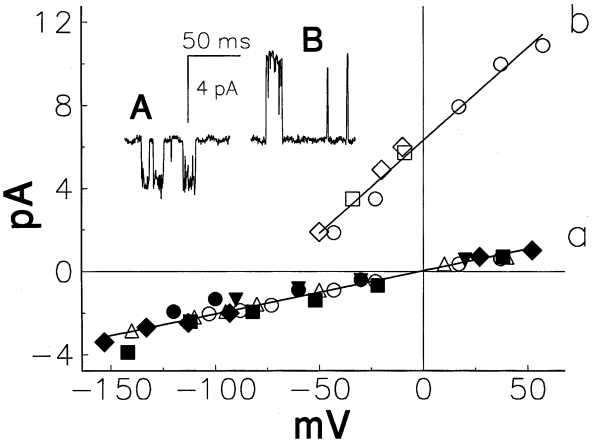

Fig. 8. Single-channel data and the *I/V* regression curves of the two observed types of SACs. Different symbols represent data from different patches. In one patch (circle), both types of SACs were present. Regression *a* gives a conductance $\gamma = 21 \pm 0.9$ pS and a reversal potential $V_r = -2.2 \pm 3.7$ mV ($n = 6$ cells) regression *b* gives $\gamma = 90 \pm 4.7$ pS and $V_r = -70 \pm 5$ mV ($n = 3$ cells). Bath and pipette: NS. *Inset:* representative currents of the 21 pS (*A*) and 90 pS (*B*) channels recorded with membrane potentials of −103 mV and −20 mV respectively.

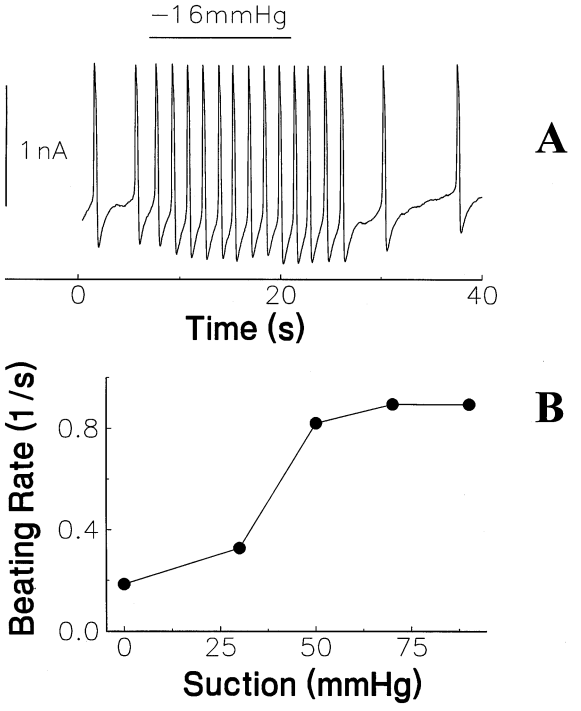

Fig. 9. Cell beating rate increased with suction in the loose-seal recording pipette (manual control of suction). (*A*) action current recording. (*B*) typical response of beating rate versus suction (different cell from panel *A*).

1:1000 dilution of *Grammastola spatulata* spider venom ($n = 3$ for both the 21 pS channel and the 90 pS channel). Since the reversal potential of the MSC fell between the reversal potentials of the two SACs, it appears that both are simultaneously activated in the whole-cell studies. The frequency of occurrence of the 21 pS channel was about three times that of the 90 pS channel.

The observation of MSCs and single SAC activity were highly correlated. In 7 out of 35 of the cell preparations we did not observe MSCs in any of the cells. Using three such preparations, we made 11 cell-attached patches and found no SAC activity. In contrast, using four preparations exhibiting MSCs, 16 out of 20 patches showed SAC activity. These results support the idea that MSCs are produced by SACs.

Discussion

Physiological Correlation of MS Currents

If SACs are active in these cells, they should be able to alter the action potentials. About ⅓ of quiescent cells could be mechanically stimulated to beat in NS bath solution at room temperature (21 out of 67 cells). However, in the presence of Gd^{3+} we observed no stimulated beating (0 out of 13), although there were spontaneously beating cells in the dishes. Consistent with these results, stretch has been shown to induce beating in the quiescent early embryonic chick heart in vivo (Rajala et al., 1977).

Because the traditional method of stimulating SACs in tight-seal patch experiments is via pipette suction, we tested its effect on cell beating. To minimize changes in the cell structure associated with patching, we made loose seals (20–40 MΩ) rather than tight seals. Applying suction to the recording pipette reversibly increased the cell beating rate (Fig. 9), though the sensitivity varied from cell to cell. We observed no significant differences when we used either NS or HK with or without Ca^{2+} in the pipette. These results suggest that it was not the specific ions but the currents injected into the cell that altered the cell beating rate.

Small Currents are Required to Alter Beating Rates

To estimate how much current was needed to significantly alter the beating rate of a cell using a loose patch, we applied voltage to the pipette in voltage-clamp mode. The cell beating rate reversibly increased with positive pipette voltages. An increase of 5 mV elevated the beating rate by 15–50%. Assuming that the resistance of the membrane inside the pipette was ~10 *G*Ω (the whole-cell resistance was ~1 *G*Ω), the injected current was <(5 mV)/(10 *G*Ω) = 0.5 pA. Thus, a current of ~0.5 pA (equivalent to ~0.1 pA/pF) can significantly alter the cell beating rate. Given that the pacemaker potential has a

slope of ~10 mV/sec, the net current necessary to produce this slope in a 5 pF cell is $I = 5\ \text{pF} \cdot 10\ \text{mV/sec} = 0.05$ pA. Thus, 0.5 pA is far in excess of that minimal required current.

To see what 0.5 pA means in terms of SAC activity, consider the following calculation. The cell has a resting potential of −70 mV, close to the reversal potential of the 90 pS channel, so that when these channels open they will carry little current. For the 21 pS channel with a reversal potential of ~0 mV, if we assume that the area of the patch is ~20 μm^2 (Sokabe, Sachs & Jing, 1991), the channel density is ~0.3/μm^2 (Ruknudin et al., 1993), and let P_o be the probability of being open. Suction produces a current of

$$I_{MS} = 20(\mu m^2) \times 0.3(/\mu m^2) \times 21\ (pS) \times 0.07\ (V) \times P_o \approx 8.8\ P_o\ (pA). \quad (4)$$

Thus, 0.5 pA requires a change of P_o of only ~6%, a value readily consistent with SAC properties (Sachs, 1992).

Comparing MSC and Voltage-sensitive Currents

The peak MSCs we observed ranged from 60–600 pS/pF, with no attempt to saturate the system. To judge the significance of these currents relative to more familiar voltage-sensitive currents, we used standard step and ramp protocols. The peak Na^+ and Ca^{2+} currents were ~500 pA/pF and 15 pA/pF respectively, and the corresponding conductances were estimated as ~4,000 pS/pF and 150 pS/pF. The inward rectifying K^+ current was ~50 pA/pF at −120 mV membrane potential, indicating a conductance of ~1,500 pS/pF. These currents are comparable to values reported by others in chick heart cells (Horres, Aiton & Lieberman, 1979; Clapham & Logothetis, 1988; Josephson & Sperelakis, 1992). Thus, the MSC conductance is smaller than the peak Na^+ conductance but larger than the Ca^{2+} conductance, and comparable to the inward rectifying K^+ (IRK) conductance, although it is much larger than IRK within the physiological potential range. The magnitude and reversal potential of the observed MSC current seem adequate to explain many of the observed effects of stretch on action potential properties in a variety of cardiac preparations (Sachs, 1994).

Although MSCs are somewhat labile, apparently varying with the preparation technique, they do not appear to be a selective property of damaged cells. Preparations that had MSCs had normal voltage-sensitive currents, however all preparations, including those without MSCs, exhibited voltage sensitive currents suggesting that they are more robust. This labile nature of mechanical responses has been seen before. In isolated heart cells, stretching was found to change the resting membrane potential in some preparations but not in others (Lab, 1980; White et al., 1993). It is known that SAC responses are dependent upon stimulation history, probably as a result of alterations in the cytoskeleton (Hamill & McBride, Jr. 1992).

The MSC is Not a Leakage Current

The current produced by mechanical deformation is not a leakage current, but is caused by specific ion channels. Fig. 2*B* and *C* illustrate that treatment with two very different reagents removes the MSCs. Also, some of our preparations had no MSCs while exhibiting normal voltage-gated currents. This conclusion is in agreement with the results of Sigurdson et al. (1992) that the mechanically-induced Ca^{2+} influx in heart cells was blocked by Gd^{3+} and the results of Morris and Horn (1991) that a variety of mechanical strains caused no change in the current of molluscan neurons.

Inactivation of Mechanosensitive Currents

Although we used a state model to describe the inactivation of the MSC, the actual origin of inactivation remains to be determined. It may result from different states of the channels (as we modeled it), relaxation of the cytoskeleton that alters the actual force applied to the channel (Pender & McCulloch, 1991; Sokabe et al., 1992), or inactivation of other conductances by second messengers (Wellner & Isenberg, 1995). Adaptation caused by a long lasting increase in Ca^{2+}/K^+ conductance, as that observed by Wellner & Isenberg (1995), is unlikely since there was no outward current upon release of the stimulus.

Inactivation has been previously observed in single MS channels (Gustin et al., 1988; Stockbridge & French, 1988; Hamill & McBride, Jr. 1992; Bowman & Lohr, 1994; Chen, Guber & Plant, 1994). In *Xenopus* oocytes, inactivation was labile, only observed at negative membrane potentials (Hamill & McBride, Jr. 1992) and only when the gigaseal was formed using gentle suction. A different dynamic behavior, delayed activation, was observed in *Lymnaea* K^+ selective SACs (Small & Morris, 1994). A delay of 1- to 4-sec was observed between the onset of the channel activity and the application of pressure jumps. Such delays were eliminated by cytochalasin D, or repeated mechanical stimuli suggesting involvement of the cytoskeleton. At present, we do not know why some cells inactivate and others do not, but macroscopic experiments have shown velocity sensitive effects on arrhythmia generation (Franz et al., 1992) but not on stretch induced potentials changes of the working myocardium (Stacy, Jr. et al., 1992). This result has been postulated to arise from a subpopulation of specialized cells.

Contribution of Both SACs to the MSCs

If the MSC reported here was due to the activity of the two types of SACs observed in these cells, then it is possible to estimate the ratio of the probabilities of the channels being open. Since we found no consistent variation of the reversal potential with the amount of strain, it would appear that the channels are acted upon by proportional forces and the probabilities of being open are also in proportion. From the definition of the reversal potential of the MSC, the following equation holds,

$$(E_r - E_{90})g_{90}N_{90}(P_o)_{90} = (E_{21} - E_r)g_{21}N_{21}(P_o)_{21}, \quad (5)$$

where, E_r is the reversal potential of the MSC, E_{90} and E_{21} are the reversal potentials of the 90 pS and the 21 pS SAC respectively, g_s are the conductance of the SACs, N_s the numbers of channels, and P_{os} the probabilities of the SACs being open. Using the observed $N_{21}/N_{90} = 3$ as the occurrence ratio of the 21 pS channel over the 90 pS channel, we can get

$$\frac{(P_o)_{21}}{(P_o)_{90}} = 5.6 \quad (6)$$

This suggests that the 21 pS channel is open 5.6 times more often than the 90 pS channel. This is somewhat higher than the rough estimates of 1:1 we made from cell-attached patches, but more detailed studies are required to see whether this ratio is preserved in the patch. Given the large deformations involved in forming a patch (Milton & Caldwell, 1990) and the sensitivity of the kinetics to cytoskeletal alteration (Morris & Horn, 1991; Hamill & McBride, Jr. 1992; Small & Morris, 1994), such a ratio need not be preserved.

Estimating the Cortical Tension Generated by the Mechanical Stimulation

The stresses produced by the stimulation are difficult to calculate in detail since both the constitutive properties of the structural elements and the detailed geometry of the strain are not known. However, if we use the common ''liquid drop'' model in which the cell is modeled as an elastic membrane covering a liquid core, we can make some estimates. Assuming that compression by the stimulation pipette transformed the spherical cell into an oblate of equivalent volume. The relative increase in area (A) for such a process is

$$\frac{\Delta A}{A} = \frac{1}{2x} + \frac{x^2}{4R} 1n \frac{1+R}{1-R} - 1, \quad (7)$$

where $R = \sqrt{1 - x^3}$, $x = b/r$, and r is the radius of the sphere and b is the semi minor-axis of the ellipsoid. The estimated area changes were <5%. Measurements of patch elasticity give values for the area elastic constant of the cell cortex in the range of 20–60 dyne/cm (Sokabe et al., 1991; Sigurdson & Sachs, 1994). These are probably upper limits since patches are normally under resting tension due to adhesion of the membrane to the walls of the pipette (Sokabe et al., 1991). For a change in area of 5%, these values would give a cortical mean tension of <1–3 dyne/cm. As shown by Sokabe et al. (1991), in patches the elasticity arises from stress in the cytoskeleton, not from the bilayer.

The above calculation assumes uniform deformation of an elastic membrane surrounding a liquid core. With an elastic core bound to the membrane, the stresses are bound to be different, but are difficult to estimate and it is unclear whether the true stresses are lower because of the sharing of elastic energy between the core and the cortex, or higher because of shear stresses near the stimulating pipette. The significance of local stresses was emphasized by Sigurdson et al. (1992) who showed that the Ca^{2+} influx arising from local mechanical stimulation occurs at the site of stimulation. To minimize the local strain, we used the side of the stimulation pipette rather than the tip to press against the cell.

Our results stand in sharp contrast to the negative results of Morris and Horn (1991) obtained on *Lymnaea* neurons, in which the inability evoke significant MSCs were presented as evidence for the artifactual nature of single-channel MS activity and for the physiological irrelevance of SACs. Although it remains unclear under what conditions it is difficult to evoke MSCs, Morris and Horn's conclusion is clearly not general. MSCs evoked by direct mechanical stimulation have been recorded in several other preparations including vascular smooth muscle (Davis, Donovitz & Hood, 1992), guinea pig heart (Sasaki et al., 1992) and bladder smooth muscle (Wellner & Isenberg, 1994). Cloning of a SAC from *E. Coli* (Sukharev et al., 1994) should be convincing evidence for the non artifactual nature of SACs.

From this work paper, SACs would appear to be capable of physiologically significant activity in the heart. While the normal physiological function of the MSC is as yet unproved, we can speculate that it serves to adjust the pacemaker potential according to the degree of filling of the atria (accounting for the old observation by Bainbridge, 1915). In ventricular cells it may serve to improve the uniformity of contraction. Weaker cells, stretched during systole, could be encouraged to contract more strongly on subsequent beats by increasing Ca^{2+} entry through direct fluxes and Na^+/Ca^{2+} exchange.

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Note: Some results have been published in abstract form (Hu & Sachs, 1994*b;* Hu & Sachs, 1994*a;* Hu & Sachs, 1995).

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