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The Touch-induced Action Potential in *Chara*: Inquiry into the Ionic Basis and the Mechanoreceptor

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Abstract

We developed an assay to characterize the mechanoreceptor for the touch-induced action potential of *Chara* internodal cells in order to compare it with the mechanoreceptor involved in sensing the gravitational pressure that induces a polarity of cytoplasmic streaming in the same cells. We show that there are at least two classes of mechanoreceptors in characean internodal cells. One class contains a RGD-binding, integrin-like protein that is localized at the end of the cells. This class of mechanoreceptors requires a turgid plasma membrane for sensing-competence and does not exhibit adaptation. We propose that this type of mechanoreceptor senses gravitational and hydrostatic pressure by sensing the magnitude and sign of its displacement relative to the extracellular matrix.

The second class of mechanoreceptors most likely does not contain RGD-binding, integrin-like proteins and does not require a turgid plasma membrane. This class is uniformly localized throughout the length of the cell and displays adaptation. We propose that this class of mechanoreceptors senses touch by sensing changes in tension which is a result of the magnitude but not the sign of the displacement of the membrane.

Introduction

Animal cells, plant cells and protozoa are capable of responding to a wide variety of mechanical stimulations, including gravitational pressure, hydrostatic pressure and touch (Julian and Goldman 1962; Kishimoto 1968; Lillywhite 1987; Machemer *et al.* 1991; Ooya *et al.* 1992). Mechanical stimulation causes a deformation of the plasma membrane and it is thought that the deformation of the plasma membrane activates mechanosensitive ion channels which lead to a given response (Sachs 1986; Falke *et al.* 1988; Morris 1990; Cosgrove and Hedrich 1991). While studying the effect of gravity on the polarity of cytoplasmic streaming in characean internodal cells, we discovered that the plasma membrane-extracellular matrix junction at the ends of the cell is essential for detecting gravitational and unidirectionally applied hydrostatic pressure (Wayne *et al.* 1990, 1992). Moreover, we have suggested that the mechanoreceptor contains an integrin-like protein (Hynes 1992) based on the ability of the tetrapeptide Arg-Gly-Asp-Ser (RGDS) to inhibit both the gravitational- and hydrostatic pressure-induced polarity of cytoplasmic streaming (Wayne *et al.* 1992).

In order to test the hypothesis that RGDS-binding integrin-like proteins serve as a component of the mechanoreceptors for all mechanical stress-induced responses, we developed an assay to characterize the mechanoreceptor for other mechanical stimulation-induced responses in characean internodal cells, particularly the touch-induced membrane depolarization response. Kishimoto (1968) discovered that mechanical stimulation induces an action potential in characean internodal cells. Here we further characterize this electrical response of characean internodal cells to touch and show that there are at least two classes

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of mechanoreceptors in characean internodal cells: one that contains a high affinity RGD-binding, integrin-like protein that senses gravitational and hydrostatic pressure and is localized at the end of the cells; and one that does not contain a high affinity RGD-binding protein, senses touch and is localized throughout the length of the cell.

Materials and Methods

Culture Conditions

Chara corallina Klein ex Willd. em. R.D.W. (= *Chara australis* R. Brown) and *Nitellopsis obtusa* (Desv. in Lois.) J. Gr. were grown in a soil/water medium under continuous fluorescent light at 25°C in a temperature-controlled room. Under these conditions, $CaCO_3$ does not deposit on the cell wall and there is not any visible banding pattern. See Wayne and Staves (1991) for details of the culture conditions.

Experimental Setup

The uppermost expanded internodal cells were used for experiments. They had a diameter of approximately 5×10^{-4} m and were 2–3×10^{-2} m in length. The internodal cells were removed from the cultures immediately before use.

The internodal cells were gently dried with a piece of toilet paper and placed in a Plexiglas chamber that was divided into two electrically isolated parts with washers and high vacuum silicone grease (Dow Corning, Midland, MI, USA; Fig. 1). The rubber washers (Step by Step, Wheeling, IL, USA) had an outside diameter of 23×10^{-3} m, an inside diameter of 15×10^{-3} m and a height of 3×10^{-3} m. The end of the cell that was mechanically stimulated by the glass rod was supported on a small piece of washer that was 2×10^{-3} m thick. The internodal cell was inserted in a 10^{-3} m deep groove cut in the washers and positioned in the chamber so that the length of the internodal cell in each of the two chambers was approximately equal. The two chambers were filled with the standard solution which contained 1 mol m^{-3} EGTA (pH titrated to 7·2 with NaOH), 2 mol m^{-3} Hepes (pH titrated to 7·2 with NaOH), 0·1 mol m^{-3} KCl, 0·1 mol m^{-3} NaCl and 10^{-2} mol m^{-3} free Ca^{2+} added as $CaCl_2$. The whole solution was titrated to pH 7·2 with NaOH. Alternatively, the cells were bathed in artificial pond water (APW; 0·1 mol m^{-3} NaCl, KCl and $CaCl_2$). The cells were allowed to recover from manipulation for several minutes after being placed in the chamber. All experiments were performed on a Zeiss IM 35 inverted microscope under normal room light at 25°C in a temperature-controlled room unless stated otherwise. Temperature was measured with a digital thermometer (Model 871; Keithley, Cleveland, OH, USA) equipped with a thermocouple probe.

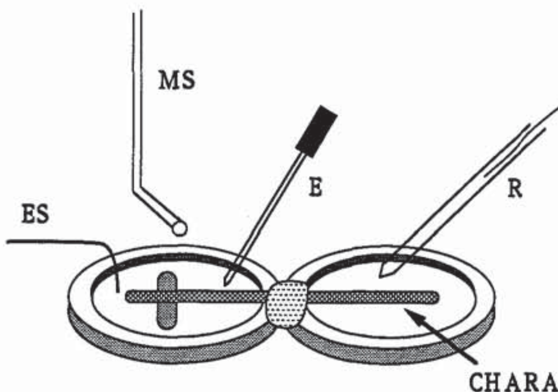

Fig. 1. Diagram of the apparatus used for mechanical (touch) stimulation. A *Chara* cell is placed in a groove between two chambers isolated by a silicone grease seal. E, microelectrode; R, reference electrode; MS, mechanical stimulator; ES, electrical stimulator.

Measurement of Potential Differences

Conventional microcapillary electrodes were not used for measuring the change in membrane potential upon mechanical stimulation in the present experiments in order to avoid any artifacts that may result from changes in the membrane seal around the electrode during and following mechano-stimulation. Consequently, we recorded the change in membrane potential as a potential difference

between chamber A and chamber B with extracellular electrodes (Kishimoto 1968). The electrode in chamber A was made out of a microcapillary pipette that contained 3000 mol m^{-3} (3 M) KCl. The microcapillary electrodes were made with a pipette puller (Model PP-83, Narishige, Tokyo, Japan). The electrode in chamber B was made from a Ag/AgCl wire embedded in 2% agar containing 100 mol m^{-3} (100 mM) KCl. The two electrodes were connected to an electrometer (Model S-7071A, World Precision Instruments, Sarasota, FL, USA) and potential differences were recorded on a strip chart recorder set at 0·5 V full scale and run at 10^{-1} m min^{-1} (Model 2125M, Allen Datagraph Inc., Salem, NH, USA).

In some cells, the resting membrane potentials were measured with the conventional intracellular microcapillary electrode method using an identical recording system.

Mechanical Stimulation

Cells were stimulated from above with a glass rod with a flattened heat-sealed end. The glass rods were made from 10^{-3} m diameter microcapillaries (Item No. 1B100F-4, World Precision Instruments). A rod was positioned with a micromanipulator (Narishige, Tokyo, Japan). The cells were stimulated by turning the z-axis knob by hand on the MN-1 manipulator which was attached to a MO-103N three-dimensional hydraulic micromanipulator. The rod was lowered a distance of 8×10^{-3} m in air before it hit the cell. It took 0·6 s to lower the rod. Thus, the rod had an average acceleration of 2×10^{-3} m s^{-2} and, assuming the acceleration was linear, a final acceleration (a) of 4×10^{-3} m^{-2} when it struck the cell. The apparent mass (m) of the rod was determined by striking an electronic balance (Model L 610, Sartorius, Brinkmann Instruments Inc., Westbury, NY, USA) placed on the microscope. The mass was $13\cdot2\pm5\times10^{-3}$ kg. Thus, the cell was struck with a force ($F=ma$) of $5\cdot28\times10^{-5}$ N. The force was applied over an area (A) of approximately 5×10^{-7} m^2 (area = diameter of rod × width of cell). Thus, the cell was stimulated by a pressure ($P=F/A$) of approximately 106 Pa for approximately 0·4 s. The kinetic energy ($KE=\frac{1}{2}mv^2$) imparted on the cell by the stimulus was $1\cdot1\times10^{-8}$ J. We are currently improving our stimulating system by using a piezoelectric bimorph element for mechanostimulation (Corey and Hudspeth 1980).

Electrical Stimulation/Cessation of Cytoplasmic Streaming Coupling Experiments

Electrical stimulation was given by applying a current of 2×10^{-6} A through the cell for a duration of 0·4 s with a Tri-Level Stimulator (Medical Systems Corp., Greenvale, NY, USA) and a homemade voltage to current converter. The current was delivered through Ag/AgCl wires. The velocity of cytoplasmic streaming was measured with a stopwatch and a calibrated ocular micrometer while viewing the cells on a Zeiss IM 35 inverted microscope equipped with a 3·2× (N.A. = 0·07) objective lens, Kpl 10×/18 oculars and brightfield optics.

Preparation of Solutions

Ca^{2+}-buffered solutions were made with the aid of a computer program (Wayne 1985). Lanthanide-buffered solutions were made with the aid of a computer program modified from the above program (available on request). The logarithms of the stability constants for the EGTA-lanthanide complexes used in the program were La, 15·55; Ce, 15·70; Pr, 16·05; Nd, 16·16; Sm, 16·88; Eu, 17·1; Gd, 16·94; Tb, 17·27; Dy, 17·42; Ho, 17·38; Er, 17·4; Tm, 17·48; Yb, 17·78; and Lu, 17·81. The pH electrodes were cleaned with 100 mol m^{-3} HCl after titrating the lanthanide buffers. Lanthanum and cerium were purchased and used as Cl^- salts and the other lanthanides were purchased as oxides and converted to NO_3^- salts by heating the oxides in nitric acid at 250°C.

Influence of Turgor Pressure

The turgor pressure was varied by changing the osmotic pressure of the external medium by adding sorbitol. The turgor pressure (P) was calculated by taking the difference between the osmotic pressure of the cell sap ($\pi_i=0\cdot635$ MPa, Staves *et al.* 1992) and the external solution π_o according to equation (1):

$$P=\pi_i-\pi_o\,. \tag{1}$$

The osmotic pressures of the solutions and the cell sap were measured with a freezing point depression osmometer (Model One-Ten; Fiske Associates, Needham Heights, MA, USA).

UV Irradiation

Cell ends were irradiated with a UV microbeam as previously described (Wayne *et al.* 1990).

Chemicals

Tetraethylammonium Cl^-, nifedipine, verapamil, ω-Conotoxin, Arg-Gly-Asp-Ser, Arg-Gly-Glu-Ser, Ser-Asp-Gly-Arg-Gly and all the lanthanides were purchased from Sigma Chemical Corp. (St Louis, MO, USA]. 9-anthracene-carboxylic acid was purchased from Aldrich Chem Co. (Milwaukee, WI, USA). Amiprophos methyl was a gift from Dr M. Parthasarathy. Cellulysin, thermolysin and proteinase K were purchased from Calbiochem Corp. (La Jolla, CA, USA). Cells treated with Cellulysin (1%, 2 h) did not survive the touch stimulus.

Statistics

Statistics were calculated with the aid of MINITAB (Minitab Inc., State College, PA, USA). Hill coefficients were determined algebraically using the method of Segel (1968).

Results and Discussion

Characean internodal cells are capable of sensing and responding to a mechanical stimulation (Kishimoto 1968). Applying a transient mechanical pressure (i.e. touch) of approximately 100 Pa to *Chara* and *Nitellopsis* internodal cells results in a transient depolarization of the plasma membrane, indicating that the plasma membrane acts as a mechanoelectrical transducer that can transduce mechanical energy into electrical energy, i.e. it acts as a piezoelectric element (Fig. 2). The transient voltage change consists of

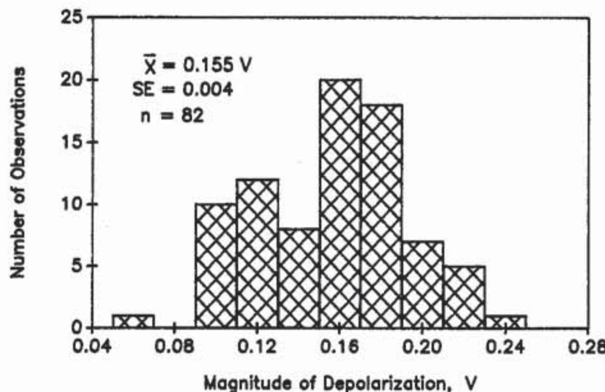

Fig. 2. Histogram showing the distribution of the magnitudes of membrane depolarization induced by touch in *Chara*. Cells were treated with the standard buffer.

a depolarization of $0 \cdot 155 \pm 0 \cdot 004$ V ($n = 82$) which is followed by a subsequent repolarization (Fig. 3*a*). The depolarization and repolarization take approximately 1 s. The resting membrane potential is $-0 \cdot 212 \pm 0 \cdot 006$ V. The transient depolarization is essentially an all-or-none response and it is transient even if the applied mechanical pressure is constant (Fig. 3*b*), indicating that the mechanoreceptor itself or subsequent steps in the signal transduction chain show adaptive behaviour (i.e. desensitization; Gustin *et al.* 1988; Assad *et al.* 1989; Hamill and McBride 1992). The removal of an applied pressure is equivalent to the addition of an applied pressure, indicating that the cell detects the change in pressure, but not the sign (Fig. 3*c*). Moreover, the transient depolarization is propagated along the length of the cell (data not shown). Given these characteristics, we consider that this transient depolarization is an action potential. The voltage changes that occur during the touch-induced action potential are identical to those observed following electrical excitation (Fig. 3*d*). Both mechanical and electrical stimulation cause a rapid (<1 s) cessation of cytoplasmic streaming which is followed by a relatively slow (≈ 250 s) recovery to the initial

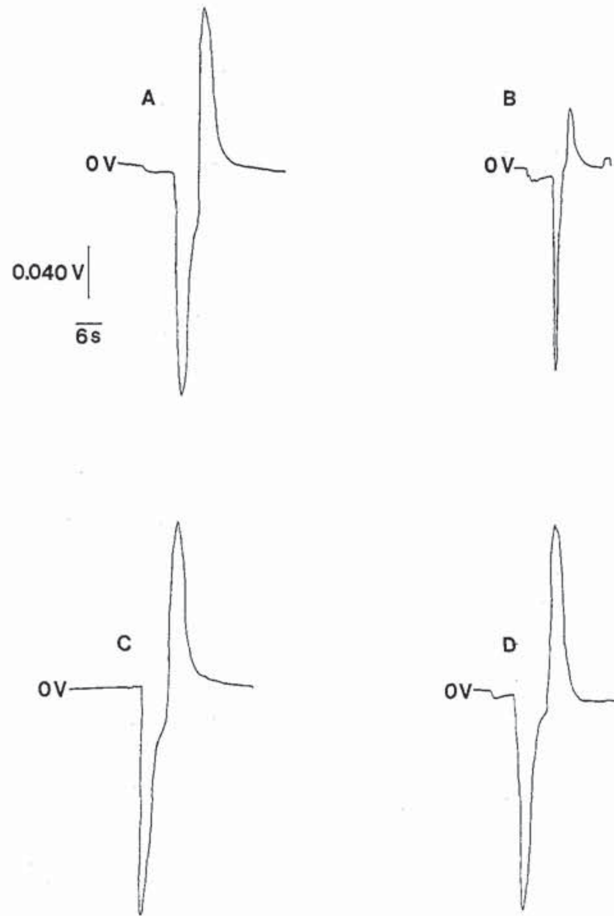

Fig. 3. Electrical tracings of action potentials induced in *Chara* internodal cells by (*a*) a transiently applied mechanical stimulus; (*b*) a constantly applied mechanical stimulus; (*c*) the removal of constantly applied mechanical stimulus; and (*d*) an electrical stimulus. The vertical bar represents 0·04 V and the horizontal bar represents 6 s. Note the biphasic nature of the action potentials which results from the propagation of the action potential from the stimulated end of the cell to the non-stimulated end of the cell. Downward deflections represent the depolarization of the stimulated side (followed by a subsequent repolarization), while upward deflections represent the depolarization of the unstimulated side (followed by a subsequent repolarization). The sign of the voltage change was determined in cells which had one end treated with either chloroform or KCl. All traces start with and return to a zero potential difference between the two chambers.

value (Fig. 4). Mechanically or electrically stimulated internodal cells are unable to generate a second action potential immediately following a first action potential. This refractory period is variable between cells, but lasts between 1 and 20 s (data not shown), indicating that there is no correlation between the recovery of excitability and the recovery of cytoplasmic streaming. The magnitude of the mechanically stimulated action potential, in

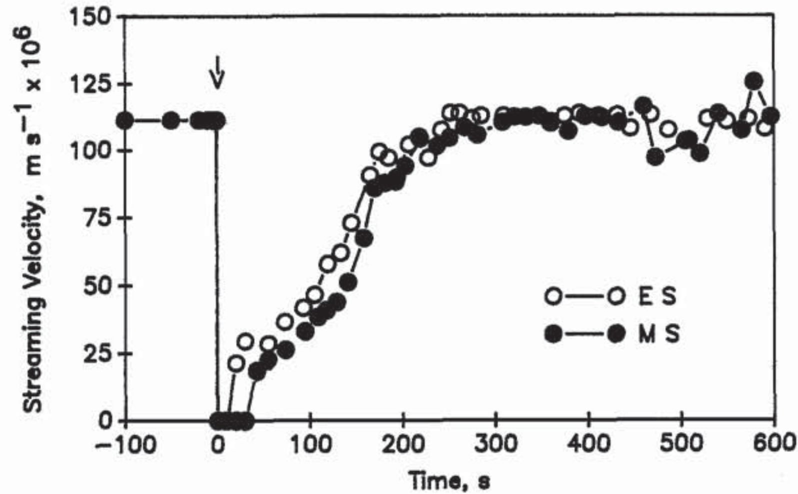

Fig. 4. The effect of mechanical and electrical stimulation on the velocity of streaming in one and the same *Chara* cell. The cell was stimulated either mechanically or electrically at time zero (arrow). MS, mechanically stimulated cell; ES, electrically stimulated cell.

contrast to the magnitude of the electrically stimulated action potential, is independent of temperature (Fig. 5). The Q_{10} values of the two responses are approximately 1 and 3·5 respectively.

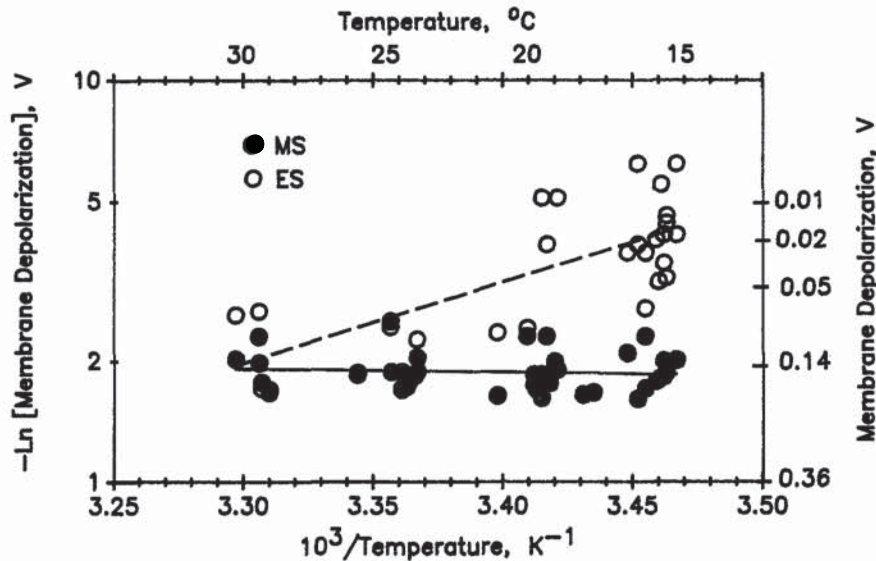

Fig. 5. The effect of temperature on the magnitude of the depolarization of mechanically stimulated and electrically stimulated action potentials. Lines were determined by linear regressions. Cells were incubated at each temperature for at least 20 min. Mean values ± s.e. are presented ($n = 14$–16).

Elucidation of the Ionic Basis of the Action Potential

As a first step in understanding the mechanical stimulation-induced action potential, we characterized the ionic requirements of the action potential and compared them with those of an electrically induced action potential. A thermodynamic analysis shows that Ca^{2+} and/or Cl^- ions are the only candidates capable of carrying the inward current that is responsible for the observed touch-induced depolarization (Findlay and Hope 1964). The equilibrium potentials (E_i) for Ca^{2+} and Cl^-, determined with the Nernst Equation, are $+0\cdot059$ and $+0\cdot103$ V respectively. Therefore, we varied the external Ca^{2+} concentration to assess whether or not Ca^{2+} is required for the touch-induced action potential.

The Effect of External Ca^{2+} on the Touch-induced Action Potential

External Ca^{2+} is required for the realization of the touch-induced action potential (Fig. 6), just as it is required for the electrical stimulation-induced action potential (Findlay and Hope 1964). The threshold concentration is approximately pCa 7 (10^{-4} mol m^{-3}) and the concentration that supports a half-maximal response is approximately pCa 6 (10^{-3} mol m^{-3}). The touch-induced cessation of cytoplasmic streaming also has a similar requirement for external Ca^{2+} (data not shown). There is also an absolute dependence on extracellular Ca^{2+} for the mechanosensitivity of hair cells (Assad *et al.* 1991; Crawford *et al.* 1991). By contrast, characean internodal cells are able to sense gravitational and hydrostatic pressure when the external Ca^{2+} concentration is as low as pCa 8 (10^{-5} mol m^{-3}), although the polarity of the response is reversed (Wayne *et al.* 1990).

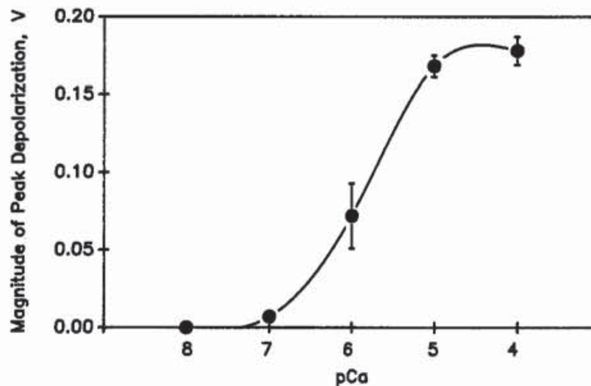

Fig. 6. The effect of external calcium on the magnitude of the depolarization induced by touch in *Chara*. Internodal cells were incubated in buffered Ca^{2+} solutions for 20 min. Mean values ± s.e. are presented ($n = 3$–9). $pCa = -\log[Ca^{2+}]$.

The Effect of Ca^{2+} Channel Blockers

Since the external calcium concentration has a profound effect on the ability of *Chara* internodal cells to produce an action potential in response to touch, we wanted to determine the effects of Ca^{2+} channel blockers on this response. Nifedipine ($0\cdot1$ mol m^{-3}, 20 min) and verapamil ($0\cdot1$ mol m^{-3}, 20 min) have a statistically significant, yet small and perhaps physiologically insignificant, effect on the magnitude of the depolarization caused by touch (Table 1). ω-Conotoxin ($0\cdot01$ mol m^{-3}, 20 min) has no effect at all. Furthermore, none of these channel blockers have any effect on the touch-induced cessation of cytoplasmic streaming (data not shown). By contrast, all these drugs inhibit the gravitational and hydrostatic pressure-induced polarity of cytoplasmic streaming (Staves *et al.* 1992). Therefore, we conclude that the nifedipine-, verapamil- and ω-Conotoxin-sensitive Ca^{2+} channels that are involved in gravisensing contribute little or nothing to the signal transduction chain involved in the touch-induced action potential.

While the organic Ca^{2+} channel blockers used have little or no effect on the touch-induced action potential, inorganic Ca^{2+} channel blockers do inhibit the touch-induced

Table 1. The effect of organic Ca^{2+} channel blockers on the magnitude of the depolarization induced by touch in *Chara*

Internodal cells were incubated for 20 min in the standard buffer followed by a 20 min incubation in the standard buffer containing: 0·1 mol m^{-3} (100 μM) nifedipine ($n=17$); 0·1 mol m^{-3} (100 μM) verapamil ($n=14$); or 10^{-2} mol m^{-3} (10 μM) ω-Conotoxin ($n=4$). Mean values ± s.e. are presented

Treatment	Magnitude of depolarization (V)
Control	0·168 ± 0·014
Nifedipine	0·118 ± 0·011
Control	0·141 ± 0·019
Verapamil	0·111 ± 0·015
Control	0·152 ± 0·019
ω-Conotoxin	0·145 ± 0·012

action potential. We find that all the stable members of the lanthanide series inhibit the touch-induced action potential (Fig. 7) and cessation of streaming (data not shown). The relative efficacy of each lanthanide varies over seven orders of magnitude. The slopes of the sigmoidal curves are relatively steep, indicating that there are at least two binding sites for the lanthanides. The average Hill coefficient for all the curves is 1·9 ± 0·1, which is close to the maximum value that could be resolved given the concentrations used. When the relative efficacy of each lanthanide is plotted against the ionic radius, a 'fingerprint', of the Ca^{2+} channel is obtained (Fig. 8; Staves *et al.*, unpublished data). This Ca^{2+} channel is very different from those involved in gravisensing, but is identical to the Ca^{2+} channel involved in the electrical stimulation-induced cessation of streaming response (Staves *et al.*, unpublished data).

Gd^{3+} is thought to be uniquely able to block mechanosensitive channels (Yang and Sachs 1989). However, our results indicate that all stable lanthanides are able to block the touch-induced depolarization of the plasma membrane. As we have shown, Gd^{3+} is among

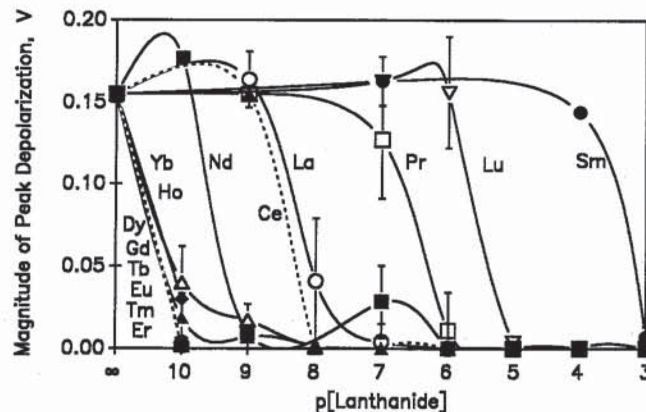

Fig. 7. The effect of lanthanides on the magnitude of the depolarization induced by touch in *Chara*. The lanthanides were added to the standard buffer. The cells were incubated in each solution for 20 min. Mean values ± s.e. are presented ($n=3$). pLn = $-\log[Ln^{3+}]$.

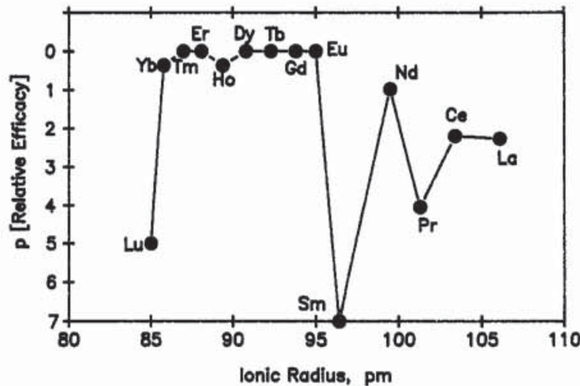

Fig. 8. The relative efficacy of the lanthanides in inhibiting the touch-induced action potential in *Chara* as a function of their ionic radius. The relative efficacy is given as p[Relative efficacy]; where p stands for 'the negative log of'. The concentration of each lanthanide required to give a 50% inhibition (I_{50}) was determined. In order to obtain the relative efficacy of a given lanthanide, the I_{50} of the most effective lanthanide was divided by the I_{50} concentration of the given lanthanide. [Ionic radii were obtained from the Handbook of Chemistry and Physics (1991–1992). (CRC Press: Cleveland, OH.).]

the most effective lanthanides in blocking this response, but several others, including Tm^{3+}, Er^{3+}, Dy^{3+}, Tb^{3+} and Eu^{3+}, are equally effective in inhibiting this response. Moreover, since Gd^{3+} and the other lanthanides inhibit the electrical stimulation-induced action potential and cessation of streaming in an identical manner to the way they inhibit the touch-induced action potential and cessation of streaming, we propose that Gd^{3+} acts not as a specific inhibitor of mechanosensitive channels, but as a Ca^{2+} channel blocker. Our model states that Gd^{3+} binds to one of three calcium binding sites in the pore of an elastic, slightly peristaltic channel protein. Normally, Ca^{2+} moves from site to site through the channel with a movement that is euphonious with that of the channel protein and consequently carries a current. By contrast the lanthanides, including Gd^{3+}, move through the channel with a movement that is discordant with that of the channel protein. The lack of resonance causes the lanthanides to remain in one of the three Ca^{2+}-binding sites within the pore for a long period of time. Depending on the resolution of the amplifier used to resolve the channel, the long residence time will appear as either a decrease in the single channel current or as a decrease in the open probability of the channel (Staves *et al.*, unpublished data).

The Effect of External Cl^- and a Cl^- Channel Blocker on the Touch-induced Action Potential

It is possible that Ca^{2+} itself carries the inward current responsible for the depolarization and/or that Ca^{2+} activates a Cl^- channel that allows the passage of a depolarizing Cl^- current (Mullins 1962; Lunevsky *et al.* 1983; Shiina and Tazawa 1987; Okihara *et al.* 1991). In order to see whether a Cl^- efflux was involved in the touch-induced action potential as it is in the electrical stimulation-induced action potential, we supplemented the medium with $CaCl_2$. We thus simultaneously increased the electrochemical potential gradient for Ca^{2+} and reduced the electrochemical potential gradient Cl^-. As a consequence of the oppositely directed electrochemical gradients for Ca^{2+} and Cl^-, we could determine which ion is carrying the inward current. If Ca^{2+} influx alone causes the depolarization, then the magnitude of the depolarization will increase with increasing $CaCl_2$ concentration; conversely, if Cl^- efflux alone is causing the depolarization, then the magnitude of the depolarization will decrease with increasing concentrations of $CaCl_2$. We find that the magnitude of the depolarization decreases as the extracellular $CaCl_2$ concentration increases (Fig. 9). Moreover, external Cl^- added as choline Cl^- inhibits the touch-induced action potential in an identical manner as Cl^- added as $CaCl_2$. These data indicate that a Cl^- efflux occurs during the touch-induced action potential. This conclusion is supported by the observation that a

Cl^- channel blocker, 9-anthracene carboxylic acid (A-9-C; 1 mol m^{-3}, 45 min; Tyerman *et al.* 1986), prevents the touch-induced action potential (Table 2).

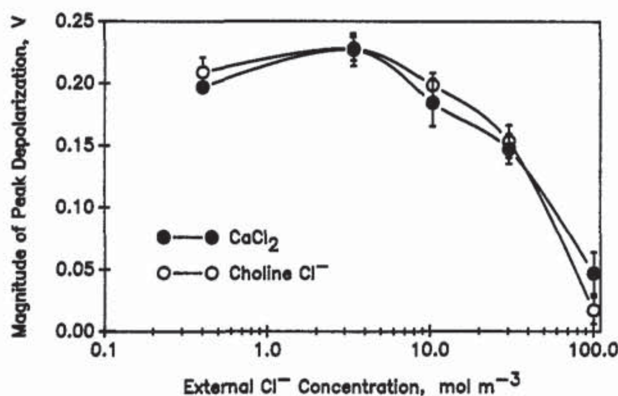

Fig. 9. The effect of external Cl^- on the magnitude of the depolarization induced by touch in *Chara*. Internodal cells were incubated in APW (0·1 mol m^{-3} of KCl, NaCl and $CaCl_2$) containing the indicated amount of $CaCl_2$ or choline Cl^- for 20 min. Mean values ± s.e. are presented (n = 3–4). mol m^{-3} = mM.

Table 2. The effect of A9C, a Cl^- channel blocker, on the magnitude of the depolarization induced by touch in *Chara*

Internodal cells were incubated for 1 h in the standard medium containing: 1 mol m^{-3} NaOH with or without 1 mol m^{-3} A9C. The stock solution of A9C was made by dissolving A9C in 10^3 mol m^{-3} (1 M) NaOH to give a stock concentration of 10^3 mol m^{-3} (1 M). Mean values ± s.e. are presented (n = 6)

Treatment	Magnitude of depolarization (V)
Control	0·173 ± 0·028
A9C	0·017 ± 0·006

The Effect of Extracellular K^+ and the K^+ Channel Blocker, Tetraethylammonium (TEA^+), on the Touch-induced Action Potential

The repolarization of the plasma membrane following electrical stimulation is a result of the efflux of K^+ due to an increase in the permeability of the membrane to K^+ and a change in the electrochemical potential gradient for K^+ (Oda 1976). The magnitude of the depolarization that we measure following a touch-stimulus is dependent on the ratio of the Cl^- to the K^+ conductance (Nosaka *et al.* 1992) where the equilibrium potential for Cl^- is approximately +0·103 V and the equilibrium potential for K^+ is approximately −0·180 V. We find that increasing the extracellular K^+ concentration from 0·1 mol m^{-3} to 1 mol m^{-3} or above completely inhibits the touch-induced action potential (Fig. 10) as well as the touch-induced cessation of streaming (data not shown). These effects are probably a result of a K^+-dependent clamp of the membrane potential to the equilibrium potentials for K^+ (E_K) which are voltages more positive than the threshold potential for the activation of the Ca^{2+} and Cl^- channels. This conclusion is supported by the observation that raising the external K^+ concentration increases the permeability of the plasma membrane to K^+ (Beilby 1986). Such an increase would cause the resting membrane potential to depolarize.

We find that the extracellular application of the K^+ channel blocker, TEA^+ (10 mol m^{-3}, 20 min), has no effect on the magnitude or the duration of the touch-induced action

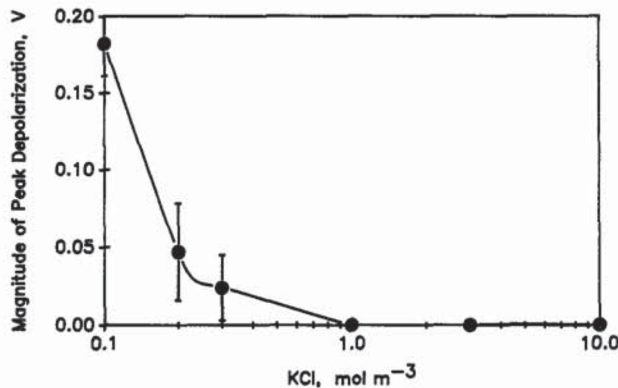

Fig. 10. The effect of extracellular K^+ on the magnitude of depolarization induced by touch in *Chara*. Various concentrations of KCl were added to APW ($0 \cdot 1$ mol m^{-3} of KCl, NaCl and $CaCl_2$). Mean values ± s.e. are presented ($n = 3$–6).

potential (data not shown). Likewise, Shimmen and Tazawa (1983) and Beilby and Coster (1979) find that extracellular TEA has no effect on the electrically induced action potential in *Chara*. By contrast, Tester (1988) finds that it prolongs the electrically stimulated action potential in *Chara*. The discrepancies in identifying TEA^+ effects on the duration of the action potential may be related to the differences in the temporal resolving power of the recording systems.

The ionic basis of the touch-induced action potential is similar to, if not identical to, the ionic basis of the electrical stimulation-induced action potential (Tazawa and Shimmen 1987; Tazawa *et al.* 1987; Wayne 1993). In both cases, there is an absolute requirement for Ca^{2+} as a regulatory ion and the inward current is carried predominantly by Cl^-. For the next phase of our investigation, we tested whether or not the mechanoreceptor involved in the touch response has similar properties to the mechanoreceptor involved in gravitational and hydrostatic pressure sensing.

The Effect of Cell Turgor on the Touch-induced Generation of an Action Potential

Gravitational and hydrostatic pressure sensing are absolutely dependent on cell turgor (Staves *et al.* 1992). If the turgor pressure drops from its normal value of $0 \cdot 635$ MPa to below $0 \cdot 34$ MPa, gravitational and hydrostatic pressure sensing is impaired, indicating that either mechanical strain in the plasma membrane or a close association between the plasma membrane and the extracellular matrix is important for gravitational and hydrostatic pressure sensing. By contrast, the ability to generate an action potential in response to touch is independent of cell turgor in the range of 10^{-5}–$0 \cdot 64$ MPa (Fig. 11). The cell loses its ability to respond to touch only after the cell is visibly plasmolyzed.

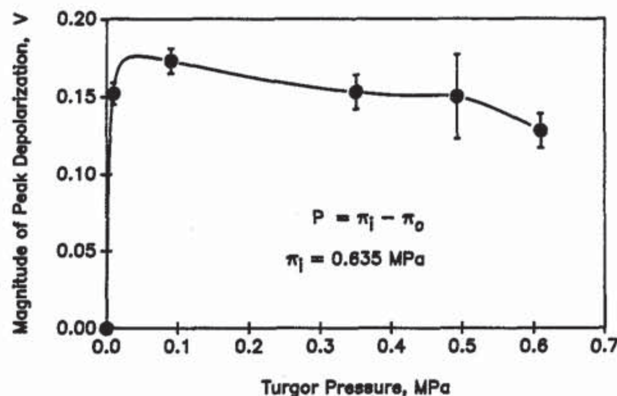

Fig. 11. The effect of turgor pressure on the magnitude of the depolarization induced by touch in *Chara*. Internodal cells were incubated in the standard buffer, containing 0, 50, 100, 200, or 255 mol m^{-3} sorbitol. Turgor pressure was determined by subtracting the osmotic pressure of the external medium from the measured osmotic pressure of *Chara* internodal cell ($0 \cdot 635$ MPa). Mean values ± s.e. are presented ($n = 3$–6).

The Participation of RGD-sensitive Binding Sites between Integrins and Extracellular Matrix Proteins in Touch-sensitive Mechanoreception

The close association of the plasma membrane and the extracellular matrix is probably important for gravitational and hydrostatic pressure sensing since integrins, proteins that link the plasma membrane to the extracellular matrix, have been implicated in gravitational and hydrostatic pressure sensing. This conclusion comes from experiments that show that Arg-Gly-Asp-Ser (RGDS), the tetrapeptide that competitively inhibits the binding of many integrins to the extracellular matrix, inhibits the gravitational and hydrostatic pressure-induced polarity of cytoplasmic streaming (Wayne *et al.* 1992). Moreover, when the aspartic acid residue is replaced with a glutamic acid residue, the resultant tetrapeptide (RGES) is completely ineffective in inhibiting the polarity of cytoplasmic streaming induced by either gravitational or hydrostatic pressure, indicating that a specific RGDS-binding site between an integrin and an extracellular matrix protein is involved in the sensing of mechanical pressure in the form of gravitational or hydrostatic pressure (Wayne *et al.* 1992).

We treated the cells with RGDS to determine whether or not a RGD-sensitive binding site between an integrin and an extracellular matrix protein is responsible for sensing the mechanical stimulation that results in the touch-induced action potential. We find that the touch-induced action potential is relatively insensitive to RGDS. The concentration needed to give a 50% inhibition is greater than $0{\cdot}5$ mol m^{-3} and requires long incubations (4 h; Fig. 12). By contrast, a 50% inhibition of gravisensing is obtained when the cells are treated with 10^{-3} mol m^{-3} RGDS for 20 min. RGDS is probably not acting specifically on the touch receptor at $0{\cdot}5$ mol m^{-3}, since RGDS at this concentration also inhibits the electrical-stimulation-induced action potential in an identical manner (data not shown). RGES ($1{\cdot}1$ mol m^{-3}, 4 h) and SDGRG ($0{\cdot}484$ mol m^{-3}, 4 h) also have little or no effect on the touch-induced action potential response (data not shown). These observations indicate that a mechanoreceptor that contains an RGD-sensitive binding site between an integrin and an extracellular matrix protein is probably not involved in sensing the touch-induced mechanical stimulation that leads to the generation of an action potential. However, we cannot eliminate the possibility that an integrin is involved in sensing touch, since one and the same integrin can bind to a number of extracellular matrix proteins, only some of which contain the RGD sequence. For example, integrin VLA-3 can bind to fibronectin, collagen and laminin, but only the interaction with fibronectin is inhibited by RGD (Elices *et al.* 1991). We can conclude, however, that there must be more than one kind of mechanoreceptor complex in characean internodal cells.

Further support that the mechanoreceptor for touch is distinct from the mechanoreceptor for gravitational and hydrostatic pressure comes from the observation that the

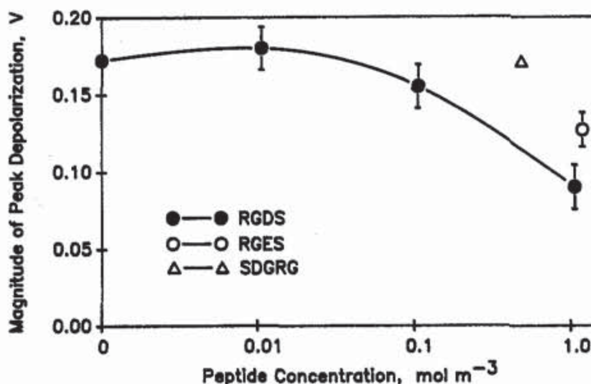

Fig. 12. The effect of the peptides RGDS, RGES and SDGRG on the magnitude of the depolarization induced by touch in *Chara*. RGDS, RGES and SDGRG were dissolved in the standard buffer. Cells were treated with the peptides for 4 h. Mean values ± s.e. are presented ($n = 3$–4).

touch-induced action potential is not sensitive to external protease treatments (Table 3). By contrast, gravitational and hydrostatic pressure sensing is inhibited by thermolysin (0·1%, 20 min) and proteinase K (0·1%, 20 min) treatments (Wayne *et al.* 1992).

Table 3. The effect of protease treatment on the magnitude of the depolarization induced by touch in *Chara*

Cells were treated for 20 min with 0·1% proteinase K or 0·1% thermolysin dissolved in pCa 5 buffer. Mean values ± s.e. are presented ($n = 3$)

Treatment	Magnitude of depolarization (V)
Control	0·116 ± 0·009
Proteinase K	0·115 ± 0·010
Thermolysin	0·133 ± 0·027

Spatial Localization of the Touch-sensitive Mechanoreceptor

The mechanoreceptor involved in gravitational and hydrostatic pressure sensing is not uniformly distributed throughout the cell but is localized at the ends of the cell. This conclusion is based upon experiments that show that UV irradiation of the ends, but not the middle of the cell, prevents the gravitational or hydrostatic pressure-induced polarity of cytoplasmic streaming. Moreover, the removal of the cell ends following cellular ligation completely inhibits gravitational and hydrostatic pressure sensing. By contrast, these treatments have no effect on the touch-induced action potential (Table 4).

Table 4. The effect of UV irradiation and cellular ligation on the magnitude of the depolarization induced by touch in *Chara*

Internodal cells were incubated in APW then ligated ($n = 3$) or irradiated ($n = 3$). Mean values ± s.e. are presented

Treatment	Magnitude of depolarization (V)
Control	0·197 ± 0·006
UV irradiated	0·218 ± 0·014
Intact control	0·204 ± 0·006
Ligated cells	0·208 ± 0·004

The Effect of Cytoskeletal Inhibitors on the Touch-induced Action Potential

Since an RGD-sensitive binding between an integrin-like protein in the plasma membrane and an extracellular matrix protein is important for gravitational and hydrostatic pressure sensing, but not for sensing the mechanical pressure that can induce an action potential, we explored the possibility that the cytoskeleton that subtends the plasma membrane may be necessary for sensing the mechanical pressure that leads to an action potential (Guharay and Sachs 1984; Ruknudin *et al.* 1991; Sokabe *et al.* 1991). In characean internodal cells, the action cytoskeleton is involved in regulating water transport across the plasma membrane (Wayne and Tazawa 1988). However, the actin cytoskeleton does not participate in the touch-induced generation of an action potential since cytochalasin D (2×10^{-2} mol m^{-3}, 20 min), at a concentration that completely inhibits actin-based cytoplasmic streaming, has no effect on the touch-induced action potential (Table 5).

The microtubule-based cytoskeleton may also be involved in the regulation of the permeability of the plasma membrane. Fisahn and Lucas (1990) show that the microtubular cytoskeleton may be involved in organizing the acid and alkaline bands in characean cells,

although these results are questioned by Wasteneys and Williamson (1992). We find that the microtubule inhibitors APM (amiprophos methyl; 10^{-2} mol m^{-3}, 4 h) and colchicine (5 mol m^{-3}, 3 h) cause a statistically significant, although small and probably physiologically insignificant, decrease in the magnitude of the depolarization induced by touch (Table 5). The cytoskeletal agents also have identical effects on the electrical stimulation-induced action potential (data not shown). Therefore, it is possible that microtubules have a minor effect on the channels involved in the action potential, but have no effect on the touch receptor itself. The possibility remains that other submembranous skeletal proteins, including spectrin, are necessary for mechanosensitivity (Sachs 1986; Sokabe *et al.* 1991). Alternatively, mechanosensitivity to touch may depend only on the mechanosensor itself and the lipid bilayer (Martinac *et al.* 1990).

Table 5. The effect of cytoskeleton inhibitors on the magnitude of the depolarization induced by touch in *Chara*

Internodal cells were incubated in the standard buffer (containing 0·1% DMSO for cytochalasin D and APM treatments) followed by incubations in the standard buffer containing: 2×10^{-2} mol m^{-3} (20 μM) cytochalasin D (20 min, $n=4$) which caused cytoplasmic streaming to stop within 10 min; 10^{-2} mol m^{-3} (10 μM) APM (4 h, $n=6$); or 5 mol m^{-3} (5 mM) colchicine (3 h, $n=4$). Mean values ± s.e. are presented

Treatment	Magnitude of depolarization (V)
Control	0·188 ± 0·003
Cytochalasin D	0·173 ± 0·011
Control	0·202 ± 0·007
APM	0·157 ± 0·017
Control	0·157 ± 0·010
Colchicine	0·102 ± 0·015

Argument for at least Two Classes of Mechanoreceptors

At the commencement of this work, we took the conservative view that there was only one type of mechanoreceptor that is involved in all forms of mechanical sensing, including gravisensing, turgor sensing and touch sensing. We believed that all mechanoreceptors would prove to contain RGD-binding, integrin-containing protein complexes whose attachment to the extracellular matrix was in a *sine qua non* for mechanoreception. We thought that it was not the *properties* of the mechanoreceptor, but the *position* (in both space and time) of the mechanoreceptor that gave specificity to a mechanical response. We believed that gravity sensing was a specialized form of mechanical sensing where the integrin-containing mechanoreceptors at the top and bottom of a vertical cell were the mechanoreceptors that experienced a change in pressure in a gravitational field, whereas the integrin-containing mechanoreceptors on the flank of the cell did not. However, the present experiments indicate that our initial hypothesis was wrong and there is more than one class of mechanoreceptors. In hindsight, this seems like a very reasonable conclusion.

Firstly, the energy available to a characean internodal cell as a consequence of its orientation in a gravitational field is approximately 10^{-15} J (Wayne *et al.* 1990). This energy is released as a consequence of the displacement in a gravitational field of the protoplast of a cell relative to the extracellular matrix. Thus, the stimulus energy comes from a change in potential energy ($PE=F\cdot d$). Arrhenius plots show that the overall process leading from gravity perception to the gravity response has an activation energy of less than 6×10^{-20} J (Staves and Wayne, unpublished observations).

By contrast, the touch response requires energies in the range of 10^{-8} J if we consider that the energy of the stimulus comes from a transfer of the kinetic energy of the stimulus to the cell as calculated from the following equation:

$$KE = \tfrac{1}{2}mv^2 = 1 \cdot 1 \times 10^{-8} \text{ J}, \quad (2)$$

where KE is the kinetic energy of the stimulus (in J), m is the apparent mass of the stimulus ($13 \cdot 2 \times 10^{-3}$ kg), and v is the velocity of the stimulus ($1 \cdot 27 \times 10^{-3}$ m s^{-1}).

The kinetic energy of the stimulus will cause a large deformation of the membrane, resulting in an increase in the potential energy of the membrane which is capable of doing work. Assuming a 10% efficiency in the conversion of kinetic energy to potential energy, the gain in potential energy as a consequence of touch is still approximately a million times greater than the energy available for gravity sensing. If we assume that any class of mechanoreceptors has a Boltzmann distribution of active and inactive states, it seems that one class of mechanoreceptors would be completely inactivated when subjected to gravitational energies, and completely activated when subjected to the energies resulting from the kinetic energy of touch. We recognize that this is not theoretically impossible. One class of mechanoreceptors could be subjected to sensitivity modulation that could vary the midpoint of a typical sigmoidal sensitivity curve over six orders of magnitude (Hepler and Wayne 1985).

It is usually considered that mechanoreceptors are activated by the tension in the membrane that is generated as a consequence of pressure (Gustin *et al.* 1988; Sokabe *et al.* 1991) and this modal can explain all forms of mechanoreception (Sachs 1986; Ding and Pickard 1990). The energy, E (in J), available to do work as a result of the tension (T') in the membrane is defined by the following equation:

$$E = T' \cdot A, \quad (3)$$

where T' is the tension in the membrane ($\approx 5 \times 10^{-3}$ N m^{-1}), and A is the area of the membrane in a patch pipette ($\approx 8 \times 10^{-13}$ m^2).

Solving this equation for typical tensions and areas of membrane in the patch pipette, we see that the mechanosensitive channels are activated by energies in the range of 10^{-15} J. This is approximately 10^4 times greater than the energy needed to activate the mechanosensitive channel in the hair cell (Howard *et al.* 1988). Again, it seems unreasonable for the same mechanoreceptor with a typical Boltzmann distribution of active and inactive states to sense energies that are so different without being completely inactive at the low energies and completely active at the high energies. Thus, it is likely that the mechanoreceptors for the 'stretch-activated channels' observed in patch clamping and the displacement-activated channels observed in hair cells are different (Howard *et al.* 1988; Hudspeth 1989; Gillespie and Hudspeth 1991; Pickles and Corey 1992).

We tentatively propose that the mechanoreceptor involved in 'stretch-activated channels' is similar in properties to the mechanoreceptor involved in the touch response in characean internodal cells. Both are activated by energies greater than 10^{-15} J, and detect *changes* in pressure but not the sign. Stretch-activated channels are usually equally activated by positive or negative pressures (Morris 1990). Stretch-activated channels are observed in protoplasts (Falke *et al.* 1988; Cosgrove and Hedrich 1991) and thus require no attachment to the extracellular matrix. They are often uniformly distributed throughout the cell. The stimulation of this mechanoreceptor may activate Ca^{2+} channels that are insensitive to organic channel blockers, but show the lanthanide fingerprint of the high-discrimination Ca^{2+} channel (Staves *et al.*, unpublished data).

We also tentatively propose that the mechanoreceptor involved in mechanosensing (including gravisensing) in hair cells is similar in properties to that involved in gravitational and hydrostatic pressure sensing in characean cells. They both are activated by low energies in the range of 10^{-20} to 10^{-19} J, have a differential response to displacement in one

direction relative to the other, require an attachment to an extracellular matrix protein (Assad *et al.* 1991) and have a non-uniform spatial localization. The stimulation of this mechanoreceptor may activate nifedipine-, verapamil- and ω-Conotoxin-sensitive Ca^{2+} channels that show the lanthanide fingerprint of the low-discrimination Ca^{2+} channel (Staves *et al.*, unpublished data).

While stretch-activated channels that are activated by pressures in the 10^3 Pa ($\approx 10^{-15}$ J) range were first discovered using patch-clamping technology (Falke *et al.* 1988; Cosgrove and Hedrich 1991), it is becoming clear that mechanoelectrical transducing channels that are activated by lower pressures in the range of 10^2 Pa also exist (Sackin 1989; Alexandre and Lassalles 1991; Filipovic and Sackin 1991). Perhaps it is just a matter of time before mechanoelectrical transducing channels that are activated by 1 Pa are detected.

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