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INVITED REVIEW

Twenty odd years of stretch-sensitive channels

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Abstract After formation of the giga-seal, the membrane patch can be stimulated by hydrostatic or osmotic pressure gradients applied across the patch. This feature led to the discovery of stretch-sensitive or mechanosensitive (MS) channels, which are now known to be ubiquitously expressed in cells representative of all the living kingdoms. In addition to mechanosensation, MS channels have been implicated in many basic cell functions, including regulation of cell volume, shape, and motility. The successful cloning, overexpression, and crystallization of bacterial MS channel proteins combined with patch clamp and modeling studies have provided atomic insight into the working of these nanomachines. In particular, studies of MS channels have revealed new understanding of how the lipid bilayer modulates membrane protein function. Three major membrane protein families, transient receptor potential, 2 pore domain K^+, and the epithelial Na^+ channels, have been shown to form MS channels in animal cells, and their polymodal activation embrace fields far beyond mechanosensitivity. The discovery of new drugs highly selective for MS channels ("mechanopharmaceutics") and the demonstration of MS channel involvement in several major human diseases ("mechanochannelopathies") provide added motivation for devising new techniques and approaches for studying MS channels.

Keywords Mechanosensitive channels · Mechanotransduction · Transient receptor potential · Patch clamp · Giga seal

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Introduction

The formation of the giga-seal depends upon applying suction to draw the membrane into the pipette. Once the seal is formed, suction is not necessary, and it should be released [53]. However, the membrane patch spanning the pipette can now be mechanically stimulated by hydrostatic or osmotic pressure gradients applied across the patch without disrupting the seal. This feature allowed the first recordings of cell-swelling and stretch-activated channel currents [50, 52]. Over the last 20-odd years, interest in mechanosensitive (MS) channels has progressed from being a possible patch clamp recording artifact to a central player in our understanding of protein–bilayer interactions and a promising new therapeutic target against several major human diseases. This article highlights some recent developments and unresolved issues regarding MS channels, with a major focus on the MS Ca^{2+} permeant cation channel (MscCa) recently identified in vertebrate cells [97].

What happens to the membrane patch in the pipette?

An important issue for MS channels is how the process of aspiration and sealing of the membrane in the pipette alters the mechanics and possible stretch sensitivity of channels in the patch. Because of the small size and inaccessibility of the patch in the pipette, a variety of techniques, including high-resolution video imaging [121, 152–154, 184], high-voltage electron microscopy [142], atomic force microscopy [70], and fluorescence-imaged microdeformation [32, 33] have been used to study the aspirated patch and its underlying cell cytoskeleton (CSK). Here we focus on results obtained on the *Xenopus* oocyte [182–185]. The first issue is whether the pressure/suction applied to the patch after seal formation somehow

induces changes in the seal resistance that appear as "MS-channel-like" events. This idea is not entirely far-fetched because video imaging indicates that suction tends to peal the membrane off the walls of the pipette [121], and gated, cation-selective channels have been recorded with patch pipettes sealed onto noncellular hydrophobic surfaces [145]. However, although strong suction can rupture the patch, it typically does not disrupt the giga-seal, thereby allowing for tight seal whole-cell and/or outside-out patch recording [53]. In addition, suction ramps applied to cell-attached frog oocyte patches reversibly activate either a saturating macroscopic current (Fig. 1a) or a unitary amplitude current event (Fig. 1b) depending upon patch area [60]. These current waveforms are consistent with multiple and single MS channel patches, respectively, but difficult to reconcile with MS changes in seal resistance. Even more compelling is that patches formed on pure liposomes fail to express MS channel currents [97, 102, 122, 160]. This absence allowed for the identification of MS channel proteins following functional reconstitution of solubilized membrane proteins from bacteria and archaea [81, 158, 159] and, most recently, from *Xenopus* oocytes [97].

Although MS channels are clearly not seal "leaks," the sealing process does change patch geometry and the underlying CSK, thereby altering patch mechanics. For example, Fig. 2a and b shows electron microscopy (EM) images of the *Xenopus* oocyte surface, indicating its extensive membrane folding and high density of microvilli (est. ~7 microvilli per square micrometer) [181, 184, 185]. This complex membrane geometry is also reflected in electrical capacitance (C_m) measurements that indicate a membrane surface area that is tenfold greater than that required for the cell's volume [184]. In contrast, high-resolution video images of cell-attached oocyte patches (Fig. 2c, d) indicate an optically smooth membrane that is pulled flat and perpendicular to the walls of the pipette [184; see also 113, 152, 153, 161]. Furthermore, C_m measurements indicate a patch area of approximately 50 μm^2, consistent with the patch geometry but inconsistent with the approximately 500 μm^2 expected if the approximately 300 microvilli and membrane folds evident in Fig. 2a and b were preserved during the sealing process [184]. Presumably, the suction used to obtain the giga-seal is sufficient to smooth out the surface folds and microvilli so that the cell membrane is now tightly stretched over an expanded CSK (Fig. 3). This smoothing out of microvilli is not an exclusive patch clamp phenomenon because a similar phenomenon has been visualized in EM studies of cells undergoing osmotic inflation [82] and spreading before cell migration [37]. In these cases, the process is presumably reversible, indicating the considerable plasticity of the microvilli and their supporting CSK.

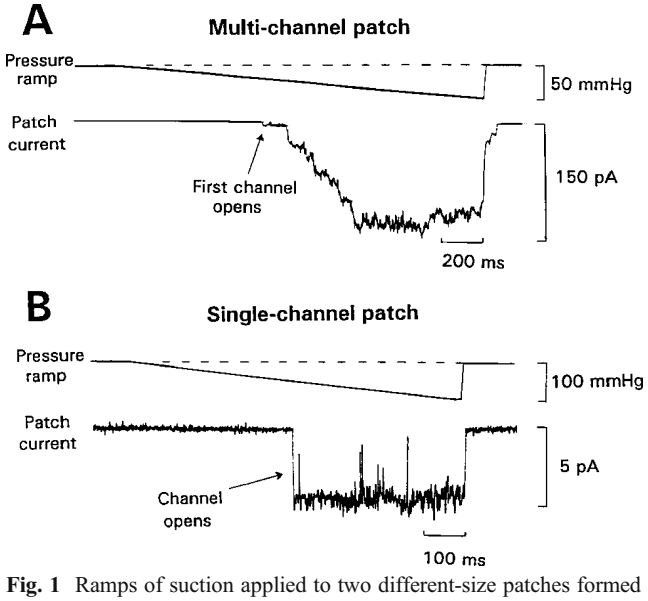

Fig. 1 Ramps of suction applied to two different-size patches formed on a *Xenopus* oocyte are consistent with a finite number of discrete MS channels but inconsistent with pressure-induced leaks in the seal. **a** A relatively large patch formed with an approximately 3-μm-diameter tip pipette shows a current that fully saturates at around 40 mmHg. The *arrow* indicates the initial opening of 5 pA single MscCa. **b** A smaller patch formed with an approximately 0.50-μm-diameter tip pipette reveals the opening of single MscCa, indicating that once open, the channel current was independent of suction. These results indicate that MscCa is either open or closed, and the saturation of current in a multichannel patch represents the balance between opening and closing rates for a finite number of channels

There are at least two related mechanisms by which changes in patch geometry will increase stretch sensitivity of channels in the patch. First, in the absence of any excess membrane, brief pressure pulses applied to the patch will rapidly flex the membrane and increase bilayer tension (Fig. 2c, d). The flexing of the membrane either outward with suction or inward with pressure results in the rapid activation (<1 ms) of inward channel currents [107, 108, 184]. In contrast, more sustained pressures would be required to inflate the oocyte and smooth out the membrane reserves to increase membrane tension (T_m) [15, 117, 184]. Second, according to Laplace's law, $P=2\ T_m/r$, the pressure (P) required to activate channels in the patch with a radius of curvature (r) of approximately 1 μm would be 1/20th of that required to activate the same channels located on microvilli that have a radii of curvature of approximately 0.05 μm. For example, stimulus–peak current relations shown in Fig. 4 indicate that half the channels in an oocyte patch are activated by a suction of approximately 10 mmHg (~1.3 kN m^{-2}), which translates to a tension of approximately 0.6 mN m^{-1} (i.e., the near-symmetrical suction/pressure relations indicate a tension-gated channel and justify Laplace's law). To achieve the same tension in microvilli would require a suction of 200 mmHg. However, this would exceed the approximately 100-mmHg that causes patch rupture (i.e., a lytic tension of ~6 mN m^{-1}) under these conditions [119].

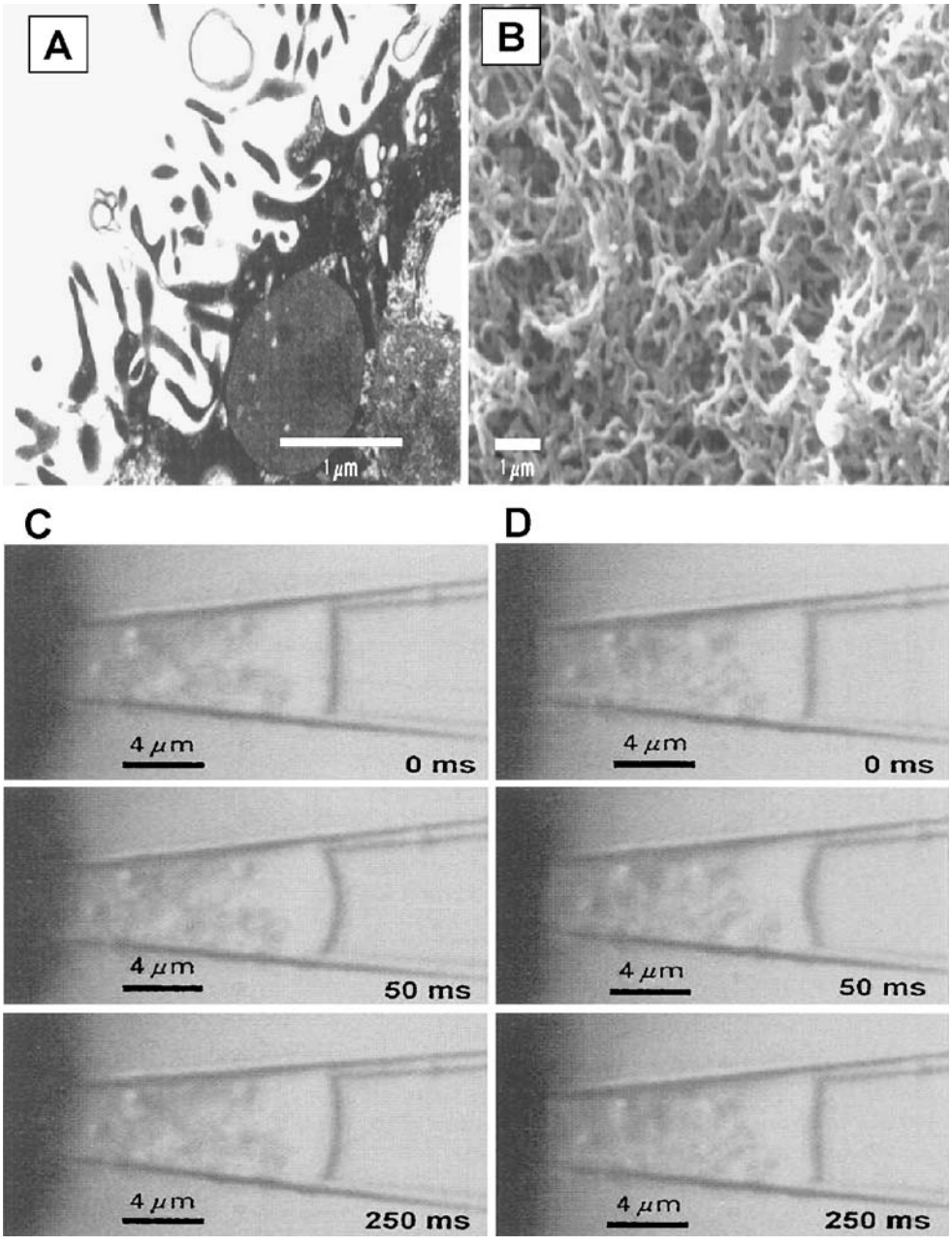

Fig. 2 Comparison of the membrane geometry of the oocyte surface and of the patch sealed in a pipette. **a** Transmission EM of the oocyte surface showing prominent microvilli containing dark cytoplasmic material. **b** Scanning EM of the oocyte surface, indicating the high density of microvilli. **c** High-resolution video images of a membrane patch before (0 ms), during (50 ms), and after (250 ms) a 100-ms suction step. **d** The same patch as in C except a 100-ms pressure step was applied. Both suction and pressure steps activated a 50-pA inward current (modified from Zhang and Hamill [184] and Zhang et al. [185] with permission)

The sealing process may also alter patch mechanics by changing the CSK structure underlying the patch [55, 63, 173]. When a gentle sealing protocol is used to achieve the giga-seal, the cortical CSK network that is pulled into the pipette may be dilated without disrupting its links with the membrane [54, 185; see also 32, 33]. However, if the suction used to draw the membrane into the pipette exceeds the strength of CSK–membrane linkages, then a CSK-free membrane (bleb) may be formed [55, 63, 112]. Similarly, after the membrane has sealed in the pipette, additional suction may cause the blebbing at the membrane cap as shown in Fig. 5 [185]. This membrane blebbing can either increase or decrease the stretch sensitivity depending upon the specific MS channel. For example, the TREK and TRAAK MscK channels show an increase in stretch sensitivity, presumably because the CSK normally acts as a constraint and prevents tension being conveyed to the bilayer [69, 151]. On the other hand, MscCa typically shows a loss of both stretch sensitivity and fast dynamics presumably because they depend upon CSK interactions with the channel/membrane (Fig. 5) [55, 156, 185].

In summary, giga-seal formation introduces significant changes in patch mechanics that can alter the mechanosensitivity of channels in the patch. The extrinsic changes in membrane geometry and CSK structure may have different effects on specific channels depending upon

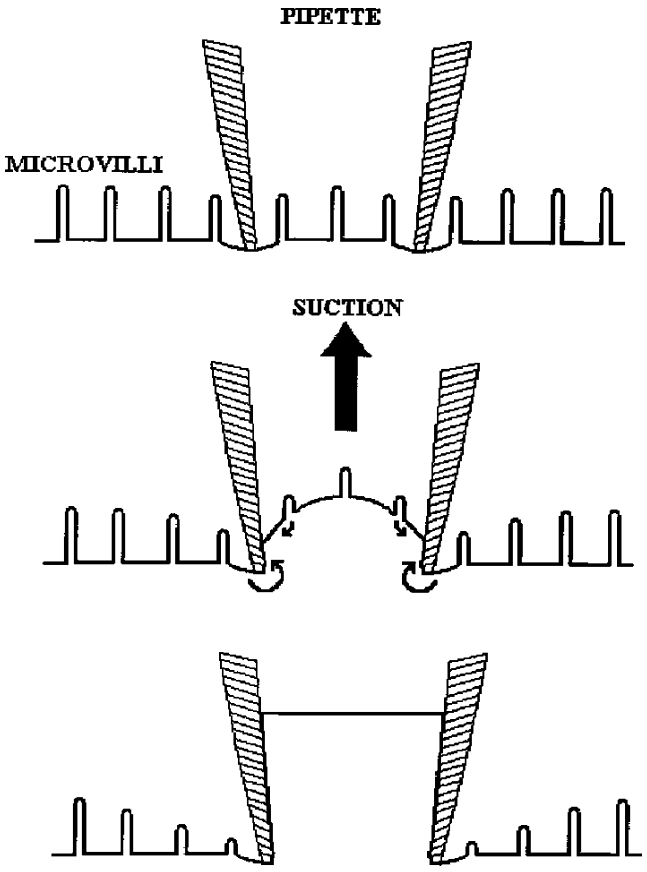

Fig. 3 Schematic illustrating the proposed smoothing out of microvilli caused by the pipette aspiration and giga-seal formation

their intrinsic properties (i.e., protein structure and protein–CSK interactions).

Do animal cells generate/experience membrane tensions that activate MS channels?

Although it has been questioned whether animal cells experience the same in-plane membrane tensions that activate MS channels in the patch [30, 149, 172], there is evidence that they can experience even larger tensions that result in membrane rupture. In particular, cell wounding events, as judged by the cell filling with the membrane impermeant fluorescein-labeled dextran, have been observed in experimentally unperturbed rodent skin, gut epithelium, cardiac, and skeletal muscle, and found to increase in frequency with mechanical loading [11, 109–111, 164]. The cell types that commonly experience wounding in vivo include epidermal cells and fibroblasts (skin), epithelial cells and smooth muscle (GI tract and respiratory system) endothelial cells, and smooth muscle (cardiovascular system) and peripheral neurons. The proportion of the cells wounded in the various systems can range from 2 to as high as 25%. In eccentrically exercised muscle (e.g., downhill running), there can be a tenfold increase in cell wounding compared with resting muscle, and in dystrophic mice that lack the CSK–structural protein dystrophin, exercise can produce massive wounding events that ultimately overload the muscle regeneration mechanisms [180]. Normal migrating cells can generate traction forces that not only lengthen and stretch the cell but also cause cell fragments to be ripped off and deposited along the migration trail [104]. Furthermore, if the normal contractile mechanisms that allow a migrating cell to retract its rear are blocked, the front of the cell can tear away from the cell body and move off as a motile cell fragment [170]. The common occurrence of these traumatic mechanical stresses under physiological and pathological conditions has presumably provided strong selective pressure for the evolution of the membrane resealing mechanism(s) that is widely expressed in eukaryotic cells [11, 109–111, 164].

Despite the above evidence of membrane rupture tensions (i.e., >5 mN m^{-1}), direct estimates of membrane tension in "resting" isolated cells indicate much lower values (<0.1 mN m^{-1}) [28–31, 116, 138]. The tension measurement involves pulling a tether from the cell surface using optical tweezers and measuring the force required to maintain it at constant length. The basic assumption is that membrane tension is contiguous over the whole surface so that pulling a tether from one region perturbs the tension in all regions of the cell membrane. From the static tether force (F_0) measured in piconewtons, one can estimate the membrane tension T_m according to the equation:

$$F_0 = 2\pi(2BT_m)^{1/2}$$

where the membrane bending stiffness (B) is assumed to be constant with a value of 2.7×10^{-19} N m^{-1}. The practical limitation of this technique is that the optical tweezers can only sustain forces up to 100 pN, which would correspond to a tension of 0.5 mN m^{-1}. For an animal cell with its cortical CSK, the measured tension is assumed to represent a combination of in-plane tension and CSK adhesion, and is referred to as the "apparent" tension. However, T_m measurements of membrane blebs that lack CSK indicate the in-plane tension contributes only 25% of the T_m value [28]. Experiments on two different cell types (RBL 2H3 cells and snail neurons) indicate that cell swelling increases steady-state tensions from approximately 0.04 to 0.12 mN m^{-1},which then returns to approximately 0.04 mN m^{-1} with reshrinking [30, 31]. However, the same cells also experience tension surges that exceed the strength of the trap (i.e., >0.5 mN m^{-1}). Based on other experiments measuring exocytosis/endocytosis as a function of membrane tension, it has been proposed that membrane surface area and tension are in a feedback loop in which high tensions favor membrane recruitment, and low tension favors membrane retrieval [31, 116, 148]. As a conse-

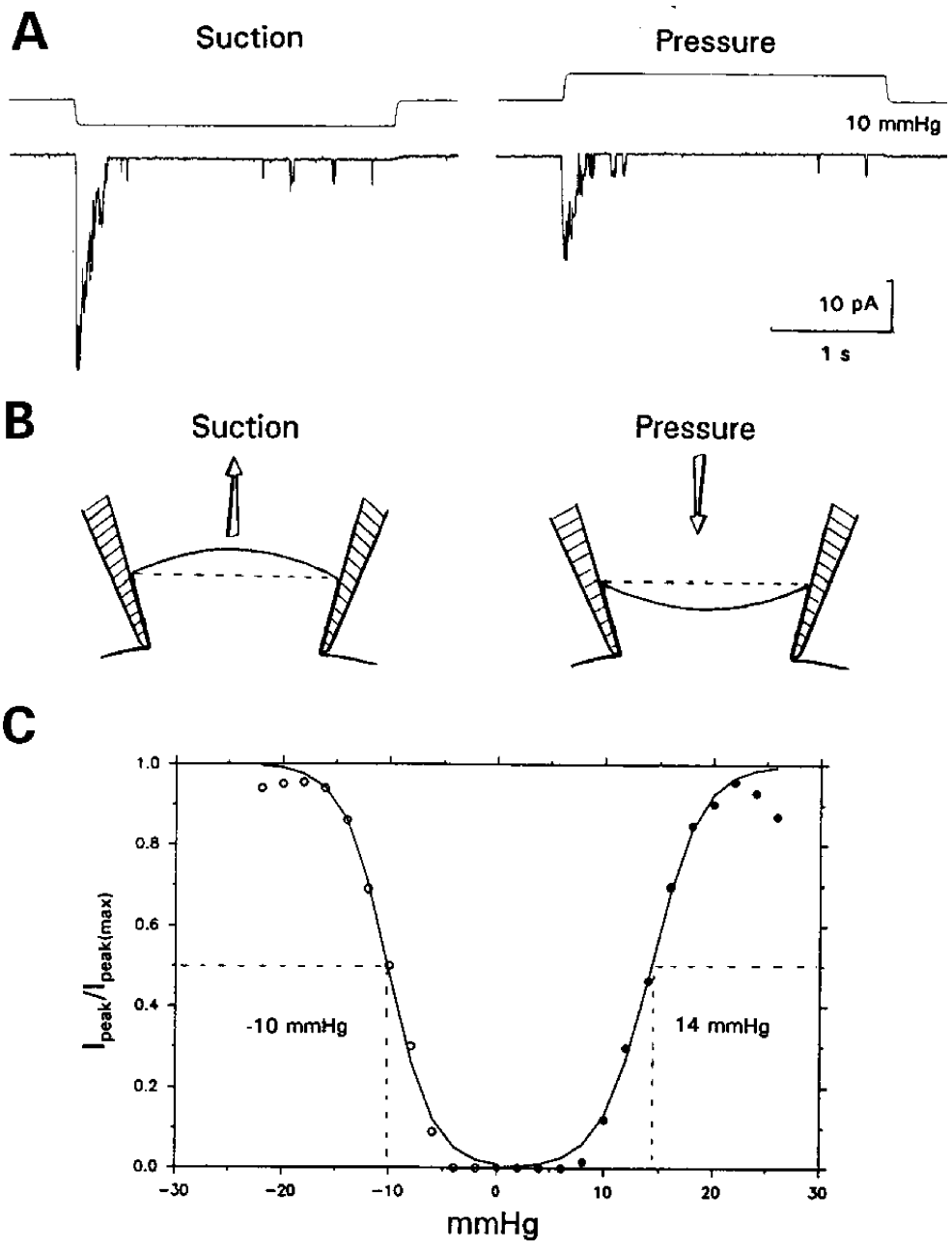

Fig. 4 Inward current responses to suction and pressure pulses applied to an oocyte patch. **a** Both suction and pressure pulses (2.5 s) result in rapid opening of MscCa that mostly close within 200 ms of the pulse. **b** Schematic showing flexion of the patch outward (suction) or inward (pressure). **c** Stimulus–response relations for suction and pressure steps. The sigmoid fits indicate that suction ($P_{0.5}$=−10 mmHg) was slightly more effective than pressure ($P_{0.5}$=14 mmHg) in activating MscCa

quence, it has been proposed that surface area regulation (SAR) maintains membrane tension around a relatively low set point of approximately 0.1 mN m^{-1}, which would be well below the lytic tension (≥5 mN m^{-1}) and the near-lytic tensions (~4 mN m^{-1}) required to activate the bacterial MS channels. However, the direct measurement of high tension surges exceeding the low set point and the occurrence of cell wounding events indicate that SAR mechanisms can be saturated. Furthermore, as indicated in Fig. 6, lower tensions are required to activate MS channels in animals cells ($T_{50\%}$ for MscK=2.4 mN m^{-1}) and (MscCa ~0.6 mN m^{-1}) compared with MscL (~4.7 mN m^{-1}) that functions as a last-resort safety valve [94]. As a consequence, MS channels in animal cells would seem more geared to regulating processes with lower tension set points such as regulatory volume decrease [20, 26, 146, 147, 169] cell locomotion [92] and, possibly, SAR via Ca^{2+}-induced exocytosis. However, it appears that integrins rather than MscCa act as the mechanosensor for MS exocytosis/membrane trafficking at the frog neuromuscular junction [24] and the oocyte [96].

"Mechanopharmaceutics"

Progress in the MS channel field would be greatly enhanced by the discovery of high-affinity agents that

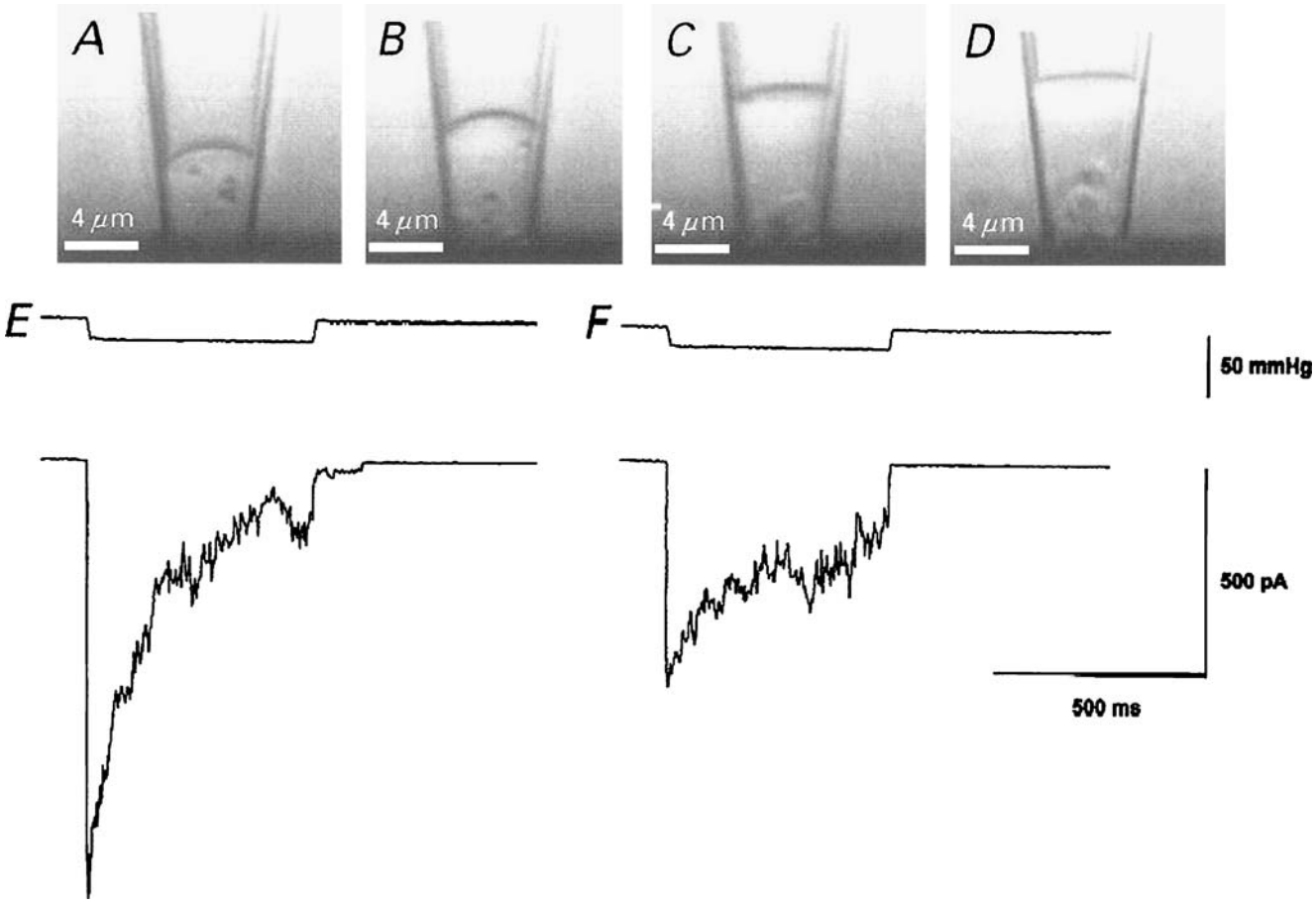

Fig. 5 Changes in the membrane and patch currents as a consequence of repetitive pressure pulses applied to the patch pipette. **a**–**d** video images of a cell-attached patch at different times after formation of the giga-seal. Between each image, suction steps (20 mmHg, 500 ms) were applied. **a** The first image taken immediately after giga-seal formation shows the patch curved outwards and located closed to the end of the pipette (~5 μm). Particles located in the cytoplasm exhibited no motion presumably because they were still constrained by the intact CSK. **b–d** repetitive suction pulse caused the patch to move up the pipette away from the cell, and a clear space developed between the membrane and the CSK remaining close to the cell. Particles that moved into this space displayed Brownian motion, indicating the loss of constraining CSK structures [153]. **e** Application of suction pulses at **a** caused a rapid opening of MscCa that closed almost completely. **f** Application of a suction pulse at **d** caused activation of a smaller more sustained current (from Zhang et al. [185] with permission)

selectivity target specific MS channels. These agents would be highly useful for following MS channel proteins during purification procedures and identifying MS channel roles in novel functions. In addition, given that MS channels may be polymodally activated (e.g., by tension, voltage, pH, temperature, Ca^{2+} store depletion, and/or lipid second messenger) [27, 125, 171], it would be advantageous to discover agents that selectively acted on MS channel only when they were mechano-gated. Although no ideal MS channel reagent has yet been discovered, a number of compounds have been identified that act as MS channel blockers or activators [48, 61, 65]. One class, amiloride and its analogs, appear to act on a traditional "lock and key" protein receptor, whereas other agents, GsMTx-4 and possibly maitotoxin, seem to act via nontraditional "receptors" at the lipid or lipid–protein interface where they may change the local bilayer mechanics and thereby modify MS channel gating. Below we briefly review their salient features.

Amiloride has been the most rigorously studied in terms of its MS channel blocking mechanism and provides an example where variations in mechanistic detail may enable discrimination between different channel families in terms of their participation in specific MS functions. In particular, the amiloride block of MscCa/TRPC-1 in *Xenopus* oocytes [56, 89–91] and MscCa in vertebrate hair cells [141] has been shown to involve basically the same unusual mechanism in which two amiloride molecules bind cooperatively to channel sites that only become accessible at hyperpolarized potentials after the channel has opened. This mechanism, referred to as "conformational" block, implies different open-state conformations at hyperpolarized vs

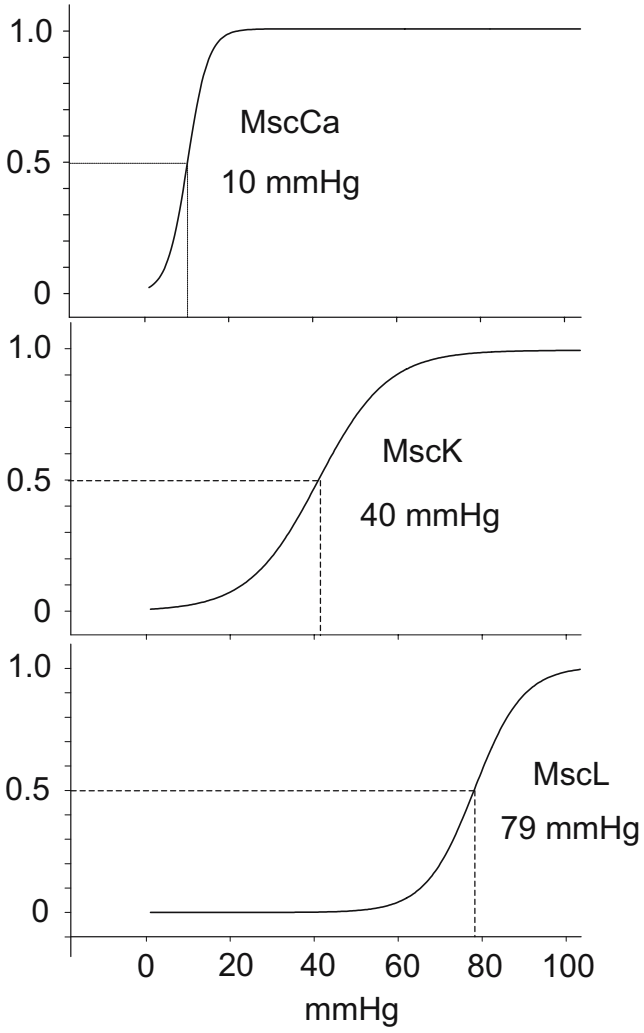

Fig. 6 Comparison of the normalized pressure–current relations for three different MS channels. MscCa curve fitted to curve Fig. 2c (*top*). MscK curve fitted to data from Ref. [69] (*middle*). MscL curve fitted to data from Ref. [54] (*bottom*)

depolarized potentials, and is distinctly different from the amiloride block of the high-affinity amiloride-sensitive epithelial Na^+ channel (ENaC) where the voltage dependency arises because the positively charged amiloride binds to a pore site that senses a fraction of the electric field [130]. A further difference is seen in the order of potencies of amiloride analogs in blocking the two channel classes (Table 1) [80]. Amiloride blocks the MEC-4 DEG/ENaC currents in touch receptor neurons in *Caenorhabditis elegans* [120] and also the mammalian arterial myogenic response, which has been used to implicate DEG/ENaC as the vascular mechanosensor [34]. However, amiloride also blocks TRPC-6, also implicated as the arterial smooth muscle mechanosensor [74, 175]. It will be interesting to determine if mechanistic differences in amiloride block (i.e., conformational vs pore block) can be used to resolve the MS Channel's identity.

Gadolinium blocks a variety of MS channels (e.g., MscCa, MscK, MscL, and MscS), several TRP channels (e.g., TRPC-1, TRPC-4, TRPC-5, and TRPV1), various voltage-gated (Ca^{2+}, Na^+, and K^+) and receptor-gated channels [e.g., *N*-methyl D-aspartate (NMDA), AChR, etc.] [61, 124, 136, 144, 165, 176, 178]. Because of its trivalency, Gd^{3+} will bind with high affinity to negative groups on proteins, lipids, and polysaccharides, as well as any inorganic anions present in solution [17, 61]. Its ionic radius (0.938 Å), which is similar to Na^+ (0.97 Å) and Ca^{2+} (0.99 Å), may also allow it to enter and bind to negatively change groups (Glu and/or Asp) within cation channels. Evidence that Gd^{3+} interacts directly with channel proteins comes from studies of specific TRP members (TRPV-1, TRPC-4, and TRPC-5) where Gd^{3+} has been shown to have dual effects, activating the channels at low micromolar concentrations (<100 μM) but blocking at higher concentrations (>300 μM). The activation of TRPV-1 depends upon binding to specific external glutamate residues that confer acid sensitivity on the channel, and neutralization of these residues blocks the activation and modifies inhibition [165]. Similar concentration-dependent potentiating and blocking effects also occur with TRPC-4 and TRPC-5 [77]. In contrast, Gd^{3+} only blocks TRPC-1 and TRPC-3 channels at relatively low micromolar concentrations [97, 166].

Table 1 Amiloride analog potency (IC_{50} amiloride/IC_{50} analog) of MS channels and the epithelial Na^+ channel

	Amiloride (IC50, μM)	DMA	Phenamil	PBDCB	Benzamil	HMA	I-NMBA	Reference no.
MscCa mouse hair cell	1 (53)	1.3	4.4	5	9.6	12.3	29	[141]
MscCa *Xenopus* oocyte	1 (500)	1.4	–	–	5.3	14.7 (BrHMA)		[90]
Epithelial Na^+ channel (high affinity)	1 (0.34)	0.04	17	–	9	0.04	7	[80]
Epithelial Na^+ channel (low affinity)	1 (10)	2.2	2.9	–	3.8	–	–	[80]

DMA Dimethylamiloride; *PBDCB* 5-(*N*-propyl-*N*-butyl)-dichlorobenzamile; *HMA* hexamethyleneamiloride; *I-NMBA* 6-iodide-2-methoxy-5-nitrobenzamile; *BrHMA* bromohexamethylene amiloride

An early view on how Gd^{3+} might block MS channels was via effects on the bilayer. Gd^{3+} has been shown to interact with black lipid membranes containing the negatively charged phosphatidylserine (PS) but not with the neutral phosphatidylcholine to increase the boundary potential and membrane tension [38]. However, whether these effects underlie MS channel block remains unclear because PS is normally restricted to the internal-facing monolayer, and Gd^{3+} acts externally. Gd^{3+} has also been shown to promote shape changes in giant unilamellar vesicles lacking PS [162]. In this case, it was proposed that Gd^{3+} bound to the hydrophilic lipid head (i.e., negative charge of the phosphate groups) of the external monolayer, and in doing so, decreased its surface area relative to the internal monolayer, thereby causing a change in membrane curvature. However, whether this effect would block MS channels remains unclear because amphipaths that also change membrane curvature usually result in MS channel activation [101, 135]. A recent study has reported that Gd^{3+} can block MS channels without altering pressure-induced changes in C_m, which would be expected if Gd^{3+} acted by altering membrane mechanics [156]. However, as pointed out by the authors, measurement of this parameter may be complicated because Gd^{3+} has multiple effects, including increasing the giga-seal [35].

GsMTx-4 is a 34-amino acid (4 kDa) peptide isolated from tarantula venom [48, 49, 155, 157]. It is amphipathic with a hydrophobic membrane face opposite a positively charged face, and it is a member of the inhibitory cysteine knot (ICK) toxin superfamily. GsMTx-4 is the most specific MS blocker identified to date. Because of its structure, it would be expected to be attracted to negative regions of proteins/lipids, and it will tend to partition into hydrophobic pockets either within the protein or at the protein/lipid interface. Unlike the nonspecific channel blocker Gd^{3+}, GsMTx-4 has so far not been reported to affect voltage- or receptor-gated channel. GsMTx-4 blocks MscCa at between 0.2 and 3 μM in chick heart, rat astrocyte, and human bladder and kidney cells [48, 49], and the crude tarantula venom also blocks MscCa in growing pollen protoplasts [36]. Most recently, GsMTx-4 has been shown to stimulate neurite outgrowth by blocking Ca^{2+} elevation in *Xenopus* spinal neurons [76]. However, GsMTx-4 does not block MscCa involved in auditory transduction [48], MscK formed by TREK (E. Honore, unpublished observations, cited in Ref. [48]) the bacterial MscL [99], and perhaps, most surprisingly, MscCa in *Xenopus* oocytes (R. Maroto and O.P. Hamill, unpublished observations). The last result is puzzling given that the oocyte MscCa is often treated as the prototypical MscCa, and TRPC-1 has been implicated as forming MscCa in the *Xenopus* oocyte and mammalian cells [97]. One possible explanation is that there are structural differences between MscCa proteins in different cell types. However, based on the observation that GsMTx-4 synthesized from D instead of L amino acids shows the same potency in blocking specific MS channels, it has been proposed that GsMTx-4 is more likely to act by binding to boundary lipids surrounding the channel, and, at least, consistent with this is that GsMTx-4 and its enantomer also alters the gating of gramicidin A, which is particularly sensitive to bilayer mechanics [157]. It may therefore be that differences in lipids between poikilotherms vs homeotherms is a factor that underlies the different GsMTx-4 sensitivities. In this case, it will be particularly interesting to determine GsMTx-4 action on MscCa/TRPCs reconstituted into defined lipid environments.

Maitotoxin (MTX) is a highly potent marine poison (LD 50 for mice 50 ng/kg) from the dinoflagellate (*Gambierdiscus toxicus)* that is responsible for Giguartera, a form of seafood poisoning [40]. It is water-soluble polyether with 2 sulfate esters, 28 hydroxyls, and 32 ether rings, and with a molecular weight of 3.4 kDa, it is the largest among the known nonbiopolymers. The hydroxyl and ionized sulfate groups makes MTX a highly polar substance, but the presence of large hydrophobic portions make it amphipathic so that it most likely inserts itself deep into the bilayer. MTX elicits Ca^{2+} influx in a variety of cell types, and the Ca^{2+} influx may lead to secondary effects, including phosphinositide breakdown and arachidonic release. Of special interest here in that cells expressing TRPC-1 show a substantial increase in MTX-initiated Ca^{2+} influx that is blocked by Gd^{3+} (K_{D50}=3 μM) and also by amiloride and benzamil but not by flufenamic acid or niflumic acid [10, 14, 174]. MTX activates 40 pS channels when applied to outside-out patches but not inside-out patches indicate that MTX acts on the extracellular face and does not require second messengers [40]. Both the conductance and pharmacological properties have led to the idea that MTX activates the MscCa channels in oocytes, which is consistent with its effect of activating similar channel currents in TRPC-1 expressing cells. On the other hand, MTX also increases Ca^{2+} influx in red blood cell (RBC) ghosts which may involve another mechanism [85]. Although it has been suggested that MTX mainly acts to increase current by stimulating insertion of channels in the oocyte membrane, the evidence is based on large rapid C_m changes that follow moment-to-moment changes in conductance induced by MTX and which are blocked by the same ions and agents that also block the conductance changes [174]. These properties indicate the C_m changes may have been contaminated by changes in membrane conductance [25]. In this case, alternative methods for measuring membrane trafficking (e.g., FMI-43 fluorescence) should be used to test whether MTX-induced

membrane conductance occurs by channel insertion and/or channel activation [40].

Mechanosensitive channel protein identification

The membrane proteins forming specific MS channels have only been recently identified, and there were several reasons for this delay, including the general low abundance of MS channels in animal cells, the absence of high-affinity MS channel agents, the inability to employ conventional expression cloning strategies because of widespread endogenous MS channel expression, and the absence of identified mutant phenotypes involving stretch-activated channels. To overcome these handicaps, a novel strategy was developed by Sukharev et al. [159, 160] that involved detergent-solubilizing and fractionating membrane proteins, reconstituting the protein fractions in liposomes, then assaying the fractions for stretch sensitivity using patch clamp recording. This technique has been used to identify a variety of MS channel proteins in bacteria and archaea [81, 103, 158, 159] and, most recently, the TRPC protein family in forming MscCa in *Xenopus* oocytes [97]. Furthermore, by demonstrating that a purified protein reconstituted in pure liposomes can retain stretch sensitivity, the technique also provided unequivocal evidence for the idea that bilayer tension alone gated MS channels [101]. Although ENaC has also been reconstituted in lipid bilayer and reported to show stretch sensitivity, it is not clear whether the proposed mechanism of stretch-induced release of Ca^{2+} channel block operates in situ [75]. At this time, the best evidence for ENaC family members forming MS channels comes from genetic studies of *C. elegans* touch-insensitive mutants [see 8, 39, 47, 62 for reviews].

The proteins forming the other major class of MS channels in animals cells, MscK [79, 118, 150, 168], were identified serendipitously, in that after the first members of the 2 pore domain K^+ (K2P) channel family had already been cloned and shown to form K^+ channels [41, 42, 93], they were subsequently found to be stretch-activated [6, 126, 129]. The recent demonstration that TREK and TRAAK retain stretch sensitivity in CSK-free membrane blebs indicates that they are also bilayer-gated channels [69]. In addition to the MS channel proteins that may function as mechanosensors in situ, there is also an increasing number of voltage-gated and receptor-gated channels as well as peptides that form simple model channels (alamethicin and gramicidin) that display mechanosensitivity [19, 102, 115, 122, 163]. Although these channels may operate on the same general principles that confer mechanosensitivity on membrane proteins, their role, if any, as mechanotransducers remains to be demonstrated.

Mechanosensitive channel dynamics: adaptation/desensitization/inactivation

Gating dynamics (adaptation/inactivation/desensitization) has been shown to play a critical role in the signaling by voltage- and receptor-gated channels and the hair cell mechanotransduction channel [67, 72] and defects in gating dynamics underlie a number of channelopathies [4]. In the initial studies of single MS channel currents, the channels seemed to obey stationary kinetics and were analyzed accordingly [46, 50, 51, 90, 114, 143, 177, 179]. However, with the ability to apply fast pressure steps to the patch [9, 59, 105–108], it became evident that MS channels also displayed dynamics in which the channels either closed reversibly (adaptation or inactivation/desensitization) or faded irreversibly (run down) with constant stimuli [55, 69, 105, 106, 156].

In principle, the reversible closure of MS channels during maintained stimulation can arise through relaxation in either the mechanical force being applied to the channel or the sensitivity to that mechanical force [54, 57] Because mechanical gating arises from the channel protein being sensitive to some mechanical-induced deformation [i.e., either in the bilayer or in CSK/extracellular matrix (ECM) elements], then closure can arise because of a relaxation in the force causing the deformation or a relaxation in the sensitivity to that deformation. For example, in the simplest case of a two-state channel in which the rate constants for channel opening (β) and closing (α) are displacement-sensitive (i.e., for a tethered MS channel) or tension-sensitive (i.e., for bilayer-gated MS channel) the probability of the channel being open (P_o) will be given by:

$$P_o = 1/(1+K)$$

where

$$K = \beta/\alpha$$

Or, in terms of displacement,

$$K = K_0 e^{s(x_0 - x)}$$

where K_0 is the equilibrium constant when the displacement x is equal to the set point x_0 and determines the number of channels open at zero relative displacement, and s is the sensitivity to the relative displacement change (x_0–x). For a bilayer-gated channel, we can substitute displacement with area change. An exponential time relaxation in either s or x_0 can produce the same adapting MS channel currents [57]. Figure 7 illustrates simulations of the stimulus-response relations made, assuming that after a step stimulus, there is an exponential change in either the set point x_0 (Fig. 7a) or the sensitivity factor s (Fig. 7b).

Although both mechanisms predict the same kinetics of channel closure, the consequences on the P_o–X curves are

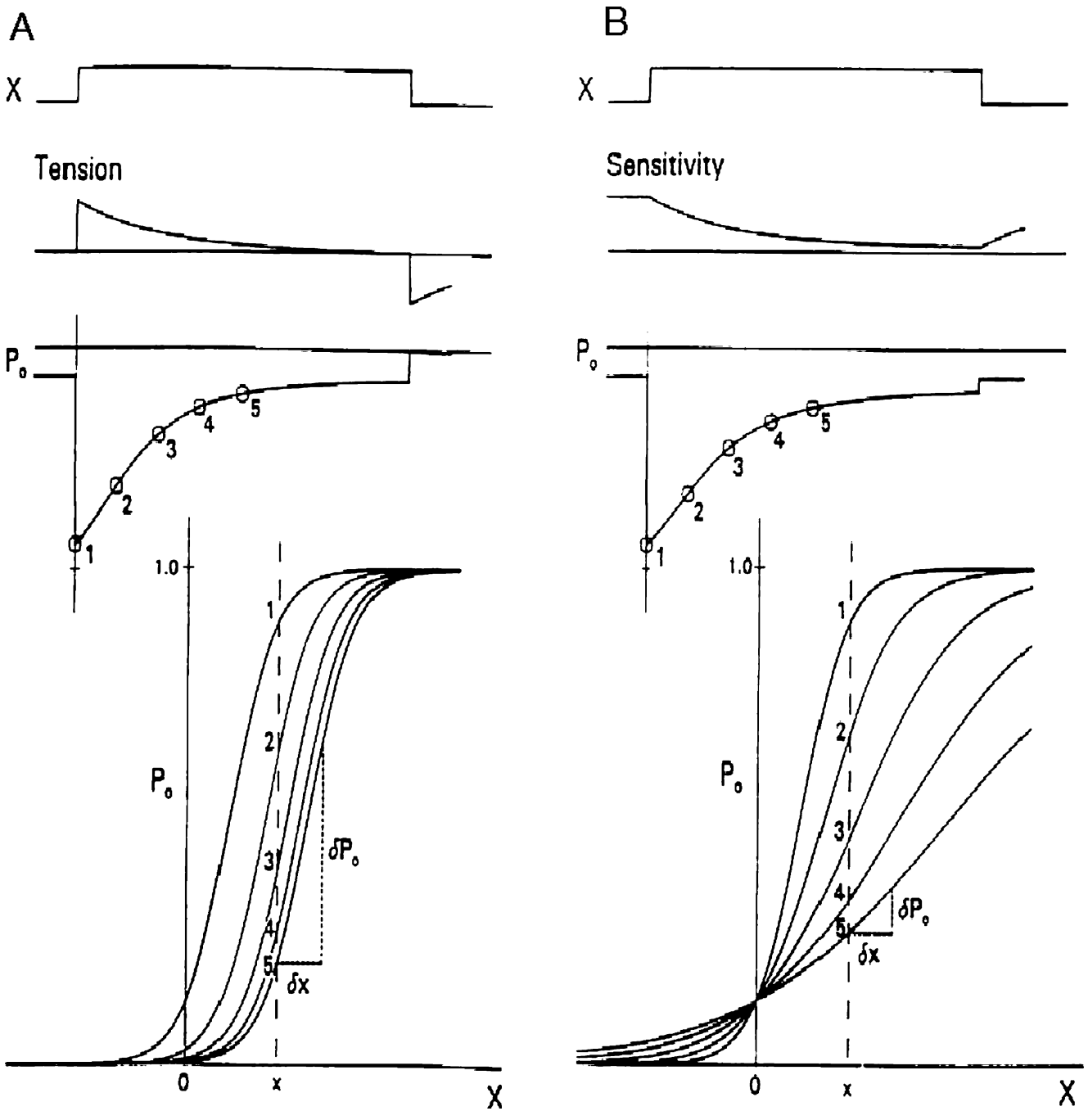

Fig. 7 Simulation of two mechanisms that results in closure of MS channels in the presence of sustained stimulation. A two-state channel is assumed, and a step displacement from 0 to x (*top*, *trace 1*) is used to activate the MS channels (**a** and **b**). *Trace 2* represents the changes in tension (**a**) or sensitivity tension (**b**). In *trace 3*, the channel currents are represented by the change in open-channel probability (P_o), with the *numbered points* (*1–5*) representing equally spaced times where P_o–X curves were generated to follow changes in the MS channel sensitivity. **a** The decay of the current is due to a change in the tension (measured as x–x_0) caused by a shift in the set point. In this case, there is a shift along the x-axis with no change in slope. Consequently, the δP_o response due to δx does not decrease during what can be considered true adaptation. **b** The decay of the current is due to a change in sensitivity in which the slopes of the P_o–X curves decrease as they pivot around a common point. As a consequence, the incremental change in the response (δP_o) for a fixed δx decreases during the current decay, which is akin to receptor desensitization. Modified from Ref. [57]

clearly different. In the first case of adaptation, the curves shift along the x-axis with no changes in slope (i.e., sensitivity) around a common set point. In the second case, the sensitivity decreases as the curves pivot around a common point. From a functional point of view, the first case is true adaptation because sensitivity is maintained

[55], whereas the second case is more akin to receptor desensitization or voltage-gated channel inactivation, where the stimulus must be removed for sensitivity to recover [69]. Below, we consider specific MS channels in terms of these general principles.

MscCa in Xenopus oocytes This channel displays different gating dynamics depending upon patch "history." In the case when the giga-seal is formed using a "gentle" suction protocol (e.g., 10 mmHg for 1–10 s), the application of a suction/pressure step produces rapid opening (<1 ms) of channels followed by a slower closure (~100 ms), although the stimulus is maintained constant. The resultant decay of MscCa current can be fitted by a single exponential with a time constant of around 100 ms at −100 mV that shows a monotonic *e*-fold increase for every approximately 100-mV depolarization. The voltage dependence of the channel closure is most evident when the voltage is switched from hyperpolarized to depolarized potential (or vice versa) during the pressure step (Fig. 8) [55, 107, 108]. The direction of this voltage dependence is similar to the voltage dependence of adaptation displayed by the hair cell mechanotransducer channel [5] and MscS (see below). The stimulus induced closure of the oocyte channel was originally referred to as adaptation because increasing the stimulus could reopen channels. In the oocyte, suctions/pressures of approximately 20 mmHg produce saturating responses (see Fig. 4), so it was assumed that any channel opening caused by an increase in suction/pressure of at least 20 mmHg would involve reopening channels that had just closed. However, a practical limitation in using these protocols on oocyte patches is that application of even larger pulses (e.g., ≥40 mmHg) that would undoubtedly activate all channels will also cause irreversible loss of the channel activity and gating dynamics as described below [55, 105, 106].

In the second case, if a more forceful suction protocol is required to achieve the seal, then more often than not, the transient current response is absent, and instead, the channels remain open for the full duration of the suction. Similarly, if after a gentle seal is formed the patch is mechanically "over-stimulated," then adaptation of MscCa activity disappears either progressively with each moderate-sized pulse (Fig. 9a) or suddenly within a single large suction (Fig. 9b). This transition from the transient mode (TM) to the sustained mode (SM) of gating is irreversible and occurs without a change in single-channel conductance [64].

The fragility of MscCa dynamics and the transition from TM to SM gating has been proposed to arise through mechanical decoupling of CSK interactions with either the channel or the membrane, which are thought to be important for TM of gating. It has been suggested that viscous elements (dashpots) in the CSK can become frozen or decoupled without disconnecting the gating springs. However, adaptation is preserved in both inside-out and outside-out patches, and in patches treated with agents that disrupt microtubules (colchicine) or microfilaments (cytochalasin D), similar to what has been reported for TRAAK desensitization [69]. In contrast, transient gating kinetics of MscCa are not retained in either "blebbed" membrane that it lacks an underlying CSK [185] or in pure liposome patches expressing MscCa activity following reconstitution of detergent-solubilized oocyte membrane proteins [97]. Furthermore, overexpression of TRPC-1 that forms the oocyte MscCa does not result in channel activity that displays TM gating. Whether the absence of adaptation reflects prior mechanodisruption or the absence of CSK remains unclear, as does the mechanism that causes irreversible run down. One possibility is that there are

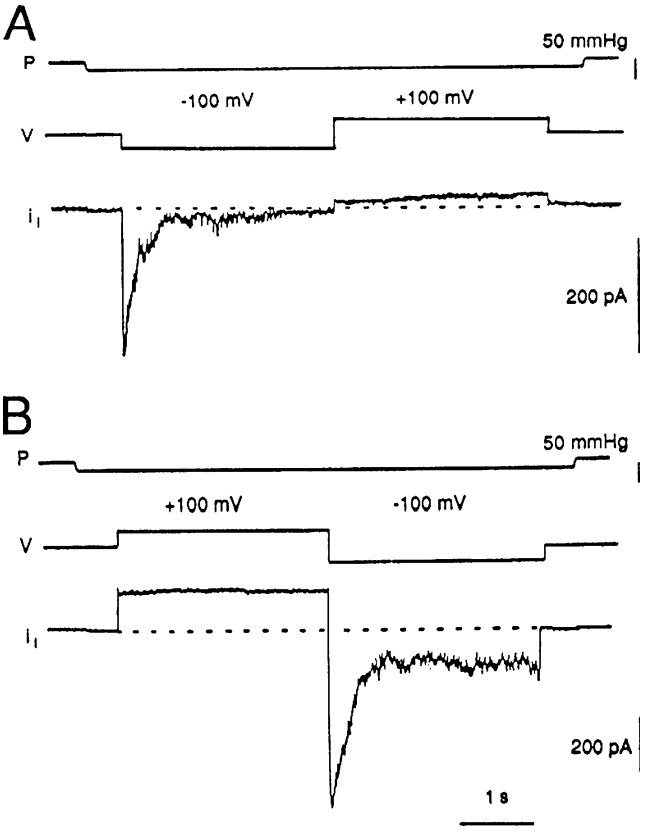

Fig. 8 Voltage dependence of pressure-induced currents recorded from cell-attached oocyte patches. In each *panel*, the *top trace* represents the pressure (suction); the *middle trace*, the voltage; and the *bottom trace*, the current. **a** The application of the suction pulse to the patch held at −100 mV caused rapid opening of channels that had nearly all closed before the voltage was switched to 100 mV while maintaining the suction pulse. **b** In this case, the suction was applied to the patch held 100 mV and produced a steady-state current. Switching the voltage to −100 mV activated a transient increase in current that decayed incompletely in the presence of maintained suction

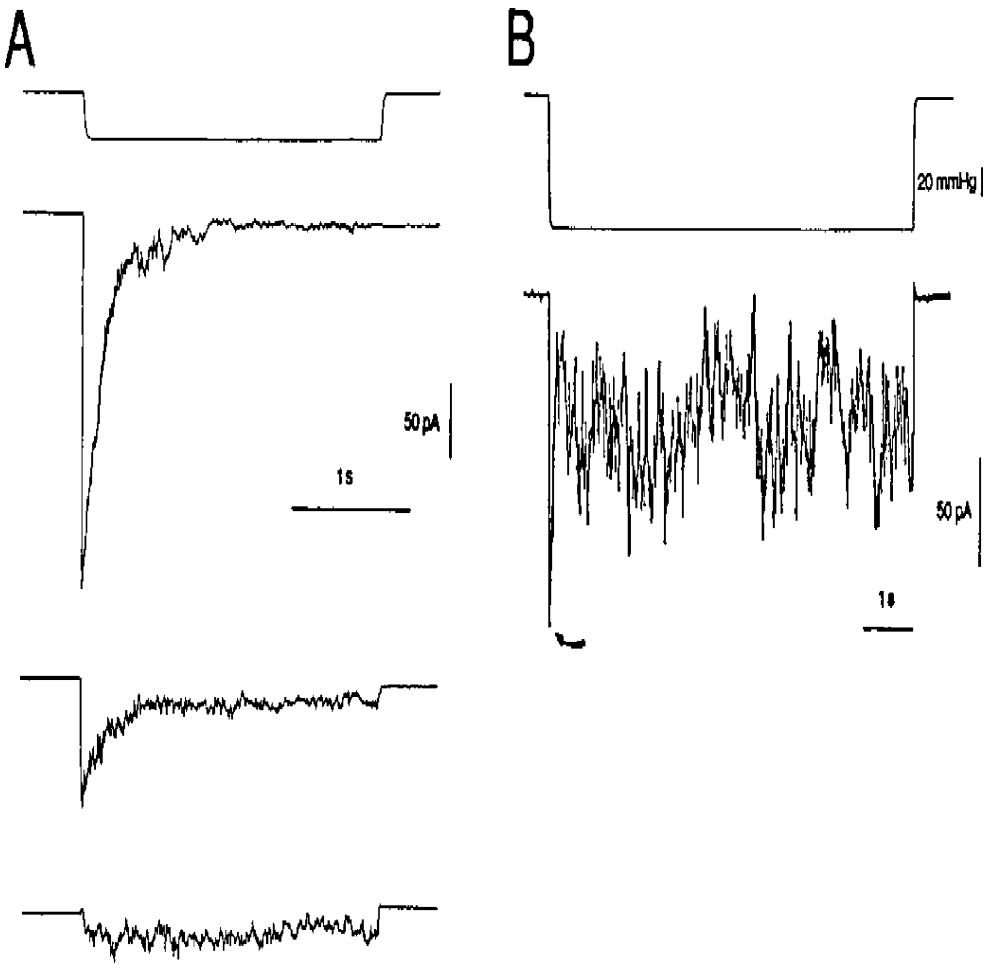

Fig. 9 Irreversible loss of transient mode gating of MscCa. **a** Three consecutive suction pulses of 30 mmHg were applied to a patch 30 s apart, causing a progressive loss of the transient gating and a decrease in the peak current. **b** A single large suction pulse (100 mmHg, 10 s) was applied to a patch and caused an initial peak current that was converted into a sustained current

irreversible changes in the membrane–glass adhesion that alters the ability to generate tension changes in the bilayer. For example, if the membrane does not reseal after mechanical decoupling of lipid–glass interface [121], then increasing pressure may draw further membrane into the pipette without increasing bilayer tension.

MscCa in rat astocytes This channel shows certain dynamic properties similar to those of the oocyte MscCa, including its voltage dependence and mechanical fragility. However, in the astrocyte, the decrease in current occurs because of increased occupancy of lower conductance states and a reduced open-channel probability [13, 156]. Furthermore, the closed channels cannot be reactivated by increasing the stimulus strength (i.e., are refractory), indicating inactivation rather than adaptation. The process was modeled as a ball-and-chain-type inactivation, in which the inactivating ball was a CSK element rather than part of the channel protein. By assuming that the binding rates of the inactivating ball were affected by the position of an intramembrane voltage-sensing subunit, one can account for the voltage dependence of inactivation. Apparently consistent with the model, it was demonstrated that a combination of agents targeting actin (cytochalasin), microtubules (colchicine), and intermediate filaments (acrylamide) was required to abolish the inactivation, but this loss might again reflect general mechanical patch damage rather than implicating specific CSK elements. In the same study, fast C_m measurements were used to monitor the change in membrane area/thickness during pressure steps and demonstrated a similar voltage-independent monotonic increase in patch capacitance at −90 and + 50 mV, which contrasted with the voltage-dependent inactivation. This observation was interpreted as indicating inactivation was due to intrinsic properties of the channel rather than relaxation of bilayer tension [156].

MscS and MscL in Escherichia coli The two predominant MS channels in *Escherichia coli*, MscS (0.5 pS) and MscL (1–3 nS) [100, 160], also exhibit transient gating dynamics [2, 66, 86, 87]. MscS currents measured in *E. coli* protoplasts in response to pressure steps undergo a pressure-induced exponential decay that appeared to be voltage-independent with a time constant of 2–3 s when measured over a narrow voltage range of ±30 mV [86]. However, when measured over a wider voltage range of

±100 mV there was a decrease in the rate of inactivation with depolarization similar to MscCa [2]. Application of the second of a double-step protocol activates fewer channels, and a finite time without stimulation is required for recovery of the full response [86]. Furthermore, suction ramps produced smaller peak responses than suction steps [2]. These features are more consistent with inactivation than adaptation. Unlike MscCa in vertebrate cells, MscS dynamics are not mechanically fragile, although pronase treatment of the intracellular membrane face abolishes the transient kinetics and, ultimately, mechanosensitivity. This last observation led Koprowski and Kubalski to propose that both activation and inactivation may depend upon interaction between a cytoplasmic (pronase-sensitive) region of the channel with the lipid bilayer [86, 87]. Note the proteolytic inhibition of MscS activity is opposite to the potentiation of MscL activity [1]. Given that a bilayer rather than a tethered mechanism gates MscS, it was proposed that inactivation might be associated with insertion of the cytoplasmic domain of MscS in the bilayer (i.e., a "hybrid" or intrinsic tethered model; see below).

MscL reconstituted in liposomes also shows a transient decay in the current with a time constant of seconds [66]. Although the distinction between adaptation and inactivation still needs to be made, the observation is significant because the clear absence of any CSK excludes its involvement in these transient kinetics. One possible explanation is time-dependent sliding/relaxation of the two monolayers that results in relaxation of the gating tension [144].

TREK and TRAAK MS channels In a recent study, pressure steps have been used to analyze the dynamics of MscK formed by cloned TREK-1 and TRAAK herologously expressed in COS cells and *Xenopus* oocytes [69]. Both channels show rapid closure (τ~20–50 ms), with constant stimulation similar to the MscCa. However, unlike MscCa, MscK gating dynamics are not voltage-sensitive, and either mechanical or chemical disruption (i.., using latrunculin) of the CSK causes "run up" rather that "run down" of the channels without removing the transient gating kinetics. Because it was clearly demonstrated that channels could not be reactivated without a finite time for recovery, the phenomenon was referred to as desensitization. The lack

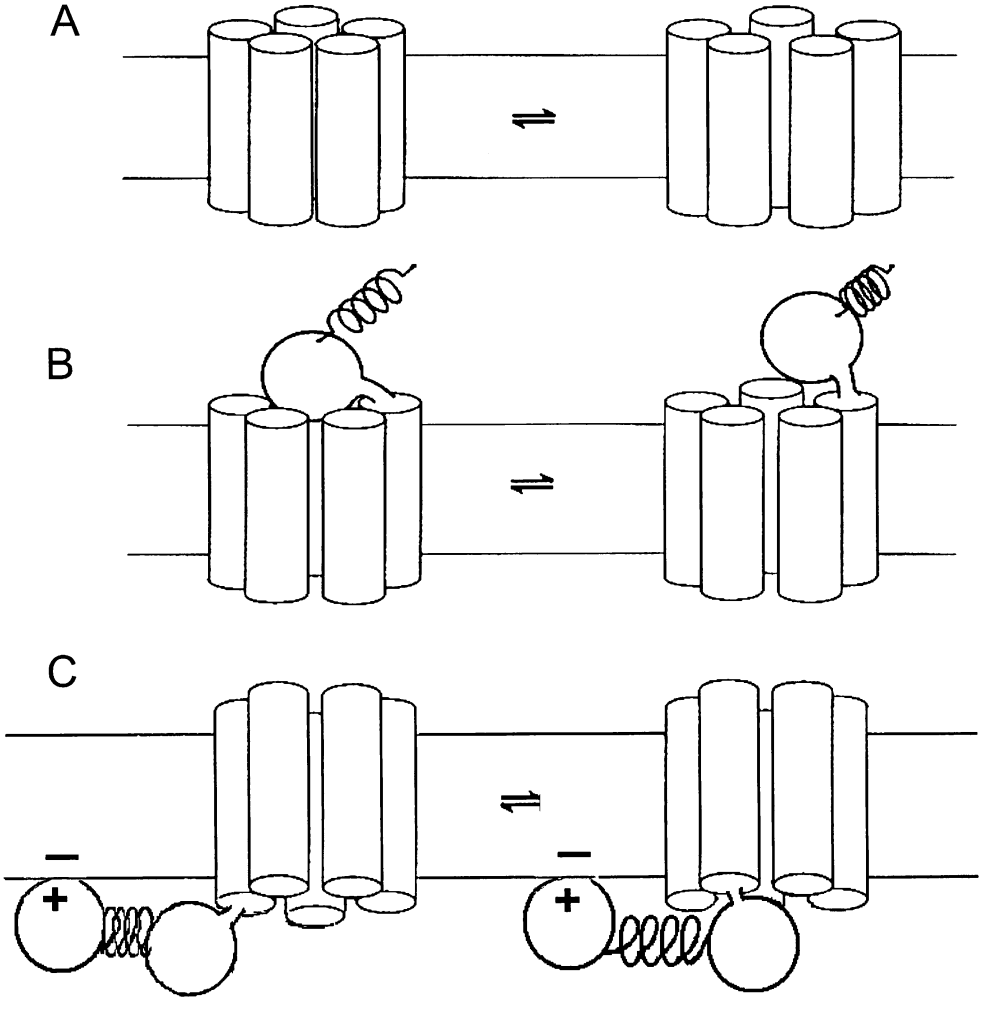

Fig. 10 Three different models of mechanosensitive channel gating **a** Bilayer. Mechanical forces are conveyed to the channel purely via the bilayer. Tension sensitivity occurs because of a difference protein area (or hydrophobic thickness and/or lateral shape) between the open and closed channel conformations. **b** Extrinsic tether. Tensions are exerted directly on the channel protein via extracellular or cytoskeletal elastic elements/gating springs. When tension is exerted on the gating spring, the open state is energetically more favorable. Intrinsic tether (hybrid). In this model, the gating spring is one of the cytoplasmic domains that binds to the phospholipids and, in this way, becomes sensitive to membrane stretch

of effects of either mechanical or chemical CSK disruption indicates that desensitization is an intrinsic property of the channel [69].

Molecular models of stretch sensitivity

There are three broad classes of mechanisms that may impart stretch sensitivity on a membrane ion channel. They will be referred to as "bilayer," "tethered," and "hybrid," and are shown schematically in Fig. 10. The models need not be mutually exclusive, and a single channel may derive its mechanosensitivity from all three mechanisms. Each mechanism can be discussed in terms of a simple two-state channel that fluctuates between a closed and open state. The bilayer mechanism applies to a variety of MS channels, as evidenced by retention of mechanosensitivity following liposome reconstitution and/or activation by amphipaths or lysophospholipids. The basic idea is that stretching the bilayer will tend to decrease its lipid packing density and thickness, so that if the channel protein undergoes a change in membrane-occupied area (Fig. 9a) and/or hydrophobic mismatch, there will be a shift in the distribution between closed and open channel conformations [54, 88, 95]. By inserting in the membrane, lysophospholipids and amphipathic molecules may cause local changes in tension and curvature at the lipid–protein interface and thereby shift the channel distribution [12, 95, 98, 99, 131–133].

In the tethered mechanism, either an extracellular or cytoskeletal protein is directly connected to the channel and acts as a gating spring [50, 58, 62, 71, 72]. When the gating spring is stretched, it favors the open state of the channel because it allows relaxation of the spring. In Fig. 9b, the gate is represented as a trapdoor that opens out, but it can well represent subunits that are either pulled apart (increased in area) or lengthened (change if hydrophobic mismatch). Evidence pro and con for the tethered mechanism has been discussed previously [54, 88].

The hybrid of the above two mechanisms depends upon stretching of the bilayer, but in this case, there are cytoplasmic domains of the channel protein that bind to phospholipids, and in this way act as intrinsic tethers or gating springs that are stretched along with the bilayer (Fig. 9c). Evidence for the hybrid model comes from the identification in the specific K2P channels of a phospholipid-sensing domain on the proximal carboxyl terminus that involves a cluster of positively charged residues that also includes the proton sensor E306 [23, 68]. Protonation of E306 drastically tightens channel–phospholipid interaction and leads to TEK-1 opening at atmospheric pressure. The carboxy terminal domain of TREK-1 interacts with plasma membrane, probably via electrostatic interaction between a cluster of positive charges (a PIP2-interacting domain) and anionic phospholipids.

Mechanosensitive channels in human diseases

An exiting development in the field has been the growing number of diseases associated with abnormalities of mechanotransduction. Donald Ingber [73], in a recent review, listed 45 diseases that may arise due to changes in cell mechanics, alterations in tissue structure, or deregulation of mechanosignaling pathways. Of these diseases, several have been directly associated with changes in expression and/or gating of MS channels, including cardiac arrhythmias [84], polycystic kidney disease [18], hyperten-

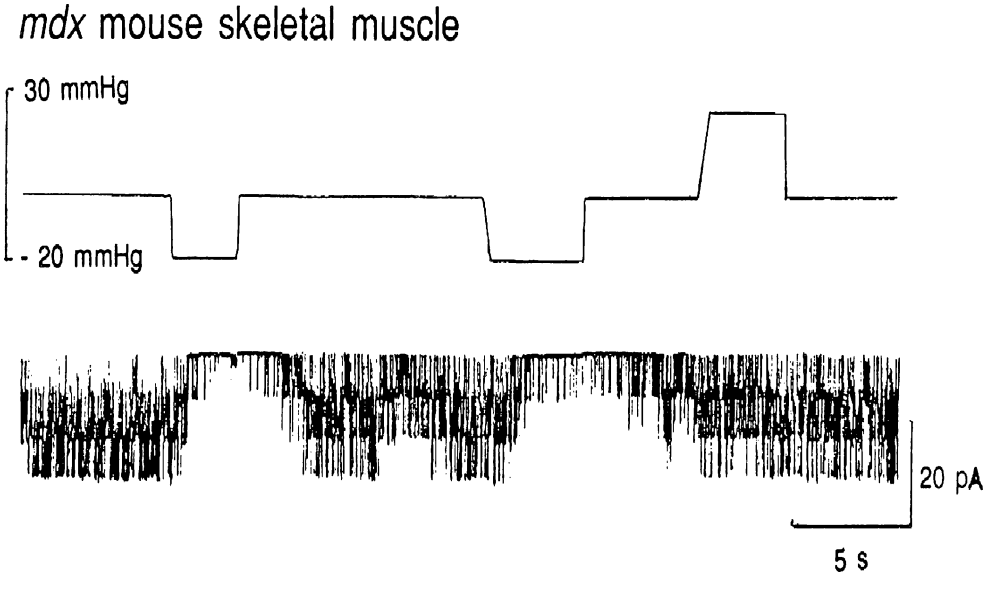

Fig. 11 Cell-attached patch recording on an *mdx* mouse myotube showing high constitutive channel activity that was reduced with suction but unaffected by positive pressure. This "apparent" stretch-inactivated channel activity was rare and seen in only 1 of approximately 100 patches. The majority of other patches showed no spontaneous channel activity, and suction activated either a transient opening of channels or channels that remained open for 10 s after the pulse (e.g., see [107])

sion [83], glioma [123], glaucoma [78] atherosclerosis [22, 134], Duchenne muscular dystrophy [44, 45], and tumorigenesis [128]. Furthermore, increased MscK activity has been shown to prevent brain ischemia [16] and promote general anesthesia [127], whereas MscCa/TRPC activity may regulate wound healing [137] and promote neuronal regeneration [76]. Of particular note is Duchenne muscular dystrophy (DMD), a devastating X-linked genetic disease that affects approximately 1 in 3,500 male births and is characterized by progressive muscle wasting and weakness (reviewed in [180]). DMD is caused by the absence of the gene product of dystrophin, a cytoskeletal protein that binds to actin and provides structural support for the membrane particularly during muscle stretching. In *mdx* muscle fibers (i.e., from the mouse model of DMD), there is increased vulnerability to stretch-induced membrane wounding, and several studies indicate elevated $[Ca^{2+}]_i$ levels in *mdx* myotubes that have been associated with increased Ca^{2+} permeant leak channel activity [43] and/or abnormal MscCa activity [44, 45]. Anti-MscCa agents, including Gd^{3+}, streptomycin, amiloride, and GsMTx-4, have been reported to block Ca^{2+} elevation and/or reduce muscle fiber degeneration [3, 56]. Based on the observation that the leak channel activity was increased by internal calcium store depletion, Vandebrouck et al. [167] proposed that a store-operated Ca^{2+} channel (SOCC) belonging to the TRPC family may be involved. To test this idea, they transfected muscles with antisense oligonucleotide designed against the most conserved region sequences of the TRPCs and showed it caused significant knockdown of TRPC-1 and - 4 but not TRPC-6 (all three were detected in wild-type and *mdx* muscle), and reduced both control and thapsigagin-induced Ca^{2+} leak channels without affecting voltage-gated Na^+ channels. The mechanosensitivity of the channels was not tested in this study. However, MscCa can show significant spontaneous opening in the absence of membrane stretch [140]. Furthermore, although Franco and Lansman [44] initially reported a stretch-inactivated Ca^{2+} channel in *mdx* mice, they subsequently concluded that the channel activity may arise from a novel gating mode of MscCa induced by membrane stress [45]. Most recently, it has been suggested that stretch inactivation in patches of *mdx* muscle and other cells may be a patch recording artifact induced when suction applied to the patch reduces a tonic tension generated by CSK forces that bend the patch toward the cell [69]. At least consistent with this notion is that suction (but not positive pressure) causes inactivation of MscCa in *mdx* patches (Fig. 11).

Conclusion and future prospects

The giga-seal patch clamp technique has been a major contributor to increased understanding of MS channels over the last 20 odd years. However, there is still somewhat a disconnect between the phenomena seen in the patch and how they translate in MS currents in the whole cell. Furthermore, given the growing evidence that MS channels are promiscuous in terms of their modes of activation, it becomes even more important to identify the exact physiological stimulus that activates the channel in specific situations. The development of new techniques that can monitor/generate membrane tension changes in normally operating cells while recording MS channel on the cell can address many of the unresolved issues. Similarly, the discovery of high affinity and selective agents that can target mechanically gated channels will represent a major breakthrough for the field. The determination of the crystal structure of bacterial MS channels [7, 21, 139] has provided a rich environment for model building and testing, and a similar trajectory is predicted for the recently identified MS channel proteins in animal cells. A key question that these studies should answer is whether a unified set of principles can account for the stretch sensitivity of channels in both prokaryotes and eukaryotes [54, 88].

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