

# Chapter 6

## Sensing Mechanism of Stretch Activated Ion Channels

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### 6.1 Introduction

Physical stresses/mechanical force such as expansile force (membrane tension), osmotic pressure, hydrostatic pressure, shear stress, and gravity are crucial stimuli for cells in various tissues to control their proliferation, differentiation, development and cell death. For instance, the transpulmonary pressure balance produced by appropriate lung fluid is crucial for normal lung growth and development in the fetal late stages (Hooper et al. 2006). The airways and alveoli of the fetal lung are filled with fluid. Tracheal ligation, which impedes the outflow of fluid from the trachea, produces large fluid-filled lungs (Alcorn et al. 1997; Carmel et al. 1965), and increases hydrostatic pressure to expand the lung. In contrast, tracheal drainage reduces the hydrostatic pressure by excreting the fluid from the inside of lung to the outside. Previous studies with fetal lamb have indicated that lung growth and development is stimulated by tracheal ligation-increased expansion of lung (Alcorn et al. 1997; Fewell et al. 1983), and is inhibited by tracheal drainage (Alcorn et al. 1997). So far, various *in vivo* and *in vitro* model systems have been developed to investigate the mechanotransduction process. In the lung epithelial cells and fibroblasts, mechanical force is mainly recognized by membrane proteins such as stretch-activated cation (SAC) channels and receptor tyrosine kinases that transduce the mechanical force into intracellular signal molecules associated with their proliferation and differentiation (Liu et al. 1995, 1999). For the cardiovascular systems, endothelial cells, smooth muscle cells and cardiac myocytes are the major cells that face mechanical force including vessel stretch and cardiac stretch. Blood pressure is the major determinant of vessel stretch, and blood volume and pressure are the major determinants of cardiac stretch. Mechanical forces in the cardiovascular systems have been categorized into at least three elements; magnitude, frequency and duration in cyclic stretch. Although cells in cardiovascular systems are exposed to a dynamic mechanical environment

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modified by pulsatile pressure and oscillatory shear forces, they should have mechanisms sensing these mechanical stimuli. Endothelial cells are mainly exposed to shear stress produced by flowing blood that is known to cause stress fibers aligned perpendicular to the stretch direction for bearing less tension and remodeling of endothelial cells (Chien 2007; Taber 1998). Mechanical stretch in vascular smooth muscle cells *in vitro* can cause a uniform alignment almost perpendicular to stretch vector (Lehoux et al. 2000; Standly et al. 2002). These phenomena show that cells in vascular system sense both magnitude and direction of mechanical force, and that cytoskeleton and extracellular matrix molecules complementarily support the basic sensing mechanism through membrane tension. Further, shear stress, osmotic pressure and hydrostatic pressure are also important stimuli for  $\text{Na}^+$  reabsorption to control extracellular fluid volume and blood pressure in renal tubule. When epithelial cells in cortical collecting duct (CCD) are exposed to the decreased plasma osmolality, epithelial  $\text{Na}^+$  channel (ENaC)-mediated  $\text{Na}^+$  reabsorption in CCD is stimulated to recover normal plasma osmolality, suggesting that epithelial cells in CCD have ability to sense changes in extracellular osmolality and precisely respond to it by increasing both translocation and gene expression of ENaC (Niisato et al. 2000; Taruno et al. 2007). On the other hand, weightlessness and being bedridden accelerate bone resorption, indicating that physical stress/mechanical force by gravity and exercise is necessary for osteogenesis (Carmeliet et al. 2001; LeBlanc et al. 2007).

As described above, it is well known that physical stress/mechanical force is a crucial signal for diverse cellular functions likely chemical stimuli. In previous studies, the existence of mechanosensitive ion (MS) channels have been recognized in various cells and tissues, and MS channels are considered to be involved in sensing physical stress/mechanical force (Arnadóttir et al. 2010; Yoshimura et al. 2010). Currently, MS channels are believed to play central roles in mechanotransduction and to convert physical stress/mechanical force into intracellular signals that contribute to physiological functions in their expressed cells and tissues.

## 6.2 MS Channels in Bacteria for Sensing Osmotic Pressure

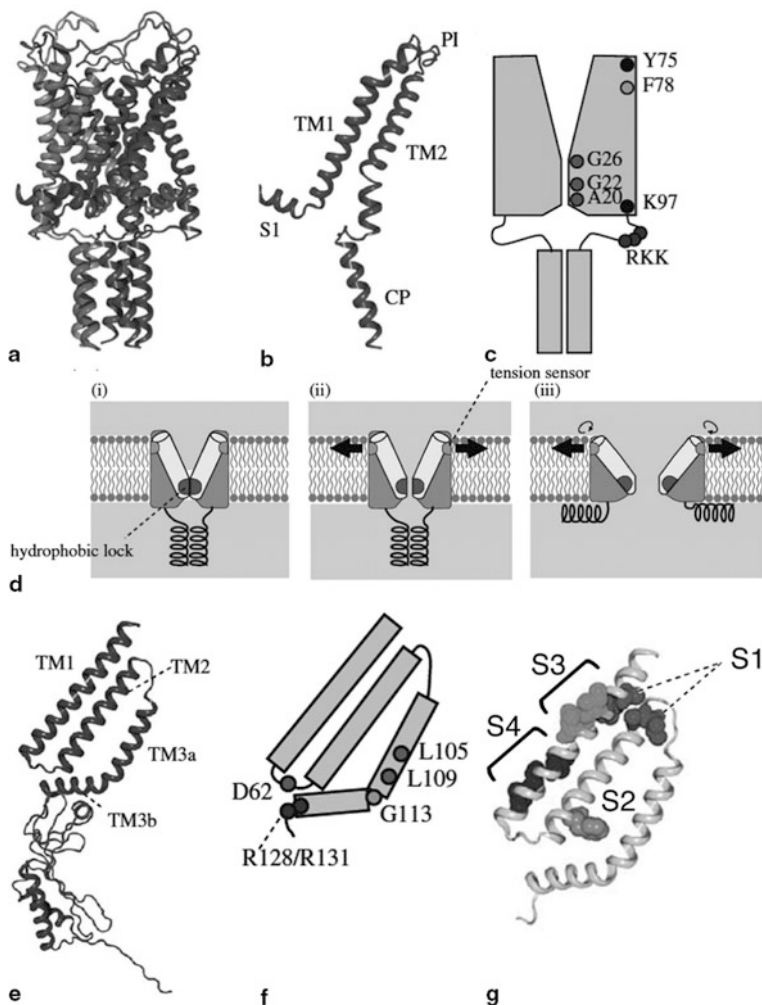
### 6.2.1 *Physiological Importance of MS Channels*

Prokaryotic cells possess MS channels that sense mechanical force to survive and adjust to a new environment. When bacteria cells are exposed to hypotonic environments (for example, exposure to rain), the cells protect themselves against the hypotonicity-caused lysis by activating MscS (mechanosensitive channel with small conductance) and MscL (mechanosensitive channel with large conductance) (Kung et al. 2010; Levina et al. 1999; Batiza et al. 2002) that release cytosolic components for reduction of intracellular osmotic pressure adapting to extracellular hypotonic environments. Especially, in bacteria cell as monad, the action of MS channel to avoid hypotonic lysis is closely related to a matter of life and death. In bacteria cells,

MS channels are considered to be activated by an increase of membrane tension due to cell swelling (Levina et al. 1999) in response to hypotonic stress and serve as ‘emergency valves’. Notably, for bacteria cells, sensing magnitudes of changes in extracellular osmolality is essential to determine amounts of cytosolic components to be released for adaptation to reduction of extracellular hypotonic pressure. Therefore, these two MS channels with different conductance, MscS and MscL, are activated at different tension thresholds and control amounts of cytosolic components to be released through MscS and MscL. Hypotonic stress is converted to cell swelling (i.e., the changes in cell volume) through osmotic pressure-dependent water influx, which in turn produces membrane tension as mechanical force. Namely, magnitudes of hypotonic stress are transduced to magnitudes of membrane tension. This means that bacteria cells can sense changes in extracellular osmolality as magnitudes of membrane tension. The MscS and MscL channels open, when the membrane tension is larger than their thresholds. If hypotonic stress is weak or moderate, only MscS with small conductance is activated, because the amount of cytosolic component to be released in response to the hypotonic stress is relatively small. On the contrary, if hypotonic stress is strong, both MscS and MscL should be activated for releasing large amounts of cytosolic component. These regulatory mechanisms of two MS channels physiologically seem to be reasonable and similar to regulatory volume decrease (RVD) as regulatory mechanisms of cell volume observed in higher organisms.

### 6.2.2 MscL (Mechanosensitive Channel with large Conductance)

MscL in *Escherichia coli* (EcMscL) and *Mycobacterium tuberculosis* (TbMscL) is one of the most studied MS channels in prokaryotic cells. MscL is a homopentamer (Fig. 6.1a) and each subunit has two transmembrane domains, TM1 and TM2 (Sukharev et al. 1994, 1999) (Fig. 6.1b). The sequence at amino-terminus and carboxy-terminus forms S1 and cytoplasmic helices (CP), respectively (Fig. 6.1b). The channel pore is lined with TM1 and the gate is probably formed between two residues, A20 and G26 (Fig. 6.1c). The region facing to the membrane (lipid bilayer) comprises the whole length of TM2 and the periplasmic end of TM1. EcMscL has a conductance of 3 nS and passes low molecular substances such as inorganic ions (Ajouz et al. 1998). It is supposed that exposure of hydrophobic surface of the gate in TM1 to aqueous environments is an energy barrier for channel gating (Fig. 6.1d). Hydrophobic lock of the gate via this energy barrier stabilizes the closed structure of EcMscL. In MS channels, membrane tension is generally an energy source for activation of channel getting over the barrier (Sokabe et al. 1991; Hamill et al. 2001). Mutants in the pore-lining residues substituted with a more hydrophilic amino acid lower the gating threshold. Substitution of Gly-22 in the gate with all other 19 amino acids reveals that hydrophilicity determines the ease of gating (Fig. 6.1c). The diameter of the open pore is estimated to be as large as 3–4 nm. This large pore is formed by an iris-like rotation of the transmembrane helices: the helices tilt, while



**Fig. 6.1** Structure and gating model of MscL and MscS channel. **a** Crystal structure of *M. tuberculosis* MscL channel. **b** Structure of single subunit of Tb MscL. S1; N-terminal helix, TM1; the first transmembrane helix, TM2; the second transmembrane helix, PL; periplasmic loop, CP; cytoplasmic helix. **c** Positions of important residues in the schematic EcMscL. The pore is constricted between A20 and G26. Gating threshold depends upon the hydrophilicity of G22 and mechanosensitivity is lost on hydrophilic substitution of Y78. **d** Gating model of MscL. (i) The closed structure is stabilized by the hydrophobic lock of the gate. (ii) Membrane tension perceived by the tension sensor opens the gate that results in the exposure of hydrophobic lock to water. (iii) On full opening, cytoplasmic helices are disassembled. **e** Structure of single subunit MscS. TM1; the first transmembrane helix, TM2; the second transmembrane helix, TM3; the third transmembrane helix, which is separated by a kink at G113 (TM3a and TM3b). **f** Important residues for MscS function. L105 and L109 form a pore of MscS. **g** The gating threshold increases on asparagine substitution at the residues indicated by S1 (A34, I37, A85, L86), S4 (I48, A51, L55) and S2 (F68). Conversely, the threshold decreases on mutation at I39, V40 and V43 (S3). (Reproduced from Yoshimura et al. 2010 with copyright permission, Royal Society Publishing)

sliding between neighboring TM1 helices occurs (Fig. 6.1d). Further, expansion in the transmembrane domain dissociates the CP helices that probably causes association between TM2 and CP. Previous studies critically indicate that MscL opens by directly sensing changes in membrane tension, since MscL keeps mechanosensing ability in its purified and reconstituted form in liposomes (Sukharev et al. 1993; Häse et al. 1995). Notably, it is revealed that the identified 21 residues in M1 and M2 domains contribute to interaction with membrane lipid (Yoshimura et al. 2004), and that this lipid-protein interaction plays a central role in sensing membrane lipid tension directly. Especially, mapping by asparagine scanning mutagenesis in the lipid-protein interface region indicates that high-impact residues (Leu-36, Ile-40 and Ile-41 in M1 domain and Phe-78, Ile-79, Phe83 and Ile-87 in M2 domain) form clusters in the lipid-protein interface probably receiving the membrane tension (stretch) from membrane lipid through hydrophobic interaction. Further, roles of the periplasmic loop are not fully understood although a number of loss-of-function mutations are detected in this region (Li et al. 2004; Yoshimura et al. 2004). Proteolysis of the periplasmic loop in MscL markedly increases its sensitivity to membrane tension by decreasing the gating threshold, suggesting that periplasmic loop might be involved in holding MscL in the closed state (Ajouz et al. 2000; Yoshimura et al. 2004). Thus, the gating of MscL is regulated in a balance between the expansible force of MscL by membrane tension and the strength of intramolecular interaction for holding the closed conformation.

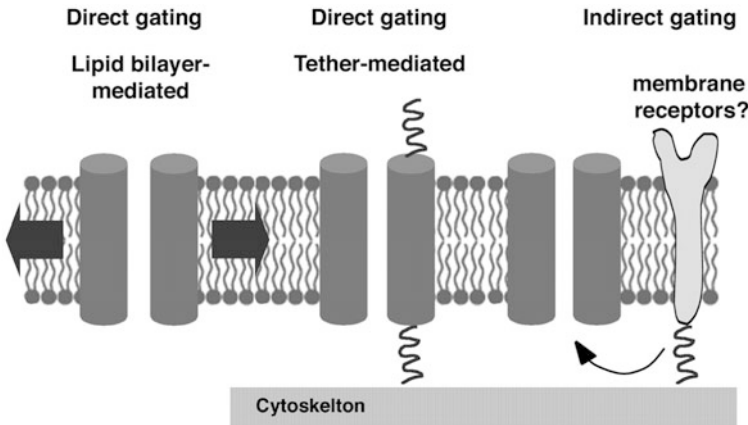
### 6.2.3 *MscS (Mechanosensitive Channel with Small Conductance)*

MscS is also a major component for adaptation of bacteria to hypotonic stress in addition to MscL (Blount et al. 1999; Levina et al. 1999). MscS is gated by both membrane tension and voltage (Martinac et al. 1987). Bass et al. have revealed that the MscS with 1 nS conductance is composed of homo-heptameric complex with three transmembrane helices in a subunit and a large cytoplasmic C-terminal domain by presenting crystallographic analysis of the 3D MscS structure (Bass et al. 2002) (Fig. 6.1e). The transmembrane domains TM1 and TM2 are thought to constitute to sensing membrane tension and voltage (Bass et al. 2002), whereas TM3, a pore lining helix, has a distinctive kink at G113, which divides TM3 into amino-terminal helix TM3a and carboxy-terminal helix TM3b (Fig. 6.1f). TM3a helices fit each other to form a tightly closed gate by inserting knobs of alanine residues into holes of glycine residues as a highly conserved region in the membrane domain (Edwards et al. 2005). The cytoplasmic domain forms a large cage. TM3b covers the upper surface of the cytoplasmic cage (Fig. 6.1e). A study by using electron paramagnetic resonance (EPR) spectroscopy indicates that one side of TM1 is exposed to membrane lipid and another side faces to other domain of the channel protein (Vasquez et al. 2008), and that TM2 is mostly buried in the protein. The gating threshold is increased by substituting four hydrophobic residues (A34, I37, A85, L86) in the periplasmic ends of TM1 and TM2 with asparagine (Fig. 6.1g; S1). Loss of hydrophobic contact between TM1

and TM2 at these ends possibly interrupts transmission of membrane tension to the channel. The mutants (I48, A51, L55) on one side of TM1 close to the cytoplasmic end also have decreased sensitivity to mechanotransduction (Fig. 6.1g; S4), leading a distorted TM1 conformation. Hydrophobic residues at border between membrane lipid and channel protein maintain the tension-sensitive conformation. Phe-68 is considered to play a crucial role in transmitting membrane tension to the gate through hydrophobic contact between TM2 and TM3a (Fig. 6.1g; S2), as Phe-68 points towards TM3 (Belyy et al. 2010). Conversely, three mutants (I39N, V40N and I43N) on the TM1 facing to lipid decrease the threshold (Fig. 6.1g; S3) (Nomura et al. 2006). Interestingly, introduction of asparagine into the most hydrophobic residues in the middle of transmembrane domains does not interrupt the mechanosensitivity. In contrast, mutations that increase the gating threshold are only at the ends of transmembrane domains. These observations suggest that residues close to the surface of the lipid bilayer are essential parts for MscS function. In previous studies, it is considered that membrane proteins are subject to a negative lateral pressure from the lipid just beneath the surface of the membrane. Therefore, proper intramolecular interactions at the level of water-lipid interface are important for the MscS function. MscS shares the same characteristics as MscL in which the interaction with lipid near the polar-apolar boundary is essential to the function.

During transition from the closed to the open state, MscS drastically changes its conformation (Miller et al. 2003, Edwards et al. 2004). TM3 is considered to be involved in the channel opening by slight iris-like rotations and tilting by expansile force (Edwards et al. 2005) likely to MscL. Further, MscS is inhibited by submillimolar concentrations of gadolinium ( $Gd^{3+}$ ) likely to other MS channels (Hamill et al. 2001). Notably,  $Gd^{3+}$  is thought to affect mechanical properties of lipid bilayer surrounding the MS channels, due to its high affinity to negatively charged lipid head groups (Ermakov et al. 1998), rather than directly acting on the MS channels. Numerous compounds are discovered to activate MS channels by modifying membrane curvature after asymmetrical insertion into the membrane, thereby changing the pressure profile in lipid bilayer (Kung 2005). Among the compounds, chlorpromazine (CPZ) and trinitrophenol (TNP) can activate the MS channel in the absence of pressure (Martinac et al. 1990). Lysolipids as conical lipids also modify membrane shape and affect MS channel gating by causing hydrophobic mismatches and/or curvature at the critical protein-lipid interphase (Martinac et al. 1990). Recently, parabens (*p*-hydroxybenzoic acid) has been found to activate MscS and MscL of *E. coli* by directly binding the gate of these channels without membrane stretch (Nguyen et al. 2005). The discovery of these compounds is expected to characterize the gating of MS channels.

As described above, MscS and MscL are channels to sense the membrane tension directly and be activated by conformational changes. The subunits of these channels tilt depending upon expansive forces of membrane through hypotonic cell swelling by iris-like rotation, resulting in opening of the MS channels. When bacteria adapts to the changes in extracellular osmolality for survival, they need to sense magnitudes of the changes in extracellular osmolality to release the proper amount of cytosolic components through MS channels. A physiological role of MscS and MscL channels



**Fig. 6.2** Models for mechanosensitive ion channels gating

is to convert the expansile force caused by changes in osmotic pressure into survival ability to release cytoplasmic components leading to reduction of cytoplasmic osmotic pressure for adaptation of cytoplasmic osmolality to extracellular hypotonic one.

### 6.3 MSCs in Eukaryotes

We can sense physical contact, gravity, sound waves, muscle stretch, fluid flow, and blood pressure via conversion of these stimuli interacting with specific sensory cells into electrical signals through MS channels. At the present, several types of MS channels in eukaryotes are recognized as candidates for transmission of mechanical force. MS channels can be gated directly with membrane tension coming from lipid bilayers themselves or from tethered extracellular matrixes and/or cytoskeletons (Fig. 6.2). An alternative indirect model involves another primary mechanosensor(s), such as a membrane receptor and associated second messenger(s) (Fig. 6.2). In recent studies, most focused MS channels in eukaryotes belong to transient receptor potential (TRP) ion channel superfamily, nonselective cation channels to permeate  $\text{Ca}^{2+}$ . The mechanotransduction for sensing mechanical forces through MS channels are mostly mediated to regulate cytosolic  $\text{Ca}^{2+}$  concentration that in turn activates downstream signal molecules to respond to the mechanical forces. Noteworthy, eukaryotic cells have extensive cytoskeleton near the lipid bilayer and contact to extracellular matrix through focal adhesion. The cytoskeletons and/or extracellular matrixes as tethers enable to sense the magnitude, direction and duration of mechanical forces and to transmit the elements of mechanical forces to various intracellular signal molecules for achievement of complicated and precise responses to diverse stimuli. In this section, we summarize the mechanical gating of the MS channels in eukaryotes and discuss physiological importance of these channels.

### 6.3.1 TRP Channels as MSCs

Transient receptor potential (TRP) ion channel superfamily is known to contribute to sensing and transduction of various external and internal stimuli including pain, temperature, pH, mechanical force, osmotic pressure, and chemical stimuli. Most of TRP channels are  $\text{Ca}^{2+}$  permeable, non-selective cation channels. They are composed of four homomeric or heteromeric TRP subunits. TRP channels in mammals are divided into several groups (TRPA, TRPC, TRPN, TRPV, TRPM, TRPP and TRPML) on basis of similarity of their amino-acid sequences. TRP channels are considered to have a common topological structure with six transmembrane domains and intracellular amino- and carboxyl-termini. Numerous evidences have recently revealed that members of TRP ion channel superfamily are potential candidates for cellular mechanosensing and mechanotransduction.

#### 6.3.1.1 TRPV Channels

Transient receptor potential vanilloid (TRPV) channels (TRPV1–6 have been identified) are widely expressed in both sensory and nonsensory cells, and are activated by various chemical and physical stimuli. TRPV4 displays significantly a stronger homology with TRPV1-TRPV3 than with TRPV5 or TRPV6. TRPV4 (OTRPC4, VRL-2, VR-OAC, and TRP12) was first described as a channel activated by hypotonicity-induced cell swelling (Liedtke et al. 2000; Nilius et al. 2001). Genetic analysis in *Caenorhabditis elegans* has revealed that OSM-9 and OCR-2 are essential for both osmosensory and mechanosensory (nose-touch) behaviors (Colbert et al. 1997; Tobin et al. 2002; Sokolchik et al. 2005). In particular, the rescue of OSM-9 and PCR-2 functions with mammalian TRPV4 and TRPV2 respectively has indicated that these two channel proteins are a central component of the sensor channel in *C. elegans*. It is now recognized that TRPV2 (Muraki et al. 2003) and TRPV4 (Strotmann et al. 2000) are sensitive to hypotonic cell swelling, shear stress/fluid flow (TRPV4), and membrane stretch (TRPV2). In the study of osmotic metabolism in TRPV4<sup>-/-</sup> mice, TRPV4 in the brain may transmit a negative signal to AVP secretion similar to an inhibitory pass through the baroregulatory system (Mizuno et al. 2003). Therefore, TRPV4 may be the hypothalamic osmoreceptor, or a component of the osmoreceptor, which controls water balance by releasing vasopressin from the posterior pituitary (Liedtke et al. 2003; Mizuno et al. 2003; Suzuki et al. 2003). Further, functional characterization of the TRPV4 has progressed owing to discovery of synthetic 4 $\alpha$ -phorbol as a direct channel activator. The mechanism to activate TRPV4 channel by cell swelling is not due to a direct sensing to mechanical stimuli, whereas it may be activated indirectly through arachidonic acid metabolic pathways. Cell swelling has been shown to activate phospholipase A<sub>2</sub> (PLA<sub>2</sub>), producing arachidonic acid and cytochrome P450 metabolite, 5', 6'-epoxyeicosatrienoic acid (5'6'-EET) that would be signals to activate TRPV4 (Basavappa et al. 1998, Thoroed et al. 1997). Moreover, abolishment of the arachidonic acid and 5'6'-EET production by preventing PLA<sub>2</sub> and cytochrome



P450 epoxygenase respectively strongly inhibited the hypotonicity-induced TRPV4  $\text{Ca}^{2+}$  influx and TRPV4 channel current (Vriens et al. 2004). Accordingly, hypotonic activation of TRPV4 channel would be mediated via indirect pathways.

### 6.3.1.2 TRPM Channels

The melastatin-related transient receptor potential (TRPM) subfamily is named based on the first discovered member, melastatin (TRPM1), which has identified in the study of melanomas. Some members of TRPM channels have been shown to relate to human tumors. Expression of TRPM8 increases in prostate carcinomas (Tsavaler et al. 2001), TRPM5 may be found in Wilms' tumours and rhabdomyosarcomas (Prawitt et al. 2000), and reduction of TRPM1 expression is linked to more-malignant melanomas (Duncan et al. 1998; Deeds et al. 2000). It is proposed that TRPM channels play a role in tumourigenesis, proliferation and differentiation, while members of TRPM channels, TRPM3, TRPM4 and TRPM7, are activated with mechanical stimuli such as hypotonic cell swelling and membrane stretch. TRPM7 is initially characterized as a  $\text{Ca}^{2+}$ -permeable, non-selective cation channel. Oancea et al. have previously indicated that shear stress activates TRPM7 channel by increasing exocytotic incorporation of TRPM7 into plasma membrane in vascular smooth muscle cells and recombinant TRPM7-overexpressing HEK293T cells (Oancea et al. 2006). On the other hand, Numata et al. have demonstrated that endogenously expressed TRPM7-like channel in HeLa cells is directly activated by membrane expansion induced by membrane stretch or osmotic cell swelling (Numata et al. 2007a). Further, Numata et al. have revealed that membrane stretch directly activates TRPM7 by increasing open probability of heterologous TRPM7 expressed in HEK 293 cells (Numata et al. 2007b). Consequently, it is considered that mechanical forces activate TRPM 7 channel by increasing both number and open probability of the channel in a cell type- and tissue-dependent manner. On the other hands, TRPM4-like channels can be activated by membrane stretch, possibly through ryanodine receptor (RyR) activation in rat cerebral artery myocytes and TRPM4B-overexpressing HEK cells. Activation of TRPM4 channel in response to membrane stretch is mostly abolished by a putative RyR antagonist in cell-attached mode patch clamp, whereas inhibitors for PLC-dependent cascade failed to suppress the effect of membrane stretch on TRPM4 channels (Morita et al. 2007). TRPM3 is also characterized as a cation channel activated by extracellular hypotonic stress (Harteneck et al. 2007). Although the mechanism of TRPM3 activation by hypotonic stress is at the present unclear, sphingosine is a possible candidate to activate TRPM3 channel. It is well known that hypotonic stress and cell swelling activate receptors for growth factors and coupled to G proteins (Franco et al. 2004; Niisato et al. 2000; Sadoshima et al. 1996; Taruno et al. 2007). Production of sphingosine is dependent upon activity of enzymes such as sphingomyelinase and ceramidase following activation by growth factors (Coroneos et al. 1995; Jacobs et al. 1993). Therefore, cell swelling and hypotonic stress might activate TRPM3 by up-regulating the sphingomyelinase/ceramidase pathway through activation of receptor tyrosine kinase for growth factors (Kraft et al. 2005).

As describe above, TRPM7 channel increases its activity by sensing membrane tension, meanwhile TRPM3 and TRPM4 is might be indirectly activated through sphingosine- and ryanosine-dependent pathway respectively. However, even in TRPM7, exocytotic insertion of TRPM7 channel to the plasma membrane induced by hypotonic stress might be regulated by intracellular signal molecules. Therefore, it is supposed that TRPM channel is likely to be indirectly regulated by hypotonic cell swelling and membrane stretch through intracellular signal cascades.

### 6.3.1.3 TRPC Channels

The subfamily of TRP channels; canonical TRP channels (TRPC) have seven members. TRPC1 and TRPC6 are non-selective  $\text{Ca}^{2+}$  permeable channels that have been recently claimed to be stretch-activated cation channels (SACs) (Maroto et al. 2005; Spassova et al. 2006). Further, these channels are related to inherited and acquired pathologies including familial focal segmental glomerulosclerosis, Duchenne muscular dystrophy (DMD), and cardiac hypertrophy (Pagnamenta et al. 2011; Ward et al. 2008; Winn et al. 2005). TRPC1 is widely expressed, and is a non-selective “store-operated ion channel” (SOC) involved in  $\text{Ca}^{2+}$  entry following  $\text{Ca}^{2+}$  depletion of the endoplasmic reticulum (ER). Maroto et al. have indicated that TRPC1 is a component of vertebrate mechanosensitive cation channels that are gated by tension in reconstituted lipid bilayers (Maroto et al. 2005). TRPC6 is another  $\text{Ca}^{2+}$ -permeable non-selective cation channel, and is activated in response to  $\text{PIP}_2$  receptors, thus called a “receptor-operated channel” (ROC). When TRPC6 is coexpressed with angiotension II type 1 receptor ( $\text{AT}_1\text{R}$ ), TRPC6 would gain ability of mechanosensitivity without any ligands in vascular smooth muscle cells (Mederos et al. 2008). TRPC6 activation by stretch or swelling is blocked by PLC inhibition or by  $\text{GDP}\beta\text{s}$  that suppresses G protein activation (Mederos et al. 2008; Park et al. 2003). Consequently, membrane stretch causes an agonist-independent conformational change of  $\text{AT}_1\text{R}$  thereby activating the downstream  $\text{Gq}_{11}/\text{PLC}/\text{DAG}/\text{TRPC6}$  signaling cascade. As mentioned above, activation of TRPC6 is mediated through an indirect pathway and a G protein-coupled receptor (GPCR) might be a sensor of membrane tension.

### 6.3.1.4 TRPP Channels

TRPP subunits are abundantly expressed in the kidney and the cardiovascular system (Bichet et al. 2006). TRPP1 (PKD1, PC1) is a large transmembrane glycoprotein with an extended N-terminal extracellular domain, 11 transmembrane domains and a short intracellular C-terminal domain (Bichet et al. 2006). TRPP channel subfamily has four members including TRPP1 and TRPP2 (PKD2, PC2) (Giamarchi et al. 2006). Surfaces of the primary cilium in renal epithelial and endothelial cells express the TRPP1/TRPP2 complex. Shear stress causes an increase in intracellular  $\text{Ca}^{2+}$  concentration in primary cilia and this response disappears in cells lacking TRPP1 or TRPP2 (Nauli et al. 2003, 2008; Praetorius et al. 2003). Sharif-Naeini et al.

have indicated that TRPP2 alone inhibits stretch-activated ion channels (SACs) (Sharif et al. 2009). This inhibitory effect is reversed by coexpression with TRPP1. On the other hand, the actin cytoskeleton is indeed implicated in SAC inhibition by TRPP2, as this effect is abolished by F-actin disruption (Sharif et al. 2009). Further, filamin A (FLNa) is a novel cytoskeletal element interacting with TRPP2 (Stossel et al. 2001). FLNa stiffens cell cortexes by virtue of its ability to crosslink adjacent actin filaments and increases actin polymerization/gelation rates in vitro (Stossel et al. 2001). Activity of nonselective SACs is reduced in the presence of FLNa, and the inhibitory effect of TRPP2 on SACs is abolished when FLNa is absent. Thus, it is now recognized that the TRPP1/TRPP2 ratio controls SAC mechanosensitivity through coupling of FLNa to the actin cytoskeleton, and affects the conversion of intraluminal pressure to local bilayer tension for modulating the arterial myogenic response to intraluminal pressure (Giamarchi et al. 2006; McGrath et al. 2003; Nauli et al. 2003; Sharif et al. 2009).

## 6.4 Degenerin/Epithelial Na<sup>+</sup> Channel (DEG/ENaC) Superfamily

*C. elegans* degenerins were named due to phenotypes of some touch-defective mutants that cause neuronal degeneration. Degenerin/epithelial Na<sup>+</sup> channel (DEG/ENaC) family is generally characterized as being selective for Na<sup>+</sup> and blocked by amiloride. DEG/ENaC subunits have two transmembrane domains with intracellular amino- and carboxyl- termini and a large extracellular loop. Channels of DEG/ENaC family are multimeric (both homomeric and heteromeric) and appear to be gated by diverse stimuli including hormones (Marunaka et al. 1991; Rossier 2002), mechanical force, low extracellular pH (Krishtal et al. 1981). Genes required for mechano (touch) sensitivity are first identified in *C. elegans* by screening mutants defective in response to gentle touch. Screened genes are named *mec* for mechanosensory abnormal and expressed in touch receptor neurons. Four *mec* genes are coded proteins involved in mechanosensory channel complex. Electrophysiological studies in live animals have revealed that touching the animal actually increases amiloride-sensitive Na<sup>+</sup> inward current that probably transduces the mechanical stress into electrical signals through mechanosensitive channels such as degenerins. However, it is still unclear how gentle touch activates the mechnosensory channel in *C. elegans*.

### 6.4.1 ASIC: Acid-Sensing Ion Channel

In 1981, Krishtal and Pidoplichko have provided the first evidence to indicate a receptor for H<sup>+</sup> that carries inward Na<sup>+</sup> currents, that is ASIC (acid-sensing ion

channel), in mammalian sensory neurons (Krishtal et al. 1981). Currently, ASIC proteins have 4 members (ASIC1, ASIC2, ASIC3, and ASIC4) and closely related to ENaC. ASIC can form homomultimeric and heteromultimeric channels. The structure of ASIC in the chicken has been revealed by X-ray crystallography, and the crystallized ASIC is composed of a homotrimer (Jasti et al. 2007). Although ASIC is also sensitive to amiloride, they need higher doses than ENaC (10- to 100-fold) to be blocked. A decrease in extracellular pH opens most of ASICs. ASIC has also been proposed as a mechanosensor. ASICs among the DEG/ENaC superfamily are identified in neuronal and neuroepithelial tissues, where they may be involved in acid taste, acid sensation, learning, and mechanosensation. Further, recent evidences suggest that ASICs are also expressed in vascular smooth muscle cells and involved in cardiovascular homeostasis through actions as mechanoreceptors in arterial baroreceptor neurons and vascular smooth muscle cells. For example, ASIC/ENaC blocker (amiloride) suppresses responses in baroreceptor neurons to mechanical stimulation (Drummond et al. 1998). In addition, mechanosensory dorsal root ganglion cells are found to express ASIC subunits (Garcia-Anoveros et al. 2001). Investigations in ASIC knock-out mice have revealed that these channels may indeed be required for touch sensitivity (Price et al. 2000, 2001). Furthermore, ASIC3 expression in transgenic mice leads to an increased sensitivity to mechanical stimuli (Mogil et al. 2005). On the contrary, more recent reports have failed to demonstrate a significant role of ASICs in mechano-sensory function (Drew et al. 2004; Roza et al. 2004). Therefore, involvements of ASICs in mechanotransduction remain controversial.

#### **6.4.2 ENaC: Epithelial Na<sup>+</sup> Channel**

ENaC plays a key role in maintenance of Na<sup>+</sup> balance and regulation of blood pressure. Generally, entry of Na<sup>+</sup> through ENaC in the apical membrane is the rate limiting step for transcellular Na<sup>+</sup> absorption in epithelium. ENaC belong to the DEG/ ENaC superfamily (Kellenberger et al. 2002; Mano et al. 1999) and is characterized to be sensitive to amiloride and its derivatives. Three homologous ENaC subunits ( $\alpha$ ,  $\beta$  and  $\gamma$ ), which has two transmembrane domains (Canessa et al. 1994) with intracellular amino- and carboxyl- termini and a large extracellular loop, assemble to form a highly Na<sup>+</sup> selective channel. Firsov et al. (Firsov et al. 1998) have indicated that ENaC stoichiometry (four subunits) is two  $\alpha$ , one  $\beta$  and one  $\gamma$  and that the channel pore consists of two  $\alpha$  subunits (Berdiev et al. 1998). The conformational analysis of ENaC by mutagenesis reveals that the region of G/SXS; G587–S589 in  $\alpha$ -ENaC forms the narrowest part of the pore, which determines ionic selectivity and unitary conductance (Kellenberger et al. 1999a, b, 2001). The binding site of amiloride, a pore blocker, is located at four residues upstream of the selectivity filter, as identified by mutations of residues,  $\alpha$ S583 and homologous  $\beta$ G525/ $\gamma$ G537 that obstruct amiloride binding (Schild et al. 1997).

It is well known that activity of ENaC is generally regulated by various factors such as hormones (Marunaka et al. 1991; Rossier 2002), kinases (Diakov et al. 2004; Nilius et al. 2001), intrinsic  $\text{Na}^+$ -dependent mechanisms (Garty et al. 1997; Turnheim 1991) and proteases (Caldwell et al. 2003; Knight et al. 2006; Rossier 2004; Vallet et al. 1997). So far, evidences implicating responses of ENaC to mechanical force including osmotic stress (Awayda et al. 1998; Ji et al. 1998; Taruno et al. 2007), hydrostatic pressure (Awayda et al. 1995; Palmer et al. 1996), and laminar shear stress (Carattino et al. 2004; Satlin et al. 2001) have been provided. When  $\alpha$ -bENaC (bovine ENaC) alone is inserted into artificial planar lipid bilayers without  $\beta$ - and  $\gamma$ -subunits, the reconstituted ion channel with a single-channel conductance of 40 pS exhibits characteristics very similar to those of stretch-activated nonselective cation channel observed in several types of tissues (Awayda et al. 1995) and is activated due to increasing open probability by exposure to a hydrostatic pressure gradient. Kizer et al. have also indicated (Kizer et al. 1997) that stretch-activated, nonselective cation channels were observed in  $\alpha$ -ENaC alone expressed in LM (mouse fibroblast cell line) cell, that the channels are activated by negative pressure applied to the cell attached patches, cell swelling, or patch excision. However, it is still controversial, because some studies have failed to indicate mechanosensitivity of ENaC (Awayda et al. 1998). Possibilities of ENaC to have mechanosensitivity are explained by reasons why some kinds of epithelia expressing ENaC are constantly exposed to mechanical forces; that is airway epithelia by air flow during breathings (Sidhaye et al. 2008; Tarran et al. 2005) and the cortical collecting duct epithelium by the tubular flows (Liu et al. 2003; Satlin et al. 2001). Shear stress such as air and fluid flows is one of possible mechanical stresses for ENaC physiologically relevant to ENaC functions in various cells. Althaus et al. have clearly indicated that laminar shear stress (LSS) significantly increases open probabilities ( $P_o$ ) of rENaC (rat ENaC) and xENaC (*Xenopus* ENaC) after LSS exposure by using single-channel recordings. Increased  $P_o$  is associated with either a significant increase of mean open time or a decrease of mean closed time. There is no significant increase in the number of active ENaCs in exposure to LSS. On the other hand, shear force also activates hENaC (human ENaC) cloned from human lung tissue that is expressed in *Xenopus* oocytes. In outside-out single channel recording experiments, it is proved that ENaC is directly activated by shear force with increases in  $NP_o$  (number of channels  $\times P_o$ ) (Froniusa et al. 2010). This observation suggests that hENaC has a crucial role in regulation of pulmonary  $\text{Na}^+$  absorption and pulmonary fluid homeostasis by directly sensing shear force. Further, ENaC subunits are revealed to be expressed in vascular tissues (Drummond et al. 2001, 2004; Jernigan et al. 2005) and sensory nerve endings (Drummond et al. 2000). ENaC expressed in vascular tissues might contribute to mechano-sensory systems that are involved in control of blood pressure (Drummond et al. 2001, 2004). These studies strongly support the possibilities that ENaC responds to some kinds of mechanical forces and plays a crucial role as a mechanosensor.

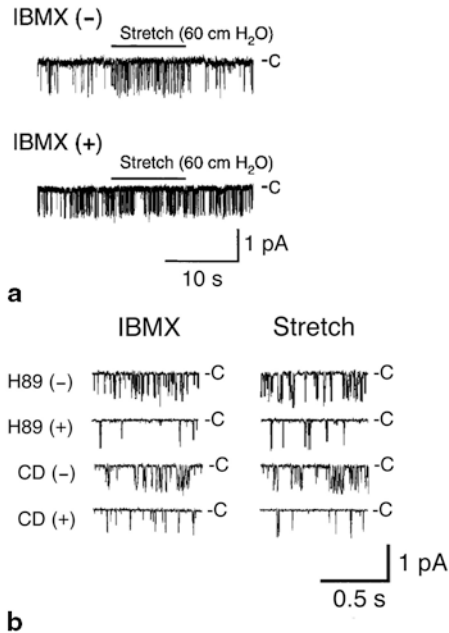
## 6.5 MS Channels in Renal Tubules

### 6.5.1 Sensing Renal Fluid Flow

It has previously reported (Engbretson et al. 1987; Malnic et al. 1989; Satlin 1994, 2001; Stokes 1993) that elevation in tubular fluid flow rate upregulates net  $\text{Na}^+$  reabsorption in the mammalian cortical collecting duct (CCD). On the other hand, we and others have indicated that ENaC and/or non-selective stretch-activated cation (SAC) channel as a  $\text{Na}^+$  permeable ion channel exist in CCD and contribute to  $\text{Na}^+$  reabsorption (Butterworth 2010; Marunaka et al. 1994, 1997; Niisato et al. 2001). Therefore, it is expected that at least either of ENaC or SAC channel in CCD should be activated in response to shear stress caused by tubular fluid flow. A flow-induced increase in net  $\text{Na}^+$  absorption in CCD or  $I_{\text{Na}}$  in oocytes expressing ENaC is supposed to be caused by increasing the number of apical channels and/or channel open probability. LSS does not enhance mutant ENaC channels ( $\alpha\beta\text{S518K}\gamma$  or  $\alpha\text{S580C}\beta\gamma$ ) that have a high intrinsic open probability, suggesting that LSS activates ENaC by increasing channel open probability (Carattino et al. 2004). A previous study has indicated that LSS alters the channel gating, especially the open probability, without affecting other parameters including amiloride binding kinetics, single-channel conductance, or ion selectivity (Carattino et al. 2005). Nevertheless, it is postulated mechanosensors might be located at the extracellular region of the channel. Namely, mechanosensors might either be parts of, or associated with, the large extracellular loops of ENaC molecules which must be coupled to the gating of the channel. This hypothesis, the large extracellular loop is a mechanosensor, is further supported by a recent study indicating that ENaC activation by LSS (fluid flow) is independent of membrane trafficking (Morimoto et al. 2006). However, additional experimental data are necessary to identify mechanosensors and to clarify this issue.

### 6.5.2 NSC Channel in *Xenopus Laevis* A6 Cells

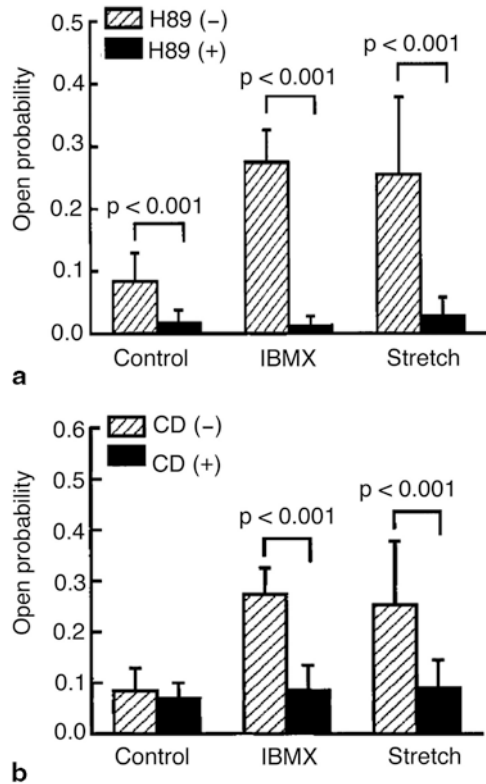
In a model of CCD cell (A6 cells) to study ENaC-mediated  $\text{Na}^+$  reabsorption, our and other laboratories have shown that A6 cells have stretch activated non-selective cation (NSC) channels that are activated by negative pressure applied to patch pipettes (Marunaka et al. 1994, 1997; Nakahari et al. 1996; Niisato et al. 2001; Urbach et al. 1999; Yu et al. 1997). In our previous reports, NSC channels have 28–29 pS conductance and  $\text{Na}^+/\text{K}^+$  permeability. Although most of SACs can permeate  $\text{Ca}^{2+}$  in addition to  $\text{Na}^+$  and  $\text{K}^+$ , 28 pS NSC channel in A6 cells hardly permeate  $\text{Ca}^{2+}$  (Nakahari et al. 1996; Marunaka et al. 1997). The NSC channel is also activated by compounds to increase cytosolic cAMP concentration. After the NSC channel is once fully activated by cAMP, negative pressure can no longer activate it and vice versa (Fig. 6.3). At the present, it is considered that cAMP and negative pressure is involved in a common mechanism for activation of NSC channels. By using



**Fig. 6.3** Single channel recording of NSC channel stimulated with IBMX and stretch in the presence or absence of H89 and cytochalasin D. **a** Effects of negative pressure (stretch) on open probability ( $P_o$ ) of NSC channels obtained from cell-attached patches formed on the apical membrane at no applied potential (the resting membrane potential) in IBMX-untreated and -treated cells. Representative traces of single channel currents with and without negative pressure of 60 cm H<sub>2</sub>O (stretch) in IBMX-untreated and -treated cells. **b** Effects of H89 (a PKA inhibitor) and cytochalasin D (CD) on  $P_o$  of NSC channels obtained in cell-attached patches of A6 cells treated with IBMX or subjected to negative pressure as stretch. (Modified from Marunaka et al. 1997)

open and closed time-interval histograms obtained from cell-attached patches, single channel kinetics in the NSC channel in the presence and absence of 3-isobutyl-1-methylxanthine (IBMX, an inhibitor of phosphodiesterase used to increase cytosolic cAMP) and negative pressure is analyzed. The open time-interval histogram obtained from an NSC channel is fitted by one exponential function. In contrast, the closed time-interval histogram is fitted by two exponential functions. The mean values of open or closed times do not change significantly with application of IBMX, although IBMX increases the open probability ( $P_o$ ) as NSC channel activity. To clarify how IBMX could increase the  $P_o$  without any detectable changes in open and closed times, the frequency of events staying at each state is compared. IBMX increased the frequency of the short closed events and decreased the frequency of the long closed events leading to an increase in  $P_o$  of the channel. In A6 cells, the NSC channel has one open and two closed states, and a linear gating kinetic model " $C_L \leftrightarrow C_S \leftrightarrow O$ " (the long closed state,  $C_L$ ; the short closed state,  $C_S$ , the open state,  $O$ ) is the most suitable one to explain observed channel behaviors. According to the Model " $C_L \leftrightarrow C_S \leftrightarrow O$ " only the leaving rate of the channel for  $C_L$  from  $C_S$  is

**Fig. 6.4** Statistical results of the effects of H89 and cytochalasin D (CD) on  $P_o$  of basal, IBMX-, and stretch-activated channels. (Modified from Marunaka et al. 1997)



decreased by application of IBMX or stretch, resulting in an increase in the  $P_o$  of the channel.

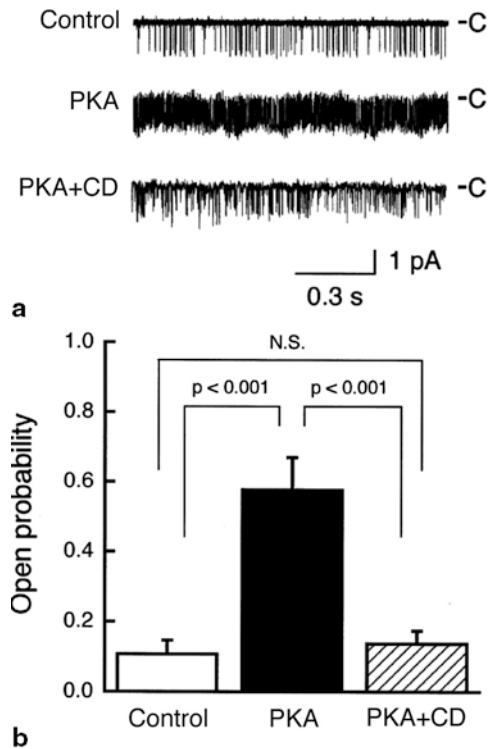
On the other hand, the actin cytoskeleton is known to contribute to sensing mechanical force and regulation of MSCs activities (Prat et al. 1993a, b). In NSC channels of A6 cells, treatment with cytochalasin D, which depolymerizes actin filaments, decreases the  $P_o$  of the IBMX- or stretch-activated channel to the basal level (Figs. 6.3 and 6.4). H89 (a PKA inhibitor) also markedly decreased the  $P_o$  of the NSC channel and abolished the response of the NSC channel to IBMX or stretch (Figs. 6.3 and 6.4). On the other hand, a PKA catalytic subunit significantly increases  $P_o$ , which is abolished by cytochalasin D (Fig. 6.5). The observations indicate that both activation of PKA and existence of polymerized actin filaments are essential for activation of NSC channels in A6 cells by IBMX and stretch.

In excised inside-out patches, catalytic subunits of PKA significantly increased  $P_o$  of NSC channels and single channel kinetics are changes to “ $C \leftrightarrow O$ ” (one closed state and one open state). It is supposed that PKA may influence only the communication between the short and long closed states without affecting the communication between  $C_S$  and  $O$ . There are two possibilities to explain this phenomenon: (i) the long closed state really disappeared (in other words, the channel does not have



**Fig. 6.5** Effects of PKA catalytic subunit and cytochalasin D (CD) on  $P_o$  of NSC channels obtained from excised inside-out patches formed on the apical membrane at a membrane potential of  $-40$  mV in the presence of 2 mM ATP.

**a** Representative traces of single-channel currents before treatment (Control), 10 min after application of PKA (10  $\mu$ g/mL) (PKA), and 10 min after PKA application with CD pretreatment (5  $\mu$ M, 10 min) (PKA + CD) to the cytosolic surface of the patch membrane. The closed level is marked by a horizontal bar and "C." **b** Statistical results of effects of PKA catalytic subunit and CD on the  $P_o$ . (Modified from Niisato et al. 2001)



access to the long closed state and has only one closed state), or (ii) the mean time of the channel staying at the long closed state becomes identical to that of the short closed state (in other words, based upon only the mean times the long closed state cannot be distinguished from the short closed state, hence giving an impression that the channel has only one closed state). Consequently, application of PKA catalytic subunit affects the transition rate of the channel from the short closed state to the long closed state, and that PKA action on this rate is abolished by treatment with cytochalasin D. Communication of the channel between the short and long closed states is affected by PKA-induced phosphorylation of polymerized actin filaments. These observations and our previous study (Marunaka et al. 1997) indicate that phosphorylation of polymerized actin filaments mimics the action of stretch, suggesting that phosphorylation might produce some stretch-like mechanical phenomenon.

It is generally considered that mechanical force initiates signal transduction via stretch-activated ion channels in the cell membrane (Gillespie et al. 2001). However, cellular mechanotransduction may involve numerous molecular mechanisms other than ion channels, such as force initiated signal transduction via changes in cytoskeletal-ECM linkages (Sawada et al. 2002; Tamada et al. 2004). In this study, negative pressure as mechanical force might be converted to chemical signals activating PKA. As reported in studies on mechanosensitivity of TRPC6 channel (Mederos

et al. 2008; Park et al. 2003), membrane tension possibly activates G protein-coupled receptor (GPCR) associated with adenylate cyclase without ligand, and the increased cAMP by membrane tension activates PKA in A6 cells. Therefore, a sensor of membrane tension in A6 cells might be GPCR but not NSC channel. However, it is still unclear that a role of the polymerized actin filament especially in excised inside-out patches. One possibility is that existence of actin filaments nearby cell membrane is essentially required but not transmit the mechanical force through cytoskeleton network to the channels. It is seemed that gating of NSC channels is “indirect gating” rather than “tether model” shown in Fig. 6.2, although further studies are needed.

## 6.6 Conclusion and Perspectives

Physical force including osmotic pressure, hydrostatic pressure, gravity, shear stress and expansile force (membrane tension) is a crucial signal to control cellular functions such as proliferation, differentiation, development and cell death. In prokaryotic cell as monad, physical stress such as osmotic pressure directly connects to life and death. In bacteria cells, MS channels simply and directly sense the magnitude of membrane tension as the osmotic pressure activating MS channels if the membrane tension is larger than its gating threshold. MscL and MscS in lipid bilayers are physically expanded by membrane tension, which in turn causes conformational changes for gating. Recent progress of crystallographic analysis and computing modeling have revealed gating mechanisms of MscL and MscS; tilting of transmembrane domains in lipid bilayer by iris-like rotations that enlarges the channel pore size through transition from the closed state to the open state. The gating of MscL and MscS seems to be simple, because bacteria cells only need to recognize the magnitude of membrane tension as osmotic pressure that determines the amount of releasing cytosolic components. In contrast, eukaryotic cells, more complex mechanisms are involved in sensing mechanical force. Mechanical force contains some kinds of elements; magnitude, direction, cycle, duration. To precisely sense mechanical force including elements, MS channels recognize the mechanical force not only through membrane tension itself but also through cytoskeletal networks, adhesion between cell and extracellular matrix and mechanosensitive membrane proteins associated with MS channels (Fig. 6.2). Basically, channel gating is tightly related to the conformation of the channel in the plasma membrane. On the other hand, cytoskeletons backup the plasma membrane by connecting to proteins in the plasma membrane. This means that modification of cytoskeletons is an alternative way to affect MS channels in the plasma membrane. Thus, indirect transmission of mechanical force via cytoskeletons might enable more fine control of activity of MS channels. One of the most interesting examples is a response to shear stress in endothelial cells. They align perpendicular to the stretch for bearing less tension. This phenomenon indicates that endothelial cell can recognize the direction of blood flow as shear stress through stress fiber reconstitution. In eukaryotic cell, cytoskeleton contributes

to recognition of the direction of mechanical force. As an indirect gating of MS channels, mechanosensitive proteins in the plasma membrane are also involved in the mechanotransduction. MS channels such as TRPC6 channel and NSC channel in A6 cells might be regulated by GPCR-dependent signal molecules and GPCR converts the mechanical force to production of its second messengers without its ligand. This means that mechanical force is a physical ligand to activate receptors located in the plasma membrane instead of chemical ligands. Based on previous numerous studies, the plasma membrane itself might be a special complex to sense physical stress and lipid bilayer is a transmitter of mechanical stress to MS channels. Further studies are necessary to reveal the complete understanding of MS channel gating.

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