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ORIGINAL ARTICLE

Tuning the mechanosensitivity of a BK channel by changing the linker length

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Some large-conductance Ca^{2+} and voltage-activated K^+ (BK) channels are activated by membrane stretch. However, the mechanism of mechano-gating of the BK channels is still not well understood. Previous studies have led to the proposal that the linker-gating ring complex functions as a passive spring, transducing the force generated by intracellular Ca^{2+} to the gate to open the channel. This raises the question as to whether membrane stretch is also transmitted to the gate of mechanosensitive (MS) BK channels via the linker-gating complex. To study this, we changed the linker length in the stretch-activated BK channel (SAKCaC), and examined the effect of membrane stretch on the gating of the resultant mutant channels. Shortening the linker increased, whereas extending the linker reduced, the channel mechanosensitivity both in the presence and in the absence of intracellular Ca^{2+}. However, the voltage and Ca^{2+} sensitivities were not significantly altered by membrane stretch. Furthermore, the SAKCaC became less sensitive to membrane stretch at relatively high intracellular Ca^{2+} concentrations or membrane depolarization. These observations suggest that once the channel is in the open-state conformation, tension on the spring is partially released and membrane stretch is less effective. Our results are consistent with the idea that membrane stretch is transferred to the gate via the linker-gating ring complex of the MS BK channels.

Keywords: BK channel, mechanosensitive channel, mechano-gating, heart

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Introduction

Mechanosensitive (MS) ion channels are found in a variety of organisms from bacteria to human. These channels transduce mechanical stimuli into intracellular signals, such as elevation in intracellular Ca^{2+} concentration and electrical activities [1-6], which are important in many physiological functions and pathological conditions, for example, sound detection [4], cell volume regulation [7], touch sensation [8], hypo-osmotic shock protection [9], cell locomotion [10], muscular dystrophy and cardiac arrhythmias [11]. The BK channel is one type of MS channels which is widely expressed in both excitable and non-excitable cells and plays an important role in, for example, vascular smooth muscle tone regulation, neuronal firing and endocrine cell secretion [12-16]. The MS properties of the BK channels expressed in skeletal muscle [17], kidney [18], smooth muscle [19], neuroepithelial cells and osteoblasts [20, 21] have been characterized. We previously identified a stretch-activated BK channel (referred as SAKCaC) in cultured chick embryo ventricular myocytes [22]. Like other BK channels, SAKCaC can be activated by membrane voltage and intracellular Ca^{2+}. However, the unique property of this channel is that it can also be activated by membrane stretch. Molecular cloning and subsequent functional characterization of the SAKCaC have shown that the channel subunit contains a 59-amino-acid insert (called stress-axis regulated exon (STREX)) in the C-terminus, and that STREX domain is the stretch sensor conferring the membrane stretch sensitivity to the channel [23, 24]. However, it is not well understood how the membrane stretch is transferred to the gate to open the channel. In the model based on the MthK channel structure,

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the pore-forming a subunit contains two regulator of K^+ conductance (RCK) domains in the intracellular C-terminus, and eight RCK domains from the tetrameric channel form a gating ring [25, 26]. The linker that connects the S6 gate to the RCK domain and the gating ring form the linker-gating complex, which functions as a passive spring and transduces the force generated by intracellular Ca^{2+} to the channel gate [27]. The STREX domain is located between RCK1 and RCK2 domains in the long intracellular C-terminus of SAKCaC (Figure 1) [19]. The present study showed that shortening the linker rendered the channel more sensitive to membrane stretch, whereas lengthening the linker resulted in the opposite effect, suggesting that, similar to Ca^{2+} activation, the membrane stretch sensed by the STREX domain seems to be transmitted to the channel gate by the linker-gating complex.

Results

Activation of SAKCaC by membrane stretch

We first characterized the activation of wild-type (WT) SAKCaC by membrane stretch in excised inside-out patches. Figure 2A shows typical single channel current traces in response to stepwise negative pressures with 10 mmHg increments. Figure 2B plots the channel open probability (P_o) against the level of pressure applied. The P_o increased with increased pressure. In contrast, there were no detectable channel activities induced by membrane stretch in mock transfected cells (data not shown). The SAKCaC activation occurred in the absence of intracellular Ca^{2+}, suggesting that membrane stretch directly activated the channel. All these results are in agreement with those reported previously [9, 23, 28].

Effect of linker length on P_o of SAKCaC

To address the role of the linker in SAKCaC activation, we produced mutant channels, in which the S6-RCK1 linker was shortened by deleting one residue or lengthened by inserting three residues. Figure 3A shows representative current traces of the mutant BK channels in response to membrane depolarization (+60 mV), and Figure 3B summarizes the P_o. It is obvious that the P_o was greatly increased by shortening the linker (–1), but decreased by lengthening the linker (+3). Similar results were reported previously in the *Slo* BK channels and the interpretation is that the linker-gating ring complex forms a passive spring to exert the force on the gate [27]. We also generated mutant subunits in which more than one residue was deleted or more than three residues were inserted in the linker. However, no detectable channel activities were observed in cells expressing these mutants, even at voltages of greater than +100 mV and in the presence of 100 μM intracellular Ca^{2+} (data not shown), possibly due to defective channel assembly and/or membrane trafficking.

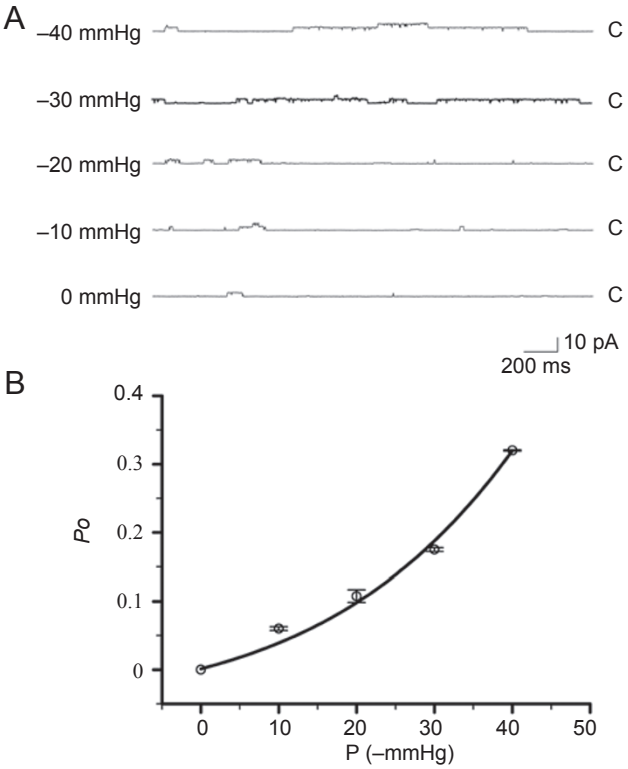

Figure 2 Pressure dependency of P_o of SAKCaC. Pipette solution contained 145 mM K^+, 0 mM Ca^{2+} (with 1 mM EGTA), bath solution contained 145 mM K^+ and 0 mM Ca^{2+} (n = 18). **(A)** Representative single-channel current traces of SAKCaC in response to membrane stretch (C denotes the closed current level). Membrane potential was held at +60 mV. **(B)** Pressure-P_o curve was fit by the Boltzmann equation.

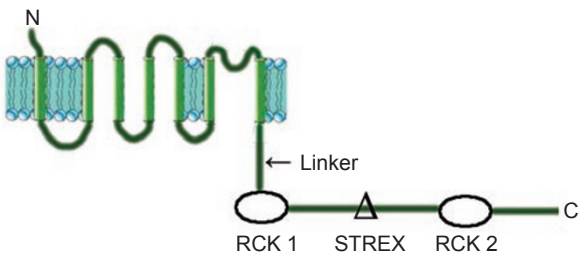

Figure 1 Schematic presentation of the linker, RCK1, RCK2 and STREX domains of BK channel α-subunit.

Effect of changing the linker length on the mechanosensitivity of SAKCaC

We next examined the effect of changing the linker length on SAKCaC activation by membrane stretch. As shown in Figure 4, membrane stretch greatly increased the

P_o of both WT and mutant channels in a dose-dependent manner. However, the mechanosensitivity was strongly enhanced by shortening the linker (–1), and decreased by increasing the linker (+3). The relationship of P_o to the pressure, resulting from membrane stretch, could be fitted with the Boltzmann equation: $P_o=1/[1+\exp(p-p_{1/2})k]$, where p is the pressure, $p_{1/2}$ is the pressure required to induce half-maximal channel activation, and k is the reciprocal of the curve steepness or slope. The $p_{1/2}$ values were 23 mm Hg, 18 mm Hg*, and 27 mm Hg*, and the k values were 22 mm Hg , 23 mm Hg , and 20 mm Hg for WT, –1, and +3 mutant channels, respectively. The $p_{1/2}$ values for both mutant channels were significantly different from that for WT (* indicate significant difference p < 0.05). The unitary channel conductance (260 ± 3.1 pS), however, remained unaltered by either membrane stretch or change in the linker length (data not shown).

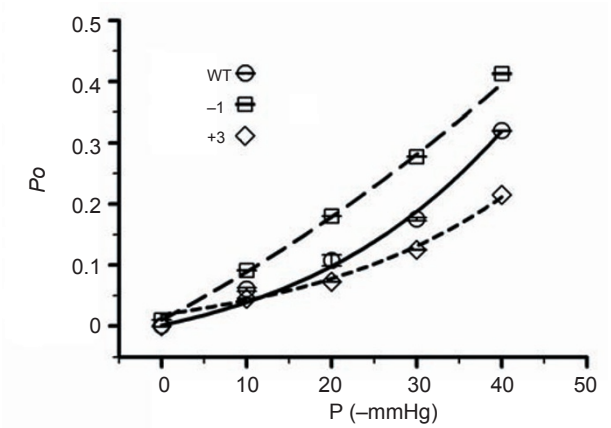

Figure 4 Mechanosensitivity of SAKCaC with linkers of different length (n = 10). The pipette and bath solution contained 145 mM K^+ with 0 mM Ca^{2+}. Membrane potential was held at +60 mV. The data points were fitted with the Boltzmann equation.

Effect of changing the linker length on the mechanosensitivity of SAKCaC at different membrane voltages and intracellular Ca^{2+} concentrations

One key feature of the BK channels is Ca^{2+} and voltage dependence of channel activation [12]. Here we therefore examined the role of the linker in Ca^{2+}-dependent SAKCaC activation in the presence of membrane stretch. As shown in Figure 5, increase in intracellular Ca^{2+} concentration from 0 to 1.2 μM greatly enhanced the channel activity of both WT and mutant channels at all the levels of membrane stretch tested. Compared to WT, the P_o-Ca^{2+} curve for the mutant channel with a shorter linker (–1) was left-shifted, and the slope was not significantly changed (Figure 5B). In contrast, the P_o-Ca^{2+} curve was shifted to the right for the mutant channel with a lengthened linker (+3), and the slope was also decreased (Figure 5C). In the presence of

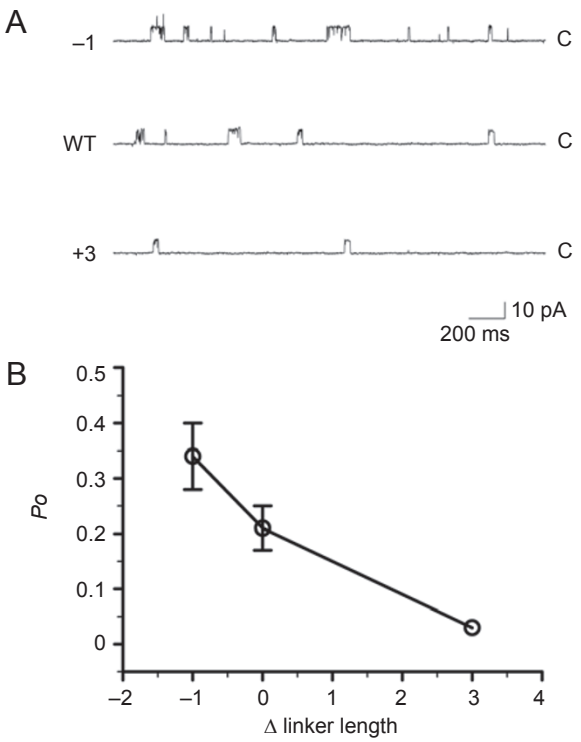

Figure 3 Change in the linker length alters the channel activity in the absence of Ca^{2+} (n = 11). **(A)** Representative recording of currents at a membrane voltage of +60 mV. **(B)** Plot of P_o versus change in the linker length.

Table 1 The effect of membrane stretch on the Hill coefficient of SAKCaC with a linker of different length

Negative pressure	Wild	–1	+3
(mm Hg)	Hill coefficient	Hill coefficient	Hill coefficient
–0	1.42	1.56	1.01
–10	1.47	1.71	1.05
–20	1.62	1.90	1.05
–30	1.63	1.90	1.09
–40	1.67	2.13	1.11

Table 2 The effect of membrane stretch on V1/2 (mV) of SAKCaC with a linker of different length

Negative pressure	Wild	–1	+3
(mm Hg)	($V_{1/2}$)	($V_{1/2}$)	($V_{1/2}$)
–0	92	55	110
–10	82	47	88
–20	50	28	82
–30	12	–25	50
–40	0	–27	46

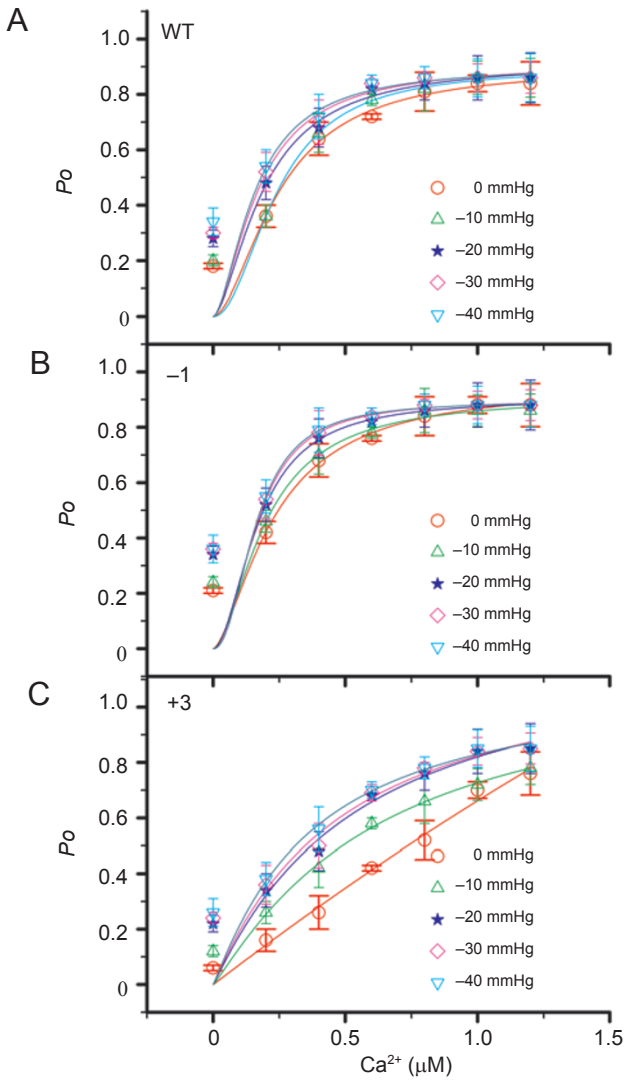

Figure 5 Effect of changing the linker length on Ca^{2+} dependence of SAKCaC in the presence of membrane stretch (n = 11). Membrane potential was held at +60 mV. The data were fitted with the Hill equation: $P_o=[Ca^{2+}]_i^{\eta H}/(K_d^{\eta H}+[Ca^{2+}]_i^{\eta H})$, where ηH is the Hill coefficient and K_d is the dissociation constant.

intracellular Ca^{2+} concentrations, application of negative pressure induced an increase in the P_o. Furthermore, for both WT and mutant channels, increase in negative pressure resulted in a parallel left-shift in the P_o-Ca^{2+} curves without obvious change in the slope (Figure 5; Table 1). Thus, the enhanced SAKCaC activation in the presence of membrane stretch seems not due to an increase in Ca^{2+} sensitivity, suggesting that change in membrane stretch does not alter the Ca^{2+} sensitivity.

We also examined whether change in the linker length and membrane stretch affects the sensitivity of SAKCaC to membrane voltage. Figure 6 plots the P_o against the membrane voltage (V) for the WT and mutant channels in the absence of intracellular Ca^{2+} and in the presence of membrane stretch ranging from 0 to 40 mmHg. These data were fitted with the Boltzmann equation to derive the voltage for half-channel activation, $V_{0.5}$. The results show that lengthening the linker increased, whereas shortening the linker decreased, the $V_{0.5}$ values (Table 2). In addition, compared to WT at all the membrane stretches tested, the P_o-V curve was left-shifted for the mutant channel with a shortened linker (Figure 6B), but right-shifted with decreased slopes for the mutant channel with a lengthened linker (Figure 6C). The P_o at fixed pressure was voltage-dependent, being higher when the membrane was depolarized.

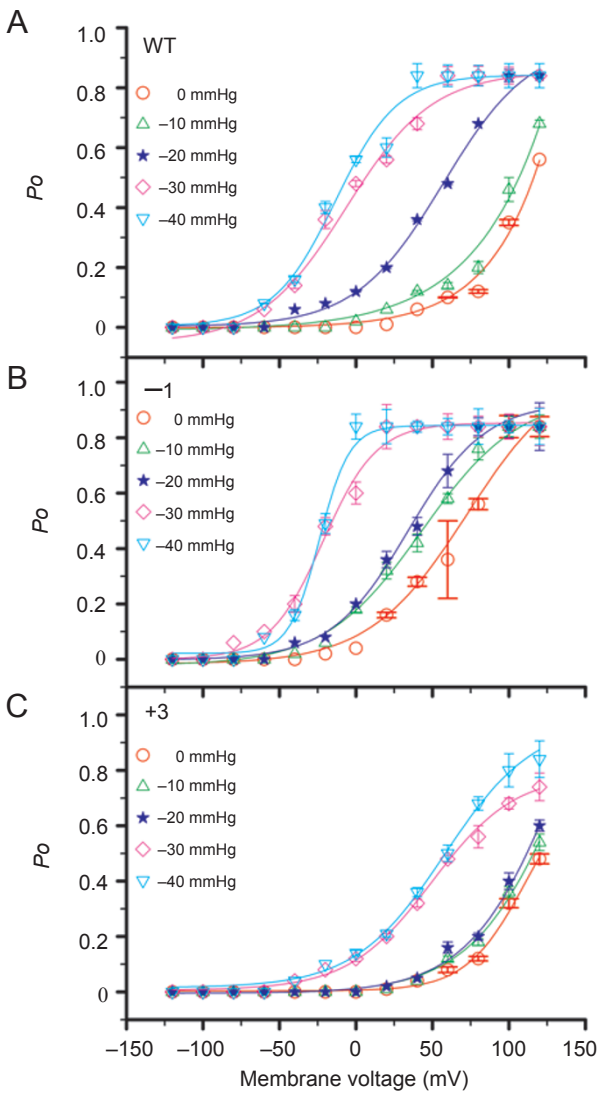

Figure 6 Effect of changing the linker length on voltage dependence of SAKCaC in the presence of membrane stretch in the absence of Ca^{2+} (n = 16). The data were fitted with the Boltzmann equation: $P_o=1/\{1+\exp[-ZF/RT(V-V_{1/2})]\}$, where $V_{1/2}$ is the membrane potential for half-maximal channel activation.

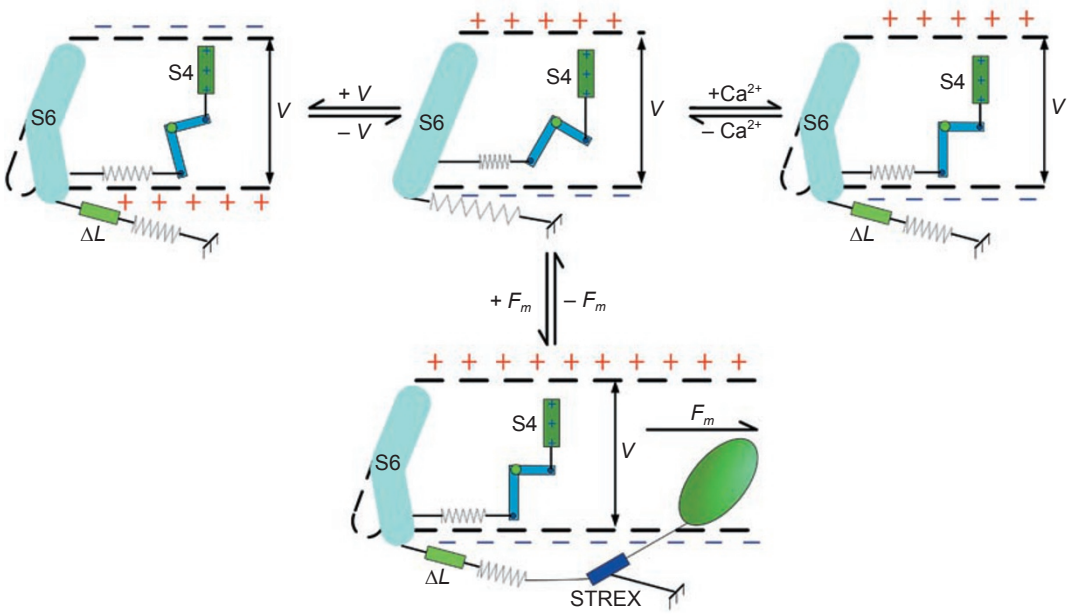

Figure 7 Regulation of BK channel gating by membrane stretch based on the spring model proposed for BK channel gating by Niu *et al* [27]. The membrane stretch (Fm) was conveyed by a membrane-associated protein and STREX, and then transferred to the gate by the linker connecting the transmembrane segment 6 (S6) gate. The charged transmembrane segment 4 (S4) voltage sensor is indirectly connected to the S6 gate through a spring. Addition of amino acids into the linker leads to changes of linker length (ΔL).

When the pressure was increased, the P_o-V curves were left-shifted. However, for both WT and mutant channels, there was almost no further increase in the slope at the relatively high membrane stretch. Our results also indicate that the channels were less sensitive to membrane stretch at relatively high intracellular Ca^{2+} concentrations (Figure 5) or membrane depolarization (Figure 6) (for original data see Supplementary information, Data S1).

Discussion

This study investigated the role of the linker in activation and particularly mechanosensitivity of SAKCaC. Membrane stretch induced an increase in the channel activity in a dose-dependent manner. The sensitivity to membrane stretch increased greatly by shortening the linker, and reduced by lengthening the linker, both in the presence and in the absence of intracellular Ca^{2+} (Figures 4 and 5). Furthermore, the SAKCaC was less sensitive to membrane stretch in the presence of relatively high intracellular Ca^{2+} concentrations or membrane depolarization. These results provide evidence consistent with the mechanical model recently proposed for the BK channel gating by Niu *et al.* [27]. In this model, eight RCK domains form the gating ring, and the linker that connects the S6 gate with RCK1 domain acts as a passive spring, transducing the force to the gate and pulling it open when the membrane is stretched. This model is able to account for the mechanosensitivity of SAKCaC. The membrane tension stretches the channel protein, in turn pulling the linker and increasing the tension on the spring. The tension on the spring opens the gate and thereby increases the SAKCaC activity. Thus we suggest that the membrane stretch is also transferred to the gate by the linker ring complex. More recently, we have demonstrated that the sensitivity of SAKCaC to membrane stretch is detected by the STREX domain locating between RCK1 and RCK2 in the intracellular C-terminus. Deletion of STREX or substitution of serine (S24) within the STREX domain diminishes the stretch sensitivity [28]. Thus, it is obvious that the mechanical forces are detected by the STREX domain and then transferred to the gate by the linker connecting the S6 gate with RCK1. Then a question arises as to how the STREX senses and transmits the force in the membrane to the channel. A simple explanation may be that the STREX domain interacts with a membrane-associated protein, which senses or transmits the force in the membrane, as illustrated in Figure 7. One notable feature of the SAKCaC channels with linkers of different lengths is the high Ca^{2+} sensitivity (Figure 5). The present study suggests that it may arise from the STREX domain, as previously described for the BK channels in human glioma cells [29]. It has been proposed that binding of Ca^{2+} to the

gating ring may increase the P_o by expanding the gating ring to pull the channel open [26, 27, 30]. Therefore, we conclude that the force generated by Ca^{2+} or membrane stretch is transferred to the gate via a common pathway.

The influence of the linker length on the Ca^{2+} and voltage dependence of SAKCaC activation in the presence of membrane stretch was also examined. The P_o versus membrane potential (V) or Ca^{2+} curves were shifted by altering the linker length; shortening the linker had little effect on the slopes of Ca^{2+}-P_o curve and V-P_o curve, suggesting that shortening linker length does not significantly affect the voltage sensitivity and Ca^{2+} sensitivity of SAKCaC. However, lengthening the linker decreased the slopes of P_o-Ca^{2+} curve and voltage-P_o curve, revealing that increasing the linker length decreases the effectiveness of Ca^{2+} and voltage activation of the channel. Furthermore, the P_o against membrane potential (V) curve was left-shifted for SAKCaC when membrane stretch was applied. However, the change in membrane stretch from –10 to –40 mm Hg had little effect on the slope over the range of P_o values, suggesting that membrane stretch does not significantly affect the voltage sensitivity of SAKCaC. These results are consistent with the gating properties observed from the BK channels in mouse skeletal muscle fibers [30, 31], which were activated by membrane stretch without altering the voltage sensitivity. We also evaluated whether the SAKCaC activation by membrane stretch was due to an increase in Ca^{2+} sensitivity. We examined the effects of Ca^{2+} on the SAKCaC gating when membrane stretch was applied. The channel activity was enhanced by membrane stretch, resulting in a parallel left-shift of the Ca^{2+}-P_o curve without affecting the slope, suggesting that Ca^{2+} sensitivity is not altered by membrane stretch. This is in agreement with the results previously described for the BK channels in smooth muscle cells [19] and skeletal muscle cells [32], whose activation in response to membrane stretch is not due to enhancement of Ca^{2+} sensitivity. Although activation of SAKCaC by membrane stretch is independent of the Ca^{2+} or voltage sensitivity, the spring-based gating model suggests that the effect of membrane stretch on the SAKCaC gating is similar to that produced by increasing intracellular Ca^{2+} or membrane depolarization, as illustrated in Figure 7. Binding of Ca^{2+} expands the gating ring and pulls the gate open, while depolarization opens the SAKCaC via the S4 voltage sensor pulling a spring to open the gate [27, 33]. It should be emphasized that tension on the spring is essential for influences of Ca^{2+}, membrane stretch, and voltage on the SAKCaC activation. The tension, caused by either membrane stretch or Ca^{2+} binding or membrane depolarization, would destabilize the closed conformation and favor the open conformation. The spring model predicts that the major effect of change in Ca^{2+} concentration or membrane potential occurs effectively only in the closed-state conformation (Figure 7). The effectiveness of SAKCaC activation by membrane stretch was reduced at relatively high intracellular Ca^{2+} or membrane depolarization, suggesting that when the channel is in open-state conformation, the membrane stretch also becomes less effective.

MS channels are found in many mammalian cell types, in which they are thought to be involved in a number of physiological or pathological functions. In the heart cells, the SAKCaC is thought to be involved in dilation or relaxation of heart. However, it still remains to be determined how SAKCaC functions in the heart under physiological and pathological conditions. The present study has provided evidence shedding light on how the channel is activated in response to membrane stretch.

Materials and Methods

Mutagenesis and expression of SAKCaC

The a subunit of the MS BK channel or SAKCaC (accession number AB 072618) was previously cloned from a chick embryonic heart cDNA library [23]. Introduction of mutations in the linker between S6 and RCK1 domain was described previously [27]. In brief, the linker was shortened by deletion of one amino acid residue from GGSYSAVSG to GGSYAVSG (referred as –1), or lengthened by insertion of three amino acid residues from GGSYSAVSG to GGSYSAAGAVSG (referred as +3), using the QuickChange site-directed mutagenesis kit (Stratagene). All mutations were confirmed by sequencing. Chinese hamster ovary (CHO-K1) cells were used to express WT and mutant BK channels as described previously [2, 23].

Electrophysiology

All recordings were performed at room temperature (22-25 °C) in the inside-out configuration of patch-clamp technique using an Axon 200-B amplifier. Patch pipettes were fabricated from disposable micro-pipettes (Drummond Scientific) in two stages on a PP-83 puller (Narishige Scientific Instrument Lab, Tokyo) to a tip diameter of 0.5-1.0 μm and fire-polished on a MG-83 microforge (Narishige Scientific Instrument Lab, Tokyo) to give an average resistance of 5-10 MΩ in recording solution. Negative pressure was applied via the patch pipette using a pneumatic transducer tester (DPM-IB, BIO-TEK Instruments INC.VT.). Currents were sampled at 5 kHz and filtered at 1.5-2.9 kHz via a four-pole low pass Bessel filter. The program package Clampfit 8.0 was used for data acquisition and analysis. Continuous recordings of 2 000-4 000 ms were used to estimate the channel open probability (P_o). The pipette solution contained 145 mM potassium-gluconate, 1 mM EGTA, 10 mM HEPES, and 5 mM glucose with pH adjusted to 7.4. To minimize potential Ca^{2+}-dependent SAKCaC activation, EGTA was used to eliminate possible cytoplasmic Ca^{2+} concentration via putative Ca^{2+}-permeable stretch-activated channels and/or stretch-induced Ca^{2+} leakage. The bath solution contained the same components as the pipette solution, except for inclusion of indicated Ca^{2+} concentrations. For Ca^{2+} concentration lower than 1 μM, proper concentrations of $CaCl_2$ and 1 mM EGTA were used, on the basis of calculation using the EQCAL program (Biosoft, MO). All reagents were purchased from Sigma.

Acknowledgments

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(**Supplementary information** is linked to the online version of the paper on the Cell Research website.)