

RESEARCH PAPER

Methionine and its derivatives increase bladder excitability by inhibiting stretch-dependent K^+ channels

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Background and purpose: During the bladder filling phase, the volume of the urinary bladder increases dramatically, with only minimal increases in intravesical pressure. To accomplish this, the smooth muscle of the bladder wall must remain relaxed during bladder filling. However, the mechanisms responsible for the stabilization of bladder excitability during stretch are unclear. We hypothesized that stretch-dependent K^+ (TREK) channels in bladder smooth muscle cells may inhibit contraction in response to stretch.

Experimental approaches: Bladder tissues from mouse, guinea pig and monkey were used for molecular, patch clamp, mechanical, electrical, Ca^{2+} imaging and cystometric responses to methionine and its derivatives, which are putative blockers of stretch-dependent K^+ (SDK) channels.

Key results: SDK channels are functionally expressed in bladder myocytes. The single channel conductance of SDK channels is 89pS in symmetrical K^+ conditions and is blocked by L-methionine. Expressed TREK-1 currents are also inhibited by L-methioninol. All three types of bladder smooth muscle cells from mouse, guinea pig and monkey expressed TREK-1 genes. L-methionine, methioninol and methionine methyl ester but not D-methionine increased contractility in concentration-dependent manner. Methioninol further increased contractility and depolarized the membrane in the presence of blockers of Ca^{2+} -activated K^+ conductance. L-methionine induced Ca^{2+} waves that spread long distances through the tissue under stretched conditions and were associated with strong contractions. In cystometric assays, methioninol injection increased bladder excitability mimicking overactive bladder activity.

Conclusions and implications: Methioninol-sensitive K^+ (SDK, TREK-1) channels appear to be important to prevent spread of excitation through the syncytium during bladder filling.

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Abbreviations: AUC, area under the curve; BK, large-conductance Ca^{2+} -activated K^+ channels; BSM, bladder smooth muscle; SAC, stretch-activated nonselective cation channels; SDK, stretch-dependent K^+ channels; SK, small-conductance Ca^{2+} -activated K^+ channel; TEA, tetraethylammonium; TREK, TWIK-related K^+ channel

Introduction

Bladder smooth muscle (BSM) is unique compared to smooth muscle in other organs of the body, as elicited or spontaneous action potentials do not readily spread throughout the tissue (Levin *et al.*, 1986; Brading, 1992). This suggests poor electrical coupling between BSM cells and has the advantage of allowing bladder filling without inducing waves of contraction. Other mechanisms are also involved to maintain a low intravesicular pressure during bladder filling (Levin *et al.*, 1986; Kinder and Mundy, 1987; Brading, 1994;

Hashitani *et al.*, 2000); however, the mechanism by which BSM cells prevent stretch-induced excitation is not clear.

A potential myogenic mechanism can be postulated as several types of K^+ channel have been identified in detrusor myocytes. K^+ channel antagonists are known to prolong action potential duration and promote contractile activity, suggesting a role for K^+ channels in BSM excitability. The repolarization phase is mediated, in part, by the activity of large-conductance Ca^{2+} -activated K^+ (BK) channels (Klockner and Isenberg, 1985; Heppner *et al.*, 1997). The small-conductance Ca^{2+} -activated K^+ (SK) channel blocker, apamin (10^{-7} M) has also been reported to increase the contraction amplitude and frequency, suggesting that SK channel may also be important to the regulation of spontaneous activity in the bladder (Herrera *et al.*, 2003).

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Stretch-dependent K^+ (SDK) conductances have been reported in a variety of tissues (Sackin, 1987; Kim, 1992; Ordway *et al.*, 1995). Their conductance(s) vary from tissue to tissue but fall within the range 20–200 pS in symmetrical K^+ conditions. Recently, we reported that SDK channels are expressed in murine and canine colonic myocytes (Koh and Sanders, 2001; Koh *et al.*, 2001). SDK channels are voltage-independent and are activated by negative pressure. The most likely molecular candidate is the TWIK-related K^+ (TREK) channel (Koh *et al.*, 2001). SDK channels in colonic myocytes were inhibited by sulphur-containing amino acids, such as methionine, which is a relatively specific blocker of SDK channels (Park *et al.*, 2005). Therefore, in this report, we investigated the molecular identification and functional role of SDK channels in bladder using various methionine derivatives.

Materials and methods

Tissue and cell preparation

The animals used for these studies were maintained, and experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Use and Care Committee at the University of Nevada, Reno. BALB/c mice (60–90 days old) and guinea pigs of either sex (200–350 g) were killed by inhalation of isoflurane (Baxter Healthcare, Deerfield, IL, USA) and CO_2 , respectively, followed by cervical dislocation. *Cynomolgus* monkeys (Charles River Laboratories, Sparks, NV, USA) of either sex (2.5–7 years of age) were initially sedated with ketamine (10 mg kg^{-1} ; Fort Dodge Animal Health, Fort Dodge, IA, USA), then were given 0.7 ml Beuthanasia-D solution (pentobarbital sodium and phenytoin sodium, Schering-Plough Animal Health Co, NJ, USA), followed by exsanguination. Bladders were cut open from bladder neck to base and rinsed free of urine with Krebs–Ringer bicarbonate buffer (see Park *et al.*, 2005). Tissues were pinned on the base of a Sylgard-coated dish, and the urothelium and suburothelium were dissected away. Muscle strips were used for isometric force, intracellular microelectrode recording and Ca^{2+} -imaging measurements. To obtain isolated smooth muscle cells, detrusor smooth muscle was digested as described previously (Koh and Sanders, 2001).

Isolation of smooth muscle cells

Small strips of smooth muscle layer from the bladder were equilibrated in Ca^{2+} -free Hank's solution for 10 min. The buffer was replaced with an enzyme solution containing (per ml) collagenase 1.3 mg, bovine serum albumin 2 mg, trypsin inhibitor 2 mg and ATP 0.27 mg. The tissue was placed in a 37°C water bath for 10–12 min without agitation. After three to four washes with Ca^{2+} -free Hank's solution (Koh and Sanders, 2001), the tissue was triturated through a series of three blunt pipettes of decreasing tip diameters. Dispersed cells were stored at 4°C in Ca^{2+} -free solution. Experiments were performed at room temperature within 6 h of cell dispersion.

Expression of TREK-1 channels

Murine TREK-1 cDNA (NM_010607) was subcloned from pCDNA 3.1 into the retroviral expression vector pLPCX (Clontech Laboratories, Mountain View, CA, USA) by restriction enzyme digestion and ligation at *Hind*III and *Not*I. Retrovirus was produced by transfecting the TREK-1/pLPCX construct into RetroPack PT67 packaging cells with TransIT reagent (Mirus Bio, Madison, WI, USA) and repeated selection of surviving clones with $4 \mu\text{g ml}^{-1}$ puromycin. A stable COS-7 cell line expressing TREK-1 was produced by transduction with the TREK-1 retrovirus and selection with $7.5 \mu\text{g ml}^{-1}$ puromycin. Following selection, surviving clones were maintained in DMEM growth medium supplemented with 10% fetal bovine serum and a single clone was chosen for continued propagation. Cells were trypsinized and replated at 10–15% confluency for 24 h in 35 mm plastic culture dishes before electrophysiological recordings.

Patch clamp techniques

The whole-cell and single-channel patch clamp techniques were used to record membrane currents from dissociated murine colonic smooth muscle cells and COS-7 cells expressing TREK-1. Currents were amplified with an Axopatch 200B (Axon Instruments). Data were digitized with either a 12 or 16-bit analogue to digital converter (Digidata 1322A and Digidata 1320A, respectively, Axon instruments, Foster City, CA, USA). Data were stored directly and digitized online using pClamp software (version 9.0, Axon instrument, Foster City, CA, USA). The data were sampled at 2 KHz for whole-cell and 5 KHz for single-channel recordings with low pass filtered at 1 KHz using an eight-pole Bessel filter. The pipette resistances were 1–3 M Ω for whole-cell and 5–8 M Ω for single-channel recordings. In most experiments, the uncompensated series resistance was between 2 and 4 M Ω , and thus voltage errors could have approached 12 mV. Voltage errors were much smaller during steps to negative potentials. We examined the single-channel slope conductance in symmetrical K^+ (140/140 mM). We also used asymmetrical K^+ (5/140 mM) solution. Unitary amplitude at various voltages was fitted to Goldman–Hodgkin–Katz equations. Negative pressure was applied to patches by suction via a 1 ml syringe and pressure–volume relationships were calibrated using a pressure transducer. To measure the dependence of SDK channel open probability on negative pressure, we applied atmospheric pressures ranging from -20 to $-80 \text{ cmH}_2\text{O}$ to patch membrane.

Mechanical responses of bladder detrusor smooth muscles

Standard organ bath techniques were employed to measure the changes in force generated by BSM strips. One end of BSM strip was attached to a fixed mount and the opposite end to an isometric strain gauge (Fort 10, WPI, Sarasota, FL, USA). A resting force of 100–600 mg was applied to set the muscles at optimum length, and the muscles were allowed to equilibrate for ≥ 1 h. Mechanical responses were recorded on a computer running Acqknowledge 3.2.6 (Biopac Systems, Santa Barbara, CA, USA) and measurements of the area under

the curve (AUC) obtained. The AUC was determined as the integral values above the baseline of selected area for 10 min recordings ($g \cdot s$). The AUC for the tissues exposed to tested drugs were compared to the AUC for tissues under control conditions, during an equivalent period of time.

Intracellular microelectrode recordings, Ca^{2+} imaging analysis in tissues and cells

After preparing the tissue, impalements of cells were made with glass microelectrodes having resistances of 80–120 M Ω . Transmembrane potentials were recorded with a standard electrometer (Duo 773; WPI, Sarasota, FL, USA). Data were recorded on a PC running Acknowledge 3.2.6 software. Calcium imaging methods in tissue and cells used in this study are described in detail by Hennig *et al.* (2002) and Bayguinov *et al.* (2000), respectively.

Reverse transcriptase-polymerase chain reaction

Single cells (approximately 60 cells) were collected through applied suction by aspirating them into a wide bore patch clamp pipette (Sutter Instruments, Novato, CA, USA), and stored at $-80^{\circ}C$ until use. Total RNA and cDNA preparation and amplification was identical to that reported by Koh *et al.* (2001). The following PCR primers were used (the GenBank accession number is given in parentheses): for human TREK-1 (KCNK2) (NM00101742Y), nucleotides 1094–1284, amplicon = 191 base pairs (bp); for mouse TREK-1 (KCNK2) (NM010607), nucleotides 1485–1664, amplicon = 180 bp; for human GAPDH (NM002046), nucleotides 403–572, amplicon = 170 bp; for mouse GAPDH (NM608084), nucleotides 345–514, amplicon = 170 bp.

Cystometrics

Mice were anaesthetized and subjected to cystometric evaluation. Briefly, the mice were catheterized with a 22 gauge angiocatheter, and a Crede manoeuvre was performed by applying gentle abdominal pressure until the bladders were emptied. The catheter was then replaced with a 24 gauge angiocatheter that was fastened to tubing connected to a three-way stopcock. One arm of the stopcock was connected to a pressure transducer (Model PT300; Astro-Med Grass Instr., West Warwick, RI, USA) and multichannel recorder (Model 7E, Grass Instruments, West Warwick, RI, USA). Krebs was infused (Model 975; Harvard Instruments, Holliston, MA, USA) at a rate of $25 \mu l \text{ min}^{-1}$ through the other arm of the stopcock and bladder pressure during filling were recorded. Recordings were terminated after a leak was observed.

Statistical analysis of electrophysiological and mechanical responses

Data are expressed as means \pm s.e.m. The Student's *t*-test was used where appropriate to evaluate differences in the data. *P*-values less than 0.05 were taken as a statistically significant differences. The *n* values reported in the text refer to the number of recordings from the muscle strips, which is

equivalent to the number of animals used unless otherwise stated.

Drugs and chemicals

Arachidonic acid, tetraethylammonium (TEA) chloride, methionine methylester, D-methionine RT and apamin were

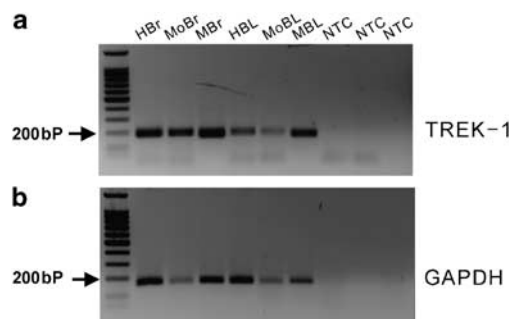


Figure 1 mRNA expression of TREK-1 in BSMs cells from mouse, monkey and human bladder. (a) Two per cent agarose/TAE gel showing results of PCR amplifications using probes specific for TREK-1. Lane 1 shows the molecular weight marker used to indicate the size of the PCR fragments. RT-PCR demonstrated the presence of TREK-1 in human brain (HBr, 191 bp), mouse brain (MoBr, 180 bp) and monkey brain (MBr, 191 bp) tissues. TREK-1 was also detected in smooth muscle cells in human bladder (HBL), mouse bladder (MoBL) and monkey bladder (MBL) using the same primers. Human primers were used for monkey tissues. Reverse transcription control on each RNA sample used a cDNA reaction as template for which the reverse transcriptase was not added (NTC), controlling for genomic DNA contamination in the source RNA. (b) GAPDH (170 bp) was used as an internal control to test for DNA contamination in the RNA preparations. BSM, bladder smooth muscle; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; TREK, TWIK-related K^{+} .

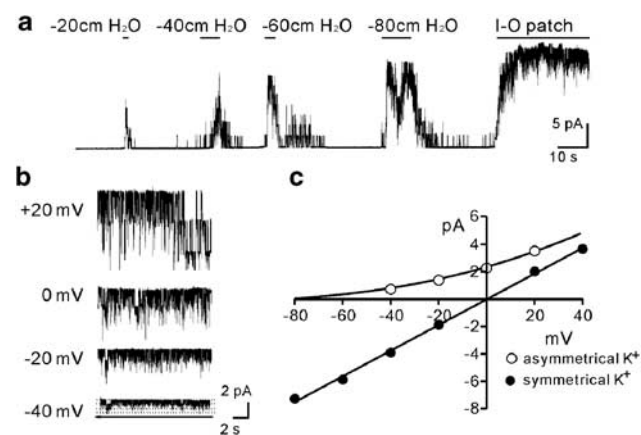


Figure 2 Negative pressure activated SDK channel in bladder myocytes. (a) Freshly dispersed bladder myocytes were exposed to pressures ranging from -20 to $-80 \text{ cm H}_2\text{O}$ at holding potential of 0 mV in asymmetrical K^{+} gradient ($5/140 \text{ mM}$). I-O patch denotes inside-out patch. (b) Representative traces in asymmetrical K^{+} gradient ($5/140 \text{ mM}$) at various holding potentials from excised patches. Solid line denotes channel close and dotted line channel open. (c) The unitary current amplitude–voltage (*I*–*V*) plot of the single-channel conductance was well fitted by the Goldman–Hodgkin–Katz equation in asymmetrical K^{+} solutions and with linear regression in symmetrical K^{+} solutions. SDK, stretch-dependent K^{+} channel.

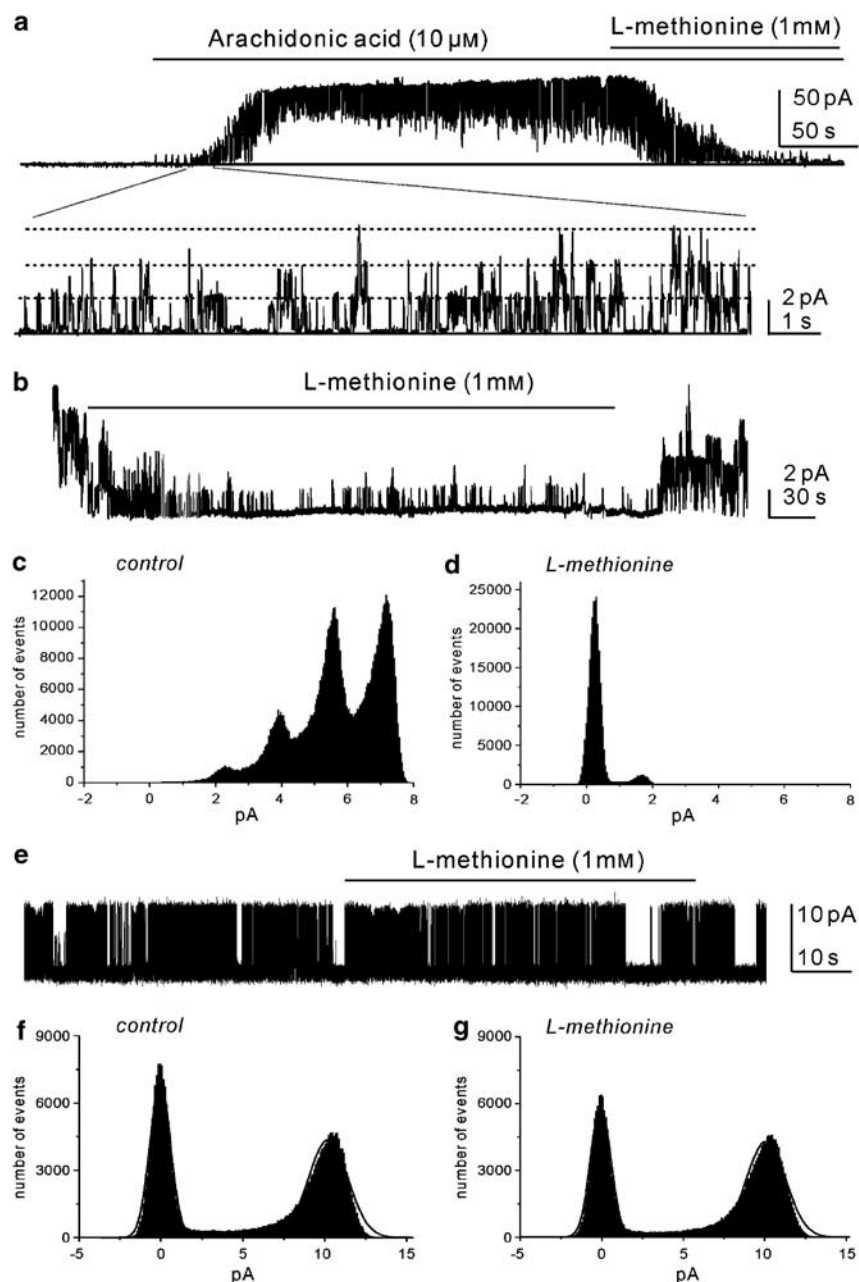


Figure 3 The effect of L-methionine on SDK and BK channels in excised patches. (a) Arachidonic acid increased the open probability of SDK channels and L-methionine decreased the open probability of SDK channels at 0 mV in asymmetrical K^+ gradients. The lower panel in (a) shows responses on an extended time scale. Solid line denotes channel closed and dotted line denotes channel openings. (b) Excised patches revealed a high open probability of SDK channels and L-methionine (1 mM) decreased the open probability of SDK channels at 0 mV in asymmetrical K^+ gradients. (c and d) The amplitude histogram was obtained from 1 min of channel recording before and after L-methionine. (e) Inside-out patches demonstrated the opening of BK channels at +20 mV in asymmetrical K^+ gradients. (f and g) The representative amplitude histogram before and after L-methionine. BK, large-conductance Ca^{2+} -activated K^+ channel; SDK, stretch-dependent K^+ channel.

obtained from Sigma Chemical Co (St Louis, MO, USA). Methioninol and L-methionine were purchased from Fluka Biochemika, Buchs, Switzerland. These drugs were dissolved in test solutions directly. Arachidonic acid was dissolved in ethanol (10 mg ml^{-1}) and stored under nitrogen at -20°C before experiments. The final concentration of ethanol was less than 0.1% and had no effect at this concentration.

Results

The expression of stretch-dependent K^+ channels in bladder smooth muscle

Smooth muscles in organs that have a reservoir function often show a SDK conductance. A possible molecular candidate for this conductance is TREK-1 channel (Koh and Sanders, 2001; Koh *et al.*, 2001). To confirm that BSM cells

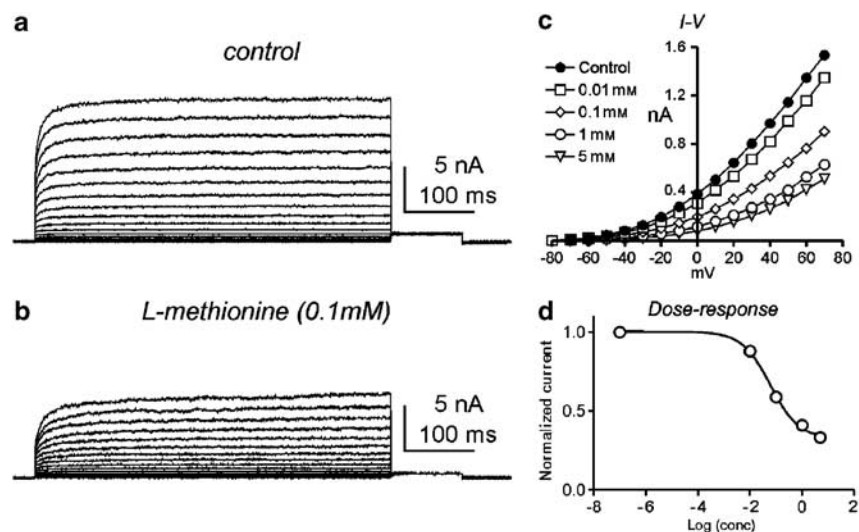


Figure 4 Concentration-dependent response of TREK-1 currents to L-methionine. (a) Representative TREK-1 currents were recorded from COS-7 cells. (b) L-methionine decreased TREK-1 currents. (c) I-V relationship of L-methionine on TREK-1 currents in various concentrations of L-methionine. (d) Dose-response relationship analysed from maximum currents at +70 mV of test potentials and fitted to a sigmoidal concentration-response function. TREK, TWIK-related K^+ .

also express TREK-1, reverse transcription-PCR was performed on isolated BSM cells from murine, human and monkey tissues. TREK-1 was expressed in all BSM cells from three species (Figure 1). TREK-2 and TRAAK, mechanosensitive two-pore K^+ channel candidates, were not expressed in murine BSM cells (data not shown).

To isolate SDK channel in murine BSM cells, we performed single-channel recordings. Single-channel currents evoked by negative pipette pressure were obtained in cell-attached patches of BSM cells. Patches were exposed to pressures ranging from -20 to -80 cmH₂O (holding potential was 0 mV in asymmetrical K^+ gradient (5/140 mM) to exclude contamination from nonselective cation conductances). Pipette solutions contained charybdotoxin (200 nM) to block BK and intermediate-conductance Ca^{2+} -activated K^+ channels. To be sure that the effects of negative pressure were reversible and lacked desensitization, incremental levels of negative pressure were applied to the same patch. Increasing the level of negative pressure increased NP_o (number of channels (N) \times open probability (P_o)) of the K^+ channels. Application of -80 cmH₂O to the cell-attached patch caused a maximal increase in NP_o (Figure 2a). When atmospheric pressure was restored, the open probability returned to near zero. The cell-free condition (inside-out patch) induced maximal channel openings (Figure 2a). Single-channel conductance in asymmetrical K^+ gradient (5/140 mM) at various holding potentials was also measured (Figure 2b). The unitary current amplitude-voltage (I-V) plot of the single-channel conductance was well fitted with the Goldman-Hodgkin-Katz equation, suggesting that currents were due to K^+ channels (Figure 2c). The open probability did not differ from negative to positive potentials (Figure 2b), suggesting that regulation of the channel was voltage-independent. Under symmetrical K^+ conditions (140/140 mM), the single-channel conductance was 89 ± 8 pS ($n = 4$; Figure 2c). These recordings demonstrate that BSM cells express SDK channels with properties similar to TREK-1

channels (Koh and Sanders, 2001; Koh *et al.*, 2001). These SDK channels in murine BSM cells were not blocked by 4-aminopyridine (up to 5 mM), TEA (up to 10 mM), glibenclamide (1 μ M), apamin (300 nM) and charybdotoxin (200 nM) (data not shown).

To investigate the role of SDK channels on BSM function, we used the amino acid methionine, which has been shown to block SDK channels (Park *et al.*, 2005) and arachidonic acid known to open TREK-1 channels (Maingret *et al.*, 1999). Cells were held at 0 mV in asymmetrical K^+ gradient (5/140 mM). The application of arachidonic acid (10 μ M) increased the activity of SDK channels (Figure 3a) and the additional application of L-methionine (1 mM) decreased the open probability of arachidonic acid-activated unitary currents. Excising patches increased the open probability of SDK channels ($NP_o = 3.2 \pm 0.7$, $n = 4$; Figure 3b). The amplitude histogram in Figures 3c and d was obtained from 1 min of single-channel recording. Application of L-methionine (1 mM) dramatically decreased the open probability of SDK channels ($P_o = 0.1 \pm 0.3$, $P < 0.01$ compared to control excised patch). The effects of L-methionine were reversible.

We also tested the effects of L-methionine on the open probability of BK channels by omitting charybdotoxin from the pipette solution. In inside-out patches, the open probability of BK channels (free $[Ca^{2+}]_i = 5 \times 10^{-7}$ M) was 0.35 ± 0.1 at 0 mV in asymmetrical K^+ gradients ($n = 4$; Figure 3e). L-methionine (1 mM) did not change P_o (0.34 ± 0.2 ; Figures 3f and g). The inactive enantiomer, D-methionine (1 mM) had no effect on SDK channels (data not shown). These data suggest that L-methionine is a specific inhibitor of the SDK channels in bladder myocytes.

To compare the properties of mTREK-1 cloned from murine smooth muscle with those of the native SDK currents, mTREK-1 was expressed in COS-7 cells. Voltage-clamp recordings from COS-7 cells expressing TREK-1 revealed a rapidly activating and non-inactivating current (Figure 4a). Similar currents were not detected in

nontransfected cells. L-methionine decreased TREK-1 currents in a concentration-dependent manner (0.01–5 mM; Figures 4b and c). The data in Figure 4d were derived from the maximum currents at +70 mV of test potential from four cells. The calculated IC_{50} of the sigmoidal concentration–response function was 0.61 mM with a slope of 0.55. These data support our hypothesis that L-methionine produces depolarization and increased excitability of murine bladder by inhibiting SDK or TREK-1 channels.

The effects of methionine and its derivatives on bladder contractility

BSM strips, isolated from mouse, guinea pig or monkey, were pretreated with tetrodotoxin (1 μ M) to exclude neural activity. At a resting tone of 100 mg, L-methionine (1 mM) caused a slow increase in tone reflected by an increase in the

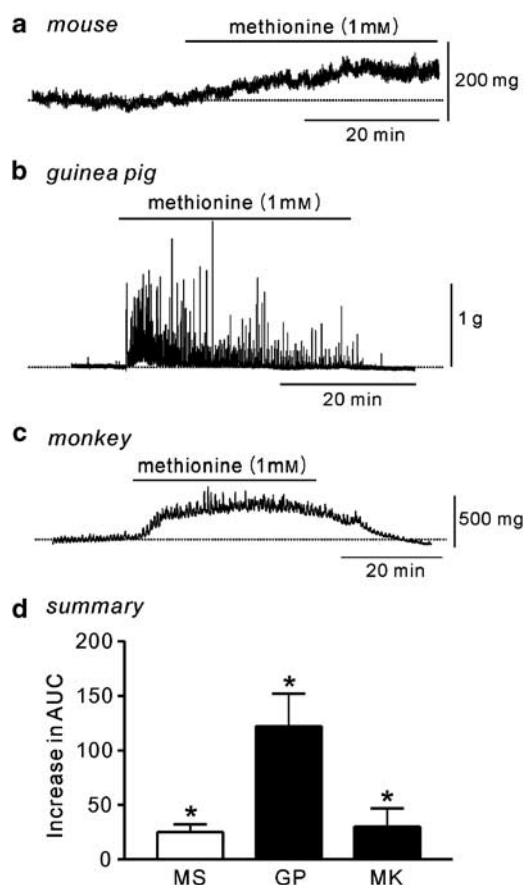


Figure 5 Effect of L-methionine on contractility of bladder strips. (a) Strips of murine bladder did not display spontaneous contractile activity. Addition of L-methionine (indicated by horizontal black bars) increased the tone. (b) Bladder strips from guinea pig showed spontaneous contractile activity that was greatly enhanced by L-methionine. (c) Bladder strips in monkey rarely showed spontaneous contractile activity. The application of L-methionine increased the tone, amplitude and frequency of contractions. (d) Summarized data show the mean increase in the AUC (mg·s) after L-methionine, compared to control, in mouse (MS), guinea pig (GP) and monkey (MK). All experiments were performed in the presence of tetrodotoxin (1 μ M). *Significantly increased over control AUC ($P < 0.05$, Student's *t*-test). AUC, area under the curve.

AUC in murine BSM strips ($n=9$; Figures 5a and d). The inactive enantiomer, D-methionine (1 mM), had no effect (data not shown). Interestingly, in BSM strips from guinea pigs, small spontaneous contractions that varied in amplitude were observed under control conditions. Addition of L-methionine (1 mM) resulted in a large increase in the frequency (from 0.7 ± 0.7 cpm (cycles per minute) to 2.9 ± 0.6 cpm) and amplitude (from 0.1 ± 0.1 to 1.1 ± 0.6 g) along with an increased AUC ($n=5$; Figures 5b and d). Monkey BSM strips were quiescent in control conditions. Addition of L-methionine resulted in an increase in tone that was maintained as long as the drug remained in the bath, leading to an increased AUC ($n=4$; Figures 5c and d).

We also investigated the effects of two methionine derivatives methioninol and methionine methyl ester on BSM contractility. Methioninol (1 mM) had an effect similar to that of L-methionine in murine BSM strips (increased AUC, $n=5$; Figures 6a and d) and guinea pig (increased AUC; frequency increased from 0.8 ± 0.4 to 3.0 ± 0.7 cpm, $P < 0.05$; amplitude increased from 0.7 ± 0.4 to 1.6 ± 0.5 g, $P < 0.05$, $n=6$; Figures 6b and d). In monkey bladder strips, methioninol also induced large phasic contractions (AUC increased; frequency increased from 0.2 ± 0.26 to 1.2 ± 0.38 cpm, $P < 0.05$; amplitude increased from 0.11 ± 0.07 to 0.23 ± 0.8 g, $P < 0.05$, $n=10$; Figures 6c and d). Methionine methyl ester also had effects similar to those of L-methionine on BSM contractility in the three species (Figures 6e–h). Furthermore, we obtained concentration-dependent effects of methioninol on murine BSM contractility (Figure 7) and found the EC_{50} of methioninol was 1.7 mM (0.88 Hill slope; Figure 7c).

The possible ionic mechanisms of methioninol on bladder excitability

We tested the effects of methioninol on bladder contractility in the presence of TEA and/or apamin. TEA (1 mM) was used to inhibit BK channels in murine BSM strips and caused by itself a small increase in tone ($n=5$; Figures 8a and d). The addition of methioninol (1 mM) to the TEA-treated strips increased tone further and phasic contractions were observed (Figures 8a and d). The increase in AUC was significantly different from that induced by TEA (1 mM) alone ($P < 0.05$, $n=8$; Figure 8d). In guinea pig BSM strips, TEA increased the amplitude of contractions from 0.2 ± 0.1 to 0.6 ± 0.3 g ($P < 0.05$) but had no effect on frequency (from 1.6 ± 0.8 to 2.2 ± 0.2 cpm, $P=0.10$), although the AUC increased ($n=5$). The addition of methioninol (1 mM) increased contractility as shown by a further increase in AUC ($P < 0.05$ compared with TEA, $n=7$), frequency (3.8 ± 0.2 cpm, $P < 0.01$ compared with TEA) and amplitude (1.2 ± 0.2 g, $P < 0.05$ compared with TEA). The effect of methioninol in the presence of TEA on monkey bladder strips was similar to those observed in murine or guinea pig tissues (data not shown).

To investigate the effects of methioninol on SK channels, we tested the effect of apamin, a blocker of these channels. Apamin (300 nM) induced spontaneous contractions with a small increase in basal tone in murine bladder strips ($n=5$; Figures 8b and e). The addition of methioninol (1 mM) to the apamin-exposed strips further increased

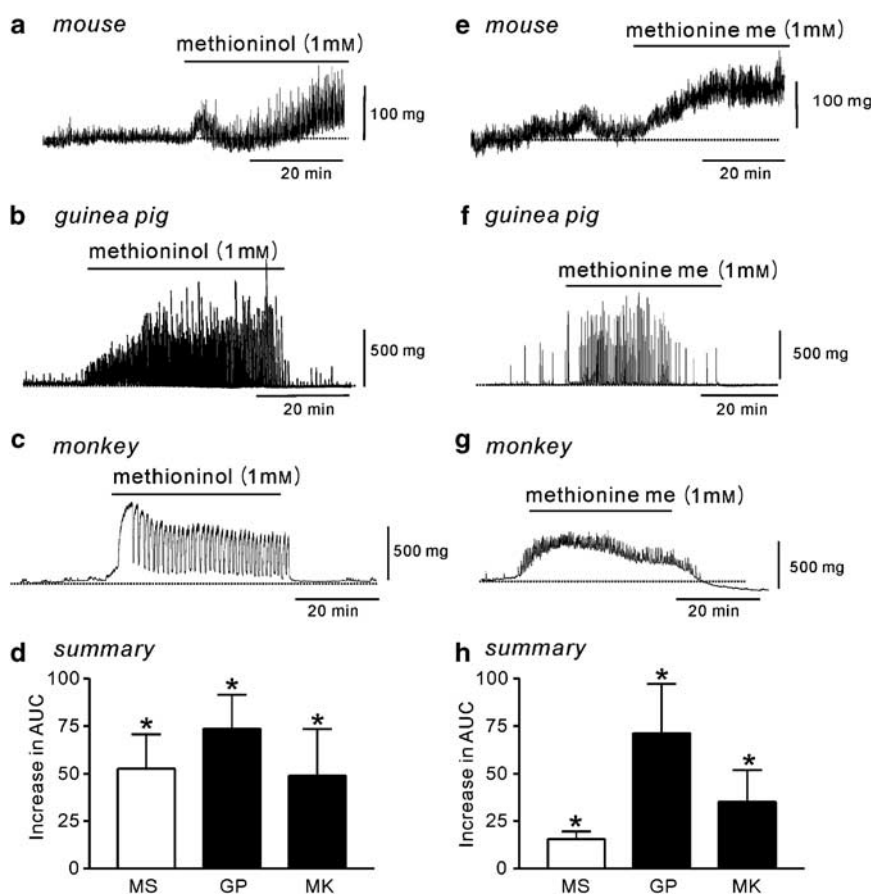


Figure 6 Effects of methioninol and methionine methyl ester on the mechanical activities of bladder strips. (a–c) Methioninol increased contractility in strips of bladder in mouse, guinea pig and monkey (indicated by black bars). (d) Summarized data show the mean increase in the AUC (mg·s) after methioninol compared with control in mouse (MS), guinea pig (GP) and monkey (MK). (e–g) Methionine methyl ester (methionine me) increased contractility in bladder strips from mouse, guinea pig and monkey. (h) Summarized data show mean increase in the AUC (mg·s) after methionine methyl ester compared with control in mouse (MS), guinea pig (GP) and monkey (MK). All experiments were performed in the presence of tetrodotoxin (1 μ M). *Significantly increased over control AUC ($P < 0.05$, Student's *t*-test). AUC, area under the curve.

basal tone ($P < 0.01$; Figures 8b and e). Treatment of murine BSM strips with a combination of TEA and apamin increased tone compared with control strips ($n = 5$; Figures 8c and f), and the further addition of methioninol (1 mM) significantly ($P < 0.05$) increased the overall muscle activity, compared with response with TEA and apamin (Figure 8f).

We also performed conventional microelectrode recordings on murine BSM cells to examine the effect of methioninol on membrane potential. Bladder tissues were electrically quiescent (18 of 25 muscle strips). Figure 9 demonstrates typical recordings of electrical activity from murine BSM showing infrequent spikes in control conditions. Charybdotoxin (200 nM) was used to block BK channels and depolarized BSM from -53 ± 2 to -46 ± 1 mV ($P < 0.05$, $n = 7$) and increased the frequency of spontaneous firing from 2.3 ± 0.3 to 8.0 ± 1.7 cpm. The further addition of methioninol (1 mM) caused BSM cells to become more depolarized (to -30 ± 1 mV, $P < 0.05$ compared with charybdotoxin alone) and showed sustained high-frequency spiking activity. Treatment of quiescent BSM with methioninol (1 mM) also induced depolarization from -51 ± 2 to -45 ± 3 mV ($P < 0.05$, $n = 18$).

The effects of methioninol on Ca^{2+} activity and cystometry in murine bladder

We used Ca^{2+} imaging to examine whether mechanical stretch of BSM was able to increase intracellular Ca^{2+} transients or induce Ca^{2+} waves through the BSM syncytium. Spatio-temporal maps showing the effect of active and maintained stretch on Ca^{2+} activity in murine BSM are shown in Figure 10. Propagating intercellular Ca^{2+} waves were rarely seen in control conditions. The majority of events observed consisted of independent Ca^{2+} transients in individual BSM cells. No propagating Ca^{2+} waves were observed in control BSM in either slack or stretched conditions (Figures 10a, b and d). After addition of TEA (1 mM), intercellular Ca^{2+} waves spread rapidly over short distances (< 6 cells wide) orthogonal to the long axis of the BSM cells (Figures 10c–e). These waves propagated at a velocity of $27 \pm 70 \mu\text{m s}^{-1}$ for $31 \pm 6 \mu\text{m}$ (range 16–33 μm ; 44 events, $n = 3$; Figure 10e), but these Ca^{2+} waves did not induce contractions. The further addition of methioninol (1 mM) induced intercellular Ca^{2+} waves that propagated longer distances across the preparation and were associated with contractions (Figures 11a–c). These waves propagated at a velocity of $241 \pm 73 \mu\text{m s}^{-1}$ for $62 \pm 32 \mu\text{m}$ (range 16–218 μm , 23 events, $n = 3$; Figures 11d–f).

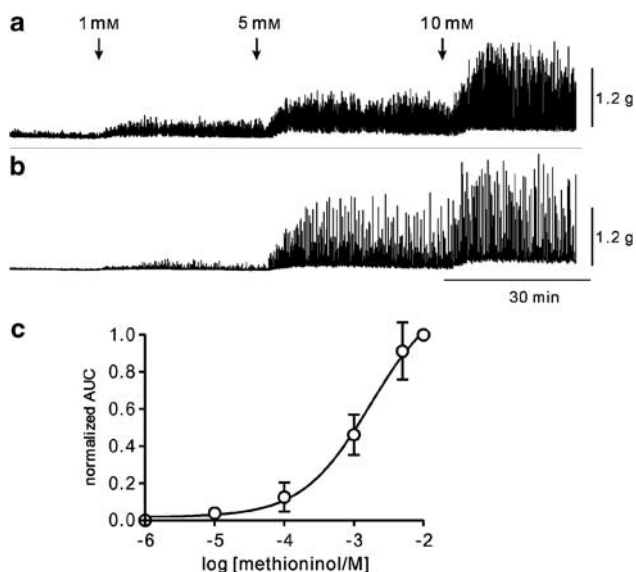


Figure 7 Effect of methioninol on murine bladder contractility. (a and b) Representative traces showing the effect of methioninol on bladder contractility in a concentration-dependent manner. (c) The AUC was normalized for the effects of methioninol (10 mM) and a concentration–response curve was fitted (Hill equation; $n = 6$). AUC, area under the curve.

We performed cystometric assays in mice to examine the functional effect of blocking SDK channels in BSM, using methioninol ($n = 5$). Methioninol injection (i.p.; 2 mg g^{-1}) induced increased contractile activity, resembling that of overactive bladder, during the filling phase (Figure 12). These data suggested that SDK (TREK-1) channels were functionally expressed in bladder and facilitated bladder compliance.

Discussion and conclusion

The urinary bladder undergoes substantial stretch during filling with only minimal increases in intravesical pressure before a contractile response is elicited. To accomplish this, the BSM must remain relaxed. In addition to neural reflexes, ionic conductances activated by distortion of the plasma membrane may be an important component of the myogenic response. Several mechanosensitive ion channels have been described in visceral smooth muscle, including swelling-activated Cl^- channels (Dick *et al.*, 1998), stretch-activated, nonselective cation channels (Wellner and Isenberg, 1993), Cl^- channels (Ji *et al.*, 2002) and Ca^{2+} channels (Farrugia *et al.*, 1999). Activation of any of these channels under physiological ionic gradients results in

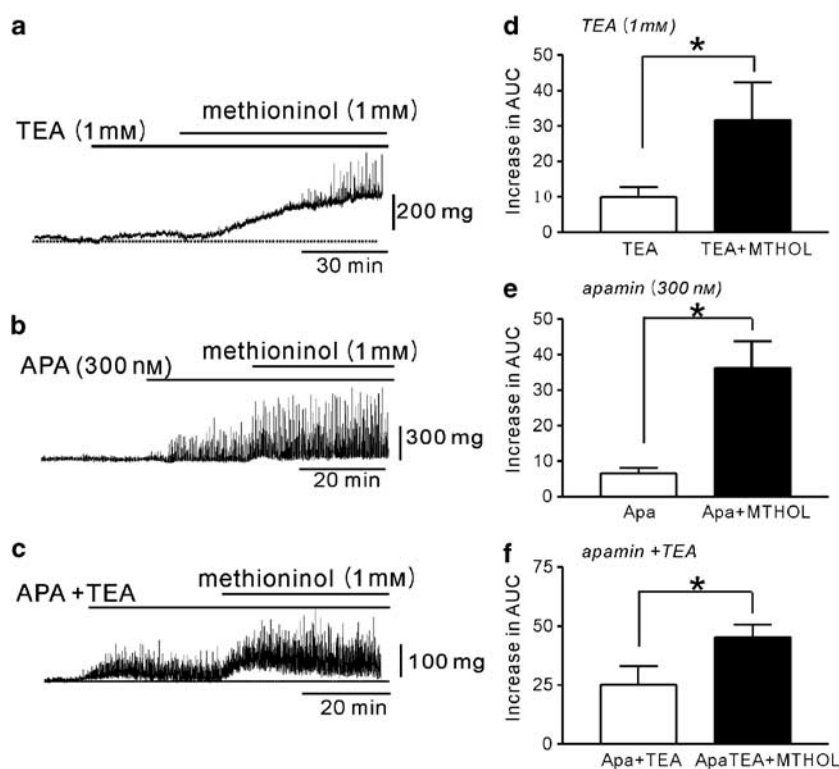


Figure 8 The effect of methioninol on bladder contractility in the presence of K^+ channel blockers. (a) Tetraethyl ammonium (TEA) increased contractility in strips of murine bladder. The application of methioninol further increased contractility in the presence of TEA. (b) Apamin (APA) increased contractility in murine bladder strips. The application of methioninol further increased contractility in the presence of apamin. (c) The application of both TEA (10 mM) and apamin (APA, 300 nM) increased contractility in murine bladder strip. The application of methioninol further increased contractility in the presence of both blockers. (d–f) Summarized data show the mean increase in the AUC ($\text{mg} \cdot \text{s}$) after methioninol (MTHOL) in the presence of TEA (d), apamin (Apa) (e) and both blockers (f). All experiments were performed in the presence of tetrodotoxin ($1 \mu\text{M}$). *Significant difference between means shown; $P < 0.05$, Students' *t*-test. AUC, area under the curve.

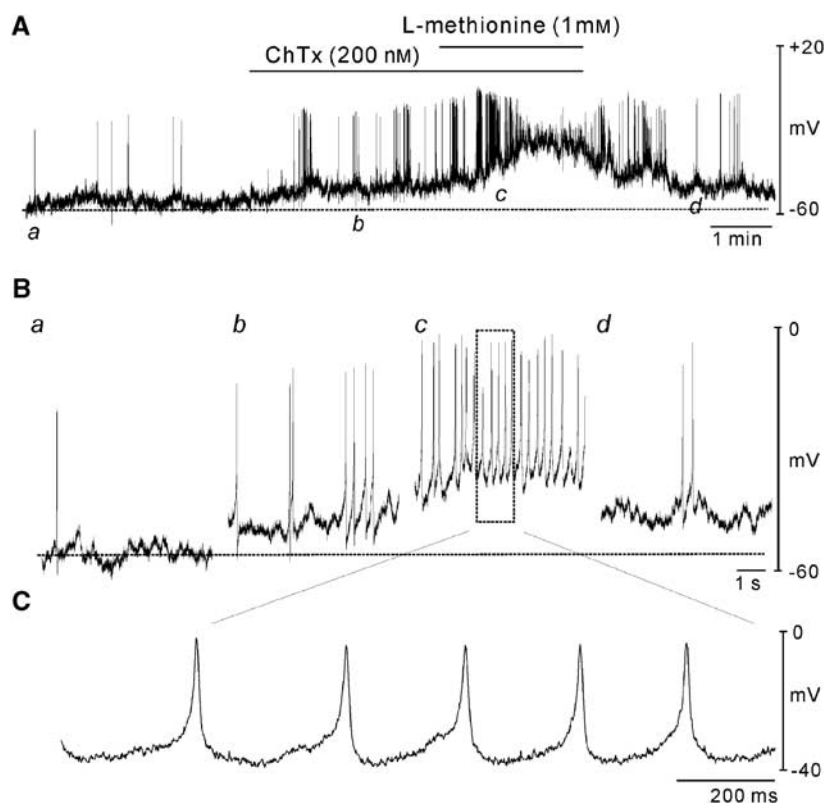


Figure 9 Effects of L-methionine on the membrane potential of murine BSM cells. (A) Intracellular microelectrode recordings were made from intact bladder muscle strips. Charybdotoxin (ChTx) induced depolarization. L-methionine caused further depolarization in the presence of ChTx. (B) Panels a–d: the lower traces (b and c) are expanded from (A). (C) L-methionine induced continuous firing of action potentials. Dashed lines in each panel denote membrane potentials under control conditions. BSM, bladder smooth muscle.

inward currents, which may lead to depolarization and muscle contraction.

There has been speculation in the literature for at least 20 years that BSMs are 'stretch receptors' for myogenic responses, but no experimental evidence exists to support this hypothesis. Our group has reported that expression of SDK channels in colonic myocytes also encoded with the TREK-1 gene as a molecular candidate (Koh and Sanders, 2001; Koh *et al.*, 2001). Membrane stretch is an effective activator of SDK and TREK channels (Maingret *et al.*, 1999). Therefore, we hypothesized that SDK (TREK) channels may provide a mechanism to inhibit contraction in response to stretch in BSM cells. In our present experiments, BSM cells from different species exhibited different patterns of electrical activity. In particular, guinea pig bladder strips were extensively studied previously, which revealed regular spontaneous mechanical and electrical activity (Hashitani *et al.*, 2000). However, monkey and murine BSM cells did not exhibit spontaneous contractions. The different contractile patterns in the three species were not due to a species-related expression of TREK-1 channels, as these were equally expressed in all three species. However, monkey and murine BSM cells did not exhibit spontaneous contractions.

We have reported that sulphur-containing amino acids may be useful blockers of SDK channels (Park *et al.*, 2005) and L-methionine was found to be a relatively selective blocker of SDK channels with little or no effect on other known K^+ conductances (Park *et al.*, 2005). In present study,

L-methionine and its two derivatives, methioninol and methionine methyl ester, were found to inhibit both the native SDK channels and the TREK-1 channels expressed in COS cells, activated by arachidonic acid and stretch. These effects corresponded to the effects of L-methionine or methioninol on BSM excitability in murine, guinea pig and monkey bladders, strongly suggesting that the increased excitability of BSM was causally related to inhibition of the SDK, and probably TREK-1, channels. However, the mechanism of methionine action needs further study.

BK channels have been shown to be involved in the regulation of membrane potential in BSM (Heppner *et al.*, 1997), and these channels are activated by depolarization and by intracellular Ca^{2+} release through ryanodine receptors (Herrera and Nelson, 2002). To exclude the effects of methioninol on BK channels, we examined its effects on contractility and electrical activity of the SDK channels in the presence of the BK channel blockers, TEA or charybdotoxin. Although both mechanical and electrical events were increased in the presence of the BK channel blockers, addition of methioninol further increased bladder contractility. In patch clamp experiments, L-methionine did not affect the open probability of BK channels, suggesting that the effects of methioninol on contractility and membrane potentials were also independent of BK channels. SK channels are also present in bladder myocytes (Herrera and Nelson, 2002; Herrera *et al.*, 2003), but methioninol in the presence of apamin (a SK channel blocker) further increased

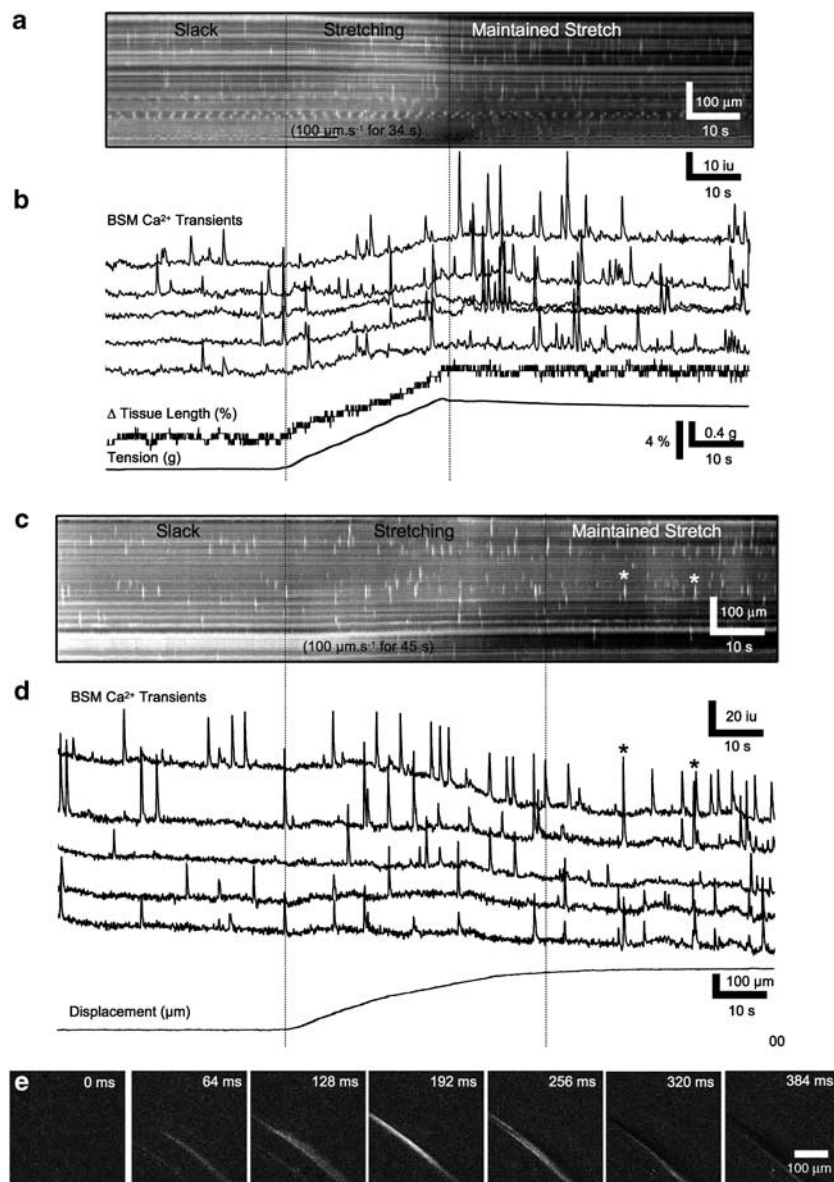


Figure 10 Effects of stretch on Ca^{2+} transients of murine BSM. (a) Shown a spatio-temporal map of Ca^{2+} transients in a flat-sheet preparation of bladder detrusor muscle during a stretch sequence. At a slack length, isolated individual firing of BSM cells prevailed (see individual Ca^{2+} transients from five cells in b). During active stretch, the pattern of firing did not change appreciably; however, when the active stretch was stopped and the length maintained, there appeared to be a brief period in which BSM firing was enhanced. (c) The response to stretch in the presence of TEA (1 mM) during which some Ca^{2+} transients appeared to propagate short distances (see asterisks). (d) Traces of individual BSM cells in TEA (1 mM). (e) Individual frames during a short propagating event (first asterisk in c). BSM, bladder smooth muscle; TEA, tetraethylammonium.

contractility, suggesting that the effect of methioninol was independent of SK channels. Furthermore, methioninol also showed significant increase of bladder contractility in the presence of both SK and BK channel blockers. In addition, spontaneous transient outward currents have been previously reported in murine BSM cells (Weidelt and Isenberg, 2000) and intracellular Ca^{2+} level can alter the open probability of BK or SK channels. However, methioninol did not affect intracellular Ca^{2+} sparks in isolated cells (data not shown). These observations clearly indicate that the effects of methioninol are not mediated through BK or SK channels

Only a small proportion of murine BSM preparations display phasic electrical activity. We can only speculate as to why some BSM strips were electrically active, whereas others remained quiescent. During the pinning of each strip, variations in the degree of tension may have altered the excitability. Similarly, each preparation may have had a slightly different stretch sensitivity. Extreme stretch can activate nonselective cation channels (Wellner and Isenberg 1993) and induce increased excitability. The mechanisms underlying phasic contractions are likely to involve coupling between BSM cells. Normally, the individual random firing of BSM cells results in an asynchronous generation of tone

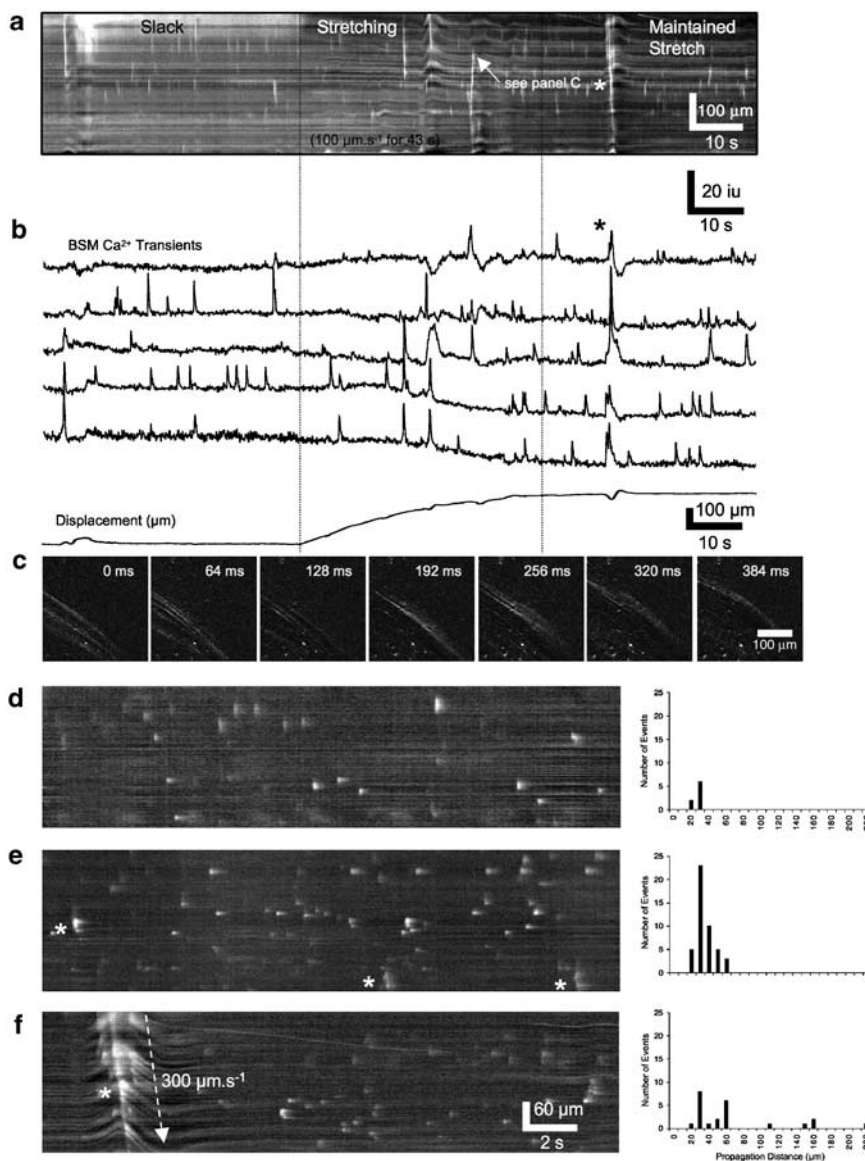


Figure 11 Effects of stretch on Ca^{2+} transients in the presence of L-methioninol. (a) A spatio-temporal map of Ca^{2+} activity in a flat-sheet preparation of bladder detrusor muscle during a stretch sequence in the presence of TEA (1 mM) and L-methioninol (1 mM). In addition to Ca^{2+} transients in individual BSM cells, during stretch, the frequency and distance of propagation of Ca^{2+} waves increased dramatically, often spreading across the entire field of view and were associated with large contractions of the tissue (see asterisk in panels a and b). An example of a propagating Ca^{2+} wave is shown in (c). (d) An example of Ca^{2+} transients during maintained stretch in control conditions. It was rare to see Ca^{2+} transients that appeared to propagate. In the presence of TEA during maintained stretch (e), occasional Ca^{2+} waves appear to propagate short distances (<4 cells; see asterisks in panel e); however, these events did not cause contraction of the tissue. (f) In the presence of TEA and L-methioninol, Ca^{2+} waves propagated large distances and were associated with considerable movements of the tissue (see asterisk in panel f). The number of events and the distance each Ca^{2+} propagated in each conditions are shown to the right of each spatio-temporal maps (d–e, $n=4$). BSM, bladder smooth muscle; TEA, tetraethylammonium.

caused by poor electrical coupling (Turner and Barding, 1997). In those situations where coupling is enhanced between BSM cells (for example, after addition of methionine), muscle action potentials are able to spread through the muscle syncytium causing a synchronous contraction of many muscle cells, giving rising to measurable phasic contractile activity.

Stretch of bladder myocytes has been shown to activate stretch-activated nonselective cation (SAC) channels (Wellner and Isenberg, 1993). This can lead to contraction via Ca^{2+} influx through SAC, which trigger Ca^{2+} action

potentials in response to depolarization and can induce Ca^{2+} release from intracellular stores. However, stretch does not activate contraction in bladder, but instead results in stabilization of membrane potential. Thus, if cation influx via SACs is fundamental to myogenic responses to stretch, then influx of these ions is counterbalanced by another mechanism that reduces excitability. This cation influx is not compatible with stabilization of membrane potentials. In our Ca^{2+} imaging experiments, to examine the response to stretch, we found that Ca^{2+} release in BSM is minimal in response to moderate levels of tissue stretch. BK channels

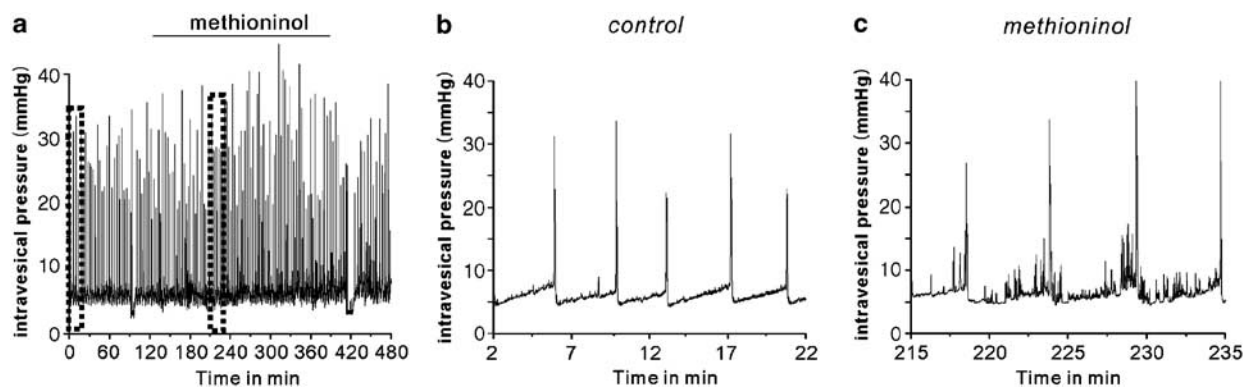


Figure 12 The effect of methioninol on cystometrograms in mice. (a) Long recordings of cystometrogram before and after methioninol (i.p., 2 mg g^{-1}). (b and c) Cystometrogram displayed on an expanded time scale before and after methioninol, respectively. Representative trace from five experiments.

were likely to be activated by stretch in these preparations as Ca^{2+} transients were significantly increased in the presence of a BK blocker. At higher degrees of stretch, BSM cells exhibit an increased frequency of Ca^{2+} transients; however, responses varied between preparations. Although we measured the overall tension on the bladder segment, the complicated non-orthogonal arrangement of muscle fibers in this tissue made it impossible to precisely determine the length/tension of cells within the field of view. Some preparations showed only small increases in BSM firing during active stretch (ramp) compared with maintained stretch and suggests the threshold required to increase the frequency of Ca^{2+} transients occurred at a higher level during active stretch (Figures 10a and b). In other preparations, an increase in the frequency of BSM Ca^{2+} transients occurred at much lower levels of stretch and was comparable to the frequency observed during maintained stretch (Figures 11a and b). However, the most interesting finding was the effect of methioninol on stretched BSM, appearing to alter the nature of BSM syncytium, allowing Ca^{2+} waves to spread long distances through the tissue. Such effects were not seen under control conditions or even after TEA. In the whole bladder, cystometric data suggested that a component of bladder overactivity may involve the inhibition of SDK channel in bladder myocytes.

In conclusion, SDK channels appear to be important to prevent spread of excitation through the syncytium during bladder filling. Further characterization of methionine compounds may be useful to develop a new group of drugs to be used clinically to target bladder excitability.

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Conflict of interest

The authors state no conflict of interest.

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