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[Original Article]

Swelling-activated and arachidonic acid-induced currents are TREK-1 in rat bladder smooth muscle cells

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Abstract

Using the perforated patch voltage clamp, we investigated swelling-activated ionic channels (SACs) in rat urinary bladder smooth muscle cells. Hypo-osmotic (60%) bath solution increased a membrane current which was inhibited by the SAC inhibitor, gadolinium. The reversal potential of the hypotonicity-induced current shifted in the positive direction by increasing external K^+ concentration. The hypotonicity-induced current was inhibited by extracellular acidic pH, phorbol ester and forskolin. These pharmacological properties are identical to those of arachidonic acid-induced current present in these cells, suggesting the presence of TREK-1, a four-transmembrane two pore domain K^+ channel. Using RT-PCR we screened rat bladder smooth muscles and cerebellum for expression of TREK-1, TREK-2 and TRAAK mRNAs. Only TREK-1 mRNA was expressed in the bladder, while all three were expressed in the cerebellum. We conclude that a mechanosensitive K^+ channel is present in rat bladder myocytes, which is activated by arachidonic acid and most likely is TREK-1. This K^+ channel may have an important role in the regulation of bladder smooth muscle tone during urine storage.

Key words : K^+ channel, TREK-1, rat urinary bladder, smooth muscle, perforated patch clamp, arachidonic acid

Introduction

Different types of stretch-activated ionic channels have been reported in a wide variety of cells (Sackin 1995 ; Hu & Sachs 1997 ; Kamkin *et al*. 2002). They are non-selective cation channels (Popp *et al*. 1992 ; Kim 1993), Ca^{2+} channels (Lansman *et al*. 1987), Cl^--selective anion channels (Tseng 1992 ; Okada 1997), ATP-sensitive K^+ channels (Van Wagoner & Russo 1992), K^+-selective channels (Guharay & Sachs 1984 ; Sigurdson *et al.* 1987), and two-pore domain K^+ channels called TREK and TRAAK family (Patel *et al*. 1998 ; Bang *et al*. 1999 ; Maingret *et al*. 1999).

SACs are present in urinary bladder myocytes and are thought to be activated during physiological bladder filling (Wellner & Isenberg 1993, 1994 ; Chambers *et al*. 1997 ; Masters *et al*. 1999). Since these reports described only non-selective cation channels, it is difficult to understand the role of SACs in bladder smooth muscle. However, if K^+-selective mechanosensitive channels are expressed in bladder myocytes, these SACs may contribute to maintenance of bladder smooth muscle relaxation during urine storage. In the present study, we determined whether there are any other types of SACs in rat bladder cells. We found that a hypotonic external solution activated a K^+ current and we examined its properties.

Materials and methods

Isolation of single smooth muscle cells

All experiments were performed with the approval of the Animal Research Committee of Fuku-

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shima Medical University.

Single smooth muscle cells were isolated from the rat bladder by the following method. Male Sprague-Dawley rats weighing 200-300 g were anaesthetized deeply with diethyl ether. The bladder was removed immediately, cut into 1 mm × 5 mm pieces and immersed overnight at 4°C in 5 ml of Mg^{2+}, Ca^{2+}-free Tyrode solution containing 10 mg collagenase (Sigma type I), 1 mg protease (Sigma type XXIV) and 100 mg bovine serum albumin (Sigma). The pieces were then incubated for 1 h at 37°C in a dispersal medium. The resulting suspension was dispersed by gentle agitation using a glass pipette, filtered through a 70-μm-meshed cell strainer (Becton Dickinson Labware, USA). Cells were recovered by centrifugation, and the pellet was transferred to Mg^{2+}, Ca^{2+}-free Tyrode solution and stirred several times to release single smooth muscle cells, which were stored in a test tube containing Mg^{2+}, Ca^{2+}-free Tyrode solution and were used within 8 h.

Solutions

The pipette solution for the perforated-patch configuration contained (mmol) : 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES (adjusted to pH 7.2 with KOH). Amphotericin B (Sigma) at 300 μg/ml was added to the pipette solution immediately before the experiment. The standard external solution contained (mmol) : 137 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 5.6 glucose, and 4.2 HEPES (adjusted to pH 7.4 with NaOH). The normotonic solution (280 mOsmol/L) contained (mmol) : 84 NaCl, 5.4 KCl, 0.5 $MgCl_2$, 1.8 $CaCl_2$, 2.8 glucose, 2.1 HEPES, (adjusted to pH 7.4 with NaOH) and mannitol 168. Hypotonic solution (168 mOsm) was made up by eliminating all the mannitol from the normotonic solution. High K^+ hypotonic solution contained 75 mM KCl instead of 84 mM NaCl. Osmolarity of each external solution was measured with an osmometer (Kyoto DAIICHI Kagaku, Kyoto, Japan) and the osmolarity was adjusted with mannitol so that the ionic strength was kept constant.

Patch-Clamp Recording

Membrane currents were recorded using the amphotericin B perforated-patch configuration of the whole-cell patch-clamp technique (Horn & Marty 1988). Pipettes were forged from borosilicate glass capillary tubing (1.5 mm outer diameter, 1.17 mm inner diameter ; Nihon Rikagaku Kikai, Tokyo, Japan) with a tip diameter of approximately 1-2 μm and a resistance of 3-5 MΩ when filled with the pipette solution. All recordings were performed at room temperature (around 22°C). A whole-cell clamp amplifier (ACT ME, TM-1000, Tokyo, Japan) was used to record the membrane current with a 16 bit A/D converter (DIGIDATA 1322A, Axon Instruments, Foster City, CA, USA). Data acquisition and analysis were carried out with a computer (DEL, USA) using pCLAMP8 software (Axon Instruments, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from bladder using the acid-guanidinium thiocyanate phenol chloroform method (Chomczynski *et al*. 1987). The first strand cDNA was prepared from 1 μg of total RNA with random primers using moloney murine leukemia virus reverse transcriptase in a final volume of 20 μl. The cDNA was then diluted with 80 μl of sterile water and used as the template in PCR. DNA amplification was carried out in 15 μl of solution containing 10 mM Tris-HCL (pH9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 125 μM deoxynucleotide triphosphates, the cDNA template (1 μl), 1 μM primer mix and 25 units/ml Taq polymerase (TaKaRa, Japan). The thermocycle consisted of one cycle of 4°C for 10 min followed by heating at 95°C for 2 min and then 33 cycles of 30 s at 95°C, 30 s at 56°C, and 10 min at 72°C. Sequences of the PCR primers were
TREK-1 (accession number AF385402) :
sense, GTCCTGCCTCCCTTGCTGAA (position 1352)
antisense, CTCAGTGGGACAGCTCAGGA (position 1644)

TREK-2 (accession number AF385401) :
sense, GTATGATTGGAGACTGGCTG (position 1331)
antisense, GAAAGCATGTCCAGTGAGTG (position 1524)

TRAAK (accession number NM_053804) :
sense, GTCCTCACTCCTACCTTCGT (position 572)
antisense, CCAGTTGCCGATAGTGGTGA (position 752)

TREK-1, TREK-2 and TRAAK were reported to be expressed in rat cerebellum (Talley *et al.* 2001). As a control for RT-PCR, rat cerebellum cDNA fragments were amplified with each sense and antisense primer. Positive control PCRs consisted of 35 cy-

cles.

PCR products were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide using a 50 bp DNA ladder as molecular weight markers. The predicted sizes (bp) of the PCR products were 331 for TREK-1, 232 for TREK-2 and 219 TRAAK.

Chemicals

Gadolinium, amphotericin B, arachidonic acid, phorbol 12-myristate13-acetate (PMA) and forskolin were purchased from Sigma (St Louis, MO, USA). All the chemicals were the highest grade available.

Data analysis

Data obtained from pCLAMP8 software were analyzed and plotted using Clampfit8 software (Axon Instruments, Foster City, CA, USA). All values are presented as means ± S.E.M. (number of experiments). Student's *t*-test and analysis of variance were used for statistical analysis. *P* values less than 0.05 were considered significant.

Results

Effect of hypotonic solution

First, we examined whether there are stretch activated ionic channels in rat bladder smooth muscle cells. Figure 1 illustrates the effects of a hypotonic external solution on the membrane current with the perforated patch. Changing the bath solution from an isotonic to a hypotonic (60%) one increased an outward current. Gd^{3+} (50 μM), a stretch activated channel inhibitor, decreased the developed current and even some of the control current. The swelling-activated current had an outwardly rectifying property and crossed with the voltage axis at −47 ± 19 mV (n = 5), which was too negative for expected reversal potentials of −12 mV for a nonselective cation current, and 0 mV for a chloride current. This suggests that there are swelling activated channels other than nonselective cation channels and chloride channels in rat bladder smooth muscle cells.

We examined the time-dependency of the stretch activated current with step voltage pulses of 200 ms duration between −120 and +50 mV from the holding potential of −60 mV. The hypotonic-induced current was not apparently time dependent (data not shown). The SAC current was time-independent and so we continued our experiments with ramp pulses.

Effect of changing external K^+ concentration on SAC current

Next we studied the ionic selectivity of the stretch-activated current by raising $[K^+]_o$ from 5.4 to 73.9 mM in the hypotonic bath solution (Fig. 2). $[K^+]_i$ in the pipette solution was 140 mM. The current-voltage (*I-V*) relationships obtained by ramp

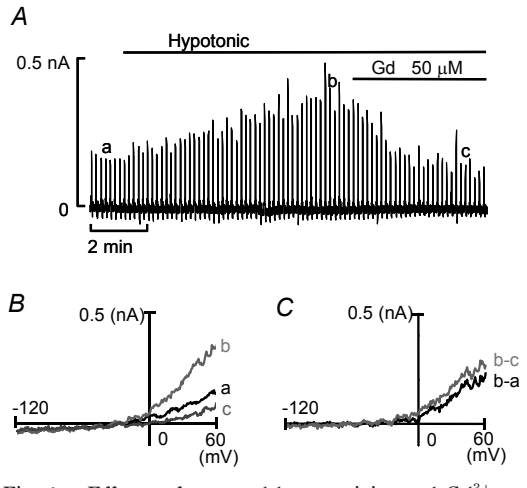

Fig. 1. Effects of external hypotonicity and Gd^{3+} on membrane current in a rat bladder smooth muscle cell.
A, a continuous recording of current in response to ramp pulses. Hypotonic solution (60% of control) increased outward current. Gd^{3+} inhibited the hypotonicity-induced current. *B*, *I-V* curves of the corresponding labels in *A*. *C*, *I-V* curves of difference currents obtained by subtraction as indicated.

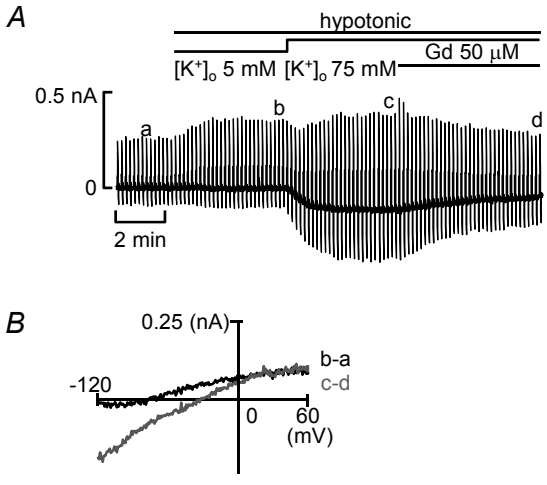

Fig. 2. Effects of $[K^+]_o$ on hypotonicity-induced current.
A, Chart recording of the current in response to ramp pulses. $[K^+]_o$ was raised from 5 to 75 mM in hypotonic solution. *B*, *I-V* curves of the net hypotonicity-induced currents obtained by subtraction were plotted at each $[K^+]_o$. Labels of the difference currents correspond to those in *A*.

pulses showed that the reversal potential shifted from -49 ± 19 mV ($n = 9$) to -23 ± 7 mV ($n = 9$). The positive shift of the reversal potential in response to higher $[K]^+_o$ indicates K^+ permeability of the swelling-activated current.

Effect of arachidonic acid on membrane current

The two-pore domain K^+ channels including TREK-1, TREK-2 and TRAAK are known to be activated by membrane stretch (Patel *et al*. 1998 ; Maingret *et al*. 1999 ; Bang *et al*. 2000) and also activated by arachidonic acid (AA) (Fink *et al*. 1998 ; Patel *et al*. 1998 ; Bang *et al*. 2000). Therefore, we examined the effect of AA on the rat bladder smooth muscle cells. AA at 50 μM in the normotonic bath solution induced a marked outward current which crossed with the voltage axis at around -60 mV (Fig. 3). The current was completely inhibited by 50 μM Gd^{3+} (Fig. 3) and it had an outwardly rectifying property. This strongly indicates that a class of TREK/TRAAK member K^+ channels exist in rat bladder smooth muscle.

Effect of acidic pH on SAC- and AA-induced currents

To assess whether the swelling-activated and the AA-activated K^+ channels are identical, we compared the pharmacological properties of the two currents. Extracellular pH 6.4 inhibits TREK-1 and TRAAK (Patel *et al*. 2001). Fig. 4 shows that extracellular pH 6.4 reduced both the hypotonicity- (Fig. 4*A-C*) and AA-induced currents (Fig. 4*D-F*). Current reductions caused by extracellular acid

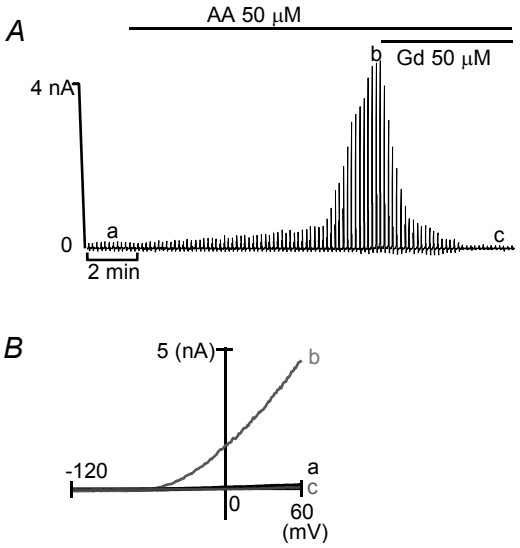

Fig. 3. Effects of arachidonic acid (AA) and Gd^{3+} on membrane current.
A, a continuous recording of the current in response to ramp pulses. *B*, *I-V* curves of the currents labeled in *A*.

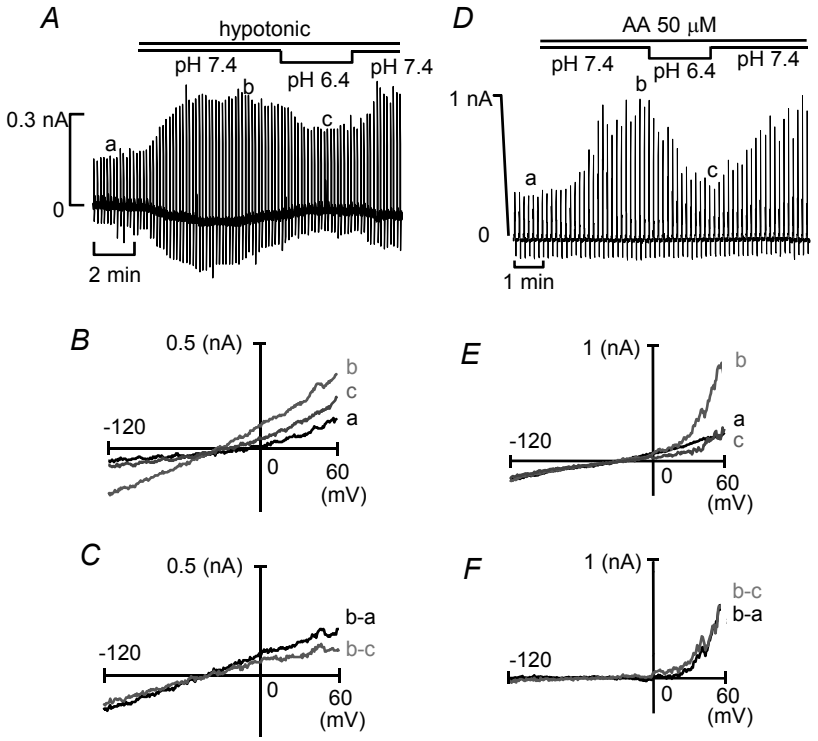

Fig. 4. Effects of extracellular acidification (pH 6.4) on SAC-induced (*A-C*) and AA-induced (*D-F*) currents.
A and *D*, continuous recordings of current in response to ramp pulses. *B* and *E*, *I-V* curves of corresponding labels in *A* and *D*, respectively. *C* and *F*, *I-V* curves of difference currents from *B* and *E*, respectively.

measured at 60 mV were 25 ± 23 % (n = 6) and 20 ± 18 % (n = 3) of the control with hypotonicity- and AA-induced currents, respectively. This result supports the possibility that the currents are identical and belongs to the class of two-pore domain K^+ channels.

Effect of forskolin on SAC- and AA-induced currents

Increased cytosolic cAMP inhibits TREK-1 and TREK-2 but not TRAAK (Reyes *et al*. 1998 ; Patel *et al*. 1998 ; Lesage *et al*. 2000). We used forskolin, a direct activator of adenylate cyclase, to increase cAMP in the bladder cells. Fig. 5 shows that 10 μM forskolin inhibited both strech- (Fig. 5*A-C*) and AA-induced currents (Fig. 5*D-F*). At 60 mV, they inhibited by 75 ± 15 % (n = 4) and 83 ± 17 % (n = 4), respectively. This result excludes the presence of TRAAK and supports the possibility of TREK-1 or TREK-2 being present.

Effect of PMA on SAC- and AA-induced currents

Among the AA-activated two-pore domain K^+ channels which include TREK-1, TREK-2 and TRAAK, only TREK-1 is inhibited by phorbol-12-myristate-13-acetate (PMA) (Fink *et al*. 1996 ; Maingret *et al*. 2000). We examined the effect of PMA on hypotonicity-induced and AA-induced currents. Fig. 6 shows that 100 nM PMA inhibited both hypotonicity-induced (Fig. 6*A, B*) and AA-induced currents (Fig. 6*C, D*). *I-V* curves are shown in Fig. 6*B* and *D*. E_{rev} of the PMA-inhibited current was −46 ± 32 mV (n = 5). PMA inhibited AA-induced and SAC currents by 75 ± 23 % (n = 5) and 81 ± 20 % (n = 6), respectively. This together with the above results supports the view that SAC channels and AA-induced channels are identical and are TREK-1, not TREK-2 or TRAAK.

Eexpression of TREK/TRAAK mRNAs in rat bladder

Since the above results suggested that the most likely candidate for the swelling-sensitive, AA-sensitive ionic channel was TREK-1, using RT-PCR, we examined whether TREK-1 mRNA is expressed in rat bladder. Figure 7 shows that TREK-1, TREK-2 and TRAAK mRNAs are all expressed in rat cerebellum, but only TREK-1 mRNA in the bladder. This result strongly suggests that the SAC in rat bladder smooth muscle cells is TREK-1.

Discussion

The only stretch-activated ionic current in the

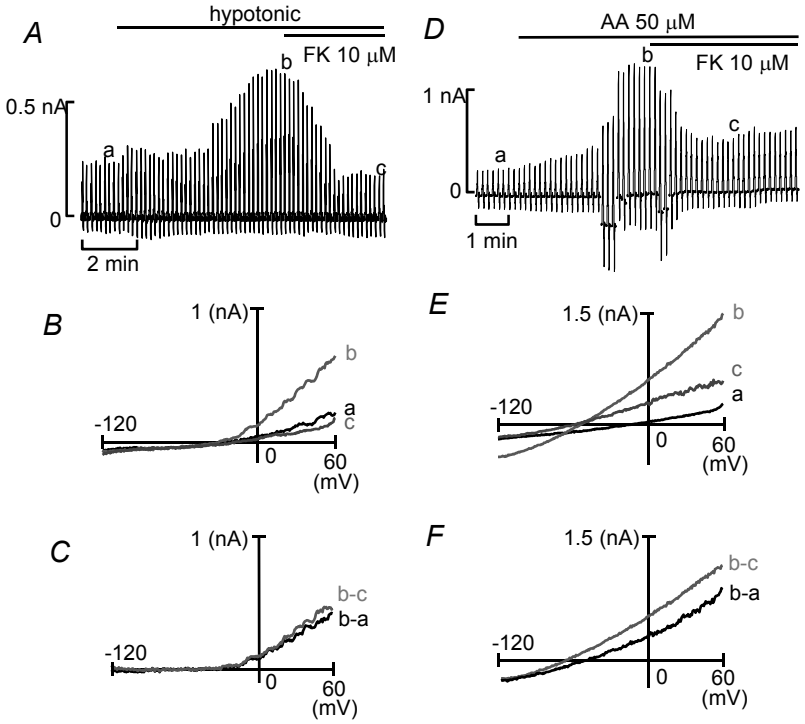

Fig. 5. Effects of forskolin (FK) on SAC-induced (*A-C*) and AA-induced (*D-F*) currents in a rat bladder smooth muscle cell.
A and *D*, continuous recordings of current in response to ramp pulses. *B* and *E*, *I-V* curves of corresponding labels in *A* and *E*, respectively. *C* and *F*, *I-V* curves of difference currents from *B* and *E*, respectively.

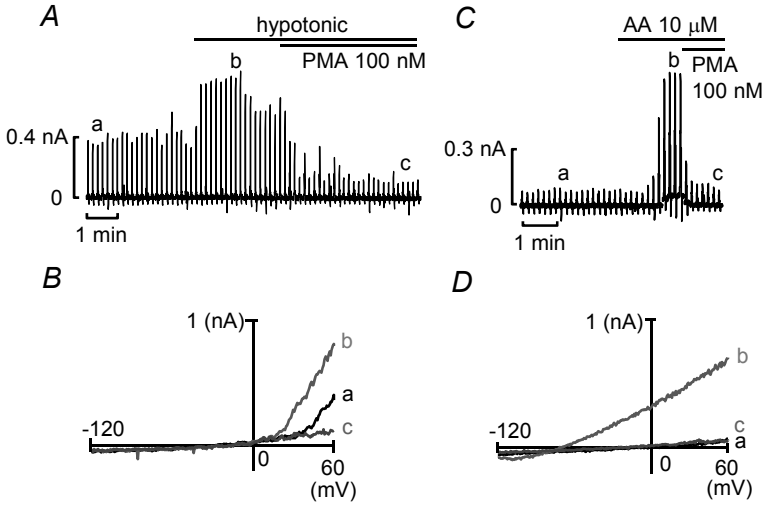

Fig. 6. Effects of PMA on SAC-induced (*A, B*) and AA-induced (*C, D*) current.
A, a continuous recording of current in response to ramp pulses. *B*, *I-V* curves of corresponding labels in *A*. *C*, a continuous recording of the current. *D*, *I-V* curves of corresponding currents in *C*.

Fig. 7. mRNA expression of TREK-1, TREK-2, and TRAAK in the cerebellum and bladder of the rat. Only TREK-1 mRNA expression was detected in the bladder in contrast to all three present in the cerebellum.

bladder reported was a non-selective cation current (Wellner & Isenberg 1993). This is the first report showing that swelling activates an outwardly rectifying K^+ current in rat bladder myocytes. This channel was activated by cell swelling caused by an external hypo-osmotic condition, and was inhibited by gadolinium (Gd^{3+}), which inhibits stretch activated nonselective cation current (Yang & Sachs 1989) and stretch activated K^+ channels (Maingret *et al*. 1999, 2000). Raising the K^+ concentration in the hypotonic bath solution without changing osmolarity shifted the reversal potential of the SAC current in a positive direction, consistent with K^+ permeability of the SAC.

The stretch-activated K^+ channels reported to date are TREK-1, TREK-2 and TRAAK, which are a mechanosensitive subgroup of the four transmembrane two pore domain K^+ channel family and are expressed in many regions in the brain as well as in certain peripheral tissues, such as ovary and small intestine (Medhurst *et al*. 2001). They behave as a background K^+ current and are modulated not only by mechanical stretch but also by unsaturated free fatty acids such as arachidonic acid, and are inhibited by Gd^{3+} (Maingret *et al*. 1999, 2000 ; Ferroni *et al*. 2003). In the present study, arachidonic acid activated a significant outwardly rectifying K^+ current in rat bladder cells which was inhibited by Gd^{3+}. This strongly indicates that TREK/TRAAK family K^+ channels are present in rat bladder.

Different pharmacological properties have been described for the TREK/TRAAK family members (Kim 2003). Although all three members are outwardly rectifying K^+ channels, TREK-1 is inhibited by PMA, extracellular acidic pH and cAMP (Fink *et al*. 1996 ; Patel *et al*. 1998, 2001 ; Maingret *et al*. 2000), while TREK-2 is not inhibited by PMA (Patel *et al*. 1998 ; Maingret *et al*. 2000) and TRAAK is not inhibited by PMA or cAMP (Fink *et al*. 1996, 1998 ; Maingret *et al*. 1999 ; Patel *et al*. 2001). In this study, both SACs and AA-sensitive channels were inhibited by PMA, extracellular acidic pH and forskolin. These data support the notion that the SAC and AA-induced channels are identical and highly likely to be TREK-1. This was confirmed by RT-PCR, because only TREK-1 mRNA, but not those for TREK-2 or TRAAK, was expressed in rat bladder. Therefore, we conclude that stretch activated TREK-1 K^+ channels are present in rat bladder myocytes. This is the first report of TREK-1 mRNA expression in bladder. However, there may be species differences, because TREK-1 mRNA was not detected in murine bladder (Koh *et al*. 2001).

Since we found TREK-1 in rat bladder in the present study, there are at least two types of K^+ channels in the bladder. In addition to TREK-1, iberiotoxin-sensitive Ca^{2+}-activated K^{2+} channels

are also present (Nakamura *et al*. 2002). Under physiological conditions, TREK-1 channel activity is elicited by increasing the mechanical pressure applied to the cell membrane and is independent of intracellular Ca^{2+} (Bang *et al*. 2000 ; Lesage *et al*. 2000 ; Patel *et al*. 1998 ; Maingret *et al*. 1999). Thus the physiological functions of these two K^+ channels may be distinctive. The Ca^{2+}-activated K^+ channels are activated when intracellular Ca^+ concentration is increased, i.e. during smooth muscle contraction and as a consequence the cells repolarize to restore the basal level of tone before contraction. In contrast, TREK-1 K^+ channels are activated by mechanical stretch which occurs during bladder filling. Thus activation of TREK-1 hyperpolarizes the myocytes and facilitates bladder wall distention.

In some cells in this study, the control currents before application of hypotonic solution were slightly decreased by Gd^{3+} (data not shown). This indicates that some TREK-1 channels may be active without being stretched when the bladder is empty. The number of open TREK-1 channels must increase as the bladder fills.

AA- and SAC-induced currents showed different *I-V* relationship. E_{rev} of AA-induced currents was close to E_K, while that of SAC was located between E_K and 0 mV which is the reversal potential of a non-specific cation currents. SAC-induced currents had a strong outward rectification. It has been reported that the outward rectification of SAC becomes less significant when external K concentration is high. We also observed that raising external K concentration revealed an inward component of SAC and outward rectification is less prominent (Fig. 2).

Lauritzen *et al.* proposed that a potential function of TREK-1 might be to monitor the levels of free fatty acids and protons in the cell, providing a protective mechanism by reducing cell excitability (Lauritzen *et al*. 2000). During ischemia, hypoxia and inflammation, phospholipases are activated, free fatty acids increase and intracellular pH falls (Bazan *et al*. 1993 ; Lipton 1999). In cystitis, for example, arachidonic acid release from bladder smooth muscle and interstitial cells is elevated and intracellular pH decreases, leading to an activation of TREK-1. This would help protect the bladder from inflammatory detrusor instability and further damage. TREK-1 also may be related to a pathological state such as overactive bladder. To date, the underlying mechanism of bladder detrusor instability is not well understood. A number of factors may be involved in its etiology. Recently, it was demonstrated that BK channel dysfunction leads to overactive bladder and urinary incontinence (Meredith *et al*. 2004). This indicates that K^+ channels play important roles in bladder function. Dysfunction of TREK-1 in the bladder may also be involved in detrusor instability.

In a variety of cells, hypo-osmotic stress activated chloride channels (Okada 1997). However, in the present study, hypo-osmolarity-induced-current in rat bladder was predominantly a K^+ current and not a chloride current, because of the positive shift of the reversal potential by raising external K^+ concentration (Fig. 2). In addition, in human small intestinal epithelial cells, volume-regulatory Cl^- current was blocked by arachidonic acid (Kubo & Okada, 1992), which was an opposite effect to that we obtained in rat bladder.

Under the hypotonic stress, intracellular Ca^{2+} concentration could rise and result in activation of Ca^+-dependent K^+ channels, whose property is similar to TREK channels. Therefore, there is a possibility that K^+ channels other than TREK-1 may also be included under hypo-osmotic pressure in rat bladder.

In conclusion, this study provides new information on the swelling-activated channels in rat bladder smooth muscle cells. Human and mouse bladder also has TREK-1 K^+ channels (Tertyshnikova *et al*. 2005 ; Baker *et al*. 2010) and so the function and modulatory mechanism of this channel needs to be further examined.

Conflict of interest

We have no conflict of interest to disclose.

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