**N-((1S)-1-[[4-((2S)-2-[[[2,4-Dichlorophenyl]sulfonyl]amino]-3-hydroxypropanoyl]-1-piperazinyl]carbonyl]-3-methylbutyl)-1-benzo thiophene-2-carboxamide (GSK1016790A), a Novel and Potent Transient Receptor Potential Vanilloid 4 Channel Agonist Induces Urinary Bladder Contraction and Hyperactivity: Part I**


**ABSTRACT**

The transient receptor potential (TRP) vanilloid 4 (TRPV4) member of the TRP superfamily has recently been implicated in numerous physiological processes. In this study, we describe a small molecule TRPV4 channel activator, (N-((1S)-1-[[4-((2S)-2-[[[2,4-dichlorophenyl]sulfonyl]amino]-3-hydroxypropanoyl]-1-piperazinyl]carbonyl]-3-methylbutyl)-1-benzo thiophene-2-carboxamide (GSK1016790A), which we have used as a valuable tool in investigating the role of TRPV4 in the urinary bladder. GSK1016790A elicited Ca2+ influx in mouse and human TRPV4-expressing human embryonic kidney (HEK) cells (EC50 values of 18 and 2.1 nM, respectively), and it evoked a dose-dependent activation of TRPV4 whole-cell currents at concentrations above 1 nM. In contrast, the TRPV4 activator 4α-phorbol 12,13-didecanoate (4α-PDD) was 300-fold less potent than GSK1016790A in activating TRPV4 currents. TRPV4 mRNA was detected in urinary bladder smooth muscle (UBSM) and urothelium of TRPV4+/− mouse bladders. Western blotting and immunohistochemistry demonstrated protein expression in both the UBSM and urothelium that was absent in TRPV4−/− bladders. TRPV4 activation with GSK1016790A contracted mouse bladders in vitro, both in the presence and absence of the urothelium, an effect that was undetected in TRPV4−/− bladders. Consistent with the effects on TRPV4 HEK whole-cell currents, 4α-PDD demonstrated a weak ability to contract bladder strips compared with GSK1016790A. In vivo, urodynamics in TRPV4+/− and TRPV4−/− mice revealed an enhanced bladder capacity in the TRPV4+/− mice. Infusion of GSK1016790A into the bladders of TRPV4+/− mice induced bladder overactivity with no effect in TRPV4−/− mice. Overall TRPV4 plays an important role in urinary bladder function that includes an ability to contract the bladder as a result of the expression of TRPV4 in the UBSM.
TRPV4 Activator GSK1016790A Induces Bladder Overactivity

Tanabe et al., 2002a). In addition, ruthenium red has been pholide (Smith et al., 2006) and the more widely used synthetic ligand 4α-phorbol 12,13-didecanoate (4α-PDD; Watanabe et al., 2002a). In addition, ruthenium red has been used as a TRPV4 channel blocker but is relatively nonselective.

TRPV4 conducts Ca\(^{2+}\) and Na\(^{+}\) across the plasma membrane of various cell types with a ~6:1 selectivity under physiological conditions (Strotmann et al., 2000; Watanabe et al., 2002a; Plant and Strotmann, 2007), which normally enhances cellular excitability. TRPV4 is activated by increased temperature with a threshold of ~27°C (Güler et al., 2002; Watanabe et al., 2002b), thereby demonstrating significant constitutive activity at physiological temperatures (Vrions et al., 2004; Shibasaki et al., 2007). TRPV4 is also activated by physical cell stress, such as that induced by hypotonic challenge (Strotmann et al., 2000; Gao et al., 2003; Liedtke and Friedman, 2003). Overall, TRPV4 activation seems to be a multimodal and complex graded process that likely serves to finely tune TRPV4 activity in different physiological and pathophysiological states.

TRP channels have been implicated in urinary bladder function, with a documented role for the TRPV1 family member in bladder sensation as a function of its expression on the urothelium and bladder afferent nerves (Birder et al., 2001, 2002). TRPV1 has been exploited clinically to desensitize bladder afferents and reduce bladder overactivity (Avelino and Cruz, 2006). More recently, TRPV4 expression has been shown in the urothelium, and it is implicated in the regulation of urothelial ATP release modulating the sensitivity of bladder afferent nerves (Birder et al., 2001; Gevaert et al., 2007). Urinary bladder smooth muscle (UBSM) elicits spontaneously occurring phasic contractions of myogenic origin that directly result from UBSM action potentials (Hashitani and Brading, 2003). A number of ion channels have been shown to modulate UBSM action potentials, including depolarizing L-type Ca\(^{2+}\) channels and various K\(^{+}\) channels that hyperpolarize the membrane potential (Heppner et al., 1997; Petkov et al., 2001; Hashitani and Brading, 2003; Thorneloe and Nelson, 2003; Thorneloe et al., 2005). In addition, nonselective cation channels, like those of the TRP family, can contribute to depolarization and bladder contraction (Thorneloe and Nelson, 2004).

In this study, we describe a novel TRPV4 channel activator GSK1016790A with significantly greater potency than those previously reported, and we demonstrate a functional role for TRPV4 channels expressed in UBSM as regulators of urinary bladder activity.

Materials and Methods

Generation of TRPV4\(^{-/-}\) Mice. Genomic fragments homologous to the TRPV4 locus were cloned by polymerase chain reaction (PCR) from the E14.1 embryonic stem (ES) cell line using the Expand long template PCR kit (Roche, Palo Alto, CA). Primers for the 5′ homology arm were VR4_5F (5′-TTC TTG ACC CAC AAG AAG CGC CT-3′) and VR4_5R (5′-ATG GTG TCG TTC CAC CCG CCG TTC CTT AGG TT-3′) spanning coding exons 3 to 4; for the 3′ arm, VR4_5F (5′-TTC TTC CAC CCC AAG GAT GAG GGA GGC T-3′) and VR4_3R (5′-AGA TTC CGG GTTC TCA TCT GTC ACC T-3′) spanning coding exons 5 to 7. The homology arms were inserted into the vector by homologous recombination in yeast as previously described (DeMarini et al., 2001) such that they flanked the positive selection cassette containing an IRES-LacZ reporter, a PGK-neo selectable marker, and the yeast URA3 selectable marker (Supplemental Fig. 1A), positioned to disrupt exons 4 and 5 and thereby interrupt the coding sequence at TM1, and Frameshifts the remainder of the transcript. The NotI-linearized targeting vector (25 μg) was electroporated into 10\(^9\) E14.1 ES cells using a Bio-Rad (Hercules, CA) Gene Pulser system set at 500 μF, 230 V. Homologous recombination in G418-resistant ES cells was confirmed by Southern blot analysis of EcoRV-digested genomic DNA using a 3′ external probe generated by PCR using primers VR4_3PF (5′-AGG CCT GTT TCG TTC AAG-3′) and VR4_3PR (5′-GCC CTG CAC AAT CTG-3′), which detects 3.75 and 3.3 kilobase (kb) bands at the wild-type and targeted locus, respectively (Supplemental Fig. 1B). Correct integration at the 5′ end was confirmed by long PCR (data not shown). Four correctly targeted ES cell clones were identified by Southern blot analysis of 400 clones in total. Targeted clones were injected into C57Bl/6J-derived blastocysts and gave rise to germline-transmitting chimeric mice. Male chimeras were crossed with C57Bl/6J females to give heterozygous offspring. These heterozygous mice were further backcrossed and interbred to generate homozygous mutants. The absence of wild-type transcript from homozygous knockout mice was confirmed by reverse transcription-PCR (RT-PCR) (Fig. 3A). Genotypic analysis was performed by PCR using DNA isolated from tails, neo-specific primers NeoF1.3 (5′-GCC GCT CTC GGG CCC AGG ATG CC-3′) and NeoR1.3 (5′-TGG CCC CCA AGA GGA AGC ACC C-3′), which produce a 202-base pairs (bp) product from the targeted allele, and wild-type-specific primers VR420L19 (5′-CAG ACA GCA ATG GGC CTT C-3′) and VR42U19 (5′-CAG CGG CAA CAT GGC TGA A-3′), which produce a 221-bp product from the wild-type allele. Interbred mice produced the expected Mendelian ratio of homozygotes to heterozygotes to wild types. Mice used in these studies were backcrossed for at least six generations, weighted 18 to 26 g, and were housed under a 12-h light/dark cycle with free access to food and water. All experiments were conducted according to the requirements of the United Kingdom Animals (Scientific Procedures) Act (1986) and strictly conformed to the ethical standards of the Institutional Animal Care and Use Committee of GlaxoSmithKline Pharmaceuticals.

RT-PCR and TaqMan. Total RNA was isolated from TRPV4\(^{-/-}\) and TRPV4\(^{-/-}\) bladders using TRI Reagent (Ambion, Austin, TX). RT-PCR was performed with M-MLV Reverse Transcriptase and SuperTaq DNA polymerase enzymes (Ambion) in conjunction with TRPV4-selective primers (Earley et al., 2005). RT-PCR products were separated on a 1% agarose gel with 1-kb ladder (Invitrogen, Carlsbad, CA) used as a standard. Images were obtained using a Kodak Image Station 440. For TaqMan studies, cDNA was synthesized using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA) used as a standard. Images were obtained using a Kodak Image Station 440. For TaqMan studies, cDNA was synthesized using MultiScribe Reverse Transcriptase (Applied Biosystems, Foster City, CA). All probe/primer sets were purchased as “assay on demand” (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase was used as the housekeeper gene. The urothelium and UBSM expression was presented relative to the average expression in UBSM.

Membrane Preparation and Western Blotting. A crude membrane preparation was prepared from frozen tissues, which were homogenized in pH 7.0 buffer containing 4 mM HEPES, 320 mM sucrose, and protease inhibitors (10 ml per gram tissue). The homogenate was centrifuged at 2000g for 10 min, and the supernatant was saved. Another equal volume of buffer was added to the pellet, which

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was rehomogenized and centrifuged. The two supernatants were pooled and centrifuged for 1 h at 100,000g. The resultant pellet was resuspended in buffer, and total protein was quantified according to the Bradford (1976) method (Bio-Rad). Forty micrograms of membrane protein from TRPV4−/− or TRPV4+/+ urothelium and UBSM were subjected to SDS-polyacrylamide gel electrophoresis with protein lysates from TRPV4-expressing human embryonic kidney (HEK293) cells used as a positive control. Proteins were then transferred to nitrocellulose membranes, which were probed with a TRPV4 polyclonal antibody (1:500 dilution) raised against CDDGHGQQYPRKWRDIAALP of the human TRPV4 C terminus (Delany et al., 2001), followed by incubation with a secondary goat anti-rabbit (926-32211; Li-Cor Biosciences, Lincoln, NE). Images of the Western blots were acquired using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

**Immunohistochemistry.** Bladders from TRPV4−/− and TRPV4+/+ mice were dissected, embedded in O.C.T. compound (Thermo Fisher Scientific, Waltham, MA), and frozen on dry ice. A polyclonal antibody specific for a synthetic peptide identical to the first 16 amino acids of rat TRPV4, MADPGDGPRAAPGDVA, was prepared in rabbits and affinity purified (Invitrogen). Eight-micron-thick cryostat sections were thaw mounted onto SuperFrost Plus (VWR, Chester, PA) slides and fixed in 3% formaldehyde in phosphate-buffered saline (PBS). Sections were rinsed in PBS, blocked in 10% normal donkey serum (Jackson Immunoresearch Laboratories Inc., West Grove, PA) to eliminate nonspecific binding, and incubated with the TRPV4 antibody (2.0 μg/ml) in PBS for 2 h at room temperature. Bound antibody was detected using a Texas Red-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA) to eliminate non-specific background (Nikon). Immunoreactivity was visualized by a combination of the appropriate fluorescein isothiocyanate or Texas Red-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Images were acquired using a Nikon E1000 microscope (Nikon, Tokyo, Japan), a DXM 1200C camera (Nikon), and Elements BR imaging program (Nikon).

**TRPV4 Ca2+ Influx.** HEK (MSRII) cells were transduced with a murine TRPV4 containing BacMam virus (6 particles/cell), then plated at 50,000 cells per well in a 96-well plate, and allowed to adhere overnight. The cells were then dye loaded with Fura-2 (Molecular Probes). In brief, cells were incubated for 1 h at 37°C in the presence of 3 μM Fura-2 in Tri-glycine (TG) buffer (145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES, pH 7.4) containing 0.02% pluronic acid (Sigma-Aldrich, St. Louis, MO). The dye-containing solution was then removed and replaced with fresh TG buffer, and the cells were incubated for an additional 30 min at 37°C to allow for hydrolysis of the dye. The buffer was then replaced with TG buffer containing 1% bovine serum albumin (Sigma-Aldrich), and the assay plate was loaded into a FlexStation (Molecular Devices, Sunnyvale, CA). The intracellular calcium concentration was monitored as the change in Fura-2 fluorescence after addition of a TRPV4 agonist.

**Electrophysiology.** Whole-cell voltage-clamp recordings were performed on HEK293 cells expressing human TRPV4 channels and on untransfected HEK cells. The pipette solution contained the following: 130 mM CsCl, 5 mM EGTA, 5.5 mM MgCl2, 5 mM Na2ATP, 0.1 mM NaGTP, and 5 mM HEPES, pH 7.2. The external solution contained the following: 135 mM NaCl, 5 mM CsCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, and 10 mM HEPES, pH 7.4. Membrane currents were recorded at room temperature using a ramp voltage-clamp protocol repeated every 10 s (holding potential of −60 mV, depolarizing from −60 mV to +60 mV in 760 ms). Peak drug responses were measured at −60 mV and normalized by the cell membrane capacitance to yield current densities.

**Urinary Bladder Contractility.** TRPV4−/− and TRPV4+/+ mice were euthanized by CO2 asphyxiation. A midline abdominal incision was made, and the bladder was dissected free from the animal. The bladders were cleaned of adherent connective tissue/fat and cut into two equal halves from bladder dome to the urethral opening. A length of 4–0 suture was tied and double knotted at the top and another at the base of each tissue. The tissue was then suspended vertically in a 15-ml organ bath by securing one suture to a stationary holder and the other to an isometric force transducer. The organ bath contained Krebs solution (38–39°C) and was constantly aerated by bubbling with 95% O2/5% CO2. One gram of tension was applied to each tissue, and the bladder strips were allowed to equilibrate for 60 min. During equilibration, the Krebs solution was changed and the tension readjusted to 1 g every 20 min. For electrical field stimulation experiments, the tissues were electrically stimulated at a frequency of 8 Hz delivered every 60 s. The stimulation parameters were as follows: 60 V; 0.5-ms pulse duration; 1-s train duration. The 8-Hz stimulation was repeated until a stable contractile response was obtained (10–20 stimulations). The stimulation was discontinued, and the tissues were allowed to recover for 60 min. A frequency-concentration curve to 1, 2, 4, 8, 16, 32, and 64 Hz was then generated in each tissue. Three consecutive responses, separated by 60 s each, were obtained for each frequency, and the average of the three contraction amplitudes was measured. In experiments involving urothelium-free bladder halves, TRPV4−/− bladders were cut in half, the urothelium was removed by sharp dissection under a dissecting microscope from one half, and compared with the paired urothelium-intact half. Force was measured in mN or as an integrated area under the curve. Phasic contractile activity was assessed by measuring the phasic area (area under the curve minus baseline).

**Conscious Cystometry.** Mouse cystometry was performed as described previously (Thorneloe et al., 2005). Mice were anesthetized using isoflurane (Abbott Laboratories, Abbott Park, IL), and the urinary bladder was exposed by an abdominal midline incision. A purse-string suture was made in the dome of the bladder into which a saline-filled bladder catheter (PE-10) was inserted and secured. The catheter was routed s.c. to the back of the neck and stored in a skin pouch. After a minimal 7-day recovery, and under light isoflurane anesthesia, the end of the bladder catheter was retrieved from the skin pouch. Saline-filled polyethylene (PE-50) tubing was used to extend the externalized catheter and connect it to the syringe pump on a Small Animal Cystometry Lab Station (MED Associates Inc., St. Albans, VT). Mice were infused with saline (25 μl/min) via the bladder catheter for a 30-min equilibration period followed by 30 min of basal, control urodynamics that were used for genotype comparison. Mice were then challenged by infusion with saline containing 0.25% acetic acid or 10 μg GSK1016790A for an additional hour. Infused maximum (volume infused to induce voiding), void volume (urine voided), pressure threshold (pressure before micturition), average filling pressure, minimal filling pressure, and peak pressure (micturition peak) were recorded. Nonvoiding contractions were assessed as increases in bladder pressure of greater than 5 mm Hg during the filling phase and were analyzed using Mini Analysis software (Synaptosoft, Decatur, GA). On completion of cystometry, mice were euthanized by carbon dioxide inhalation followed by diaphragm disruption.

**Statistical Analysis.** Data are expressed as the mean ± S.E.M. GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA) was used for graphing and statistical analysis. Two-way repeated measures analysis of variance was used for multiple group comparisons. Student’s paired or unpaired t tests were used as appropriate. P < 0.05 was deemed statistically significant.

**Results**

**Identification of the TRPV4 Activator GSK1016790A.** As part of a small molecule screening effort, GSK1016790A was identified as a novel TRPV4 channel activator (Fig. 1A).
GSK1016790A potently induced Ca$^{2+}$ influx in HEK cells expressing mouse TRPV4 channels with an EC$_{50}$ value of 18 nM (Supplemental Fig. 2). GSK1016790A demonstrated a similar potency at human TRPV4 channels (EC$_{50} = 2.1$ nM). GSK1016790A was inactive against TRPV1 channels (see accompanying article, Willette et al., 2008), which, based on sequence homology, is the TRP superfamily member closest to TRPV4. In addition, we tested GSK1016790A against human TRPM8 and TRPA1 channels at concentrations up to 20 $\mu$M demonstrating no modulation of Ca$^{2+}$ influx. GSK1016790A elicited a steep, dose-dependent activation of whole-cell currents in TRPV4-transfected HEK cells at concentrations of 3 nM and above, reaching a current density of 710 ± 144 pA/pF measured at +60 mV with 10 nM GSK1016790A ($n = 6$; Fig. 2, A, C, and D). By comparison, the TRPV4 activator 4α-PDD was ineffective at 0.3 $\mu$M evoking current at 1 $\mu$M and above, reaching 323 ± 88 pA/pF at 10 $\mu$M ($n = 6$; Fig. 2, A, C, and D). Whereas 10 nM GSK1016790A did not activate currents in untransfected HEK cells, 10 $\mu$M 4α-PDD elicited a small nonspecific current (Fig. 2, B and D; 55 ± 11 pA/pF). This suggests that 4α-PDD at a concentration of 10 $\mu$M may modulate other ion channel activities in addition to TRPV4.

**TRPV4 Expression in the Urinary Bladder.** RT-PCR performed on total RNA isolated from intact TRPV4$^{+/+}$ bladders yielded a ~500-bp product (Fig. 3A) consistent with the predicted 478-bp TRPV4 product. The TRPV4 product was absent from TRPV4$^{-/-}$ bladder samples when the reverse transcriptase enzyme was omitted from the reaction and when total RNA from TRPV4$^{-/-}$ mouse bladders was used as substrate (Fig. 3A). TaqMan for TRPV4 on isolated urothelium and UBSM tissues demonstrated that TRPV4 is expressed in both of these tissues and that mRNA levels are ~20-fold higher in the urothelium than in the UBSM (Fig. 3B). As expected, TaqMan on TRPV4$^{-/-}$ urothelium and UBSM yielded no amplification products. Western blotting for TRPV4 on crude membranes isolated from TRPV4-expressing HEK cells demonstrated two bands just above 98 kDa (Fig. 3C), which was not present in untransfected cells. Likewise, this doublet was observed in crude membranes prepared from both TRPV4$^{+/+}$ UBSM and urothelial tissues but not in TRPV4$^{-/-}$ UBSM and urothelial membranes (Fig. 3C). Immunohistochemistry was performed on fresh-frozen sections of urinary bladders isolated from TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice. TRPV4 immunofluorescence was detected in both the urothelium and UBSM layers of the bladder wall in TRPV4$^{+/+}$ sections, but not in the suburothelial space between these layers, and was absent in TRPV4$^{-/-}$ sections (Fig. 4A). Colabeling demonstrated overlap with the urothelial cell marker uroplakin III and TRPV4 in the urothelium, indicating that TRPV4 is expressed on urothelial cells (Fig. 4B). In addition, TRPV4 immunofluorescence was observed on spindle-shaped cells within the UBSM layer, typical of smooth muscle cell morphology (Fig. 4C). Consistent with this colabeling for the smooth muscle marker, smooth muscle α-actin and TRPV4 demonstrated significant overlap (Supplemental Fig. 3). Immunofluorescence intensity was higher in the urothelium compared with the UBSM (Fig. 4A), consistent with the significantly higher mRNA expression levels determined by TaqMan in the urothelium (Fig. 4A). Supporting a functional presence of TRPV4 in mouse UBSM cells, GSK1016790A activated cation currents from freshly isolated guinea pig UBSM cells (10 nM, $n = 16$) that were inhibited by the TRPV4 channel blocker ruthenium red (10 $\mu$M, $n = 6$; Supplemental Methods and Supplemental Fig. 4). These results indicate that TRPV4 is expressed in the UBSM and urothelium of TRPV4$^{+/+}$ bladders and confirm an absence of TRPV4 expression in TRPV4$^{-/-}$ bladders.

**Role of TRPV4 in in Vitro Bladder Contractility.** An in vitro contractile response was observed in TRPV4$^{+/+}$ bladders with GSK1016790A (10–100 nM; Fig. 5). This effect was absent in TRPV4$^{-/-}$ mouse bladders. GSK1016790A (100 nM; Fig. 6, A and B) and carbachol (1 $\mu$M, $n = 4$; data not shown) contractions were not affected in paired bladder halves upon removal of the urothelium. These GSK1016790A-induced contractions were also not different when normalized to the carbachol response, 29 ± 4 and 38 ± 6%, respectively, in the urothelium-free and intact bladder halves. Therefore, urothelial TRPV4 channels did not appear to contribute to contractions induced by GSK1016790A. GSK1016790A-induced contractions were reversed ~80% by 1 $\mu$M nifedipine in both urothelium intact and urothelium-free bladder halves (Fig. 6C). Consistent with the weak ability of 4α-PDD to evoke TRPV4 currents in TRPV4 HEK cells (Fig. 2C), 4α-PDD demonstrated a poor ability to contract bladder halves, eliciting only small contractile responses at 10 and 100 $\mu$M (Fig. 6D). This is in sharp contrast to the larger contractions observed with a 100-fold lower concentration of GSK1016790A (100 nM). GSK1016790A-induced contractions were blocked by ruthenium red pretreatment (10 $\mu$M, $n = 3$), but they were insensitive to ω-conotoxin GVIA (0.3 $\mu$M, $n = 3$), tetrodotoxin (1 $\mu$M), atropine (1 $\mu$M, $n = 5$), or desensitization of TRPV1 and purinergic P2X channels with capsaicin (10 $\mu$M, $n = 5$) and αβ-methylene ATP (10 $\mu$M, $n = 4$), respectively. In addition, the amplitude and frequency dependence of nerve-mediated bladder contractions evoked by electrical field stimulation (1–64 Hz) were not different in TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice (n = 4 mice/genotype). Taken together, these results suggest that the contractile effects of GSK1016790A are due to a direct action on the UBSM and not an indirect effect on neurotransmitter release.

**Urodynamics in TRPV4$^{-/-}$ Mice.** Conscious cystometry was performed to evaluate the bladder function of unrestrained, age, weight, and sex-matched TRPV4$^{+/+}$ and TRPV4$^{-/-}$ mice during intravesical saline infusion. The urodynamic threshold, peak micturition, and average filling pressures were not significantly different between genotypes (Table 1). However, the bladder capacity of TRPV4$^{-/-}$ mice was greatly enhanced compared with TRPV4$^{+/+}$ mice. This was indicated by TRPV4$^{-/-}$ mice requiring significantly larger infused volumes to initiate a...
micturition event and demonstrating larger micturition void volumes (Fig. 7; Table 1). Nonvoiding contraction frequency and amplitude, measured as bladder pressure oscillations during the filling phase of cystometrograms, were not different between TRPV4/H11001/H11001 and TRPV4/H11002/H11002 mice (TRPV4/H11001, 0.013 ± 0.002 Hz, n = 15; TRPV4/H11002, 0.013 ± 0.003 Hz, n = 11, P = 0.98; TRPV4+/+, 9.1 ± 0.4 mm Hg, n = 15; TRPV4−/−, 8.0 ± 0.6 mm Hg, n = 11, P = 0.11). Cystometric measurements were made during infusion of GSK1016790A into the bladders of TRPV4+/+ and TRPV4−/− mice. No significant effect was observed with 1 µM GSK1016790A (n = 3); however, 10 µM GSK1016790A evoked a robust reduction in infused and voided volumes, and it enhanced the average and minimal filling pressures in TRPV4+/+ mice (n = 8; Fig. 8; Table 2). Effects of GSK1016790A on bladder volumes and bladder pressures were completely absent in TRPV4−/− mice (Fig. 8; Table 2). In additional studies, TRPV4+/+ (n = 7) and TRPV4−/−

Fig. 2. Comparison of TRPV4 current activation by GSK1016790A and 4α-PDD. A and B, example current density/voltage relationship induced by a depolarizing ramp voltage-clamp protocol in TRPV4-expressing HEK cells (A) and in untransfected HEK cells (B), before and after TRPV4 channel activation with 10 nM GSK1016790A (left) and 10 µM 4α-PDD (right). C, concentration-dependent increases of current density measured at +60 mV by GSK1016790A and 4α-PDD in HEK293 cells expressing TRPV4; n values are indicated for each dose. D, comparison of current density activated by 10 nM GSK1016790A or 10 µM 4α-PDD at +60 mV in HEK293 cells expressing TRPV4 and untransfected HEK293 cells; n values are indicated for each.
(n = 11) mice were infused intravesicularly with saline followed by 0.25% acetic acid to induce bladder overactivity (Supplemental Table 1). Bladder overactivity was apparent as acetic acid greatly reduced bladder capacity, indicated by reductions in infused and voided volumes in both TRPV4+/+ and TRPV4−/− mice. In addition, average filling and threshold pressures were increased by acetic acid for TRPV4+/+ and TRPV4−/− mice, although the increase in threshold pressure did not reach significance for TRPV4+/+ (P = 0.10). A comparison of the normalized response to acetic acid in TRPV4+/+ and TRPV4−/− mice revealed no significant differences between genotypes (Supplemental Table 1).

**Discussion**

GSK1016790A is a novel TRPV4 channel activator that is ~300-fold more potent than the commonly used TRPV4 activator 4α-PDD. We have used molecular techniques, TRPV4−/− mice, in vitro contractility, and in vivo urodynamics in conjunction with GSK1016790A to support a role for TRPV4 in UBSM. TRPV4 mRNA was amplified by PCR, and
protein expression was demonstrated by Western blot analysis and immunohistochemistry in UBSM and urothelium. GSK1016790A contracted bladder independent of the urothelium and induced bladder overactivity in vivo. Effects of GSK1016790A on contractility and urodynamics were absent in TRPV4−/− mice, indicating the responses to be TRPV4 mediated. Inhibition of TRPV4 function, via knocking out of the TRPV4 gene, resulted in an enhancement of bladder capacity without alterations in bladder pressures. These data demonstrate the functional selectivity of the TRPV4 channel activator GSK1016790A, and they implicate the functional expression of TRPV4 channels in UBSM as regulators of bladder contractility.

GSK1016790A elicits Ca\(^{2+}\) influx through mouse and human TRPV4 channels in HEK cells with EC\(_{50}\) values of 18 and 2.1 nM, respectively. These values are consistent with our electrophysiological recordings, demonstrating a 3-nM threshold for TRPV4 activation. In contrast, we show that under identical conditions, \(\geq 1 \mu M\) 4α-PDD is required to activate TRPV4, demonstrating that GSK1016790A has at least 300-fold greater potency for activating TRPV4 than 4α-PDD. This was confirmed in functional bladder contractility studies in which \(\sim 1000\)-fold higher concentrations of 4α-PDD (10 \(\mu M\)) were required to provide a similar potentiation of force as with GSK1016790A (10 nM). A previous report measuring Ca\(^{2+}\) entry and TRPV4 current activation with 4α-PDD suggested an EC\(_{50}\) of 0.3 \(\mu M\) (Watanabe et al., 2002a). Our electrophysiological data would suggest a \(\sim 10\)-fold weaker potency (Fig. 2C). These differing values for 4α-PDD may be attributable to differences in recording conditions and/or expression systems. GSK1016790A is also more potent than the natural product bisandrogapholide that activates TRPV4 with an EC\(_{50}\) value of 0.95 \(\mu M\) (Smith et al., 2006). To our knowledge, GSK1016790A is by far the most potent TRPV4 channel activator reported to date.

We have detected TRPV4 mRNA expression in mouse UBSM and urothelium, with \(\sim 20\) fold higher levels in the urothelium. In addition, we have demonstrated TRPV4 protein expression in both tissues by Western blotting and immunohistochemistry. In all cases mRNA and protein expression was absent in TRPV4−/− samples demonstrating the TRPV4 specificity of the antibody signals and PCR amplifications. TRPV4 immunofluorescence was more robust in the urothelium than in the UBSM, consistent with our quantitative PCR results. This study provides the first evidence supporting TRPV4 protein expression and function in UBSM. A recent report using semiquantitative RT-PCR demonstrated that TRPV4 mRNA is present in rat urothelium and at a lower level in UBSM (Birder et al., 2007). They detected TRPV4 by immunohistochemistry in rat urothelium; however, TRPV4 protein expression in UBSM was not documented. Subsequently, TRPV4 immunofluorescence was confirmed in rat and mouse urothelium; however, no specific UBSM TRPV4 protein expression was observed (Gevaert et al., 2007). The use of different TRPV4 antibodies and/or stringency conditions may account for discrepancies between the current study demonstrating UBSM TRPV4 protein expression and previous reports not detecting TRPV4 protein in UBSM (Birder et al., 2007; Gevaert et al., 2007).

GSK1016790A contracted bladder with a dependence on TRPV4 expression because the response was absent in TRPV4−/− bladders. GSK1016790A contractions were unaffected by urothelium removal. Pharmacological modulators inhibiting neurotransmitter release from nerve terminals, or blockade/desensitization of cholinergic/purinergic receptors, had no effect on GSK1016790A contractions. This suggests a lack of a role for TRPV4 expressed in the urothelium and/or in nerves within the bladder in mediating GSK1016790A contractions, and it implicates the TRPV4 protein expression detected in UBSM as the contractile mediator. TRPV4 contraction of airway smooth muscles has also been reported (Jia et al., 2004). The ability of TRPV4 activation to contract UBSM is consistent with identified constitutive and stretch-activated nonselective cation channels in UBSM cells (Wellner and Isenberg, 1993; Thorneloe and Nelson, 2004), because TRPV4 channels demonstrate constitutive activity (Watanabe et al., 2002b; Vriens et al., 2004) and sensitivity to stretch (Liedtke et al., 2000; Strotmann et al., 2000; Gao et al., 2003). In addition, we have observed a small contractile response to 4α-PDD at concentrations \(\geq 10 \mu M\) in TRPV4−/− mouse bladders, concentrations consistent with those shown to activate TRPV4 in electrophysiological recordings. Despite the conclusions presented by Birder et al. (2007) that concentrations up to 100 \(\mu M\) 4α-PDD did not affect basal tone of rat bladder strips, it seems from the data presented in Fig. 4 in their publication that 10 \(\mu M\) and above did elicit a small dose-dependent increase in baseline force, which is consistent with our 4α-PDD data on mouse bladder strips.

Nifedipine-sensitive, L-type Ca\(^{2+}\) channel (Cav1.2) mediated Ca\(^{2+}\) entry is the major contributor to mouse UBSM

**Fig. 5.** TRPV4 activation with GSK1016790A contracts mouse urinary bladder. A, effect of GSK1016790A (10, 30, and 100 nM) on bladder halves from TRPV4+/+ and TRPV4−/− mice. B, average change in force as a percentage of baseline (baseline force \(\sim 10\) mN) \(n = 6\) per group; two-way repeated measures analysis of variance, \(P = 0.009, **, P < 0.01\) versus TRPV4−/−.
constrictions (Wegener et al., 2004). In contrast to Cav1.2−/− mice (Wegener et al., 2004), TRPV4−/− mice do not demonstrate an overt loss in UBSM contractility, as indicated by normal contractile responses to electrical field stimulation and carbachol in vitro and normal peak voiding pressures in vivo. This is consistent with a modulatory role for TRPV4 in bladder contractility in vitro and bladder function in vivo. Nifedipine reversed GSK1016790A contractions, indicating a prominent role for L-type Ca2+ channels in mediating TRPV4 contractions, and suggesting that a direct supply of Ca2+ through TRPV4 channels is likely to play a minor role, if any, in the contraction. It seems that TRPV4 channel activation depolarizes the UBSM membrane potential, thereby activating L-type Ca2+ channel entry of contractile Ca2+. However, we can not completely eliminate a contributory role for an expression of TRPV4 in other cell types present within the UBSM layer including fibroblasts and interstitial cells (Davidson and Mccloskey, 2005). Conceivably, TRPV4 activation in these cells could also play a role and indirectly modulate UBSM contractility via electrical and/or chemical signaling that leads to nifedipine-sensitive contracture. These data suggest that TRPV4 plays a role in regulating the UBSM membrane potential and perhaps responds to urinary bladder distension during bladder filling, because TRPV4 channels are activated by stretch (Stromm, et al., 2000; Gao et al., 2003; Liedtke and Friedman, 2003).

Our urodynamic characterization of TRPV4−/− mice demonstrated an increased bladder capacity based on larger voided urine volumes and a requirement of a larger infused volume to elicit micturition events compared with TRPV4+/− mice. This phenotype is similar to that described in a distinct TRPV4−/− mouse (Gevaert et al., 2007). However, we do not feel that the urodynamic in TRPV4−/− mice represent a manifestation of an incontinent phenotype as concluded by Gevaert et al. (2007). In fact, our interpretation of both the bladder phenotype described here and by Gevaert et al. (2007) is consistent with a beneficial effect on bladder function, with the loss of TRPV4 resulting in an enhancement of bladder capacity equating to a reduced frequency of urination.

TRPV4 channels are expressed in sensory afferent neurons and have been implicated in thermal and mechanical hyperalgesia as well as pressure and heat sensation (Suzuki et al., 2003; Todaka et al., 2004; Lee et al., 2005; Alessandri-Haber et al., 2006; Grant et al., 2007). This suggests a potential for expression of TRPV4 in bladder-specific sensory afferent nerves functioning as regulators of in vivo bladder activity. In addition, TRPV4-mediated ATP release from the urothelium is proposed to sensitize sensory bladder afferents as a mechanism contributing to TRPV4-induced bladder hyperactivity in vivo (Birdet et al., 2007; Gevaert et al., 2007). We have shown that acetic acid sensitization of bladder afferents remains unaltered in TRPV4−/− mice. This finding would not support a prominent role for TRPV4 in vivo bladder affe-

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
<th>Pressure Threshold</th>
<th>Pressure Peak</th>
<th>Pressure Average</th>
<th>Volume Infused</th>
<th>Volume Void</th>
<th>Body Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV4+/− (n = 15)</td>
<td>17 ± 1</td>
<td>35 ± 2</td>
<td>11.6 ± 0.8</td>
<td>152 ± 18</td>
<td>134 ± 25</td>
<td>29.8 ± 0.6</td>
</tr>
<tr>
<td>TRPV4−/− (n = 11)</td>
<td>19 ± 2</td>
<td>34 ± 2</td>
<td>12.1 ± 2.2</td>
<td>259 ± 41*</td>
<td>254 ± 41*</td>
<td>28.2 ± 0.5</td>
</tr>
</tbody>
</table>

* P < 0.05 vs. TRPV4+/−, unpaired t test.
Fig. 7. TRPV4+/- mice have an enhanced bladder capacity. A, representative cystometric profile from TRPV4+/+ (top) and TRPV4-/- (bottom) mice. B, average volume infused to elicit micturition (infused) in TRPV4+/+ and TRPV4-/- mice. C, average volume voided (void volume) from TRPV4+/+ and TRPV4-/- mice. (*, significantly different, unpaired Student’s t test, TRPV4+/+ versus TRPV4-/-).

Fig. 8. Intravesicularly infused GSK1016790A reduces the bladder capacity of TRPV4+/+ but not TRPV4-/- mice. A and B, cystometric profile from a TRPV4+/+ and TRPV4-/- mouse, respectively, under control conditions (top) and in the presence of intravesicularly infused TRPV4 agonist GSK1016790A (10 μM) (bottom). Arrows indicate voiding events. C and D, average infused bladder volumes in the absence and presence of GSK1016790A in TRPV4+/+ (n = 8) and TRPV4-/- (n = 3) mice, respectively.
ent signaling under conditions of acidic/chemical inflammatory bladder irritation. However, our data are consistent with the lack of an altered response to acetic acid reported in urodynamic characterizations of TRPV1−/− mice (Birder et al., 2002). TRPV4-dependent release of ATP from the urothelium functioning to sensitize bladder afferents is not functional in vivo studies of bladders denuded of the urothelium, in which we have clearly identified a functional role for TRPV4-regulating contraction. Therefore, we would propose that the GSK1016790A-induced bladder overactivity in vivo (absent in TRPV4−/− mice) is a result of the functional role of TRPV4 in both the urothelium and the UBSM, causing a release of ATP from the urothelium-sensitizing bladder afferents (Birder et al., 2007; Gevaert et al., 2007) and direct contraction of UBSM, respectively.

In summary, we have described a novel TRPV4 activator GSK1016790A that is at least 300-fold more potent than the TRPV4 activator 4e-PDD. GSK1016790A along with TRPV4 antibodies have enabled a clear identification of the expression and function of TRPV4 channels in UBSM. Modulation of TRPV4 channel activity demonstrates the ability to negatively and positively regulate in vivo bladder function, because TRPV4 channel activation with GSK1016790A reduces bladder capacity, and loss of TRPV4 channels in TRPV4−/− mice enhances bladder capacity. We propose that TRPV4 plays a modulatory role regulating bladder contractility and compliance during filling.

References


TABLE 2 Effect of GSK1016790A (10 μM) on TRPV4−/− and TRPV4+−/− bladder pressures (mm Hg) and volumes (μl).
2 sensitizes the transient receptor potential vanilloid 4 ion channel to cause mechanical hyperalgesia in mice. J Physiol 578:715–733.


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