Previously we have shown that the transient receptor potential vanilloid 4 (TRPV4) channel regulates urinary bladder function, and that TRPV4 is expressed in both smooth muscle and urothelial cell types within the bladder wall. Urothelial cells have also been suggested to express TRPV1 channels. Therefore, we enzymatically isolated guinea-pig urothelial cells in an attempt to record TRPV4 and TRPV1-mediated currents. The identity of the isolated cells was confirmed by quantitative PCR for the urothelial marker uroplakin 1A. Whole-cell patch-clamp recordings with the TRPV4 agonist, GSK1016790A, activated urothelial currents with an EC50 of 11 nM that were completely inhibited by the TRPV4 inhibitor ruthenium red (5 μM). Urothelial currents were also activated by challenge with hypotonic extracellular solution (220 mOsm) known to activate TRPV4 channels. However, the TRPV1 agonist capsaicin, which activated TRPV1 currents in HEK cells expressing TRPV1, was unable to evoke current in these freshly isolated guinea-pig urothelial cells. We demonstrate that TRPV4 channels are functionally expressed at the plasma membrane of freshly isolated, guinea-pig urothelial cells, further supporting the important role of TRPV4 in urinary bladder physiology.

**Introduction**

Great interest has surrounded a role for the transient receptor potential (TRP) superfamily of ion channels in urinary bladder function. Specifically the TRPV1 isoform has been implicated in regulating urodynamics, based on a functional response to desensitizing TRPV1 activators infused into the bladder of numerous species including humans (reviewed in refs. 3 and 4). Although TRPV1 is expressed in bladder sensory afferents, a portion of this response has been suggested to occur as a result of TRPV1 expression in bladder urothelial cells. More recently, we and others have identified a role for the TRPV4 isoform in bladder function. TRPV4 expressed in bladder smooth muscle mediates contraction, and TRPV4 in the bladder urothelium regulates ATP release. Here we have utilized enzymatic dissociation techniques to freshly-isolate guinea-pig urothelial cells in order to record native urothelial TRPV4 and TRPV1 currents.

**Results**

**Urothelial cell identification.** Freshly isolated guinea-pig urothelial cells demonstrated a round to oval shape, a relatively smooth plasma membrane and a large nucleus centered in the middle of the cell. Cells were collected and pooled via suction into a wide-bore patch pipette. Bladder smooth muscle cells were enzymatically isolated along side the urothelial cells and collected as a comparator cell type. Taqman for the urothelial cell marker uroplakin 1A amplified transcripts from the urothelial cell RNA, detecting a 254-fold higher expression than from smooth muscle cell RNA. Furthermore amplification of the smooth muscle marker, smooth muscle alpha-actin, from urothelial cells revealed an mRNA expression level 0.006 times that in smooth muscle cells. TRPV4 expression was detected in both urothelial and smooth muscle cells with a 36-fold higher expression in urothelial cells.

**Urothelial whole-cell currents.** The average guinea-pig urothelial cell capacitance in the current study was 19 ± 2 pF (n = 17). The TRPV4 agonist GSK1016790A and TRPV1 agonist capsaicin were utilized to detect TRPV4 and TRPV1 currents, respectively (Fig. 1). 10 nM GSK1016790A evoked robust currents with an average current density of -79 ± 35 pA/pF and 168 ± 40 pA/pF (n = 5) at -60 mV and +60 mV, respectively.
Urothelial TRPV4 currents

(Fig. 1E). The average reversal potential for the GSK1016790A-activated current was -4.2 ± 2.6 mV (n = 5), and similar to the reversal potential predicted for monovalent cations based on solution composition (-4 mV). The EC₅₀ for GSK1016790A in activating urothelial TRPV4 was determined to be 11 nM, and GSK1016790A currents were completely blocked by the TRPV4 blocker ruthenium red (Fig. 2).

Capsaicin (10 μM) did not elicit any increase in ramp current (n = 4, Fig. 1), suggesting a lack of functional TRPV1 channels on the urothelial plasma membrane. However, capsaicin (1 and 10 μM) was able to activate robust TRPV1 currents in all TRPV1 expressing HEK cells (n = 4 for 1 μM, and n = 3 for 10 μM), which were blocked by capsazepine (10 μM, Fig. 3).

The TRPV4 activator hypotonicity⁹ also induced currents in the isolated guinea-pig urothelial cells (n = 4, Fig. 4), demonstrating properties consistent with TRPV4 and similar to those observed in response to GSK1016790A. Hypotonicity evoked currents were also blocked by the TRPV4 blocker ruthenium red (5 μM).
Urothelial TRPV4 currents

To our knowledge this is the first report of the successful recording of plasma membrane currents from freshly isolated urothelial cells, the identity of which was confirmed by Taqman expression of the urothelial cell marker, uroplakin 1A. Functional TRPV4 channels in the plasma membrane of these guinea-pig urothelial cells, was substantiated by currents evoked in response to the potent TRPV4 agonist, GSK1016790A. The GSK1016790A EC$_{50}$ of 11 nM determined here in guinea-pig urothelial cells is comparable to the potency we have measured previously using recombinant human TRPV4 channels, in both whole-cell patch clamp and Ca$^{2+}$ imaging experiments.1,8 Furthermore, currents consistent with TRPV4 were recorded from these cells in response to hypotonic stimulation, a known activator of the TRPV4 channel,9 and both hypotonicity and GSK1016790A-induced currents were inhibited by the TRPV4 blocker ruthenium red. These electrophysiological recordings indicating functional TRPV4 channels are supported by Taqman data demonstrating TRPV4 mRNA expression within these isolated urothelial cells.

Our detection of functional TRPV4 channels in the plasma membrane of guinea-pig urothelial cells is consistent with the presence of TRPV4 protein in rat and mouse urothelium, as assessed by western blotting and/or immunohistochemistry.1,5,6 The heightened expression of TRPV4 mRNA in guinea-pig urothelial cells as compared to smooth muscle cells reported here (36-fold), is analogous to Taqman results obtained with mouse urothelium and bladder smooth muscle tissues demonstrating an approximate 20-fold higher urothelial TRPV4 expression.1 Furthermore, the TRPV4 current density reported here in guinea-pig urothelial cells is substantially larger than those recorded previously from guinea-pig bladder smooth muscle cells.1 The TRPV4 expression difference between the smooth muscle and urothelium observed in mouse and guinea-pig appears to hold true across numerous species, as we have also observed a similar difference in expression between these cell types using laser microdissection and Taqman on human bladder samples (36-fold; Liu AX, et al. unpublished data).

The functional implication for urothelial TRPV4 channels in regulating urinary bladder activity is based on in vitro observations using rodent urothelial preparations in which TRPV4 activation, via stretch or the pharmacological activator 4alphaPDD, enhances ATP release.5,6 This release of ATP is hypothesized to activate purinergic receptors located on bladder afferents, which are in close proximity.
apposition to the urothelium. Purinergic receptor activation has been shown to facilitate afferent excitatory signaling and increase bladder reactivity. This mechanism is thought to support at least a portion of the in vivo response that is observed with infusion of TRPV4 activators into the bladder, although a direct contractile response of the bladder smooth muscle is likely also involved. We have also observed that prostaglandin E2 production is increased in response to urothelial TRPV4 channel activation (Edwards, et al. unpublished data). Therefore, in vivo urothelial TRPV4 activation in response to increased bladder pressures may play a significant role in enhancing bladder activity, via PGE2 and ATP mediated sensitization of bladder afferents.

We were unable to induce TRPV1 currents in these freshly isolated, guinea-pig urothelial cells using the selective TRPV1 agonist capsaicin, at concentrations that provided a robust activation of recombinant TRPV1 channels expressed in HEK 293 cells. TRPV1 expression in intact rat bladder urothelium was first reported by Birder and colleagues, using RT-PCR and immunolabeling. Additionally there is evidence suggesting an expression of TRPV1 in human urothelial cells. Recently in mouse bladder urothelium TRPV1 mRNA amplification was shown to be very low, protein expression was not observed, and a complete lack of urothelial Ca\(^{2+}\) influx in response to capsaicin was reported. In this same study a robust expression of TRPV4 mRNA, protein, and an influx of Ca\(^{2+}\) in response to TRPV4 activation was documented. These findings of Yamada et al. in mice are consistent with our data reported here in the guinea pig. In another recent study whole-cell electrophysiology was used to assess of TRPV1 and TRPV4 currents in cultured rat urothelial cells. Approximately 95% of cells responded to the TRPV4 activator 4alphaPDD with large currents, whereas the TRPV1 activator capsaicin elicited small currents in only 25% of cells evaluated. This electrophysiological study is somewhat consistent with our findings reported here in freshly-isolated guinea-pig cells, in which we show a clear contribution of TRPV4 channels to the membrane current and a lack of functional TRPV1 channels. The differences between the current study and Kullman et al. may be explained by the species difference (guinea-pig versus rat), different cell isolation techniques, or may reflect culture of the rat urothelial cells by Kullman et al. prior to recording currents.

In conclusion our data further support a role for urothelial cell TRPV4 channels in urinary bladder physiology, and suggest a lack of functional TRPV1 channels in the plasma membrane of native guinea-pig urothelial cells.

**Methods**

Urothelial and smooth muscle cell isolation. The urothelial layer was dissected from guinea-pig urinary bladders, cut into small pieces and incubated in nominal Ca\(^{2+}\) free solution composed of (in mM): 137 NaCl, 5 KH\(_2\)PO\(_4\), 1 MgSO\(_4\), 10 Glucose, 5 HEPES, 8 taurine and 1 mg/mL bovine serum albumin (pH = 7.4) on ice for 10 min. The tissue pieces were then transfected to an enzyme solution composed of the above buffer with 50 μM CaCl\(_2\), 1.5 mg/mL collagenase type II (Worthington Biochemical Corporation) and 0.25 mg/mL trypsin inhibitor (Sigma). The urothelial tissue in enzyme solution was incubated at room temperature and agitated using a Pasteur pipette. The solution was examined under a microscope at multiple time points until single urothelial cells were visible (incubation time ~15 minutes). Cells were retrieved from the enzyme solution by centrifugation followed by resuspension in enzyme free solution with 0.5 mM CaCl\(_2\). Isolation of guinea-pig bladder smooth muscle cells was performed as previously described. Cells were utilized for experiments on the same day of isolation.

**Taqman.** Freshly isolated guinea-pig urinary bladder urothelial and smooth muscle cells were collected for Taqman analysis using a wide-bore patch pipette. Total RNA was isolated using the RNAeasy (Qiagen), and RNA was reverse transcribed using the High-Capacity cDNA Archive (Applied Biosystems). A pre-amplification PCR of 14 cycles was performed using Applied Biosystems Preamp technologies prior to transcript quantification by Taqman. Beta-2-microglobulin was used as a housekeeper in quantifying the expression of Uroplakin 1A, smooth muscle alpha-actin (ACTA2) and TRPV4. Transcript expression levels were adjusted to housekeeper and presented as a ratio of urothelium/smooth muscle.

**Whole-cell recordings.** The conventional whole-cell voltage clamp technique at room temperature was used to record cell membrane currents. A ramp protocol was utilized from a holding potential of -60 mV, depolarizing from -60 mV to +60 mV over 760 ms, and elicited every 10 seconds. Current amplitudes were measured at -60 mV and +60 mV and normalized by the cell capacitance to obtain current density. For all urothelial cell experiments the internal solution was (in mM): 140 CsCl, 1 MgCl\(_2\), 4 Na\(_2\)ATP, 0.9 CaCl\(_2\), 10 EGTA, 10 HEPES, and adjusted to a pH of 7.2 using CsOH. Urothelial cells were initially bathed in external solution composed of (in mM): 140 NaCl, 4 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 10 Glucose, 10 HEPES (pH = 7.4 with NaOH). After establishing the whole-cell recording configuration, the cells were perfused with external solution lacking CaCl\(_2\) and experimental challenges were then performed. For osmotic challenge experiments, hypotonic bath solution (220 mOsm) was (in mM): 100 NaCl, 4 KCl, 1 MgCl\(_2\), 10 Glucose, 10 HEPES; pH = 7.4 and isotonic bath solution contained an additional 80 mM mannitol (300 mOsm). Hypotonic and isotonic solution osmolalities were verified by osmometer. For current recordings from HEK293 cells stably expressing human TRPV1 channels the internal solution was composed of (in mM): 140 KCl, 5 EGTA, 1 MgCl\(_2\), 0.2 CaCl\(_2\), 5 HEPES, 5 MgATP (pH = 7.2), and external solution was the same as that used for urothelial cells with 2 mM CaCl\(_2\).

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