Polyamines are important endogenous regulators of ion channels and are known to modulate inflammation and nociception. Here we investigated effects of polyamines on the capsaicin receptor TRPV1, a major ion channel expressed in nociceptive sensory afferents. Extracellular spermine, spermidine and putrescine directly activated TRPV1 in a charge-dependent manner, both in heterologous expression systems and sensory neurons. The threshold for activation by spermine was ~500 µM at room temperature. At lower concentrations, spermine enhanced capsaicin-evoked currents with an EC50 of ~5µM. Further, polyamines freely permeated TRPV1 (estimated relative permeabilities compared to Na+ were between 3 and 16) and spermine reduced the single channel conductance from 96 to 49 pS. Experiments with TRPV1 mutants identified extracellular acidic residues critical for polyamine regulation. Neutralization of aspartate 646 (D646N) abolished direct activation by spermine while neutralization of this same aspartate (D646N) or glutamate 648 (E648A) inhibited spermine-induced sensitization. These data show that polyamines, by virtue of their cationic charge, can regulate the activity of TRPV1. Extracellular polyamines are present in considerable concentrations in the gastrointestinal tract and at synapses, and these levels increase during inflammation and cancer. Therefore, polyamine regulation of TRPV1 in these tissues may be relevant to a variety of physiological and pathophysiological states.

Polyamines are abundant organic cations essential for normal cell division and growth. At physiological pH, polyamines are positively charged and thus can interact electrostatically with negatively charged nucleic acids and proteins, including ion channels. Indeed, polyamines have a demonstrated role in ion channel regulation. Intracellular spermine and spermidine contribute to rectification of inward rectifier K+ channels (1,2) and Ca2+ permeable AMPA and Kainate glutamate receptors (3,4). In addition, intracellular and extracellular polyamines can respectively block the Transient Receptor Potential Melastatin channels, TRPM4 (5) and TRPM7 (6). Furthermore, extracellular spermine produces complex effects on NMDA receptors, both stimulating activity (7-9) and inducing a voltage-dependent block (10). Finally, extracellular polyamines serve as ligands for the calcium sensing receptor (CaR) (11), a G-protein coupled receptor that contributes to the regulation of calcium homeostasis.

 Interestingly, polyamines are known to regulate inflammation and pain signaling. Levels of polyamines are raised at inflammatory sites of infection, trauma and cancer (12) and intrathecal administration of spermine in mice produces nocifensive behaviors (i.e. scratching, licking and biting) characteristic of noxious stimuli (13). The target(s) for these effects of spermine has not been clearly defined. In this study, we investigated effects of polyamines on TRPV1, a major ion channel expressed in nociceptive primary afferent neurons. Significantly, TRPV1 is regulated by protons (14,15) and by extracellular cations including Na+, Mg2+ and Ca2+ (16). These cations sensitize TRPV1 to ligand activation, and divalent cations (>5 mM) directly activate the receptor and induce pain-related behaviors in mice (16). We therefore hypothesized that cationic polyamines could regulate TRPV1 activity. We show that extracellular spermine, spermidine and putrescine activate TRPV1 in a charge-dependent manner.
Moreover, spermine potently sensitizes TRPV1, enhancing capsaicin responses with an EC\textsubscript{50} of ~5 µM, a concentration that falls within the range of normal serum levels (17). These responses are abolished in TRPV1 mutants lacking key acidic residues near the extracellular pore-forming region. Thus, extracellular polyamines may represent a new class of endogenous TRPV1 ligands.

**MATERIALS AND METHODS**

All experimental procedures involving animals were approved by the Georgetown University Animal Care and Use Committee and conform to NIH guidelines.

*Oocyte electrophysiology—* *Xenopus* laevis oocytes were injected with ~10 ng of wild-type rat TRPV1 cRNA or mutants E600Q, D646N and E648A (gifts from David Julius). Double electrode voltage clamp was performed using a Warner amplifier (Warner Instruments, OC725C). Oocytes were superfused (5 ml/min) with Ca\textsuperscript{2+}-free solution containing (in mM): 100 NaCl, 2.5 KCl, 5 HEPES, 1 MgCl\textsubscript{2} and titrated to pH 7.3 with ~5 mM NaOH. For the solution containing 10 mM spermine, the concentration of NaCl was reduced to 80 mM. For solutions < pH 6.0, HEPES was replaced with 5 mM MES.

*Sensory neuron and HEK293F cell culture and electrophysiology—* Nodose ganglia were obtained from adult mice (C57Bl6/J) and mice lacking the TRPV1 receptor (B6.129S4-Trpv1tm1Jul/J), cut, digested with collagenase, and cultured in Neurobasal +2% B-27 medium (Invitrogen), 0.1% L-glutamine and 1% penicillin/streptomycin on poly-D-lysine-coated glass coverslips at 37°C in 5% CO\textsubscript{2}. Neurons were used within 24-36 hr of culture. HEK 293F cells (Invitrogen) were cultured in DMEM supplemented with 1% non-essential amino acids and 10% fetal calf serum and were cultured on poly-D-lysine-coated glass coverslips. Cells were transfected with rat TRPV1 and GFP cDNA using Lipofectamine\textsuperscript{TM} Transfection Reagent (Invitrogen) or Lipofectamine 2000\textsuperscript{TM} (Invitrogen) according to the manufacturer’s instructions.

Whole-cell patch clamp and excised patch recordings were performed using an EPC8 amplifier (HEKA). The current signal was low-pass filtered at 1-3 kHz and sampled at 4 kHz. The bath solution contained (in mM): 140 NaCl, 4 KCl, 1 MgCl\textsubscript{2}, 1 EGTA, 10 HEPES, 10 glucose, pH 7.3 (290 mOsm). The pipette solution contained (in mM): 140 CsCl, 10 NaCl, 10 HEPES, 5 EGTA, 2 MgATP and 0.3 GTP, pH 7.3.

For measurements of polyamine permeability the bath contained (in mM): 30 spermine, spermidine or putrescine, 10 HEPES, pH 7.3 (titrated with HCl) and osmolarity was adjusted to 300 mOsm with mannitol; the pipette solution contained (in mM): 150 NaCl, 5 EGTA, 10 HEPES, pH 7.3, buffered with NaOH. Liquid junction potentials were measured (spermine, -3 mV; spermidine, -1 mV; putrescine, +1 mV, and for comparison 140 mM K-gluconate, +12 mV) and subtracted as previously described (18). Permeability ratios were calculated as follows: $P_{X}/P_{Na} = [Na^+]_o \exp(\Delta V_{rev}F/RT)/(1 + \exp(\Delta V_{rev}F/RT))/z^2[X]_o$, where X represents a polyamine cation of valency, z. Ion activity coefficients were 0.75 for Na\textsuperscript{+}, 0.1 for spermine, 0.195 for spermidine and 0.5 for putrescine estimated from published values for polyvalent ions at 50 mM (19).

**RESULTS**

Polyamines activate TRPV1 in HEK293 cells and oocytes

We examined the effects of spermine on the function of TRPV1 expressed in HEK293 cells. Figure 1A shows that extracellular spermine activated inward currents in voltage-clamped cells. This activation occurred in a dose-dependent manner, 500 µM and 5 mM spermine induced responses that were ~9 and 29 % of that produced by 30 nM capsaicin (Fig. 1B). Spermine activated currents in all 65 cells tested. In contrast, no responses to spermine were observed in cells transfected with GFP alone (n=7, data not shown).

To quantify further activation by spermine we compared the magnitude of the spermine-evoked currents to the response evoked by a saturating concentration of capsaicin (1µM). In these experiments, spermine was included in the capsaicin solution to control for possible effects on ion permeation. This analysis revealed that 5 mM spermine activated ~15 % of the maximal TRPV1 activity (Fig 1C). Significantly, this increased to 42 % after stimulation of PKC, which is known to
sensitize TRPV1 to agonists (20,21). We also observed spermine activation of TRPV1 in *Xenopus* oocytes. In this system however, TRPV1 exhibited an apparent lower sensitivity. Under control conditions, 1 and 10 mM spermine evoked only small currents at positive potentials (Fig 1D), but much larger responses were evident after activation of PKC (Fig. 1E). Next, we tested the ability of the related polyamines, spermidine and putrescine, to activate TRPV1 in HEK293 cells. Figure 2A shows that 5 mM concentrations of these polyamines activated TRPV1 in a charge-dependent manner; spermine (+4) was approximately twice as effective as spermidine (+3), while putrescine (+2) evoked little detectable current. To explore the permeability of TRPV1 to polyamines, we measured the current-voltage relationship under bionic conditions with NaCl in the pipette and with extracellular solutions containing 30 mM of spermine, spermidine or putrescine as sole charge carrier. Spermine and spermidine provoked pronounced inward currents and shifted the reversal potentials to more positive values (~19 and 14 mV respectively, Fig. 2C). These values correspond with a relative permeability of polyamines to Na⁺ (Pₓ/Pₙa) of ~15.9 and 10.5 respectively (assuming polyamine activity co-efficients of 0.1 and 0.195). Putrescine alone evoked little detectable inward current but increased the current evoked by 30 nM capsaicin and produced a small negative shift in the reversal potential of -2 mV (Fig. 2D). This corresponds to a Pₓ/Pₙa of ~3.4 (using an activity co-efficient of 0.5). Thus, polyamines permeate TRPV1 and the channel exhibits a high degree of selectivity towards these cations.

**Spermine excites sensory neurons via TRPV1**

Next, we examined the ability of spermine to regulate TRPV1 in cultured sensory neurons. Similar to the effect seen in HEK293 cells, spermine activated inward currents in capsaicin-sensitive neurons (n=26), with 500 µM and 5 mM spermine eliciting responses ~8 and 28 % of 30 nM capsaicin (Figs 3A&B). Significantly, no responses to spermine were observed in capsaicin-insensitive neurons (n=18, data not shown) or in neurons cultured from TRPV1-null mice (Fig. 3D, n=6). Further, the currents evoked by spermine (500 µM) were fully blocked by the TRPV1 antagonist, capsazepine (5 µM, Fig. 3C, n=5).

Taken together, these data confirm that spermine selectively activates TRPV1 in sensory neurons. We also studied the effects of spermine in current-clamped neurons. Spermine (500 µM) produced a membrane depolarization and action potentials in capsaicin-sensitive neurons (Figure 3E, n=3) but failed to do so in capsaicin-insensitive cells (n=4, data not shown). Thus, spermine can excite nociceptive neurons via the activation of TRPV1 and this is effective at concentrations that do not inhibit impulse generation.

**Spermine sensitizes TRPV1 to capsaicin in sensory neurons and oocytes**

We have previously shown that cations can sensitize TRPV1 to various ligands and this occurs with cations concentrations that are insufficient to directly gate the channel (16). We therefore tested whether lower concentrations of spermine could similarly enhance capsaicin-evoked responses. In sensory neurons, 100 µM spermine elicited no detectable current but markedly enhanced the currents evoked by 10 nM capsaicin (Fig. 4A; 2.8± 0.3 fold, n=11). The EC₅₀ for this effect was ~5µM (Fig. 4B). Normal serum levels of polyamines are in the low micromolar range (17). Thus, spermine is a potent and physiologically relevant modulator of TRPV1 sensitivity. This sensitization was dependent on the capsaicin concentration. In TRPV1-expressing oocytes, spermine enhanced responses to a sub-saturating concentration of capsaicin (500 nM) but reduced the response to a saturating dose (10 µM, Fig. 4C-E). At intermediate concentrations near the EC₅₀ for capsaicin (1-2.5 µM) the net effect of these opposing actions was balanced and spermine produced little change in current (Fig 4E). This agrees with a recent study by Kitaguchi and Swartz (22) who also found, using the oocyte-expression system, that 1 mM spermine did not alter TRPV1 currents activated by 1 µM capsaicin. The decrease in current with higher concentrations of capsaicin likely reflects a spermine-induced reduction in single channel conductance rather than an alteration in open probability. Indeed single channel recordings (data not shown) revealed that spermine (5 mM) reduced the unitary conductance from 96±5 pS to 49±5 pS (n=3; chord conductance at +50 mV and driving force ~50 mV and 45 mV respectively).
Spermine interacts at glutamate (E648) and aspartate (D646)

We next examined the mechanism underlying spermine regulation of TRPV1. In contrast to effects seen with capsaicin, spermine failed to increase the current evoked by either protons (pH 4) or Mg$^{2+}$ (70 mM) (Figure 5A&B). These stimuli produce sub-maximal TRPV1 currents ~30% of the maximal capsaicin response (16,23) and therefore the lack of a synergistic effect cannot be attributed to a ceiling response. Both protons and divalent cations are known to regulate TRPV1 via extracellular glutamate residues (E600 and E648) (16,23), and therefore their ability to occlude spermine suggests that the latter may also act at these sites. To test this further, we examined the responses to spermine in TRPV1-mutants lacking these key glutamates (E600Q and E648A). We also considered a mutant receptor lacking aspartate 646 (D646N) because our preliminary studies indicate this residue may also contribute to regulation by cations (Ahern unpublished observations). To examine sensitization, we measured the potentiation of capsaicin-evoked response produced by 1 mM spermine (a concentration we found was insufficient to directly activate TRPV1 in oocytes at -60 mV, see Fig. 1D). Capsaicin concentrations were chosen to approximate the EC$_{50}$ values for the respective TRPV1 receptor (wild-type, 500 nM; E648A, 500 nM; D646N, 250 nM and E600Q, 25 nM) (23,24). Figure 5C shows that sensitization by spermine was significantly reduced in receptors lacking either E648 or D646 whereas in E600Q this effect was enhanced ~8 fold. To test the involvement of these residues in direct activation, we examined responses to 1 mM spermine after PKC stimulation, which reduced the concentration required to directly activate TRPV1 (see Fig. 1E). We used this approach because we observed that higher concentrations of spermine produced inactivation of TRPV1 currents in oocytes (note that this was not observed in mammalian cells). Figure 5D shows that spermine-evoked currents were unaffected by substitution of E648 but were almost abolished by removal of D646. In contrast, spermine evoked substantially larger (~4-fold) currents in E600Q mutants than in wild-type receptors. In addition, spermine activated currents in these E600Q mutants in the absence of PKC stimulation (data not shown). Taken together these data indicate an important role for the acidic residues located near the pore forming region, D646 and E648, in modulation of TRPV1 by spermine. In contrast, E600 appears to play little role in polyamine regulation. Consequently, neutralization of this residue synergistically enhances rather than blocks the response to spermine.

DISCUSSION

This study identifies polyamines as novel regulators of TRPV1 channel activity. We show that spermine and spermidine directly activate TRPV1 both in TRPV1-expressing HEK293 cells and in sensory neurons. At room temperature, this activation occurred with concentrations greater than 500 µM; however the temperature dependent sensitivity of TRPV1 suggests that polyamines may be considerably more potent at physiological 37°C. In addition to direct activation, we show that polyamines can sensitize TRPV1. Spermine potentiated capsaicin-evoked responses in sensory neurons with an EC$_{50}$ of ~5 µM. In contrast to both spermine and spermidine, putrescine failed to directly activate TRPV1 at concentrations as high as 30 mM but still enhanced capsaicin-evoked responses (Fig. 1). These data are consistent with polyamines regulating TRPV1 in a charge-dependent manner (spermine, 4+ > spermidine, 3+ > putrescine, 2+). This agrees with our prior observations that extracellular cations can activate and sensitize TRPV1 (16).

Similar to divalent cations, our data indicate that spermine interacts with extracellular site(s) on TRPV1. First, sensitization by spermine was prevented in the presence of high concentrations of protons or Mg$^{2+}$. Protons and cations are known to act at extracellular glutamate residues (16,23) and therefore this result suggests that spermine interacts at similar sites. Second, experiments with mutant TRPV1 channels confirmed the involvement of two acidic residues, E648 and D646, with the latter alone being critical for direct activation. Unlike divalent cations or protons, spermine does not appear to directly interact with E600. Interestingly, interactions with acidic residues are also believed to underlie polyamine regulation of K$^+$ channels and glutamate receptors (9).
Moreover, we show that polyamines, in addition to regulating channel activity, can permeate TRPV1. This appears to be an essential condition for activation/sensitization of TRPV1 by cations; non-permeant cations would instead block the channel. Accordingly, TRPV1 can be activated/sensitized by various permeant cations including H⁺, Ca²⁺, Mg²⁺, Na⁺, Li⁺ and polyamines. Conversely, the trivalent Gd³⁺ capable of activating TRPV1 at 10-100 µM, blocks at >100 µM, presumably via a pore-block mechanism (25). Similarly, several acylpolyamines isolated from spider venom block TRPV1 in a voltage-dependent manner (22). Our data reveal a high selectivity of TRPV1 for polyamines and this suggests that TRPV1 may contribute to the transport of polyamines across cell membranes. Although an active polyamine uptake system is clearly demonstrable in many mammalian cells, the molecular machinery has not yet been clearly identified (26). A plasma membrane diamine transporter may mediate the efflux of diamines and acetylated polyamines (27). However, little is known about the cellular efflux pathways for free polyamines. In this respect, TRPV1 and other TRP channels may represent novel candidates for polyamine transport.

Although abundant in the intracellular milieu, considerable quantities of polyamines may be found in extracellular fluids. Basal serum levels of polyamines are reported to be in the low micromolar range (17). These concentrations are capable of modulating NMDA receptors (7). Based on the data we report here, micromolar levels of spermine would also be sufficient to profoundly sensitize TRPV1. In addition, very high concentrations of polyamines (up to 1 mM) may be found in inflamed tissues (12). Thus, TRPV1 could be directly activated by polyamines at these inflammatory sites. Polyamines can also accumulate in synaptic vesicles and may be released from neurons via Ca²⁺-triggered exocytosis (28). Such a mechanism could produce high local concentrations in the synaptic cleft, sufficient to activate TRPV1, which is located presynaptically in the spinal cord and in many regions of brain. In addition, dietary intake and bacterial metabolism generate high concentrations of polyamines in the gut (17). These polyamines could regulate TRPV1 expressed in epithelial cells lining the stomach and small intestine, or in nearby sensory neurons. These direct or neurogenic signaling pathways could contribute to the well-known mitogenic effects of polyamines in the gastrointestinal tract (29).

Interestingly, spermine is implicated in nociception and exogenous application of spermine produces pain-related behaviors in mice (13). These have been attributed to activation of NMDA receptors because they are inhibited by NMDA receptor antagonists. Our results suggest that activation of TRPV1 on primary sensory afferents contributes to the nociception induced by spermine. An involvement of TRPV1 in this process would be consistent with sensitivity to NMDA antagonists, since TRPV1 activation occurs upstream of glutamatergic transmission in the spinal cord.

In summary, we show for the first time that extracellular polyamines activate, sensitize and permeate the TRPV1 ion channel. We propose that polyamines may represent a new class of endogenous TRPV1 ligands.

REFERENCES


FOOTNOTES

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FIGURE LEGENDS

Figure 1. Spermine activates TRPV1 in HEK293 cells and oocytes.
A. A representative trace from a voltage-clamped HEK293 cell (-50 mV) shows currents evoked by 500 µM and 5 mM spermine (Spm) and 30 nM capsaicin (Cap). B. Mean current in HEK293 cells induced by 500 µM (n=7) and 5 mM (n=6) spermine as percentage of 30 nM capsaicin. C. Mean current produced by 5 mM spermine normalized to the maximal response evoked by 1 µM capsaicin (+ 5 mM spermine), under control conditions (n=5) and after pretreatment with 200 nM PDBu (n=4, * p<0.05 compared to control). D&F. Current-voltage relationships in TRPV1-expressing oocytes in response to 1 and 10 mM spermine, under control conditions and after pretreatment with PDBu. The baseline currents (pre-PDBu) were subtracted.

Figure 2. Relative activation and permeation of TRPV1 by polyamines
A. Structures of the polyamines, spermine (Spm), spermidine (Sdp) and putrescine (Put). B. Summary of currents produced by 5 mM concentrations of spermine, spermidine and putrescine in HEK293 cells.
Currents are expressed as a percentage of the response to spermine and were obtained from 3-4 cells for each point. C. Current-voltage relationship for responses produced by 30 mM concentrations of either spermine or spermidine with the pipette solution containing 150 mM NaCl (liquid junction potentials were subtracted as described in Methods). Mean reversal potentials were 19.2 ± 2.8 mV (n=5) and 14.3 ± 1.7 mV (n=7) for spermine and spermidine respectively. D. Current-voltage relationship for responses evoked by 30 nM capsaicin (in Na+ medium) and 30 mM putrescine + capsaicin (bold). Reversal potentials were 0 mV and -2 ± 2 mV (n=3) respectively for capsaicin and putrescine + capsaicin.

Figure 3. Spermine activates TRPV1 in sensory neurons.
A. Representative currents evoked by spermine (500 µM and 5 mM) and capsaicin (30 nM) in a voltage-clamped neuron (-60 mV). B. Mean current induced by 500 µM (n=13) and 5 mM (n=9) spermine as a percentage of 30 nM capsaicin. C. The current induced by 500 µM spermine was fully blocked by capsaizepine (5 µM). D. Spermine (500 µM and 10 mM) failed to evoke any current in a neuron obtained from a TRPV1-null mouse. The trace is a representative recording from one of six neurons all of which responded to 100 µM ATP. E. Spermine (500 µM) depolarized a neuron under current clamp. The arrowhead indicates -60 mV.

Figure 4. Spermine increases capsaicin-evoked currents.
A. Spermine (100 µM) increased the current evoked by capsaicin (10 nM) in a cultured sensory neuron (-60 mV). Note that 100 µM spermine alone produced no current (data not shown). B. In neurons, spermine dose-dependently increased the capsaicin-evoked current over the range 1-100 µM with an EC50 of 4.8 µM. Each point is the mean (±se) of 3 to 4 cells. C&D. Current-voltage relationships in TRPV1-expressing oocytes stimulated with 500 nM or 10 µM capsaicin alone (dotted lines) or capsaicin plus spermine (1 mM, bold lines). Note that 1 mM spermine alone produced no current at -60 mV in oocytes (see Fig. 1D). E. Summary of potentiation produced by 1 mM spermine at varying concentrations of capsaicin. Data are mean of 3-9 oocytes.

Figure 5. Spermine sensitizes and activates TRPV1 via acidic residues near pore forming region.
A&B. Current-voltage relationships in TRPV1-expressing oocytes during application of either protons (pH 4) or Mg2+ (70 mM) alone (dotted lines) or in combination with spermine (1 mM, bold lines). Note that both treatments occluded potentiation by spermine. C. Summary of spermine-induced potentiation of currents evoked by capsaicin in oocytes expressing wild-type, E648A, D646N and E600Q TRPV1 channels. The capsaicin concentrations used were 500nM for WT and E648, and 250 nM for D646N and 25 nM for E600Q and were selected based on reported EC50 values for these receptors (23,24). Control data representing two successive applications with capsaicin alone was combined from all groups. The mean fold potentiation is shown for 4-9 oocytes (* p<0.01 ** p<1E-6 compared to control). D. Summary of direct spermine activation in wild type and mutant TRPV1 channels. Oocytes were pretreated with PDBu (200 nM, 3 min) prior to application of 1 mM spermine. Data are mean from 7-13 oocytes expressed as a percentage of saturating 10 µM capsaicin (plus 1 mM spermine). (* p<0.01, ** p<2E-6 compared to wild-type TRPV1). E. Model of TRPV1 showing key acidic residues (grey) identified as important for spermine regulation of TRPV1.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Polyamines are potent ligands for the capsaicin receptor TRPV1
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