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Report

TRPV1 Gene Required for Thermosensory Transduction and Anticipatory Secretion from Vasopressin Neurons during Hyperthermia

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SUMMARY

Increases in core body temperature promote thermoregulatory cooling by stimulating sweat production and preemptive renal water reabsorption through the release of vasopressin (VP, antidiuretic hormone). The mechanism by which the hypothalamus orchestrates this anticipatory VP release during hyperthermia is unknown but has been linked to a central thermosensory mechanism. Here, we report that thermal stimuli spanning core body temperatures activate a calcium-permeable, ruthenium red- and SB366791-sensitive nonselective cation conductance in hypothalamic VP neurons. This response is associated with a depolarizing receptor potential and an increase in action potential firing rate, indicating that these neurons are intrinsically thermosensitive. The thermosensitivity of VP neurons isolated from *trpv1* knockout (*Trpv1*$^{-/-}$) mice was significantly lower than that of wild-type counterparts. Moreover, *Trpv1*$^{-/-}$ mice showed an impaired VP response to hyperthermia in vivo. Channels encoded by the *trpv1* gene thus confer thermosensitivity in central VP neurons and contribute to the thermal control of VP release in vivo.

INTRODUCTION

Mammals respond to a rise in core body temperature (hyperthermia) by promoting evaporative heat loss through panting and the production of sweat (Shibasaki et al., 2006). Remarkably, the potential impact of this response on body fluid balance is blunted due to a simultaneous increase in the release of vasopressin (VP, antidiuretic hormone) from the neurohypophysis (Segar and Moore, 1968). How this important anticipatory homeostatic response is achieved is not known, but previous work has indicated that a central thermosensory mechanism may be required for this effect to occur (Szczepanska-Sadowska, 1974; Forsling et al., 1976). Indeed, VP release can be stimulated by thermode heating of the hypothalamus (Szczepanska-Sadowska, 1974), and the anticipatory release of this hormone in hyperthermic animals can be blunted by thermode cooling of this part of the brain (Forsling et al., 1976). Interestingly, the VP-releasing neurons of the hypothalamus were recently shown to express a splice variant of the heat-gated channel TRPV1 (Sharif-Naeini et al., 2006). We therefore examined whether expression of the *trpv1* gene might render VP neurons intrinsically thermosensitive and be required for VP release during hyperthermia in vivo.

RESULTS

VP Neurons Display a Heat-Activated Current

To determine whether VP neurons can be specifically activated by increases in temperature, whole-cell voltage-clamp recordings were obtained from VP neurons acutely isolated from the rat supraoptic nucleus (SON) and from non-neurosecretory neurons isolated from the adjacent perinuclear zone (PNZ), which do not express TRPV1 (Sharif-Naeini et al., 2006). Each cell was thermally challenged by gradually increasing the temperature of the perfusate from 25°C to 39°C over a period of ~2 min. In contrast to PNZ neurons, which showed a small and quasilinear increase in holding current over the entire range of temperatures tested (n = 13), VP neurons displayed a prominent increase in temperature-sensitive inward current at temperatures above 35°C (n = 15; Figures 1A and 1B). Although similar increases in holding current could be evoked reproducibly in single VP neurons (data not shown), the increase in holding current induced by consecutive heat stimuli rarely showed a complete recovery (average recovery was 63.7% ± 6.5%). Because the effect of increasing heat was repeatable, we focused our analysis on this part of the response rather than on the protracted and incomplete recovery phase. For quantification purposes, heat-evoked changes in holding current were expressed in Arrhenius plots (e.g., Figure 1 B), and the absolute slope of a linear regression through the data points was used to quantify the thermal current coefficient (i.e., Q_{10}) of the response above and below 35°C. Whereas both types of cells showed similar slopes in the 25°C–35°C range (Figure 1B), the mean values of Q_{10} observed in the 35°C–39°C range were significantly greater in VP neurons (13.1 ± 1.0, n = 15) than in PNZ neurons (2.9 ± 0.4, n = 13, $p = 3 \times 10^{-8}$).

VP Neurons Are Thermosensitive

To determine whether the heat-activated current generated in VP neurons can mediate thermosensitive changes in electrical activity, we examined the effects of thermal stimuli applied under

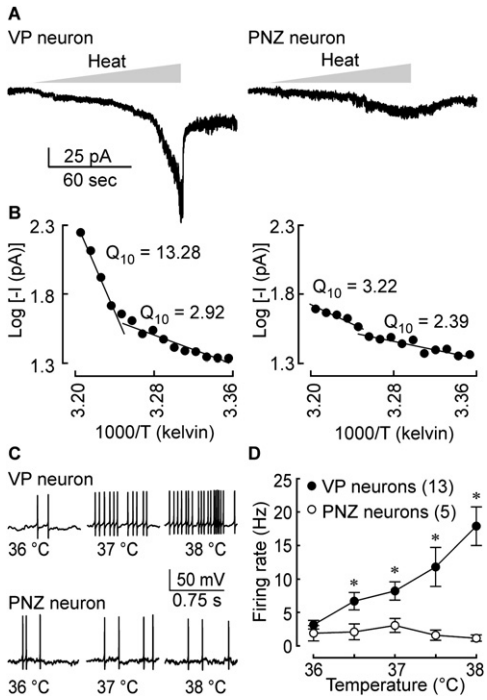

Figure 1. Vasopressin Neurons Are Thermosensitive

(A) Sample traces showing the effects of raising temperature (T) from 25°C to 39°C (heat, gray ramps) on holding current (V_H −60 mV) in single VP and PNZ neurons.

(B) Arrhenius plots of the data in (A). The Log of the average absolute current at each temperature point was plotted against 1000/T (in Kelvin). Separate linear regressions were fit through data taken between 25°C–35°C and between 35°C–39°C. The absolute value of the slope is the thermal coefficient, Q_{10}. Note the greater thermosensitivity of the VP neuron above 35°C.

(C) Sample traces demonstrate the effects of temperature on electrical activity in single VP and PNZ neurons recorded in current clamp.

(D) Mean (±SEM) effect of temperature on action potential firing rate in VP (filled circles) and PNZ (empty circles) neurons (*$p < 0.05$).

current clamp. Indeed, increasing the temperature of the perfusate from 36.0°C to 38.0°C over a period of ~2 min caused a significant increase in the firing rate of VP neurons (from 2.91 ± 0.77 Hz to 17.89 ± 2.93 Hz, p = 0.001, n = 10) but had no effect on the firing rate of PNZ neurons (from 1.87 ± 1.17 Hz to 1.12 ± 0.41 Hz, p = 0.65, n = 5; Figures 1C and 1D). Linear regression analysis indicated that the thermal activity coefficient of VP neurons (+5.6 ± 1.1 Hz/°C, n = 10) was well above + 0.8 Hz/°C, a criterion commonly used to define a neuron as being warm sensitive (e.g., Boulant and Dean, 1986), whereas PNZ neurons were not thermosensitive (thermal activity coefficient −0.3 ± 0.5 Hz/°C, n = 5, data not shown). Previous studies have shown that heating does not cause a significant depolarization of warm-sensitive neurons in the preoptic area (Zhao and Boulant, 2005). Rather, the thermosensitivity of these neurons appears to be caused by a heat-induced increase in the rate of inactivation of the transient potassium current and a corresponding reduction of the interspike interval (Griffin et al., 1996; Zhao and Boulant, 2005). However, in contrast to warm-sensitive preoptic neurons (Zhao and Boulant, 2005) and temperature-insensitive PNZ neurons (1.17 ± 0.40 mV/°C, n = 5, data not shown), thermally stimulated VP neurons displayed a significant membrane depolarization in response to heat (+5.59 ± 1.31 mV/°C, p = 0.03, n = 10). Together with the positive thermal current coefficient reported above, these observations suggest that an active depolarizing receptor current underlies the thermosensitivity of VP neurons in the SON.

VP Neurons Express Heat-Activated Calcium-Permeable Cation Channels

To determine the nature of the ionic current responsible for the thermosensitivity of VP neurons, we performed steady-state current-voltage (I-V) analysis and ion substitution experiments under whole-cell voltage clamp. Thermal stimulation of rat VP neurons was associated with an increase in membrane conductance (Figure S1 available online), indicating that ion channels were activated by heat. When compared to that observed in control solution (−24.2 ± 2.9 mV, n = 6), the reversal potential of the thermosensitive current was not different when measured in a low-chloride solution (−23.7 ± 2.8 mV, n = 5, p = 0.75) or in a solution comprised mainly of $CaCl_2$ (−29.3 ± 8.2 mV, n = 5, p = 0.60). In contrast, the reversal potential of the heat-evoked current was significantly more negative when measured in the absence of external sodium (−60.4 ± 9.3 mV, n = 3, $p = 2 \times 10^{-4}$). Thus, the thermosensitive current of VP neurons is mediated by calcium-permeable nonselective cation channels, in agreement with the permeability features of TRPV channels (Venkatachalam and Montell, 2007). If such channels are involved, then the thermosensitivity of these neurons should be inhibited by ruthenium red (RuR), a generic blocker of TRPV channels (Ramsey et al., 2006). Indeed, the heat-induced increase in membrane conductance recorded in VP neurons was significantly smaller in the presence of 10 μM RuR (18 ± 5 pS, n = 6) than in control conditions (183 ± 30 pS, n = 7, $p = 4 \times 10^{-4}$; see Figure S1).

trpv1 Gene Expression Is Required for Thermosensory Transduction in VP Neurons

To determine whether expression of the *trpv1* gene is specifically required for thermosensitivity, we first compared the thermal responses of VP neurons obtained from wild-type (WT) and *Trpv1*$^{-/-}$ mice. As illustrated by the current-temperature (I-T°) relations shown in Figures 2A and 2B, heat stimuli were less effective at evoking responses in VP neurons from *Trpv1*$^{-/-}$ animals than from WT animals. Indeed, the mean thermosensitivity of VP neurons between 35°C and 39°C was significantly greater in WT mice (Q_{10} of 19.3 ± 2.8, n = 19) than in *Trpv1*$^{-/-}$ mice (Q_{10} of 8.5 ± 1.2, n = 13, p = 0.002; Figure 2C). The impaired thermosensitivity of VP neurons in *Trpv1*$^{-/-}$ animals is consistent with a *trpv1* gene product acting as an integral part of the heat-sensing channel. However, an alternate possibility is that the heat-sensing channels do not comprise a product of the *trpv1* gene but that expression of the latter is required for specification of a thermosensory phenotype during development or for correct production or targeting of the channel in mature neurons. Therefore, as a second approach, we performed an acute loss-of-function analysis using the selective TRPV1 channel antagonist SB366791 (Gunthorpe et al., 2004). As illustrated in Figure 2, I-T° relations and average Q_{10} values were significantly smaller in WT VP neurons tested in the presence of 1.5 μM SB366791 than under control conditions. Moreover, addition of

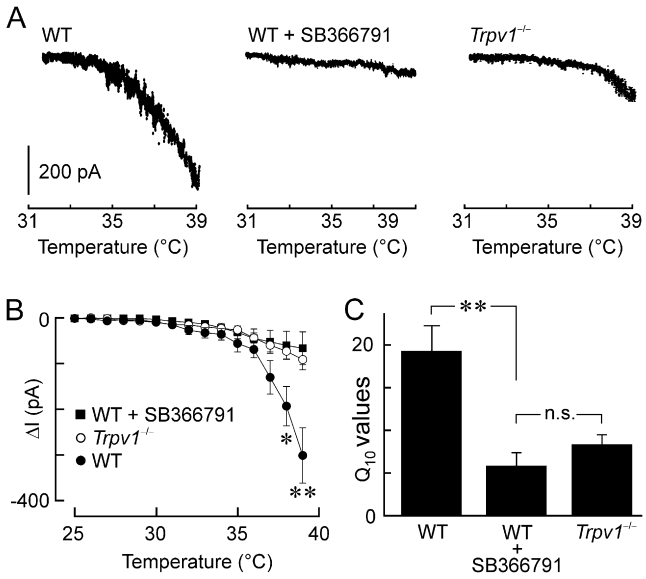

Figure 2. The *trpv1* Gene Contributes to Thermosensitivity in Isolated Mouse VP Neurons

(A) Sample I-T° relations measured in VP neurons obtained from WT mice in the absence (WT) or presence of SB366791 (1.5 μM) and from *Trpv1*$^{-/-}$ mice.

(B) Plots show mean (±SEM) I-T° relations measured in VP neurons from WT mice in the absence (WT) or presence of 1.5 μM SB366791 or from *Trpv1*$^{-/-}$ mice (*p < 0.05, **p < 0.01 compared to *Trpv1*$^{-/-}$ group).

(C) Mean (±SEM) Q_{10} values observed in the three different groups between 35°C and 39°C (WT: 19.3 ± 2.8, n = 19; WT + SB366791: 5.8 ± 1.5, n = 10; *Trpv1*$^{-/-}$: 8.5 ± 1.2, n = 13; **p < 0.01).

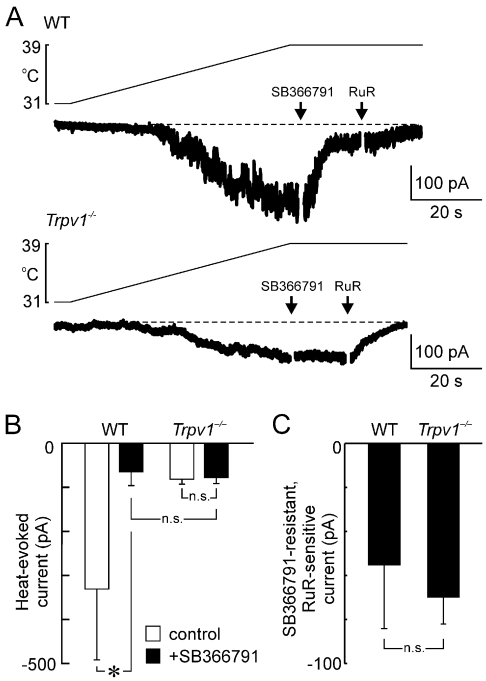

Figure 3. SB366791 Inhibits the Heat-Activated Current in VP Neurons from WT but Not *Trpv1*$^{-/-}$ Mice

(A) Whole-cell current recordings (V_{hold} −60 mV) show the effects of heat on VP neurons isolated from a WT (upper) and *Trpv1*$^{-/-}$ mice (lower). Arrows indicate where SB366791 (1.5 μM) or RuR (10 μM) were added to the bath (small gaps are segments removed to eliminate artifacts caused by the addition of drug). Note that SB366791 inhibited the heat-evoked current in the WT neuron but not the *Trpv1*$^{-/-}$ neuron and that RuR blocked all of the residual current in both genotypes (dashed lines show baseline holding current).

(B) Bar graphs show mean (±SEM) current amplitudes measured at 39°C in the absence (control) or presence of SB366791. SB366791 significantly reduced the heat-evoked current in WT (control −331.3 ± 134.1 pA versus −65.0 ± 31.3pA in SB366791, n = 8, p = 0.03) but had no effect in *Trpv1*$^{-/-}$ neurons (control −82.2 ± 11.0 pA versus −78.4 ± 13.4 pA in SB366791, p = 0.4, n = 7).

(C) Mean (±SEM) amplitude of the RuR-sensitive current measured in the presence of SB366791 in VP neurons obtained from WT (55.4 ± 28.7pA, n = 8) and *Trpv1*$^{-/-}$ mice (70.1 ± 12.0 pA, n = 7, p = 0.66). n.s., not significant.

SB366791 could reverse the inward current induced by heating WT VP neurons, but not that of VP neurons from *Trpv1*$^{-/-}$ mice (Figure 3A), confirming that the effects of this compound are mediated specifically via blockade of channels encoded by the *trpv1* gene. Interestingly, mean values of Q_{10} (Figure 2C) as well as the mean amplitudes of heat-evoked current (Figure 3B) were respectively equivalent in VP neurons taken from *Trpv1*$^{-/-}$ mice and in WT VP neurons tested in the presence of SB366791. These observations indicate that the reduced thermosensitivity of VP neurons in *Trpv1*$^{-/-}$ mice reflects an absence of heat-sensitive channels comprising pore-forming protein subunits encoded by the *trpv1* gene.

Contribution of Other RuR-Sensitive Channels to Thermosensitivity

As illustrated in Figure 3A, addition of RuR abolished the residual heat-activated current recorded from VP neurons treated with SB366791, regardless of genotype, indicating that other subtypes of RuR-sensitive channels mediate the residual thermosensitivity of VP neurons. Although the nature of the channels involved remains to be determined, the average magnitude of the SB366791-insensitive current blocked by RuR was not significantly different in VP neurons obtained from WT or *Trpv1*$^{-/-}$ mice (Figure 3C), suggesting that the density or properties of these other channels are not altered as an attempt to compensate for the thermosensory deficit of VP neurons in adult *Trpv1*$^{-/-}$ animals.

trpv1 Gene Contributes to Anticipatory VP Release during Hyperthermia

To determine whether expression of the *trpv1* gene contributes to anticipatory VP release during hyperthermia in vivo, we measured the relationship between serum VP concentration ([VP]) and core body temperature in groups of control and thermally challenged WT and *Trpv1*$^{-/-}$ mice. Because increases in serum osmolality associated with dehydration are a potent stimulus for vasopressin release (e.g., Bourque, 1998), it was important to induce hyperthermia in the absence of a significant change in serum osmolality. As illustrated in Figure 4A, exposure to a warm (40°C–41°C) environment for 15 min significantly increased core body temperature in both WT (from 37.5°C ± 0.2°C, n = 4, to 38.7°C ± 0.1°C, n = 4, p = 0.001) and *Trpv1*$^{-/-}$ mice (from 37.8°C ± 0.1°C, n = 8, to 39.1°C ± 0.1°C, n = 8, p < 0.001), without provoking concurrent changes in serum osmolality (p = 0.66 for WT and p = 0.931 in *Trpv1*$^{-/-}$). In the WT animals, hyperthermia caused a significant increase in average serum [VP] (from 163 ± 16

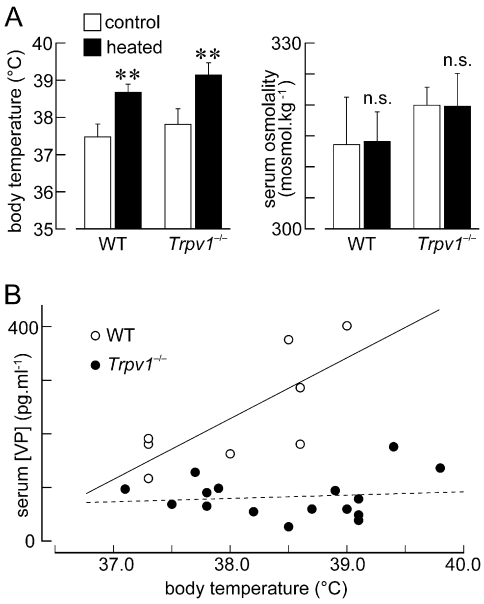

Figure 4. *Trpv1*$^{-/-}$ Mice Show Impaired Heat-Evoked VP Release In Vivo

(A) Bar histograms compare mean (±SEM) values of body temperature (left panel) and serum osmolality (right panel) observed in groups of unheated (control, open bars) and heated (filled bars) WT (n = 8) and *Trpv1*$^{-/-}$ mice (n = 16). The heat treatment significantly increased body temperature in both genotypes (**p < 0.005) but had no effect on serum osmolality (n.s., not significant, p > 0.05). Note that basal serum osmolality is higher in *Trpv1*$^{-/-}$ mice, as previously reported (Sharif-Naeini et al., 2006).

(B) Linear regression analysis of the relation between serum [VP] and core body temperature in the WT (open circles) and *Trpv1*$^{-/-}$ mice (filled circles) reported in (A). The solid line is the best fit through the WT data obtained by linear regression (slope 113.4 ± 40.5 pg.°C.ml^{-1}, n = 8, r^2 = 0.567), whereas the dashed line is the best fit through data obtained from *Trpv1*$^{-/-}$ mice (slope 6.2 ± 13.3 pg.°C.ml^{-1}, n = 16, r^2 = 0.015). The slopes of the lines are statistically different (p = 0.0046).

to 311 ± 50 pg.ml^{-1}, p < 0.05), whereas no significant change could be detected in *Trpv1*$^{-/-}$ mice (from 78 ± 11 to 86 ± 17 pg.ml^{-1}, p > 0.05). Indeed, linear regression analysis of the combined data (i.e., data from control and heated animals) revealed that the slope of the relation between serum [VP] and core body temperature was significantly lower in *Trpv1*$^{-/-}$ mice (6 ± 13 pg.°C.ml^{-1}, n = 16) than in WT mice (113 ± 41 pg.°C.ml^{-1}, n = 8, p < 0.005; Figure 4B). Thus, *Trpv1*$^{-/-}$ mice display a significant deficit in anticipatory VP secretion during hyperthermia.

DISCUSSION

The primary function of circulating VP is to promote water reabsorption from the kidney when body fluid volume is reduced and plasma osmolality is elevated, as occurs during dehydration (Bourque, 1998; Verbalis, 2006). Interestingly, increases in core body temperature caused by exposure to heat can provoke VP secretion well before changes in either of these parameters can be measured (Forsling et al., 1976). This release, therefore, confers a preemptive osmoregulatory benefit by reducing the ultimate impact of thermoregulatory evaporative water loss on body fluid balance. How the brain performs this anticipatory thermal modulation of VP release is poorly understood. Our results demonstrate that hypothalamic VP neurons are intrinsically thermosensitive and that expression of the *trpv1* gene is essential both for thermosensory transduction in these cells and for heat-induced VP secretion in vivo. Moreover, we found that the thermosensitive current could be inhibited by acute exposure to SB366791, a compound that selectively inhibits heat-activated currents in cells expressing recombinant TRPV1, but not TRPV4 (Gunthorpe et al., 2004). This finding suggests that the *trpv1* gene encodes a protein that contributes to the pore of the thermosensory channel rather than a protein that is indirectly required for thermosensitivity.

Thermal activation of VP neurons was observed at temperatures above 35°C. Although the apparent temperature threshold for TRPV1 activation in recombinant systems is commonly reported to be near 42°C (Caterina et al., 1997), the temperature dependence of this channel's activity is known to extend well below this value (Voets et al., 2004; Caterina, 2007). Indeed, the thermal threshold of TRPV1 can be lowered to physiological temperatures through sensitization of the channel by various intracellular mediators, such as PKC (Numazaki et al., 2002; Premkumar and Ahern, 2000; Vellani et al., 2001), PKA (Moriyama et al., 2005), or PIP_2 (Chuang et al., 2001; Prescott and Julius, 2003). It is also possible that alternative splicing of TRPV1 might lower the thermal activation threshold of TRPV1 (Caterina, 2007). Indeed, VP neurons in the SON do not express the full-length capsaicin receptor, but a variant of TRPV1 that lacks part of the N terminus (Sharif-Naeini et al., 2006). Finally, the TRPV1 variant expressed in VP neurons may coassemble with other TRPV subunits, as does TRPV1 (Rutter et al., 2005; Liapi and Wood, 2005; Hellwig et al., 2005; Cheng et al., 2007), and might thus form a channel with significant thermosensitivity in the 35°C–39°C range. Indeed, other TRPV channels are known to be expressed in VP neurons (e.g., TRPV2; Wainwright et al., 2004). Identifying the molecular structure of the TRPV1 variant expressed in VP neurons is now required to determine whether its expression alone, or in combination with other Trpv subunits, can encode a channel with the appropriate thermosensitivity.

Our experiments on VP neurons isolated from *Trpv1*$^{-/-}$ mice indicate that RuR-sensitive channels other than TRPV1 contribute to the residual thermosensitivity of these neurons (Figure 3). Although RuR can affect mitochondrial calcium homeostasis, this compound does not easily cross the cell membrane and commonly has to be dialysed into cells to affect mitochondrial calcium transport (e.g., Tang and Zucker, 1997). Because the blocking effect of RuR could be observed within seconds of exposure to extracellular RuR (e.g., Figure 3), we believe that the effects of RuR on heat-induced currents are more likely to be due to channel blockade than to an effect on mitochondrial transport. The heat-activated and RuR-sensitive channels TRPV2, TRPV3, and TRPV4 (Dhaka et al., 2006) are thus possible candidates for the residual thermosensitivity observed in *Trpv1*$^{-/-}$ mice. Additionally, three other TRP channels, namely TRPM2, TRPM4, and TRPM5 have been shown to be thermosensitive (Togashi et al., 2006; Talavera et al., 2005) and blocked by RuR (Ramsey et al., 2006). However, TRPM2 is poorly expressed in the hypothalamus (Nagamine et al., 1998), making it an unlikely

thermosensor in VP neurons. TRPM4 and TRPM5, for their part, are essentially impermeable to calcium (Launay et al., 2002; Nilius et al., 2003; Hofmann et al., 2003; Liu and Liman, 2003). In VP neurons, not only were heat-evoked currents still observed in external solution containing Ca^{2+} as the only inorganic cation (Figure S1), but this current could be blocked (by 66% ± 10%) by SB366791. Because this fraction is equivalent to the effect of the drug in normal solution (e.g., Figure 3B), we conclude that the majority of the residual heat-activated current is Ca^{2+} permeable and, thus, unlikely to be carried by TRPM4 or TRPM5. Additional experiments will be required to identify the nature of the SB366791-resistant thermosensitive channels in VP neurons.

We have shown previously that the intrinsic sensitivity of VP neurons to changes in external osmolality also requires expression of the *trpv1* gene (Sharif-Naeini et al., 2006). Together with the present results, these observations indicate that the central integration of thermal and osmotic signals can take place within single VP neurons and might be mediated by a single molecular complex incorporating TRPV1 protein. Previous studies have shown that thermal (Tominaga et al., 1998) and osmotic (Liu et al., 2007) stimuli can sensitize the responsiveness of TRPV1 channels to other sensory modalities. Thus, when both hyperthermia and hyperosmolality are present, these stimuli may enhance each other's effect on the activity of VP neurons. Indeed, studies in humans have shown that the slope of the relation between plasma VP and osmolality is significantly enhanced by small increases in core body temperature (Takamata et al., 1995). Although the mechanism underlying this synergism is unknown, it is tempting to speculate that this effect may involve a temperature-dependent modulation of the osmosensory channel. Evidence for a similar interaction has been provided by the demonstration that TRPV4-expressing cells show a noticeably greater response to hypotonicity at 37°C than at room temperature (Liedtke et al., 2000). Further studies will be required to investigate interactions between thermosensitivity and osmosensitivity in VP neurons.

EXPERIMENTAL PROCEDURES

Preparation and Perfusion of Isolated Neurons

For rats, tissue blocks (~1 mm^3) comprising the SON and PNZ were isolated from the brains of adult (100–130 g) male Long Evans rats according to procedures reported previously (Zhang and Bourque, 2006). Blocks were incubated for 60–90 min in oxygenated PIPES solution (33.5°C, pH 7.0) containing (in mM) NaCl 120, KCl 3, $MgCl_2$ 1, $CaCl_2$ 1, glucose 20, PIPES 10, and 0.7 mg/ml trypsin, then washed in trypsin-free PIPES (22°C) solution, triturated, and plated on glass coverslips. Cells were recorded within 30–120 min of trituration. For mice (10- to 12-week-old male C57/BL mice and *Trpv1*$^{-/-}$ mice [BL126 S4]), blocks containing SON were incubated for 30 min in an oxygenated PIPES solution (23°C, pH 7.34) containing (mM) NaCl 130, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10, PIPES 20, and 0.5 mg ml^{-1} protease X and protease XIV. Blocks were then washed in protease-free PIPES solution, triturated, and plated on glass coverslips. Coverslips were placed in a Leiden chamber (internal volume 500 μl) perfused with HEPES-buffered saline containing (in mM) NaCl (140) (150 for mice HEPES), KCl 3, $MgCl_2$ 1, $CaCl_2$ 1, glucose 10, HEPES 10 (298 mosmol.kg^{-1} in rat experiments and 312 mosmol.kg^{-1} in mouse experiments). Where required, RuR or SB366791 (both from Sigma Chemical Co.) were dissolved in HEPES before the experiment.

Whole-Cell Recording

Cells were patch clamped with glass pipettes containing (in mM) K-gluconate 120, $MgCl_2$ 1, EGTA 1, HEPES 10, Na_2-ATP 4, Na-GTP 1, adjusted (280 mosmol.kg^{-1} for mouse experiments and 265 mosmol.kg^{-1} for rat experiments) with mannitol (pH 7.34). All recordings were made using an Axopatch-200B amplifier (Molecular Devices Inc.). In current-clamp experiments, all cells were initially held at a membrane potential of approximately −50 mV, and firing frequency was measured at each 0.5°C. The perfusate was heated using a TC-324B temperature controller and SH-27B solution heater (both from Warner Instruments Inc.). To examine the temperature responses of VP and PNZ neurons in the physiological range, perfusate temperature was initially increased to approximately 32°C over 20 s (0.35°C/s), then slowly ramped to 39°C over 100 s (0.07°C/s). The temperature of the solution was monitored with a miniature thermocouple placed within 1 mm of the patch-clamped cell.

Immunocytochemistry

Recorded neurons were identified post hoc as VP or PNZ by immunocytochemistry. Following whole-cell recording, isolated SON neurons were incubated in 4% paraformaldehyde (1 hr, 4°C), then in blocking solution (2% normal goat serum, 0.2% Triton-X, 1 hr, 4°C), and finally in a mixture of polyclonal rabbit anti-VP (1/1000) and monoclonal anti-oxytocin-associated neurophysin (1/150) overnight at 4°C. Following three washes, cells were incubated (2 hr, 23°C) with a mixture of secondary antibodies: Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (both at 1/200 dilution). Following three washes, cells were visualized under a fluorescence microscope.

Induction of Hyperthermia without Osmotic Stress In Vivo

Because hyperosmolality stimulates VP release (e.g., Bourque, 1998), it was important to induce hyperthermia without a rise in serum osmolality. Cages housing single 10- to 12-week-old WT or *Trpv1*$^{-/-}$ mice were placed inside a warm incubation chamber with an ambient temperature between 40°C and 41°C (measured inside the cage). Preliminary experiments with WT mice revealed that a 20 min heat exposure significantly increased average serum osmolality (from 314 ± 4 mosmol.kg^{-1} in controls, n = 9, to 323 ± 2 mosmol.kg^{-1} after 20 min, n = 5, $p < 0.05$), whereas a 15 min exposure did not (see Figure 4A). A 15 min stimulus was therefore used in these experiments. Following the incubation period, animals were quickly anesthetized with halothane and killed by decapitation. Trunk blood was immediately collected, and core body temperature was determined by inserting a digital thermometer (L-0509, Conglom Inc., Canada) inside the body cavity.

Serum VP Measurements

Serum VP concentration was determined using an enzyme-linked immunosorbant assay (Assay Design, USA) based on a colorimetric reaction read at 405 nm using a 96-well microplate reader (EAR400AT, SLT-Instruments, Austria). For each mouse, 0.5 ml of blood was collected in a tube containing 500 kIU aprotinin (Sigma-Aldrich, Canada), placed on ice for 30 min, and then centrifuged at 1600 × g. Serum samples were assayed for VP concentration and osmolality (both in duplicates). To minimize the impact of individual differences in serum osmolality, we rejected data obtained from mice displaying serum osmolality values more than 6 mosmol.kg^{-1} higher than the average osmolality of control mice in each genotype (i.e., above 320 and 326 mosmol.kg^{-1} in WT and *Trpv1*$^{-/-}$ mice, respectively).

Statistical Analysis

All values are reported as mean ± SEM. Comparisons of linear regressions and fits through the data were performed using Prism (v5.01, GraphPad Software Inc., San Diego CA). Comparisons of the means observed in different groups were performed using a Student's t test or an analysis of variance (ANOVA), as appropriate (SigmaStat 2.03; SPSS Inc., Chicago IL).

SUPPLEMENTAL DATA

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/58/2/179/DC1/.

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