Heat-evoked activation of TRPV4 channels in a HEK293 cell expression system and in native mouse aorta endothelial cells

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ABSTRACT

We have compared activation by heat of TRPV4 channels, heterogeneously expressed in HEK293 cells, and endogenous channels in mouse aorta endothelium (MAEC). Increasing the temperature above 25^{o}C activated currents and increased $[Ca^{2+}]_i$ in HEK293 cells transfected with TRPV4 and in MAEC. When compared with activation of TRPV4 currents by the selective ligand 4αPDD (α-phorbol 12,13-didecanoate), heat activated currents in both systems showed the typical biophysical properties of currents through TRPV4, including their single channel conductance. Deletion of the three N-terminal ankyrin binding domains of TRPV4 abolished current activation cells by heat in HEK293. In inside-out patches, TRPV4 could not be activated by heat but still responded to the ligand 4αPDD. In MAEC, the same channel is activated under identical conditions as in the HEK expression system. Our data indicate that TRPV4 is a functional temperature-sensing channel in native endothelium, that is likely involved in temperature dependent Ca^{2+} signalling. The failure to activate TRPV4 channels by heat in inside-out patches, which responded to 4αPDD, may indicate that heat activation depends on the presence of an endogenous ligand, which is missing in inside-out patches.

INTRODUCTION

Sensing of temperature in the body and the environment is one of the most essential mechanisms for controlling the homeostasis of several regulatory pathways in the mammalian body (1). In recent years, unravelling of thermosensing mechanisms has been very successful, inasmuch as at least four members of the TRPV subfamily of transient receptor potential cation channels, TRPV1,2,3 and 4, and a more distantly related protein TRPM8 have been identified (for a unified nomenclature see 2,3) as sensors of temperature. Proteins of this subfamily typically contain three to six ankyrin repeats in the N-terminus, and six transmembrane segments with a pore region between segment 5 and 6. The first identified non-mammalian member of this subfamily, the *C.elegans* OSM-9 channel, is activated by changes in osmolarity (4). The second protein of the TRPV family that has been identified is the mammalian vanilloid receptor channel VR1 (TRPV1), which is activated by vanilloid compounds such as capsaicin, pepper, hot chili, moderate heat or protons (5). Unlike TRPV1, another close relative of this channel, TRPV2, is constitutively activated by growth factors (6) or by noxious heat (7). TRPV1 and TRPV2 are activated by temperatures above 43^{o}C and 52^{o}C, respectively (5,7). Currents through TRPV3 exponentially increase at temperatures above 35^{o}C (8-10). It has also been shown in current measurements on oocytes and by cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$) measurements in HEK cells that TRPV4 is activated in both expression systems at temperature above 30^{o}C (11). TRPM8 is activated by temperatures below 22 ^{o}C and is therefore a candidate for cold reception (12,13).

So far, the molecular mechanism of channel activation by heat for any of these channels is not known and functional measurements of heat-activated channels in native tissue have only been performed for TRPV1. We have studied in the present report

activation by heat of TRPV4 (also known as OTRPC4, VR-OAC or TRP12), which was originally thought to be a channel sensing changes in cell volume (14-17) by an unknown mechanism. This activation caused an increase in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. We show here that TRPV4 channels are activated by heat above a threshold temperature of 25^{o}C. Activation requires the intact N-terminal ankyrin repeats. Single TRPV4 channels can be activated by heat in cell-attached patches but not in cell-free inside-out patches. Interestingly, as shown by Northern blot analysis, TRPV4 is highly expressed in endothelial cells from mouse aorta (14). We show here for the first time in a native cell that heat activates a channel in mouse aorta endothelial cells with identical properties as the heterologously expressed channel.

METHODS

Cell culture

Human embryonic kidney cells, HEK293, were grown in DMEM containing 10 % (v/v) human serum, 2 mM L-glutamine, 2 U/ml penicillin and 2 mg/ml streptomycin at 37ºC in a humidity controlled incubator with 10 % CO_2. HEK293 cells were transiently transfected with the pCINeo/IRES-GFP/rbECaC vector using previously described methods (15).

Transient expression of mTRP12

We used the recombinant bicistronic expression plasmid pdiTRP12, which carries the entire protein coding region for murine TRPV4 (mouse mTRP12, GenBank accession number is CAC 20703) and the green fluorescent protein, GFP (14) for transient transfection. A mutant was tested in which all three ankyrin binding repeats were deleted (deletion between residues 235 and 398). Mutations were made using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). The nucleotide sequence of the mutants has been verified by sequencing the corresponding cDNAs. HEK293 cells, transiently transfected with the above-described vector, were visually identified in the patch clamp set up by their green fluorescence. GFP was excited at a wavelength between 425 and 475 nm. The emitted light was passed via a 495 nm dichroic mirror through a 500 nm long-pass filter. Non-transfected cells from the same batch were used as controls.

Endothelial cells

The “primary explant technique” was used to study freshly isolated endothelial cells from mouse aorta. This method is described in detail elsewhere (18-20). These cells express TRPV4, as previously shown with Northern blot analysis (14).

Solutions and heat application

For electrophysiological measurements, the standard extracellular solution contained in mM: 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.5 or 5 $CaCl_2$, 10 glucose, 10 HEPES, buffered at pH 7.4 with NaOH. The osmolality of this solution, as measured with a vapour pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 320 ± 5 mOsm. The pipette solution was composed of (in mM): 20 CsCl, 100 Cs-aspartate, 1 $MgCl_2$, 4 Na_2ATP, 0.08 $CaCl_2$, 10 BAPTA, 10 HEPES, pH adjusted to 7.2 with CsOH. Extra- and intracellular solutions contained Cs^+-salts to inhibit K^+ currents, which are present in some but not all HEK293 cells. For single channel measurements, the pipette solution contained (in mM): 150 NaCl, 1 $MgCl_2$, 10 HEPES, pH adjusted to 7.4 with NaOH. The bath solution contained 150 KCl, 5 $MgCl_2$, 10 HEPES, 10 glucose for cell-attached patches and 140 CsCl, 1 $MgCl_2$, 1 Na_2ATP, 0.034 $CaCl_2$, 5 EGTA, 10 HEPES for inside-out patches. The pH of the bath solution was adjusted to 7.4 with KOH for cell-attached patches and to 7.2 with CsOH for inside-out patches. Alpha-phorbol 12,13-didecanoate (4αPDD), a non-PKC-activating phorbol ester, was applied at a concentration of 1 μM from a 10 mM stock solution in ethanol. Control experiments were performed at room temperature (22–25°C). In some experiments, the bath solution was cooled to 14°C and then warmed up to 43°C in 50 seconds (temperature ramp) using a water jacket device. The bath temperature was monitored with an electronic thermometer (Comark, England) and simultaneously recorded with the whole cell or single channel currents. Data sampled at the rising phase of temperature ramp were pooled for every 0.2 s. The temperature coefficient Q_{10} was used to characterize the temperature dependence of the membrane current and was calculated by the following equation,

$$Q_{10} = (I_2/I_1)^{10/(T2-T1)} \quad (1)$$

where I_1 and I_2 represent the current amplitudes at the lower (T_1) and higher temperature (T_2). Operationally, we plotted the current, normalized to its value at 25 ^oC, on a log scale as a function of temperature, and estimated the Q_{10} value from the slope.

Electrophysiological recordings

The patch-clamp technique was used in the whole cell, cell-attached and inside-out configurations. Currents were monitored with an EPC-9 (HEKA Elektronik, Lambrecht, Germany, 8-Pole Bessel filter 2.9 kHz).

Whole-cell membrane currents were measured using ruptured patches. Patch electrodes had a DC resistance between 2 and 4 MΩ. An Ag-AgCl wire was used as a reference electrode. Capacitance and access resistance were monitored continuously. Between 50% and 70% of the series resistance was electronically compensated to minimise voltage errors. If not mentioned otherwise, we have applied a ramp protocol consisting of a voltage step to –100 mV for 20 ms followed by a 180 ms linear ramp to +100 mV, and sampled the current at 0.1 ms intervals. This protocol was repeated every 5 s. Single channels measurements were performed using a sampling interval of 200 μs. Filter setting was 2 kHz. Each trace consists of 2048 data points. Interval between the traces was 0.67s.

Ca^{2+} measurements

For $[Ca^{2+}]_i$ measurements, single cells were loaded with Fura-II by incubating them for 25 min at 37^oC in a bath solution containing 2 μM Fura-II/AM. The dye was excited alternately at wavelengths of 360 and 390 nm through a filter wheel rotating at 2 cycles/second. The fluorescence was measured at 510 nm and corrected for auto fluorescence, measured from the cell free background. Apparent free $[Ca^{2+}]_i$ was calculated from the fluorescence ratio R by $[Ca^{2+}]_i = K_{eff} (R-R_0) / (R_1-R)$, where K_{eff} is the effective binding constant, R_0 the fluorescence ratio at zero Ca^{2+}, and R_1 that at high Ca^{2+}. These

calibration constants were determined experimentally for the given set-up and the actual experimental conditions used.

Data analysis

Electrophysiological data were analysed using the WinASCD software (G. Droogmans, Leuven). Pooled data are given as mean ± S.E.M. from *n* cells. Significance was tested using Student's paired *t* test ($p<0.05$ are marked with an asterisk).

RESULTS

Heat activates an identical current as the TRPV4 agonist 4αPDD

The phorbol derivative 4αPDD activated a robust transient outwardly rectifying cation current in HEK cells expressing TRPV4, but not in non-transfected cells (Fig. 1A, C) (21), which was accompanied by a shift of the reversal potential to more positive values. Heating the bath solution from room temperature to 38^{o}C also activated an outwardly rectifying current (Fig. 1B, D). The features of both currents were nearly identical: a) the activated current was outwardly rectifying, b) the reversal potential was +17.8 ± 0.8 mV (n = 8 for 1 μM 4αPDD) and +16.3 ± 1.7 mV (n=6 for heat), c) both responses appeared with a delay, d) both currents were blocked in a voltage dependent manner by ruthenium red. However, the current densities were quite different: -110 ± 24 pA/pF for the current activated by 1 μM 4αPDD at –80 mV (n = 8, Fig. 1E), –22 ± 5 pA/pF for the heat activated current (n = 6, Fig. 1F). Substitution of extracellular Na^+ by $NMDG^+$ abolished inward currents (data not shown). The responses to heat were very small in non-transfected cells. The holding current at 0 mV was even increased, shifting the reversal potential to more negative potentials. This small current has been identified as a Ca^{2+} activated Cl^- current (data not shown).

TRPV channels comprise at least three ankyrin binding repeats in the N-terminus. We have tested a TRPV4 mutant in which all three ankyrin binding repeats have been deleted (deletion between residue 235 and 398), and found that heat could not activate a current in HEK cells transfected with this mutant (Fig. 1 F).

To estimate the temperature threshold for TRPV4 activation, we have simultaneously recorded the changes in bath temperature and current (Figure 2). At temperatures above 25^{o}C the TRPV4 current increased exponentially (Fig.2A, B). The current plotted as a function of temperature showed hysteresis (Fig. 2C). Ruthenium red, a selective voltage-dependent blocker of TRPV channels, rapidly inhibited the current (Fig. 2D). Pooled data from 5 cells are shown in figure 2E. The Q_{10} value was assessed from the slope of the log(I) versus temperature plot (see equation (1)). Obviously, at low temperatures this slope is small (Q_{10} = 1.6 ± 1.0) but increases strongly at temperatures above 24^{o}C to a Q_{10} value 19.1 ± 1.1. The threshold value of 24°C was obtained from the cross-over of both regression lines. Up to 43^{o}C no saturation was observed in these experiments. The currents activated by heat are smaller than those during stimulation with 4αPDD, indicating that further heating may recruit even more channels.

The same behaviour has been observed at the single channel level in cell-attached patches. Application of 1 μM 4αPDD to the bath induced a delayed activation of single channel currents (Fig. 3A). The single channel current amplitude, as estimated from the amplitude histogram, was –3.55 ± 0.21 pA at –60 mV (n = 10), corresponding to a chord conductance of 59.1 pS. From the I-V curve of the TRP4 single channel currents, we calculated a single channel slope conductance of 61.4 ± 4.8 pS (n = 5) for inward currents and 98.9 ± 7.3 pS (n= 5) for outward currents (Fig. 3C). Figure 3B shows heat activated (38^{o}C) single channels with a similar amplitude and chord conductance (-3.29 ± 0.10 pA and 54.8 pS ± 1.8 pS at -60 mV, +6.30 ± 0.41 pA and 105 ± 6.9 pS at +60 mV, n = 5, Fig.

3C). The incidence of channels activation by heat in cell attached patched was 42% (10 out of 24 cells).

In cell-free inside-out patches, 4αPDD or heat stimuli were applied about 40 s after excising the membrane patch. 4αPDD could still activate TRPV4 currents. (Fig. 4A). The response to 4αPDD in inside-out patches was even prolonged. The amplitude histogram from 6 patches is shown in figure 4C. The single channel current amplitude was –3.52 ± 0.12 pA (chord conductance 58.6 ± 2.1 pS) at –60 mV. The incidence of single channel currents activated by 4αPDD in cell free inside-out patches was 75% (6 out of 8 cells). However, heat did not activate TRPV4 channels in inside-out patches (Fig. 4B, D). In 21 patches we could not detect any single channel opening.

As already shown, activation of TRPV4 by 4αPDD was always accompanied by an increase in $[Ca^{2+}]_i$ (21). The basal Ca^{2+} concentration was significantly higher in TRPV4 transfected cells (Fig.5 A and B), and increased further by raising the bath temperature from 25°C to 43°C (Fig. 5 A and D). This increase did not occur in non-transfected cells (control, Fig. 5D). Deleting the ankyrin binding repeats abolished the $[Ca^{2+}]_i$ response to heat (Fig. 5C, D). The increase in $[Ca^{2+}]_i$ did not occur in Ca^{2+}-free bath solutions (data not shown).

Because sensitisation during successive heat challenges has been observed in TRPV1, TRPV2 and TRPV3 (7,13,22), we have also studied the influence of repetitive stimulation with 4αPDD and heat on TRPV4 current. The responses of TRPV4 expressing cells to repetitive stimulations with 1 μM 4αPDD were gradually reduced (Fig. 6A). The response to a subsequent heat challenge was also diminished (Fig. 6B). When normalized to the peak current elicited by the first stimulus, the second peak current was reduced by 32.1 ± 4.7% (n = 5, for 1 μM 4αPDD) and 47.4 ± 15.7% (n = 4, for heat) (Fig.6C). The threshold

temperature for TRPV4 activation during the second heat stimulus was shifted toward higher values (~30 ^{o}C).

Activation of endogenous TRPV4 channels in native aorta endothelial cells by heat

We have already demonstrated that 4αPDD activates in mouse aorta endothelial cells (MAEC), which express TRPV4 endogenously (14), a current with the functional hallmarks of the current activated in TRPV4-transfected HEK293 cells (21). Figure 7 shows an experiment to determine the heat sensitivity of native currents in MAEC. Because of the larger background currents in these cells, we measured difference current from the current traces at high and low temperatures. Typically, heating the bath solution from room temperature to 40^{o}C activated an inward current at –50 mV (Fig. 7A). The I-V curve of this current, calculated from the difference currents at room temperature and 40°C, reverses at positive potentials (Fig. 7B). The average reversal potential of the heat-activated current was 14.8 ± 3.9 mV (n = 5), which is comparable to that in TRPV4 expressing HEK293 cells. As already reported for activation by 4αPDD (21), the current densities were much smaller in these native cells than in the expression system. In cell-attached patches heat also evoked single channel activity with a chord conductance of 59.7 ± 1.2 pS (n = 4 cells) at –60 mV, a value comparable to that observed in the HEK expression system (Fig. 7C, D).

Because TRPV4 channels are Ca^{2+} permeable, we have measured changes of $[Ca^{2+}]_i$ induced by heat in MAEC. Raising the bath temperature from room temperature to 37^{o}C clearly increased $[Ca^{2+}]_i$ in the presence of 1.5 mM $[Ca^{2+}]_e$ (Fig. 8A), but not in a nominally Ca^{2+} free solution (Fig. 8B). Figure 8C summarizes the heat-induced changes in $[Ca^{2+}]_i$ in Ca^{2+} containing and Ca^{2+} free solutions. Likely, this increase reflects heat-activation of TRPV4.

DISCUSSION

TRPV4, a member of the family of vanilloid receptor channels, is widely expressed in mammalian tissues, including heart, brain, endothelium, kidney, sensory neurons, sympathetic nerves, fat tissue, gut, salivary gland, lung, skin, sweat glands, the inner ear and keratinocytes (11,14-17,23). Originally, these channels were considered as Ca^{2+} permeable sensors of cell volume, because they can be activated by cell swelling (14-17). The mechanism of activation by cell swelling is not known, however, we have hypothesized that cell swelling may produce an endogenous ligand that in turn activates the channel (21). The existence of such a ligand is very likely because several members of the TRPV family are agonist-gated channels, which are opened by binding of capsaicin and related compounds (5,24-26) and we have also shown that TRPV4 can be activated by the phorbol ester 4αPDD (21). Very recently, data from the Caterina laboratory have shown that TRPV4 can also be activated by heat (11). We show here that the currents activated by heat and 4αPDD are indeed identical. In addition, we present novel data, which might indicate that TRPV4 cannot be activated in cell free inside-out patches and is present as a channel activated by heat in native endothelial cells.

Are 4αPDD and heat activated currents identical?

4αPDD-activated currents through TRPV4 channels show the following hallmarks (21,27): 1. a current-voltage relationship with inward and outward rectification over a wide voltage range, and outward rectification between –100 and +100 mV, 2. a reversal potential between 14 and 20 mV in 150 mM Na^+ and 5.0 mM Ca^{2+} containing extracellular solutions, 3. Ca^{2+} permeates the channel, 4. voltage dependent block by ruthenium red, 5. rapid inactivation of the current during prolonged stimulation, and 6. desensitisation following repetitive stimulation. Currents with almost identical features were observed in

heat-activated TRPV4 expressing HEK 293 cells, but were absent in non-transfected cells. In addition, the conductance of the single channels activated by 4αPDD and heat are nearly identical, i.e. approximately 60 and 100 pS for inward and outward currents, respectively. These values are in agreement with those of TRPV4 channels activated by cell swelling (17). A heat-evoked increase in $[Ca^{2+}]_i$ dependent on extracellular Ca^{2+} is consistent with Ca^{2+} permeation through these channels. We have also identified a similar current in isolated endothelial cells from mouse aorta, which endogenously express TRPV4.

Temperature range of TRPV4 activation

In our TRPV4 expression system, we have observed current that increases exponentially at temperatures above 25°C. The Q_{10} value of this heat sensitive current above this threshold was about 19 compared to a sub-threshold value of 1.5. This value suggests somewhat higher temperature sensitivity than that of TRPV3, as shown in a recent report (8). We did not observe current saturation at temperatures up to 43°C. Because heat activates only a fraction of the channels that can be activated by 4αPDD, the recruitment of channels by temperature might be less efficient than by the phorbol-derivative. Compared with other temperature sensitive channels of the TRPV family, TRPV1,2,3 (5,7-10), TRPV4 channel activation occurs in a physiological temperature range, and they are already activated at normal body temperature. This is a surprising result and may indicate that these channels may serve as constitutively open Ca^{2+} entry channels sensitive to small rises or falls in temperature.

Mechanisms of TRPV4 activation by heat

So far, the mechanism(s) by which channels respond to heat has not been identified. A conformational change seems to be likely, but a heat dependent production of a messenger that activates TRPV4 cannot be excluded. It has been shown that a N-terminal splice

variant of TRPV1 does not respond anymore to capsaicin and heat (28). We have therefore studied heat-responses of a deletion mutant. Heat was unable to activate a current and to increase $[Ca^{2+}]_i$ in the ankyrin-deletion mutant. Liedtke *et al.* (16) showed that deletion of the ankyrin binding repeats in TRPV4 channels does not prevent activation by cell swelling, i.e. the channels are still functional. In addition, the truncated channels are inserted in the plasma membrane as shown by confocal microscopy. Therefore, the lack of response to heat points to a defective channel rather than to defective trafficking of the channel to the surface membrane. We hypothesise that the ankyrin repeats are necessary to stabilize a channel conformation, which is permissive for activation by heat.

Interestingly, 4αPDD can activate TRPV4 channels in both the cell free and cell-attached patch clamp configuration indicating that 1) the ligand probably reaches the channel from the inside and 2) the channel is still functional in cell free patches. However, heat only activates channels in the cell-attached mode. We failed to see any activation in cell free inside-out patches. These data might indicate that heat produces a ligand rather than activating the channel directly or enhances activation by a diffusible ligand that is already present. This is in contrast to TRPV1 channels where clear single channel activity in response to heat activation can be recorded in inside-out patches (29).

Recently, several reports have indicated that repetitive activation of TRPV channels by heat resulted in a decreased or even an increased response. However, data are variable and there is little agreement on how current amplitude and threshold temperature change with repetitive heat challenges. Sensitisation has been observed in TRPV1, 2, 3 heterologously expressed in CHO cells or in *Xenopus* oocytes (7,8,10,22). On the other hand, the studies using primary sensory neurones (30,31) have reported desensitisation of heat-evoked currents. Interestingly, Tominaga *et al.* (29) showed sensitisation in TRPV1 expressing *Xenopus* oocytes, but desensitisation in HEK293 transfected with the same channel. These

observations that accessory proteins or differences in the signalling pathways in the host cell might contribute to both the sensitisation and desensitisation mechanisms. In TRPV4 expressing HEK293 cells, we showed a diminished response to a subsequent heat application. This mechanism is mediated by a process, which is activated during the first response and continued for at least 4 min. However, the mechanism underlying this desensitisation is still obscure.

Any physiological significance of TRPV4 activation by heat ?

Reminiscent of TRPV1, TRPV4 shows some unexpected gating promiscuity: it can be activated by cell swelling, by a ligand and by heat. However, it is possible that all mechanisms refer to the same mode of activation, namely the production of an endogenous ligand. Such a mechanism has been discussed elsewhere (21). Cell swelling may activate PLA_2 thereby producing arachidonic acid or a down-stream metabolite as a possible ligand (32-34). Indeed, we have recorded channel activation by arachidonic acid (Watanabe, Nilius, unpublished results). Because heat does not evoke channel activity in inside-out patches, a likely explanation might be the loss of the metabolic pathway responsible for activation of a possible messenger. In this view, the promiscuous gating behaviour of TRPV4 might be explained by a ligand dependent gating. Whatever the precise mechanism of activation might be, the high Q_{10} value demonstrates that this system is acting as a very efficient thermosensor rather than reflecting the normal temperature sensitivity of biological reactions.

The temperature dependence of TRPV4 is – at least in this range – an unexpected result. It must be concluded that constitutive open Ca^{2+} channels exist in cells expressing TRPV4. Such cells should have an elevated intracellular Ca^{2+} concentration. So far, the physiological significance of this thermosensitive property of TRPV channels has been linked to primary nociceptive neurons. We have shown here in addition that TRPV4 plays

a central role in temperature dependent Ca^{2+} homeostasis in endothelium. We observed indeed that $[Ca^{2+}]_i$ was elevated at 37°C in mouse aorta endothelial cells which express TRPV4. Such an elevation could have important consequences, e.g. for a steady state production of nitric oxide. Cooling of peripheral blood vessels could therefore induce vasoconstriction and vice versa warming up, vasodilatation (35). Therefore, it seems likely that TRPV4 could work as both a cold and a warm receptor. In addition, endothelial the temperature sensitivity of TRPV4 may suggest a role in mediating inflammatory pathophysiology in fever, e.g. by changing barrier properties which depends on Ca^{2+} influx (36).

In general, the existence of a constitutive open Ca^{2+} entry pathway might be important to boost Ca^{2+} signalling by changes in membrane potential, lower the threshold for effects evoked by activation of another Ca^{2+} entry pathway or by intracellular Ca^{2+} release, and might therefore function as a Ca^{2+} amplifier in several issues. Interestingly, TRPV4 channels are also inactivated by elevation of $[Ca^{2+}]_i$ and are desensitised due to prolonged or consecutive stimulation. We have recently reported that the IC_{50} for intracellular inhibition of TRPV4 by $[Ca^{2+}]_i$ is 406 nM. Such a mechanism would prevent Ca^{2+} overload by TRPV4 and might be efficiently involved in fine-tuning the Ca^{2+} influx via TRPV4.

In conclusion, we show here that the Ca^{2+} entry channel TRPV4 can be gated by heat in a temperature range between 25 and 43°C. This is an additional mechanism for activation of a Ca^{2+} entry pathway. It is, however, not unlikely that the diverse modes of channel activation may converge to a common mechanism, namely binding of a still elusive endogenous ligand.

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FIGURES

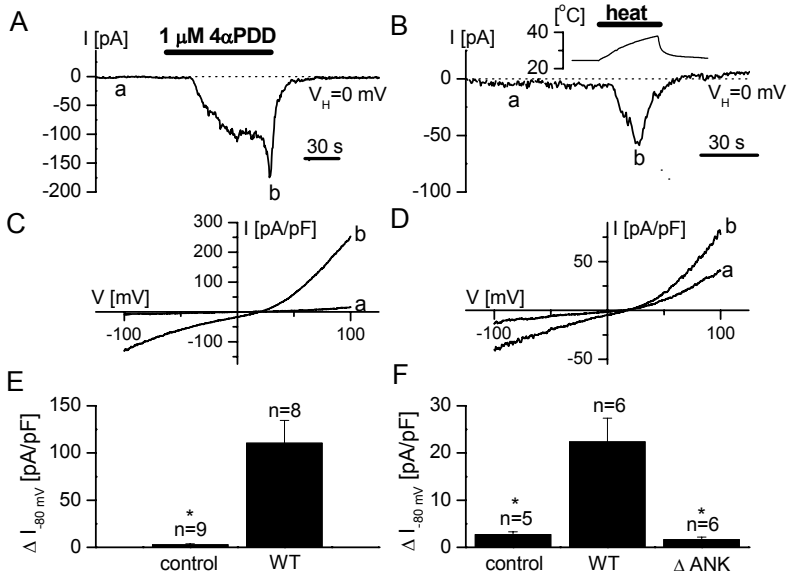

Figure 1: Activation of identical currents in HEK 293 cells expressing TRPV4 by 4α-PDD and heat.

A. The holding current at 0 mV is shown. At the time indicated, 1 µM-4αPDD was applied and induced a large inward current in HEK293 cells transfected with wild type TRPV4.

B. Time course of the holding current at 0 mV in response application of heat (inset).

C. I-V curves obtained from voltage –100 to +100 mV voltage ramps measured at the times indicated in A. Note that a large outwardly rectifying current is activated during application of 4α–PDD.

D. I-V curves show activation of an outwardly rectifying current by heat stimuli. Data from the time indicated in B.

E. Average densities of the 4αPDD-activated current at –80 mV for non-transfected cells (control) and TRPV4 wild type. *: for comparison with the TRPV4 wild type, $p<0.01$.

F. Average densities of the heat-evoked current at –80 mV. Note the absence of current activation in the mutants. ΔANK describes data from the deletion mutants of the three ankyrin binding repeats

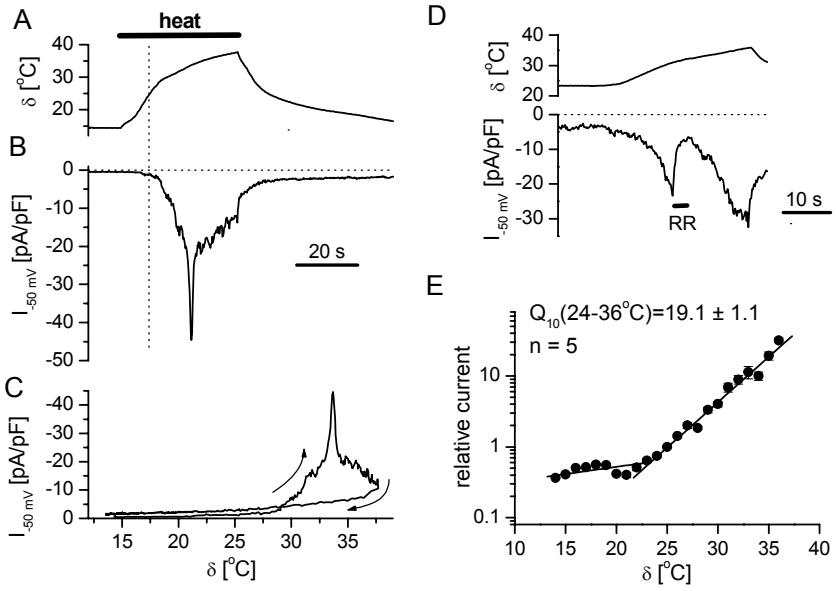

Figure 2: Analysis of the heat activated current in TRPV4 expressing HEK293 cells.

A. Temperature ramp from 14 °C to 38°C in 45 s.

B. Representative current response of TRPV4 to the indicated change in temperature. The holding potential is –50 mV. The vertical dashed line indicates the threshold temperature for TRPV4 channel activation. Note that the heat-evoked current undergoes rapid inactivation in response to excessive heat stimuli (>34°C). $[Ca^{2+}]_e$ = 5 mM.

C. Plots of the current through TRPV4 in response to an increase in temperature for the record in A, B. Arrows indicate the increase and decrease of temperature.

D. Effect of ruthenium red on heat-evoked TRPV4 current. Ruthenium red (RR, 1 μM) induced a rapid decrease of the holding current at –50 mV. After washing out of RR, the heat evoked current re-appears .

E. Semi-logarithmic plot in which bath temperature (abscissa) was related to the average current normalized to that at 25 ^{o}C (ordinate). Two lines with different slopes were extrapolated. The lower slope exhibited Q_{10}=1.6; the higher slope Q_{10}=19.1. From the intersection of these two lines, the threshold temperature for TRPV4 was estimated to be 24 ^{o}C. Data were obtained from 5 cells.

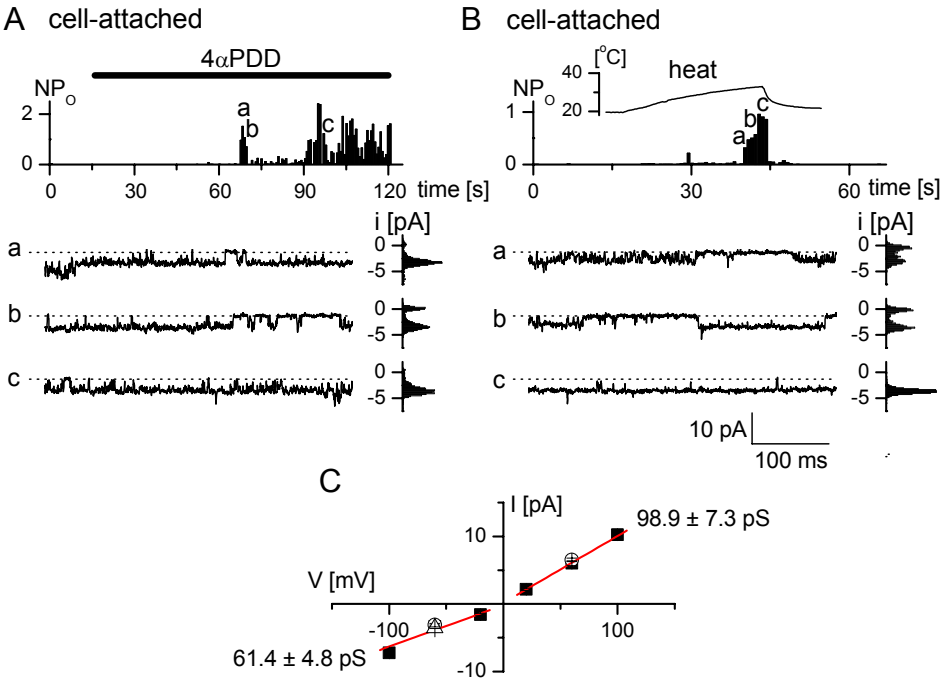

Figure 3: Heat and 4α-PDD activate identical single channel currents in TRPV4 expressing HEK 293 cells.

A, B. Time course of the channel activity evoked by 4αPDD (A) and heat (B) in cell-attached patches. The cell membrane was held at –60 mV. The pipette contained NaCl solution and the bath was perfused with KCl solution. 4αPDD or heat was applied to the bath during the period indicated by the horizontal bar. Upper trace in B shows the time course of temperature. Channel activity in the ordinate was assessed from NP_o. Current traces obtained at different times labelled a-c are shown in the lower rows. Dashed lines indicate the zero current level. The amplitude histograms of the respective traces are shown at the right hand side.

C. I-V relationship of 4αPDD-activated channel (closed square) and heat-evoked channel (open circle) in cell-attached patches, 4αPDD-activated channels (open triangle) in inside-out patches. The average of single current amplitude obtained from 5 cells was plotted against the holding potential.

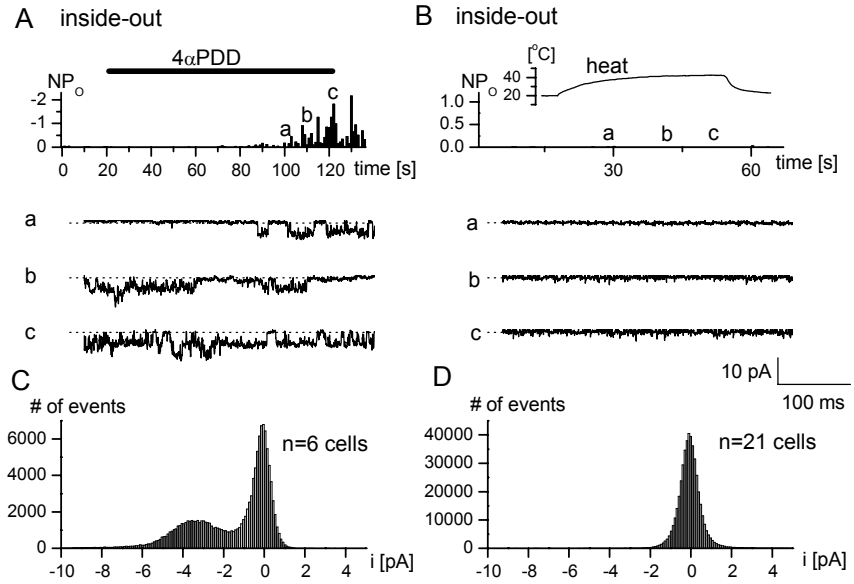

Figure 4: 4α-PDD but not heat activate single channel currents in cell-free inside-out patches of TRPV4 expressing HEK 293 cells.

A, B. Time course of the channel activity evoked by 4αPDD (C) and heat (D) in inside-out patches. The cell membrane was held at –60 mV. The pipette contained NaCl solution and the bath was perfused with CsCl solution containing 5 mM EGTA. Note that heat does not activate a channel in inside-out patches.

C, D. Pooled amplitude histograms from 6 and 21 cells for stimulation of TRPV4 in inside-out patches by 4αPDD and heat, respectively. Note that no openings are detectable by heat stimulation of inside-out patches.

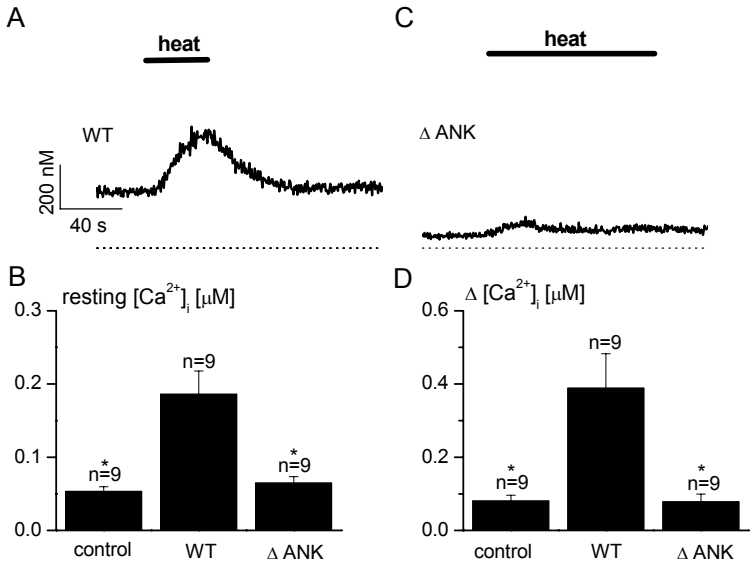

Figure 5: Heat increases the intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$ in TRPV4 expressing HEK293 cells.

A, C. $[Ca^{2+}]_i$ was monitored using Fura-II/AM before and after the application of heat ramp (from 25 to 43°C). Heat stimuli increased $[Ca^{2+}]_i$ in wild type (A) but not in ΔANK mutant (C). $[Ca^{2+}]_e$ = 5 mM.

B. Average of resting $[Ca^{2+}]_i$ for non-transfected cell (control), wild type and ΔANK mutant. Note that the resting $[Ca^{2+}]_i$ is significantly higher in wild type than in control.

D. Increase above the resting level of $[Ca^{2+}]_i$ evoked by heat stimuli. Heat stimuli do not increase $[Ca^{2+}]_i$ in the ΔANK mutant. *: $p<0.05$, comparison with wild type.TRPV4.

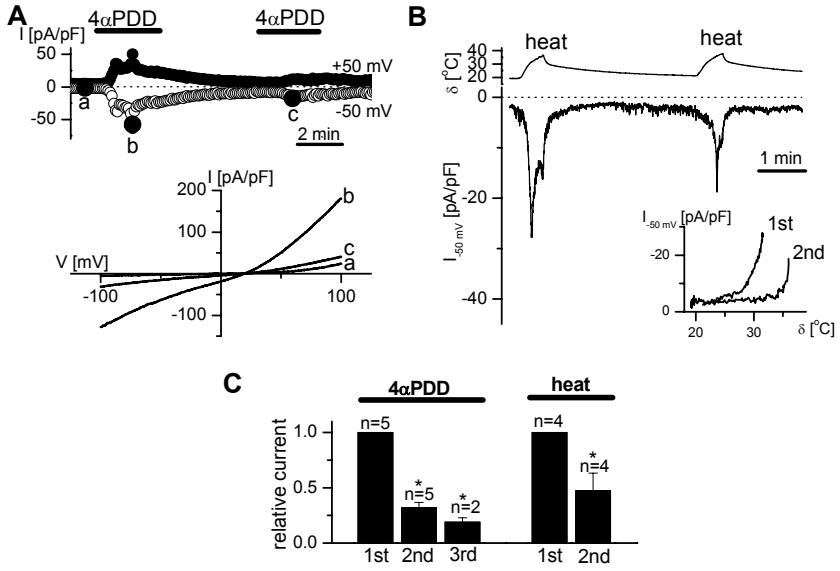

Figure 6: Desensitisation of TRPV4 current evoked by 4αPDD and heat.

A. An example of desensitisation to repetitive application of 1 μM 4αPDD (horizontal bars) at 4 min interval. *Upper trace*: time course of the whole cell current measured at –50 and +50 mV from linear voltage ramps. *Lower trace*: current voltage relationships measured at the time a, b, c indicated in upper trace. Repeated application of 4αPDD diminished the current response.

B. Heat-evoked TRPV4 currents show a similar desensitisation as during agonist stimulation. *Upper trace*: the repetitive heat ramps from 19 to 38 ^{o}C. *Lower trace*: Time course of the holding current at –50 mV in response to a subsequent heat challenge at 4 min interval. Responses to a second heat ramp showed desensitisation. *Inset*: current-temperature plots of the first and second responses. Note that the TRPV4 current is activated at a threshold of approximately 30^{o}C at the second heat application.

C. Average densities of TRPV4 currents at –50 mV during consecutive applications normalized to the maximum response of the first application for each cell. Experiments were performed in the presence of 5 mM Ca^{2+} in the bath solution.

*: $p<0.05$, comparison with the first response.

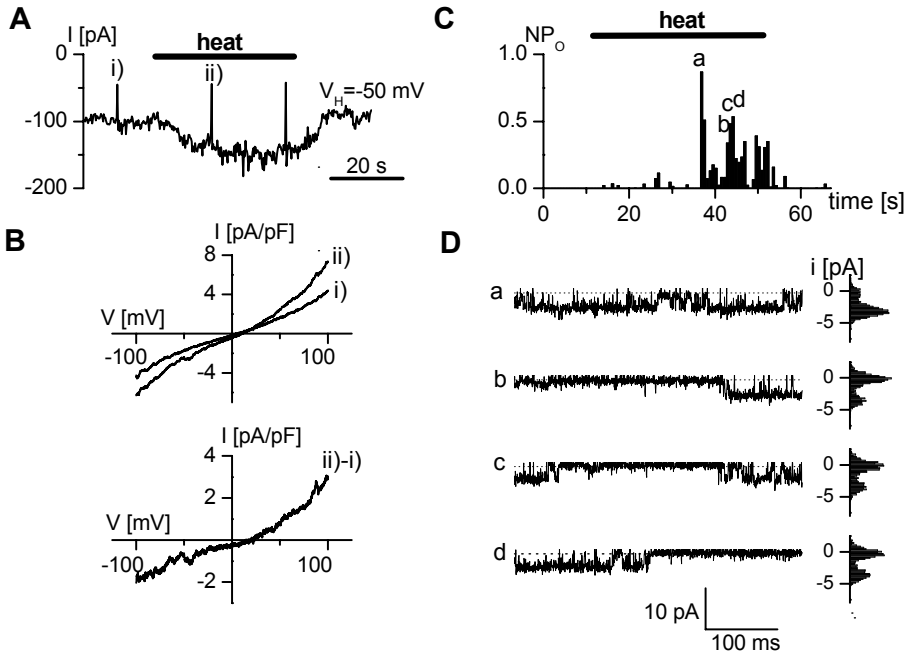

Figure 7: Activation by heat of whole cell and single channel currents in freshly isolated mouse aorta endothelial cells (MAEC).

A. Time course of the holding current at –50 mV activated by a heat ramp to ~40°C in MAEC. The deflections represent unresolved currents during voltage ramps from –100 to +100 mV. Note that heat activated an inward current.

B. The upper trace shows I-V relationship measured at the time i, ii indicated in A. The lower trace shows the difference current (ii-i), representing the I-V curve of the heat evoked current. The reversal potential is +18 mV.

C. Time course of the channel activity evoked by heat (~43°C) in cell attached patches. The cell membrane was held at –60 mV.

D. Current traces obtained at different times labelled a)-d) in C . The single channel chord conductance is 59 pS (similar to TRPV4 in HEK cells, see also figure 3). Amplitude histograms of the respective traces are shown at the right hand side.

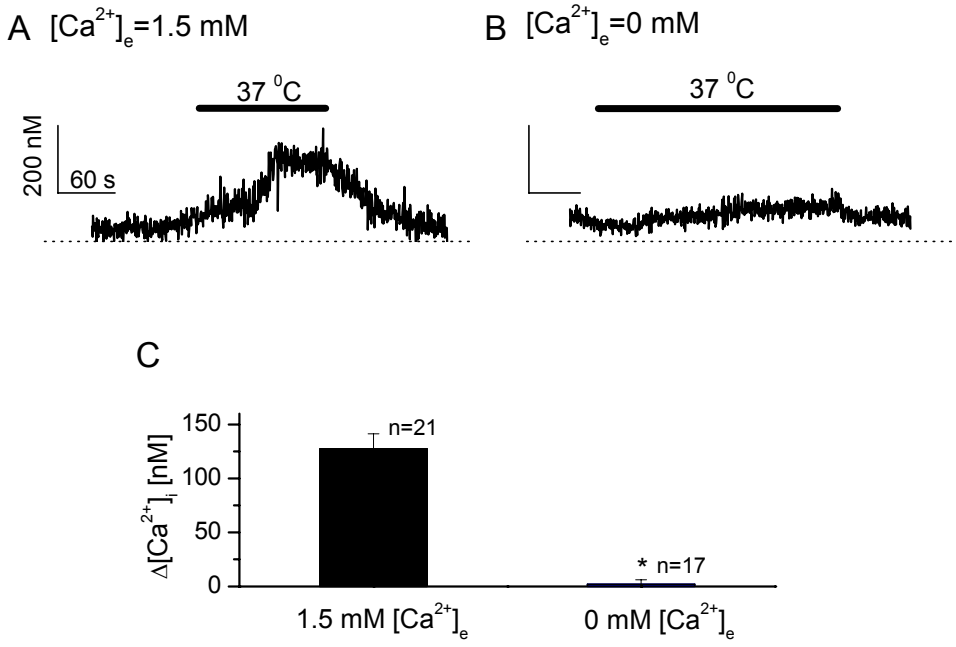

Figure 8: Increase of intracellular Ca^{2+} concentration by heat in mouse aorta endothelial cells.

A, B. Change in $[Ca^{2+}]_i$ on heat application (~37°C) in the presence (A) and absence (B) of 1.5 mM $[Ca^{2+}]_e$. Heat evoked $[Ca^{2+}]_i$ increase in the presence of $[Ca^{2+}]_e$ but not in the absence of $[Ca^{2+}]_e$.

C. Comparison of heat stimulation in the presence and absence of extracellular Ca^{2+}. This increase depends on the presence of extracellular Ca^{2+}.

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