Mammalian survival requires constant monitoring of environmental and body temperature. Recently, several members of the transient receptor potential vanilloid (TRPV) subfamily of ion channels have been identified that can be gated by increases in temperature into the warm (TRPV3 and TRPV4) or painfully hot (TRPV1 and TRPV2) range. In rodents, TRPV3 and TRPV4 proteins have not been detected in sensory neurons but are highly expressed in skin epidermal keratinocytes. Here, we show that in response to warm temperatures (>32 °C), the mouse 308 keratinocyte cell line exhibits nonselective transmembrane cationic currents and Ca²⁺ influx. Both TRPV3 and TRPV4 are expressed in 308 cells. However, the warmth-evoked responses we observe most closely resemble those mediated by recombinant TRPV4 on the basis of their electrophysiological properties and sensitivity to osmolarity and the phorbol ester, 4α-phorbol-12,13-didecanoate. Together, these data support the notion that keratinocytes are capable of detecting modest temperature elevations, strongly suggest that TRPV4 participates in these responses, and define a system for the cell biological analysis of warmth transduction.

The perception of ambient temperature is a physiological process critical to the maintenance of body temperature and the avoidance of painful or dangerous thermal extremes. Since the identification of the heat-sensing ion channel TRPV1 (1), which is activated by temperatures above 42 °C, there has been a significant focus on potential thermosensory functions for this and other ion channels of the transient receptor potential family. TRPV2 (2) and TRPV3 (3–5) were first described as heat transducers operative at very hot (>52 °C) and moderately warm (>34 °C) temperatures, respectively. TRPV4, which was originally identified as an osmosensory ion channel (6–9), can also be activated by warm temperatures (>34 °C) (10, 11). In addition, two TRP proteins outside of the TRPV subfamily, TRPM8 (12, 13) and ANKTM1 (ankyrin repeat/transmembrane-containing ion channel) (14), have been identified as cold-activated ion channels expressed in sensory neurons.

In mammals, the skin is extremely important for the transmission of thermal information and its transmission to the central nervous system. Cutaneous thermosensation has been largely attributed to the sensory nerves that innervate the dermal and epidermal layers of the skin. Recent reports, however, have suggested the possibility that other skin components, most notably keratinocytes, might also participate in temperature sensation. TRPV1 and TRPV2 are highly expressed in distinct subsets of sensory neurons (2). In humans, TRPV3 is also expressed in sensory neurons (5). In contrast, attempts to detect TRPV4 (9, 10) and TRPV3 (3) at the protein level in rodent sensory neurons have been unsuccessful. Rather, immunohistochemical studies of mouse and rat skin have revealed that keratinocytes exhibit the greatest degree of cutaneous TRPV3 (3) and TRPV4 (10) expression. In addition, although peripheral sensory neurons cultured from TRPV1 knockout mice exhibit profound deficits in heat-evoked activation, the deficits observed in the intact knockout mice or in skin-nerve explants derived from these animals are considerably more modest (15, 16). These findings suggest that TRPV3 and/or TRPV4 might mediate warmth detection and, possibly, painful heat detection at the level of the epidermal keratinocyte.

The objectives of this study were to determine whether keratinocytes in vitro exhibit responsiveness to innocuous warmth and, if so, whether these responses are mediated by one or more heat-sensitive TRPV family members. We found that in the mouse 308 keratinocyte cell line (17), mild heating evokes a rise in intracellular calcium ([Ca²⁺]ᵢ) as well as a transmembrane cationic current, with a threshold of ~33 °C. Moreover, the pharmacological and electrophysiological properties of these responses suggest that they are mediated, at least in part, by the activation of TRPV4.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The 308 cell line is a papilloma-derived keratinocyte cell line from 7,12-dimethylbenz[a]anthracene-treated adult BALB/c mouse skin (17). 308 cells were maintained at 37 °C in keratinocyte medium, which contains a 3:1 (v/v) mixture of Dulbecco's modified Eagle's medium and Ham’s F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum, 5 μg/ml insulin, 0.4 μg/ml hydrocortisone, 5 μg/ml transferrin, 2 × 10⁻¹⁰ M 3,3′,5-triiodo-t-thyronine, 10⁻¹⁶ M cholera toxin, 10 ng/ml epidermal growth factor, 60 μg/ml penicillin, and 25 μg/ml gentamicin (17). Cells were treated with 0.05% trypsin with 1 mM EDTA and replated onto glass coverslips 24–32 h prior to each experiment. Medium additives and other chemicals were obtained from Sigma unless otherwise indicated.

**Ca²⁺ Imaging**—Normal bath solution (290 mM) used for Ca²⁺ imaging contained 130 mM NaCl, 3 mM KCl, 2.5 mM CaCl₂, 0.6 mM MgCl₂, 10 mM HEPES, 1.2 mM NaHCO₃, and 10 mM glucose, adjusted to pH 7.45 with NaOH. To test the effect of osmolarity, 250 mM solution was made by reducing the concentration of NaCl to 105 mM. Mannitol was added to achieve 290 or 330 mM. Osmolarity was measured using a vapor pressure osmometer (Wescor, Logan, UT). For Ca²⁺-free solution, CaCl₂ was replaced with 10 mM EDTA. Cells were loaded with 10 μM Fura-2/AM (Molecular Probes, Inc., Eugene, OR) in normal bath solution containing 0.02% pleuronic acid (Molecular...
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Probes, Inc.) at 32 or 24 °C for 40 min. Coverslips containing Fura-loaded cells were continuously superfused with both solution unless otherwise indicated. Ratiometric Ca2+ imaging was performed using an inverted fluorescence microscope (Nikon, Melville, NY), excitation filter changer (Sutter, Novato, CA), and cooled CCD camera (Roper, Tucson, AZ). Paired images (340- and 380-nm excitation, 510-nm emission) were collected every 2 s with RatioTool software (See Imaging, Raleigh, NC). Fura ratios (emission at 340 nm excitation/emission at 380 nm excitation) were converted to absolute [Ca2+]i following system calibration (18). Heat stimuli were delivered using an in-line heater (Warner Instruments, Hamden, CT), monitored with a thermocouple (Physitemp, West Grove, PA) as described (2). Staining specificity was confirmed by signal ablation after incubation of antibodies with antigenic peptide or peptide conjugated resin. Samples were imaged using an Ultraview laser-scanning confocal microscope (Nikon).

Electrophysiology—For whole-cell recording, the recording pipette was filled with internal solution containing 120 mM cesium aspartate, 10 mM CsCl, 1 mM MgCl2, 5 mM EGTA, and 10 mM HEPES (pH 7.4 adjusted with CsOH, 285 mOsM adjusted with mannitol). Cells were initially superfused with a solution containing 130 mM sodium aspartate, 6 mM NaCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES (pH 7.4 adjusted with NaOH, 305 mOsM adjusted with mannitol). After the establishment of whole-cell mode, the solution was exchanged with a recording solution containing 130 mM sodium aspartate, 10 mM NaCl, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES (pH 7.4 adjusted with NaOH, 305 mOsM adjusted with mannitol). For single-channel recordings, pipette contained a solution composed of 130 mM sodium aspartate, 12 mM NaCl, 10 mM HEPES at pH 7.4 adjusted with NaOH (325 mOsM with mannitol). Cells were superfused with a solution containing 130 mM potassium aspartate, 10 mM KCl, 1 mM MgCl2, 10 mM glucose, 10 mM HEPES (pH 7.4 with KOH, 305 mOsM with mannitol) in cell-attached recordings. This perfusate was changed to internal solution (described above for whole-cell recording) just prior to patch excision for inside-out recordings. Because of their extremely flat morphology, cells were treated briefly with trypsin-EDTA just before the experiment to facilitate seal formation and measurement of single-cell membrane capacitance in whole-cell recordings. Cell-attached and inside-out recordings were performed without prior trypsinization. An Axopatch 200B amplifier (Axon Instruments, Union City, CA) connected to a Digidata 1200A analog/digital converter (Axon Instruments, Union City, CA) was used to acquire and analyze data. Patch electrodes were fabricated from borosilicate glass and had a tip resistance of 1.5–2.5 MΩ for whole-cell measurement and 2–5 MΩ for single-channel recording when filled with pipette solution. We recorded the whole-cell current only when the series resistance was less than 5 MΩ without compensation. An agar salt bridge containing 3 M KCl or NaCl was used throughout the experiment. Data were corrected for liquid junction potential calculated using pClamp. Membrane capacitance was measured using a capacitance neutralization circuit. Data were low pass-filtered at 2 kHz and digitized at 5 kHz in single-channel recordings and 3.3 kHz in whole-cell recordings.

RESULTS

Warm Temperatures Evoke a Rise in [Ca2+]i in Mouse 308 Keratinocytes—We performed microscopic Ca2+ imaging on the mouse 308 keratinocyte cell line to determine whether these cells would respond to a heat stimulus with a change in [Ca2+]i. Rapid but modest increases in bath temperature (from 25 to 38 °C in 10 s) (Fig. 1A) elicited substantial increases in [Ca2+]i (5.7 ± 1.2-fold, from 38 ± 5 nM to 189 ± 17 nM, p < 0.05, n = 6 coverslips) in 27% of cells. Over the entire population, the mean [Ca2+]i, increased by 2.8 ± 0.4-fold (from 33 ± 3 to 87 ± 8 nM, p < 0.001, n = 6). In nearly all responsive cells, restoration of bath temperature to 25 °C caused [Ca2+]i, to return to values at or near the initial baseline within 30–60 s. By applying extracellular bath solution at various peak tempera-
tured to parallel sets of cells (Fig. 1B), we determined that these responses were temperature-dependent and that their threshold was ~34°C, regardless of whether it was calculated on the basis of percentage of responsive cells or mean response amplitude. Application of slower temperature ramps from 25 to 40°C over 2 min (Fig. 1C) also produced reversible increases in [Ca²⁺], in many cells. Despite the monotonic shape of these temperature ramps, responses appeared only once the ambient temperature exceeded 32°C (a threshold just slightly lower than that observed in the rapid heating experiments) and increased in prevalence and size thereafter. At 39°C, 46±6% of cells exhibited a significant change in [Ca²⁺], with an increase among responders from 33±2 to 140±10 nM and an overall population increase from 31±1 to 108±6 nM. Sustained heat stimuli (25 to 37 ± 1°C within 10 s, followed by a 50-s plateau) (Fig. 2A) produced prolonged [Ca²⁺] responses, many of which commenced during the first 20–30 s of the heat stimulus, with others appearing later. The averaged population response decreased only slightly during the sustained phase of heating. However, some cells exhibited marked desensitization of heat-evoked [Ca²⁺] responses during this period. In addition, when we applied several brief heat stimuli at intervals of 3 min (Fig. 2B), responses observed during the second and third stimuli were diminished in amplitude by ~40 and ~60%, respectively.

In order to determine the source of the heat-evoked rise in 308 cell [Ca²⁺], we performed heat stimulation after removal of extracellular Ca²⁺ from the bath solution. Under these conditions (Fig. 3A), the heat-evoked change in [Ca²⁺], was extremely small (19% of control, n = 6, p < 10⁻⁶), suggesting that Ca²⁺ influx, rather than Ca²⁺ release from intracellular stores, was primarily responsible for the effects we observed. Further support for this notion comes from the observation that depletion of inositol 1,4,5-trisphosphate-sensitive Ca²⁺ stores by pretreatment of cells with the endoplasmic reticulum Ca²⁺ pump inhibitor, thapsigargin (1 μM, 15 min) failed to inhibit the heat-evoked Ca²⁺ responses (data not shown). Together, these results demonstrate that warm temperatures evoke Ca²⁺ influx in 308 keratinocytes.

**Mouse Keratinocytes Express Both TRPV3 and TRPV4**—Given the results described above and previous demonstrations of TRPV channel expression in keratinocytes in vivo (3, 10, 20, 21), we explored whether any of the four known heat-sensitive TRPV subtypes were expressed in 308 cells. After isolation of poly(A)⁺ RNA, we performed reverse transcription-PCR using...
primers specific for mouse TRPV1–4 (Fig. 4A). DNA bands of the appropriate size and sequence for each subtype could be amplified from these samples, indicating the expression of all four at the mRNA level. Immunofluorescence microscopy using affinity-purified polyclonal peptide antibodies specific for each subtype (Fig. 4B) revealed that TRPV3 and TRPV4 proteins were both expressed at readily detectable levels in 308 keratinocytes. Curiously, the distribution of immunoreactivity for both TRPV3 and TRPV4 was most prominent in apparently intracellular compartments, especially structures in the perinuclear area. However, TRPV4 immunoreactivity was also detected on or near the plasma membranes of many cells. In contrast, immunoreactivity for TRPV2 or TRPV1 was much less convincing, despite the presence of mRNA encoding these species; whereas very weak TRPV2 immunoreactivity could be detected in some cells, no clear evidence of specific TRPV1 immunoreactivity was observed (data not shown). To verify the specificity of the TRPV3 and TRPV4 antibodies used in these experiments and their ability to recognize the corresponding mouse orthologs, we performed immunoblot analysis on whole-cell extracts from HEK293 cells transfected with cDNAs encoding mouse orthologs, we performed immunoblot analysis on whole-cell extracts from HEK293 cells transfected with cDNAs encoding mouse orthologs, we performed immunoblot analysis on whole-cell extracts from HEK293 cells transfected with cDNAs encoding mouse orthologs, we performed immunoblot analysis on whole-cell extracts from HEK293 cells transfected with cDNAs encoding mouse orthologs, we performed immunoblot analysis on whole-cell extracts from HEK293 cells transfected with cDNAs encoding mouse orthologs, we performed immunoblot analysis on whole-cell extracts from HEK293 cells transfected with cDNAs encoding mouse orthologs. Both antibodies recognized the correct recombinant mouse protein, whereas neither exhibited cross-reactivity with other subtypes. Similarly, antibodies against mouse TRPV1 and TRPV2 recognized only the appropriate subtype (not shown). Finally, we sought to confirm the expression of TRPV3 and TRPV4 in 308 cells by immunoblot analysis (Fig. 4D). Both anti-TRPV3 and anti-TRPV4 antibodies recognized protein bands with apparent molecular weights similar to those of recombinant TRPV3 and TRPV4. The high molecular weight band marked with an asterisk in the TRPV3 blot may represent a multimeric form of this protein. The TRPV4 antibody recognized a doublet in the 308 cell extracts. A similar TRPV4 doublet has been described by others, with the higher molecular weight band proposed to represent a glycosylated form (22).

TRPV4 Probably Mediates the Heat-evoked [Ca\(^{2+}\)]i Increase Observed in Mouse Keratinocytes—To further examine whether one or more member(s) of the TRPV family might be responsible for the influx of Ca\(^{2+}\) evoked by exposure to warmth, we tested the effect of ruthenium red (RR), a channel blocker that has been shown to potently inhibit heat responses mediated by all four TRPV subtypes (1–3, 10). The [Ca\(^{2+}\)]i increase evoked by a 37 °C heat stimulus in the presence of RR (1 μM) was significantly smaller than that observed in cells heated in the absence of RR (25% of control, p < 10\(^{-4}\), n = 6) and comparable with that observed in the absence of extracellular Ca\(^{2+}\) (Fig. 3A). Even when applied after the initiation of the heat response (Fig. 3B), RR produced a rapid decline in response amplitude. These results are consistent with the involvement of TRPV channels in the heat-evoked [Ca\(^{2+}\)] influx we observed.

We next tested the effects of capsaicin, a selective agonist for TRPV1 (1). Consistent with the absence of significant detectable TRPV1 protein in the this cell line, treatment with capsaicin (10 μM) produced no visible increase in [Ca\(^{2+}\)]i (n = 5, data not shown). Hypoosmolarity and hyperosmolarity have been reported to enhance and inhibit, respectively, the heat-evoked activation of TRPV4 (10). In contrast, no effects of osmolarity on TRPV1 (6, 7, 10), TRPV2 (7), or TRPV3 (4) have been detected. We therefore examined the effects of extracellular osmolarity on the heat-evoked [Ca\(^{2+}\)]i responses of 308 cells by applying slow heat ramps under isotonic (290 mOsm), hypertonic (330 mOsm), or hypotonic (250 mOsm) conditions (Fig. 5A and B). No change in [Ca\(^{2+}\)]i was observed during a 2-min pretreatment with hypotonic or hypertonic bath solution at 24 °C. However, a superimposed heat stimulus evoked an increase in [Ca\(^{2+}\)]i, whose amplitude was very sensitive to osmolarity. At all temperatures >31 °C, heat-evoked response amplitudes were significantly enhanced under hypotonic conditions and inhibited under hypertonic conditions, relative to isotonic controls (mean [Ca\(^{2+}\)]i, at 39 °C: hypotonic, 160 ± 8 nM; isotonic, 113 ± 7 nM; hypertonic, 42 ± 6 nM; p < 10\(^{-7}\), n = 6, one-way ANOVA). Comparable changes were observed in the proportion of heat-responsive cells (at 39 °C: hypotonic, 85 ± 2%; isotonic, 55 ± 4%; hypertonic, 19 ± 4%; p < 10\(^{-7}\), n = 6, one-way ANOVA). These effects resemble those described for heterologously transfected TRPV4 (10).

Given these findings, we tested the effect of 4α-phorbol-12,13-didecanoate (4α-PDD) on 308 cells. This phorbol ester is an apparently selective TRPV4 agonist that is incapable of activating protein kinase C (23) or TRPV3 (4). Superfusion of 1 μM 4α-PDD was also greatly reduced by the overall [Ca\(^{2+}\)]i response (area under the [Ca\(^{2+}\)]i curve during the 3-min peristimulus period) was reduced by ~80%. The response to 4α-PDD was also greatly reduced by the removal of extracellular Ca\(^{2+}\). Under these conditions, however, some transient [Ca\(^{2+}\)] spikes were observed, probably reflecting Ca\(^{2+}\) release from intracellular stores. Upon drug washout and replacement of extracellular Ca\(^{2+}\) ions, a robust [Ca\(^{2+}\)]i increase was observed that most likely reflected residual activity of TRPV4 channels (23) and/or Ca\(^{2+}\) influx through store-operated channels.

Finally, we asked whether subthreshold concentrations of...

![Graph](image1.png)

**Fig. 3.** Extracellular Ca\(^{2+}\) dependence and RR inhibition of the heat-evoked [Ca\(^{2+}\)]i response. A, left, heat-evoked (37 °C prolonged stimulus, horizontal bar) [Ca\(^{2+}\)]i change under control conditions (circles), in the absence of extracellular Ca\(^{2+}\) (squares) or in the presence of RR (1 μM, triangles). Data are represented as mean ± S.E. from six coverslips. Right, comparison of peak [Ca\(^{2+}\)]i change under each condition. *, p < 10\(^{-4}\), unpaired Student’s t test, n = 6 coverslips. B, acute inhibition of the heat-evoked (37 °C prolonged stimulus, horizontal bar) [Ca\(^{2+}\)]i response by superfusion of RR (1 μM, squares) without pretreatment. Only cells showing heat responses before the application of RR were selected for averaging (n = 6 coverslips).
4α-PDD could augment the responsiveness of 308 keratinocytes to heat (Fig. 6B). Cells were pretreated with 4α-PDD at a concentration (50 nM), which alone produced no significant rise in [Ca$^{2+}$]. While the drug remained present, the bath temperature was raised from 25 to 40 °C. Under these conditions, both the amplitude of the heat-evoked change in [Ca$^{2+}$], and the proportion of heat-responsive cells was significantly greater than in the absence of 4α-PDD. At 39 °C, the mean response amplitude was 53 ± 7 and 102 ± 12 nM in the absence versus presence of 4α-PDD (p < 0.01, n = 7 coverslips, one-way ANOVA), whereas the proportion of heat-responsive cells rose from 30 ± 4 to 57 ± 5% (p < 0.001, n = 7, one-way ANOVA). This finding suggests that heat and 4α-PDD act supra-additively on 308 cells, as reported for recombinant TRPV4 (24) and analogous to the supra-additive effects of capsaicin, heat, and protons on cells expressing TRPV1 (1, 19). Together, these results confirm the functional expression of TRPV4 in mouse 308 keratinocytes and support the notion that this protein participates in the heat-evoked responses observed in these cells.

Heat-evoked Currents in Mouse Keratinocytes—To further explore whether TRPV4 might contribute to the heat-evoked Ca$^{2+}$ increases described above, we recorded heat-evoked whole-cell currents in 308 cells. Application of a heat ramp from 25 to 39 °C evoked temperature-dependent increases in currents assayed during repetitive voltage ramps from −100 to +100 mV at 1 Hz (Fig. 7A). These currents inactivated slightly during the heat stimulus and reverted to base line upon return to 25 °C. Current density at peak temperature was significantly greater than that recorded at 25 °C (−11.0 ± 3.6 versus −4.7 ± 2.1 pA/pF at −80, p < 0.01 in paired Student’s t test, n = 15; 16.0 ± 4.0 versus 7.0 ± 2.5 pA/pF at +80 mV, p < 0.01 in paired Student’s t test, n = 15). The threshold for activation of heat-evoked currents was −31–33 °C (Fig. 7B, B and C), comparable with that obtained from Ca$^{2+}$ imaging experiments. These currents were associated with a shift in reversal potential ($E_{rev}$) in the depolarized direction by 5.5 ± 1.1 mV (from −6.9 ± 1.2 to −1.4 ± 1.3 mV, p < 0.001 in paired Student’s t test, n = 15). To calculate the current component activated by heat stimulation ($I_{heat}$), base-line currents at 24 °C were subtracted from those recorded during heating (Fig. 7D). The current-voltage relationship of $I_{heat}$ showed an $E_{rev}$ of

Fig. 4. TRPV mRNA and protein expression in 308 cells. A, top, ethidium bromide-stained agarose gel of reverse transcription-PCR products amplified from 308 cell total RNA. Bottom, parallel samples generated in the absence of reverse transcriptase. DNA size standards (base pairs) are shown at the left. B, immunofluorescence analysis of TRPV3 and TRPV4 expression in fixed, permeabilized 308 cells without (left) or with (right) antigenic peptide competition. The arrow indicates apparent plasma membrane localization of TRPV4. C, immunoblot analysis of recombinant mouse TRPV3 and TRPV4. Whole-cell extracts of HEK293 cells transfected with control vector (pCDNA3) or subcloned mouse TRPV1, TRPV2, TRPV3, or TRPV4 cDNAs were separated on 8% SDS-PAGE gels and immunoblotted using antibodies directed against TRPV3 (left) and TRPV4 (right). D, immunoblot analysis of 308 cell extracts. TRPV3 (left) and TRPV4 (right) were assayed in the absence (−) or presence (+) of competing antigenic peptide. The arrowheads indicate TRPV3 and TRPV4 bands. An asterisk indicates a possible multimerized form of TRPV3. Molecular mass markers are included in C and D.

Fig. 5. Modulation of heat-evoked response by osmolarity. A, representative [Ca$^{2+}$], plots (black) derived from independent groups of cells exposed to a slow heat ramp (red) at 250 mOsm (top), 290 mOsm (middle), or 330 mOsm (bottom). B, mean heat-evoked [Ca$^{2+}$], change (top) and fraction of responsive cells (bottom) calculated at 2 °C intervals from 28 to 40 °C at 250 mOsm (circles), 290 mOsm (squares), and 330 mOsm (triangles). *p < 0.05; **p < 0.01; ***p < 0.001; one-way ANOVA, n = 5–6 coverslips.
3.8 ± 2.0 mV (n = 15) and weak outward rectification; outward currents at +80 mV (9.0 ± 2.3 pA/pF, n = 15) were larger than the inward currents measured at −80 mV (−6.4 ± 1.8 pA/pF, n = 15) by 1.9 ± 0.3-fold (n = 15).

To evaluate whether \( I_{\text{heat}} \) represents a cationic current, we tested the effect of replacement of extracellular Na\(^+\) and Mg\(^{2+}\) with the impermeant cation NMDG (Fig. 8A). After activation of \( I_{\text{heat}} \) at 38 °C, NMDG-containing solution was superfused onto the cells. The result was a virtual elimination of the inward current component (to levels at or below the base line measured at 24 °C) with little effect on the outward current and a significant shift to more negative reversal potentials by −23.1 ± 6.5 mV (from 1.5 ± 1.0 to −21.6 ± 6.3 mV, n = 5, \( p < 0.05 \) in paired Student’s t test). When 35 mM Ca\(^{2+}\) was used to replace Na\(^+\) as the major cation in the bath solution (Fig. 8B), the \( I_{\text{rest}} \) of \( I_{\text{heat}} \) was shifted to more depolarized potentials by 21.4 ± 2.3 mV (from −8.6 ± 2.5 to 12.8 ± 0.9 mV, n = 5, \( p < 0.001 \) in paired Student’s t test). Together, these findings suggest the involvement of a Ca\(^{2+}\)-permeable nonselective cationic current in \( I_{\text{heat}} \).

Next, we evaluated the effect of RR on \( I_{\text{heat}} \). 1 μM RR delivered after the activation of \( I_{\text{heat}} \) decreased the inward component of the heat-evoked current to 28.1 ± 8.9% of control (at −80 mV, n = 5) (Fig. 8C and D). However, we observed no inhibition over the range of depolarized potentials. Instead the outward current amplitude was increased to 305.8 ± 57.1% of control at +80 mV (n = 5). Both effects of RR reversed slowly after washout. The inhibitory effect of RR on the inward current component was even greater (−81% reduction at −80 mV) when the heat stimulus was delivered after 1 min of RR pretreatment (−3.9 ± 0.5 pA/pF in six control cells, −0.7 ± 0.2 pA/pF in five RR-treated cells, \( p < 0.001 \)). Under these conditions, \( I_{\text{heat}} \) was evident only at potentials higher than −80 mV.

Because we had observed a robust 4α-PDD-induced [Ca\(^{2+}\)] increase in the 308 cells, we looked for 4α-PDD-mediated currents using whole-cell voltage clamp. When a voltage ramp was applied at 0.1 or 0.2 Hz during the superfusion of 1 μM 4α-PDD, a slowly developing current was observed (Fig. 9A). The current activated by 4α-PDD (\( I_{\text{PDD}} \)) showed a nearly linear current-voltage profile with a rectification ratio of only 1.1 ± 0.1 (at ±80 mV, n = 7) (Fig. 9B). The mean current density after subtraction of base-line current (−24.5 ± 8.6 and 28.7 ± 12.3 mV in 4α-PDD and control, respectively) evoked by slow heat decreases and modulation of heat-sensitive current obtained by the subtraction of background current measured at 24 °C from the current at 38 °C. n = 10 cells.

![Image](https://example.com/figure6.png)

**Fig. 6.** 4α-PDD-evoked [Ca\(^{2+}\)] increases and modulation of heat-evoked responses. A, representative increases in [Ca\(^{2+}\)], in response to the application of 4α-PDD alone (top left), in the presence of 1 μM RR (top right) or under Ca\(^{2+}\)-free conditions (bottom left) in separate experiments. 1 μM 4α-PDD was superfused during the indicated 90 s. Area under the [Ca\(^{2+}\)] change plot during the 3 min following application of 4α-PDD (bottom right, arbitrary units) was averaged over 4–5 coverslips. *, \( p < 0.001 \), unpaired Student’s t test. B, enhanced heat responsiveness in the presence of a subthreshold concentration of 4α-PDD. **, \( p < 0.01; \***, \( p < 0.001; \) unpaired Student’s t test). Together, these findings suggest rising phase of heat stimulus. C, representative current responses observed during voltage ramp pulses at various temperatures, and used to derive data in A and B. D, averaged I-V relationship of heat-sensitive current obtained by the subtraction of background current measured at 24 °C from the current at 38 °C. n = 10 cells.
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**Fig. 8.** $I_{\text{heat}}$ is a nonselective cationic and RR-sensitive current. *A*, left, change in current amplitude at ±80 mV in response to a prolonged heat stimulus and the replacement of all external cations with NMDG. Right, representative current traces evoked by voltage ramp pulses at points a, b, and c, marked at the left. B, heat-evoked currents in a representative cell subjected to external solution containing 150 mM Na$^+$ and, subsequently, 0 mM Na$^+$/H$_9$262 during a single, prolonged heat stimulus (38 °C). C, left, change in current amplitude at ±80 mV in response to a prolonged heat stimulus and the addition of RR (1 μM) to the external solution. Right, representative current traces evoked by voltage ramp pulses at points a, b, and c, marked at left. D, averaged I-V relationship of heat-evoked currents obtained from control (open circles, n = 19) and RR-pretreated (filled circles, n = 5) cells.

**Fig. 9.** 4α-PDD evoked currents ($I_{4\alpha\text{-PDD}}$) in 308 cells. *A*, representative delayed onset increase in whole-cell current amplitude at ±80 mV in response to 4α-PDD (1 μM). Replacement of all external cations with NMDG and the addition of RR (1 μM) were performed at the indicated times. Data are plotted at 0.2 Hz. B, representative current traces evoked by voltage ramp pulses at points a, b, c, and d, marked in *A*. pA/pF at −80 and +80 mV, respectively, n = 7) was far greater than that of $I_{\text{heat}}$. The current reversed at about −1.2 ± 1.9 mV (n = 7), which was significantly depolarized from the $E_{\text{rev}}$ of current before 4α-PDD stimulation (−4.2 ± 1.5 mV, n = 7, p < 0.001) by 3.0 ± 0.7 mV (n = 7). When extracellular cations were replaced with NMDG, inward $I_{4\alpha\text{-PDD}}$ decreased to an amplitude similar to or smaller than that of baseline current with a significant shift in $E_{\text{rev}}$ to more hyperpolarized potentials by −22.7 ± 4.4 mV (from −2.2 ± 1.9 to −24.9 ± 3.8 mV, n = 5, p < 0.01). Application of RR (1 μM) after the development of $I_{4\alpha\text{-PDD}}$ inhibited inward currents to an extent similar to that observed with $I_{\text{heat}}$ (31.5 ± 8.3% of control at −80 mV, n = 5). Like $I_{\text{heat}}$, $I_{4\alpha\text{-PDD}}$ also exhibited an RR-mediated enhancement of the outward current component at strongly positive potentials (173.4 ± 43.0% of control at −80 mV, n = 5).

Finally, we performed electrophysiological recordings on cell-attached membrane patches in 308 cells. Upon an increase in bath temperature from 26 to 38 °C, single-channel currents were observed that became more prevalent as a function of temperature (Fig. 10A). Whereas a few such current responses were observed at temperatures slightly above 25 °C, the open probability increased most dramatically above 33 °C. Heat-evoked single-channel currents were characterized by intermittent bursts of brief, flickery openings. The unitary current amplitudes, estimated from amplitude histograms, averaged 9.3 ± 0.4 pA at ±60 mV (n = 7 at 34–36 °C, corresponding to a cord conductance of −150 pS) and were greater at positive potentials than at the corresponding negative potentials. These currents were characterized by multiple subconductance levels that were more evident at higher temperatures and in the negative potential range. 4α-PDD (1 μM at 25 °C) evoked single channel openings of a similar size (8.6 ± 0.2 pA at +60 mV, n = 7, cord conductance −140 pS) in cell-attached patches (Fig. 10B). These opening events were typically more protracted than those evoked by heat. The similar conductances of heat- and 4α-PDD-evoked currents are consistent with their being mediated by the same or similar channels. Because the conductances of these currents were intermediate between those reported for either recombinant TRPV3 (170 pS) (4) or TRPV4 (105 pS) (6, 11), however, we explored the possibility that the amplitudes we measured in the cell-attached configuration might be influenced by the ion or protein composition of the 308 cell cytosol. We therefore excised inside-out membrane patches from the 308 cells and examined heat- and 4α-PDD-evoked current responses in this context. 4α-PDD remained capable of activating single-channel openings in this recording mode. Interestingly, the amplitude of these currents was 5.4 ± 0.1 pA at ±60 mV (n = 5, cord conductance 90 pS), much closer to that reported for recombinant TRPV4 than currents recorded in the cell-attached mode (Fig. 10C). In contrast, we failed to observe any heat-evoked currents in excised membrane patches (n = 11). Even in five of these patches that had previously exhibited heat-evoked currents in the cell-attached configuration, patch excision resulted in a loss of heat-evoked channel openings (Fig. 10D). This latter finding is consistent with the report that whereas TRPV3 can be activated by heat in excised patches (4), TRPV4 cannot (11). Together, these findings support the idea that TRPV4 is a contributor to warmth-evoked responses of 308 cells.

**DISCUSSION**

Using fluorescent Ca$^{2+}$ imaging and patch clamp methods, we have found that low intensity heat stimuli (between 33 and 38 °C) evoke rapid and reversible transmembrane cationic currents and elevations in intracellular Ca$^{2+}$ in mouse 308 keratinocytes. Whereas one previous study explored the effects of very intense heat (90 °C for 2 min) on the human HaCaT keratinocyte cell line (25), this represents the first demonstration, to our knowledge, of keratinocyte responses to gentle heating. Furthermore, several observations lead us to propose that the heat-sensitive, nonselective cation channel, TRPV4, contributes to the 308 cell responses: 1) TRPV4 protein and...
mRNA are both detectable in 308 cells; 2) both \([\text{Ca}^{2+}]\), and \(I_{\text{heat}}\) increase with temperature, exhibiting an activation threshold of 31–33 °C, similar to that of heterologously expressed TRPV4; 3) RR inhibits the temperature-dependent \([\text{Ca}^{2+}]\), increase and voltage-dependently inhibits \(I_{\text{heat}}\) at concentrations that inhibit TRPV4; 4) the warmth-evoked \([\text{Ca}^{2+}]\), increase depends upon extracellular \(\text{Ca}^{2+}\); 5) the warmth-evoked \(\text{Ca}^{2+}\) increase is enhanced by hypoosmolarity and inhibited by hyperosmolarity like that mediated by heterologously expressed TRPV4; 6) 4α-PDD, a selective agonist of TRPV4, evokes RR-sensitive \([\text{Ca}^{2+}]\), increases and transmembrane cationic currents in these cells; 7) 4α-PDD at subthreshold concentrations augments the \([\text{Ca}^{2+}]\), response to heat; 8) warmth and 4α-PDD activate single-channel currents with similar amplitudes in cell-attached patches; and 9) in excised patches, 4α-PDD evokes currents similar in size to those reported for recombinant TRPV4, whereas heat fails to evoke currents in this setting.

The functional expression of TRPV1 has previously been demonstrated in human keratinocyte cell lines (20, 21). However, a significant involvement of TRPV1 in the warmth-evoked responses described in this study can most likely be excluded, given the following: 1) TRPV1 protein was not readily detectable in 308 cells; 2) capsaicin, a selective and efficacious TRPV1 agonist, failed to evoke a visible \(\text{Ca}^{2+}\) response at doses that are saturating for neuronal or recombinant TRPV1; and 3) the heat activation threshold we observed in keratinocytes was −9 °C lower than that exhibited by TRPV1 in native (26, 27) and recombinant (19) systems. The appearance of ruthenium red-insensitive \(\text{Ca}^{2+}\) responses at temperatures greater than 40 °C prevented us from extending our analysis into the range of TRPV1 responsiveness. The reported activation threshold of recombinant TRPV2 (53 °C) (3) and its apparently low expression level in our cells similarly argue against the involvement of this subtype. Our data do not allow us to rule out a contribution of TRPV3 to the heat-evoked responses of 308 cells. TRPV3 has previously been reported to be expressed in mouse skin keratinocytes (3), a result we have confirmed in rat and mouse skin and now in 308 cells. Moreover, the reported threshold temperature for activation of this subtype is approximately identical to that of TRPV4 (3–5). At present, no specific agonists or antagonists are available for TRPV3. However, the effects of osmolarity and 4α-PDD on the heat responses observed in this study, the reported failure of these stimuli to activate TRPV3 (4), and the single channel current data outlined above argue that whereas there may certainly be a role for TRPV3 in keratinocyte warmth detection, any such role appears to be shared with TRPV4. Perhaps TRPV3 and TRPV4 form heteromultimeric channels in these cells that pharmacologically resemble TRPV4. Alternatively, TRPV3 may exist at a lower density than TRPV4 in the plasma membrane of 308 cells. The two TRPV subfamily members not addressed in our study, TRPV5 and TRPV6 (28, 29), have not yet been examined for heat responsiveness or skin expression. However, TRPV5 is reportedly insensitive to osmolarity (7), and the relative insensitivity of TRPV6 to RR (30) is inconsistent with the potent inhibitory effects of this compound on heat-evoked 308 cell responses. Thus, TRPV4 remains the most viable candidate mediator of heat responses in 308 cells. A more definitive resolution of this matter must await the development of subtype-selective antagonists and/or subtype-selective protein knockdown approaches such as RNA interference or gene disruption.

Despite their similarities, it is unclear whether \(I_{\text{heat}}\) and \(I_{\text{PDD}}\) are produced by exactly the same mechanism. Heat-evoked activation of endogenously expressed TRPV4 has also been reported in cultured mouse aortic endothelial cells (11). There, as in 308 cells, whole-cell currents evoked by 4α-PDD were significantly larger than those evoked by heat. In addition, single-channel openings evoked by heat in 308 cells tended to be briefer in duration and more irregular in amplitude than those evoked by 4α-PDD. As reported for recombinant TRPV4 (11), we were able to observe 4α-PDD-evoked currents but not heat-evoked currents in excised membrane patches. Finally, \(I_{\text{heat}}\) showed weak outward rectification, whereas \(I_{\text{PDD}}\) displayed little outward rectification under the

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2 M. K. Chung and M. Caterina, unpublished observation.

3 H. Lee and M. Caterina, unpublished observation.
same conditions. Differences between chemical- and temperature-evoked ion channel activation would not be unique to TRPV4. For example, the responses of TRPV1 to heat versus capsaicin differ with regard to ion selectivity, calcium dependence of desensitization, and efficiency of antagonist blockade (19), whereas the desensitization of TRPM8 differs, depending upon whether it is activated by menthol or cold (13). In the case of TRPV4, these differences may reflect distinct modes of activation by 4α-PDD versus heat, such as direct gating versus gating by diffusible factors, as suggested (11), or a differential influence of association with other cellular proteins. Such environmental factors may also account for the differences in unitary amplitude we observed in 4α-PDD-evoked currents between cell-attached and excised membrane patches.

Voltage-dependent block by RR has thus far been reported only for TRPV4 (23) and a plant vascular Ca$^{2+}$ channel (31). Our finding that RR actually increases $I_\text{heat}$ and $I_{\text{PDD}}$ at strongly positive potentials has not been reported for recombinant TRPV4. The fact that this effect was observed on both responses reinforces the notion that they are mediated by the same channel. However, the mechanism behind this peculiar effect and the reason it has not been observed until now remain unclear. La$^{3+}$, which, like RR, appears to interact with the pore regions of certain nonselective cation channels, can augment current responses through TRPC4 and TRPC5 (32). It remains to be determined, however, whether the phenomenon we observe with RR represents a similar effect and whether it is attributable to the association of TRPV4 with cell type-specific proteins, posttranslational modifications, the secondary activation of a distinct conductance, or the particular conditions of our recordings.

Most of the immunoreactivity for TRPV4 (and TRPV3) that we observed in the 308 cells was located in an apparently intracellular vesicular compartment. In addition, whereas the warmth-evoked responses of these cells were entirely dependent upon extracellular Ca$^{2+}$ and may therefore result entirely from Ca$^{2+}$ influx, application of 4α-PDD produced oscillations of [Ca$^{2+}$]i, even in the absence of extracellular Ca$^{2+}$. This latter finding is consistent with coupling from the cell surface to intracellular stores and/or a functional role for the intracellular TRPV4 pool. We previously demonstrated that expression of TRPV4 in keratinocytes of the rat plantar epidermis was not confined to the plasma membrane but was diffusely distributed throughout the cytoplasm, excluding the nucleus (10). Other investigators have demonstrated that a pool of recombinant TRPV4 is expressed in an apparently intracellular compartment in transfected fibroblasts (24). TRPV3 appears to exhibit a similar pattern in skin sections (3), a result we have confirmed.2 Functional intracellular TRPV1 has been detected in sensory neurons (33–35) and heterologously transfected cells (36). In urinary epithelial cells, TRPV1 immunoreactivity has also been detected in an intracellular, apparently vesicular compartment (37). Moreover, TRPV2 has been shown to translocate from an intracellular compartment to the plasma membrane upon growth factor receptor stimulation in several cell types (38, 39). The potential functional significance and regulation of TRPV3 and TRPV4 subcellular localization should therefore be explored.

The precise threshold for TRPV4 activation appears to depend upon its cellular context and environmental history. Whereas we have previously reported the threshold of this molecule to be ~34 °C when expressed in HEK293 cells (10), Watanabe et al. (11) obtained lower threshold values when the starting temperature was lowered to 14 °C. Moreover, cells plated on the same coverslip showed heterogenous heat responses with different thresholds, onset times, response amplitudes, and durations. The plasticity of these temperature response properties is further supported by our observation that specific plating conditions and temperature exposure protocols influenced the proportion of heat-responsive 308 cells. Activity-dependent changes in channel state, channel phosphorylation/phosphorylation (22), changes in osmolarity, activation of downstream signaling pathways, and protein-protein interactions such as heteromultimeric channel formation may all conspire to produce such effects. Nevertheless, what is most clear is that TRPV4 maintains a substantial degree of temperature-dependent activity over the range of temperatures that can be considered “warm” (i.e. between 30 and 40 °C) under many different experimental conditions (10, 11).

Together with our previous findings (10), the results presented in this study raise the possibility that TRPV4 in keratinocytes contributes to warmth perception and/or cutaneous thermoregulation. An analogous role for TRPV4 has been proposed in temperature-mediated regulation of vascular function by endothelial cells (11). Since the basal temperature of the rodent skin surface can readily range from 27 to 35 °C (40), TRPV4 seems optimally poised to translate even modest temperature increases into changes in [Ca$^{2+}$]i. As suggested (3), the activation of keratinocytes by warmth might result in the release of a soluble messenger, such as ATP, that can be detected by ATP-gated ion channel receptors on underlying sensory neurons (41). A similar mechanism exists in the mouse urinary bladder, where urinary epithelial cell TRPV1 is required for hypoosmolarity-evoked release of ATP (37), which in turn appears to be detected by P2X3 (ATP-gated ion channel subtype three)-containing nerve terminals in the bladder wall (42). Alternative candidate heat-evoked signaling molecules include nitric oxide, which also undergoes TRPV1-dependent release from urinary epithelial cells (43), as well as prostaglandins and platelet-activating factor, which have been shown to be released from keratinocytes in response to UV radiation (44), extreme heating, oxidative stress (25), or, in human keratinocytes, capsaicin-evoked activation of TRPV1 (21). Biochemical analysis of supernatants from warmth-treated keratinocytes would allow the “soluble messenger” hypothesis to be tested experimentally, as would the analysis of thermosensation and thermoregulation in mice lacking TRPV4 selectively in keratinocytes. The latter experiments would also serve to rule out the possibility that TRPV4 heat sensation is an artifact of the cell culture context. It should be noted that keratinocyte-mediated warmth detection is not mutually exclusive with the direct detection of warmth by sensory neurons. Indeed, the expression of TRPV3 protein in human sensory neurons (5), despite its apparent absence from mouse sensory neurons (3), suggests that peripheral neurons can act as direct sensors of innocuous warmth.

The temperature-dependent activation of TRPV4 might also participate in keratinocyte Ca$^{2+}$ homeostasis, especially given that a basal level of TRPV4 activity is likely to exist at normal skin temperature. The growth and differentiation state of keratinocytes is profoundly influenced by intracellular and extracellular [Ca$^{2+}$] (45–47). At relatively low extracellular [Ca$^{2+}$] (0.09 mM), primary keratinocytes maintain an undifferentiated proliferative phenotype, whereas exposure to higher [Ca$^{2+}$] (1.44 mM) triggers a halt in proliferation, cell morphology changes, and the stage-specific expression of several markers of terminal differentiation (45, 48). Parallel gradients of keratinocyte differentiation state and intracellular and extracellular [Ca$^{2+}$] are observed in vivo as one traverses the skin epithelium from the apical to basal side (49), as are gradients of TRPV4 (10) and TRPV3 (3) expression level. Along these same lines, the variability that we observe in 308 cell TRPV3 and
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TRPV4 expression levels and subcellular localization, as well as the failure of some cells to respond detectably to heat, is consistent with the observation that these cells exhibit a spectrum of differentiation states under typical culture conditions. The possibility of an interplay among local temperature, TRPV3 and TRPV4 expression, and keratinocyte differentiation therefore appears worthy of further study.

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