Brief Communication

2-Aminoethoxydiphenyl Borate Activates and Sensitizes the Heat-Gated Ion Channel TRPV3

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Six of the mammalian transient receptor potential (TRP) ion channel subtypes are nonselective cation channels that can be activated by increases or decreases in ambient temperature. Five of them can alternatively be activated by nonthermal stimuli such as capsaicin (transient receptor potential vanilloid 1 (TRPV1)) or hypo-osmolarity (TRPV2 and TRPV4). No nonthermal stimuli have yet been described for TRPV3, a warmth-gated ion channel expressed prominently in skin keratinocytes. Here, we demonstrate that 2-aminoethoxydiphenyl borate (2-APB), a compound used to inhibit store-operated Ca²⁺ channels and IP₃ receptors, produces robust activation of recombinant TRPV3 in human embryonic kidney 293 cells with an EC₅₀ of 28 µM. 2-APB also sensitizes TRPV3 to activation by heat, even at subthreshold concentrations. In inside-out membrane patches from TRPV3-expressing cells, 2-APB increases the open probability of TRPV3. Also, whereas heat alone is capable of activating TRPV3-mediated currents in only a small proportion of primary mouse keratinocytes, 2-APB activates heat-evoked, TRPV3-mediated currents in the majority of these cells. Together, these findings identify 2-APB as the first known chemical activator of TRPV3 and enhance the notion that TRPV3 participates in the detection of heat by keratinocytes.

Key words: 2-APB; TRPV3; heat; thermosensation; keratinocytes; temperature

Introduction

The mammalian nervous system evaluates temperature over a range extending from extreme cold to extreme heat. This capacity involves the selective activation of neuronal subpopulations that fire over discrete ranges of skin temperature. The identification of temperature-gating nonselective cation channels of the transient receptor potential (TRP) family has provided candidate mediators of this sensory diversity (Patapoutian et al., 2003). Transient receptor potential vanilloid 1 (TRPV1) is activated by temperatures >42°C and is expressed most highly in a subpopulation of nociceptive neurons (Caterina and Julius, 2001). TRPV2, which is expressed in a distinct neuronal subpopulation, is activated at higher temperatures (>52°C) (Caterina and Julius, 2001). TRPV3 (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002) and TRPV4 (Guler et al., 2002; Watanabe et al., 2002) both exhibit thresholds in the warm range (>32°C). Although these two proteins have been detected immunologically in sensory neurons (Smith et al., 2002; Alessandri-Haber et al., 2003), their most robust cutaneous expression is in keratinocytes (Guler et al., 2002; Peier et al., 2002b), leading to speculation that keratinocytes cooperate with sensory afferents to signal elevated temperature. Transient receptor potential melastatin 8 (TRPM8) (McKemy et al., 2002; Peier et al., 2002a) and transient receptor potential ankyrin 1 (TRPA1) (Story et al., 2003) are reportedly activated by cool (<28°C) and cold (<20°C) temperatures, respectively.

Most thermosensitive TRP channels can alternatively be activated by nonthermal stimuli. TRPV1 can be activated by capsaicin, protons, or endocannabinoids (Caterina and Julius, 2001). TRPV2 can be activated by hypo-osmolarity (Muraki et al., 2003) or growth-factor signaling (Kanzaki et al., 1999), and TRPV4 can be activated by hypo-osmolarity, arachidonic acid metabolites, or 4α-phorbol dibutyryl didecanoate (Watanabe et al., 2003; Nilius et al., 2004). TRPM8 and TRPA1 can be activated by menthol (McKemy et al., 2002; Peier et al., 2002a) and pungent isothiocyanates (Bandell et al., 2004; Jordt et al., 2004), respectively. The prevalence of polymodality among these channels is consistent with the fact that they are expressed not only in external tissues but also internally, where temperature is less variable.

Given these findings, it might be expected that TRPV3, too, can be activated by nonthermal stimuli. However, no such stimuli have been reported to date. Another intriguing observation is that although TRPV3 immunoreactivity is detectable in a majority of cultured primary keratinocytes, <3% of these cells exhibit TRPV3-like heat-evoked currents (Chung et al., 2004). To address these issues, we examined the possibility that known modulators of ion-channel activity might unmask or facilitate TRPV3 gating. We discovered that 2-aminoethoxydiphenyl borate (2-APB), a compound commonly used to inhibit IP₃ receptor-mediated Ca²⁺ release or store-operated Ca²⁺ entry (Bootman...
et al., 2002), can activate recombinant TRPV3 and sensitize recombinant and native TRPV3 to heat in transfected cells and primary keratinocytes, respectively.

**Materials and Methods**

**Cell culture.** Wild-type C57BL/6 mice and TRPV4−/− mice (Suzuki et al., 2003) were used. Procedures were approved by the Johns Hopkins Animal Care and Use Committee. Chemicals and culture reagents were obtained from Invitrogen (Carlsbad, CA) or Sigma (St. Louis, MO). Human embryonic kidney (HEK) 293 cells and primary mouse keratinocytes were cultured as described previously (Chung et al., 2003, 2004). cDNAs encoding mouse TRPV1, TRPV2, and TRPV3 (Chung et al., 2003), mouse TRPV4 (gift from Veit Flockerzi, Universitat des Saarlandes, Homburg, Germany), and human TRPV3 (gift from David Clapham, Harvard Medical School, Boston, MA), subcloned into a control vector (pCDNA3), were cotransfected with green fluorescent protein into HEK293 cells using Fugene 6 (Roche, Palo Alto, CA) and as directed for liquid junction potential. Membrane capacitance was measured using the following equation:

\[ E_{\text{eff}} = \frac{E_{\text{mem}}}{1 + \frac{E_{\text{ag}}}{E_{\text{eff}}}} \]

where \( E_{\text{mem}} \) is the maximal effect and \( x \) is the agonist concentration.

**Results**

**2-APB activates TRPV3 in HEK293 cells.** We examined the effects of agents known to influence other TRP channels on recombinant TRPV3. One of these was 2-APB, a modulator of store-operated Ca\(^{2+}\) influx and IP\(_3\) receptor-mediated Ca\(^{2+}\) release (Bootman et al., 2002). 2-APB (32 \( \mu \)M) evoked a slowly developing, robust, outwardly rectifying current when administered to mouse TRPV3-transfected HEK293 cells at 24°C. Like heat-evoked TRPV3 currents (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002), 2-APB-evoked currents were reversible and sensitized with successive challenges. 2-APB also sensitized TRPV3-transfected cells to a subsequent heat stimulus (Fig. 1A). Cells expressing human TRPV3 exhibited similarly strong 2-APB-evoked currents (Fig. 1B). In contrast, no 2-APB-evoked (100 \( \mu \)M) currents were observed at 24°C in pCDNA3-transfected HEK293 cells or cDNAs encoding the related channels, TRPV2 or TRPV4 (Fig. 1B). TRPV1-transfected cells exhibited a very weak 2-APB-evoked increase in outward current at strong positive potentials (Fig. 1B). At 24°C, small TRPV3 currents were observed in response to 3.2 \( \mu \)M 2-APB, and the responses increased dose dependently (EC\(_{50}\) = 28.3 at +80 mV and 41.6 \( \mu \)M at −80 mV), with apparent saturation between 100 and 320 \( \mu \)M (Fig. 1C). At low 2-APB concentrations, TRPV3 currents exhibited strong outward rectification, like those evoked by heat. However, as 2-APB was increased to >10 \( \mu \)M, this outward rectification gradually evolved into dual rectification, with a clear difference from heat-evoked currents at negative potentials (Fig. 1A–C). 2-APB also sensitized heat-evoked TRPV3 current responses. At a dose (1 \( \mu \)M) insufficient to evoke significant

**Figure 1.** 2-APB activates recombinant TRPV3 in HEK293 cells. A, Top, Sensitizing whole-cell current responses of TRPV3-transfected HEK293 cells during successive applications of 2-APB (32 \( \mu \)M) and subsequent challenge with heat (+80 mV, upward; −80 mV, downward). Traces were produced by connecting current amplitude (black line) or temperature (gray line) measurements recorded every 2 sec. Middle, Mean current amplitude change normalized to membrane capacitance at +80 mV (upward, filled bars) and −80 mV (downward, open bars) (\( \Delta I_{\text{norm}} \)) after application of 2-APB, at indicated times (\( n = 5; * p < 0.05; \# \text{one-way ANOVA} \)). Bottom, I–V traces obtained at times a–e (shown at the top) during repetitive 200 msec voltage ramps from −100 to +100 mV. The dashed line indicates zero current or potential level. V, Membrane potential; I, current. B, \( \Delta I_{\text{norm}} \) recorded from HEK293 cells transfected with mouse TRPV1 (V1), TRPV2 (V2), TRPV3 (V3), and TRPV4 (V4), human TRPV3 (hV3), and pCDNA3 (P3). For V1, V2, and V4, currents were evoked by 100 \( \mu \)M 2-APB (filled bars) followed by heat (open bars; V1, 47°C; V2, 45°C; V3, 43°C; V4, 40°C) after washout. For V3 and hV3, 2-APB (gray bars) was applied after heat (43°C, open bars). Filled bars for V3 indicate 2-APB addition without previous heat application. n = 6 in each group. Scale bars, V1, 0.5 nA; V2, V4, P3, 50 pA; V3, 0.1 nA. C, Top, \( \Delta I_{\text{norm}} \) evolved in TRPV3-expressing HEK293 cells at 24°C by different concentrations of 2-APB (−80 mV, squares; +80 mV, circles); n = 6. Bottom, Representative traces from TRPV3-transfected cells at indicated 2-APB concentrations. Dashed lines indicate zero current or potential level.
TRPV3 currents at 24°C, 2-APB produced an approximately sixfold supra-additive enhancement of warmth (37°C)-evoked currents (Fig. 2A). Fluorescent Ca$^{2+}$ imaging of HEK293 cells revealed robust 2-APB-activated increases in intracellular Ca$^{2+}$ in cells transfected with TRPV3, but not pCDNA3, at 24°C. This increase was reversibly abolished by elimination of extracellular Ca$^{2+}$, suggesting that it arises from Ca$^{2+}$ influx through TRPV3, rather than release of internal Ca$^{2+}$ stores (Fig. 2B). We next examined the effects of ruthenium red (RR), an antagonist of ion influx through most TRPV channels, including TRPV3 (Peier et al., 2002b; Smith et al., 2002; Xu et al., 2002). After several exposures to 2-APB, 10 μM RR decreased the basal inward current exhibited by TRPV3-expressing HEK293 cells and potentiated the outward current at 24°C (Fig. 2C). Superimposition of 2-APB onto RR resulted in no detectable inward current. Outward current increased slightly, but not as much as it had in the absence of RR (Fig. 2C, bar c vs bar a). Application of 2-APB after RR washout resulted in large inward and outward currents. No TRPV3 activation was observed in response to 1-[β-3-(4-methoxyphenyl)propoxy]-4-methoxymethyl-1H-imidazole hydrochloride (40 μM), another inhibitor of store-operated Ca$^{2+}$ entry, or diphenhydramine (100 μM), a molecule structurally similar to 2-APB.

We have reported previously that mouse keratinocytes exhibit two distinct current responses to warm temperatures: a TRPV4-dependent desensitizing response observed in most cells and a sensitizing, strongly rectifying response that appears to be mediated by TRPV3. Immunofluorescence staining revealed that most keratinocytes expressed TRPV3. However, only ∼3% of them exhibited convincing TRPV3-like heat-evoked currents (Chung et al., 2004). We therefore examined the effects of 2-APB on primary mouse keratinocytes. At 24°C, 2-APB (100 μM) produced a slight increase in current noise at strong positive potentials but no measurable change in whole-cell current (Fig. 4A). However, concomitant heating (42°C) evoked a response distinct from that observed in the absence of 2-APB. In 22 of 27 2-APB-treated keratinocytes, repetitive heat challenges resulted in sensitizing, outwardly rectifying current responses. In some of these cells, the first heat response was biphasic, with an initial desensitizing, strongly rectifying response that appears to be mediated by TRPV3. This pattern strongly resembles that observed in keratinocytes exhibiting an additive TRPV4-like and TRPV3-like heat response (Chung et al., 2004).
2004). Given that TRPV3 immunoreactivity in primary keratinocytes is primarily intracellular (Chung et al., 2004), we asked whether the effect of 2-APB was attributable to increased TRPV3 surface expression. Contrary to this prediction, 2-APB failed to visibly alter the keratinocyte TRPV3 immunostaining pattern (data not shown).

2-APB (100 μM) application to keratinocytes derived from TRPV4-/- mice again evoked only miniscule current responses at 24°C. However, superimposed heat stimuli evoked large, outwardly rectifying currents (60.1 and 10.8 pA/pF at +60 mV, n = 7; E_rev = 1.3 ± 1.1 mV, n = 12) in 23 of 30 cells (Fig. 4B) that sensitized with repeated heating. After 2-APB washout, baseline current at 24°C subsided, and only small heat-evoked responses were recorded. Addition of 2-APB in the middle of a heat challenge rapidly evoked large, outwardly rectifying current responses in these cells (Fig. 4C). As in TRPV3-transfected HEK293 cells, RR (10 μM) inhibited inward currents evoked by 2-APB at 42°C in TRPV4-/- keratinocytes (Fig. 4D). When 2-APB was present at 24°C, RR evoked an outward current (Fig. 4D, bar c) not seen previously without 2-APB (Chung et al., 2004) but dampened outward current responses to a superimposed heat stimulus (Fig. 4D, bar b vs bar d). The former effect appears to reflect a synergistic interaction of subthreshold concentrations of RR and 2-APB that we observed in TRPV3-transfected HEK293 cells and TRPV4-/- keratinocytes (supplemental Fig. 1, available at www.jneurosci.org). In transfected cells, the magnitude of this complex interaction varied as a function of drug dose and TRPV3 expression level (data not shown). Together, these properties lead us to conclude that TRPV3 is the likely mediator of

Figure 3. Single-channel currents evoked by heat and 2-APB in inside-out patches from TRPV3-transfected HEK293 cells. A, Changes in the open probability of multiple channels (NPo) after heating (left) or addition of 1 μM 2-APB (right). Alternating 540-msec-step pulses to ±60 mV were repeated every 1.5 sec. Dotted lines indicate the closed channel state. Arrowheads indicate multiple-channel open states. At the bottom, a–d and e–h indicate currents recorded at +60 mV and −60 mV, respectively, at the corresponding time points shown at the top. B, Representative single-channel currents evoked by heat (39°C) or 2-APB (1 μM at 25°C) at various voltages. Dashed lines indicate closed channel state. C, I–V relationship of single-channel currents evoked by heat (circles) or 2-APB (squares). n = 5 patches per symbol. Small, filled squares and circles at ±60 mV indicate values derived from linear fit in E at 25 and 39°C, respectively. D, E, Temperature dependence of unitary TRPV3 current amplitude. D illustrates representative currents evoked by 2-APB or heat. In E, unitary current amplitude is plotted as a function of temperature. To facilitate observation of channel openings over a broad temperature range, heat-evoked currents were recorded during both ascending (plus signs) and descending (triangles) phases of heat stimulus. Circles indicate 2-APB-evoked currents recorded at room temperature. Data were derived from eight independent cells. Lines indicate linear fit of heat-evoked current amplitudes (slope = 0.59, Q10 = 2.0 over 29–39°C at +60 mV; slope = 0.63, Q10 = 1.6 at −60 mV).
2-APB-sensitized keratinocyte responses to heat and that such responses do not require TRPV4.

Discussion

2-APB has been used extensively as an inhibitor of store-operated Ca$^{2+}$ influx and IP$_3$-mediated Ca$^{2+}$ release (Maruyama et al., 1997; Bootman et al., 2002). Although some have interpreted the effect on store-operated Ca$^{2+}$ entry as reflecting direct action on cell-surface channels (Prakriya and Lewis, 2001), others have suggested an indirect effect, mediated through a common regulatory component that couples Ca$^{2+}$ influx and Ca$^{2+}$ release (Ma et al., 2002; Schindl et al., 2002). In some cases, 2-APB has been shown to stimulate rather than inhibit IP$_3$ receptor-mediated Ca$^{2+}$ release from intracellular stores, cell-surface store-operated Ca$^{2+}$ channels, and a nonselective cationic current of unknown function (Prakriya and Lewis, 2001; Ma et al., 2002; Braun et al., 2003). Thus, the precise molecular target(s) and mechanism(s) of action of this drug remain unclear.

Among the proposed mediators of store-operated Ca$^{2+}$ entry are TRP channels of the transient receptor potential canonical and TRPV subfamilies, some of whose activities can be modulated by 2-APB. Although most of these effects are inhibitory, one example of a positive effect of 2-APB on channel activity is that of TRPV6. Constitutive TRPV6 currents recorded in the presence of extracellular Ca$^{2+}$ increase by 25% on addition of 2-APB (50–75 μM) (Voets et al., 2001; Schindl et al., 2002). 2-APB-evoked activation of TRPV3 is considerably more robust but exhibits a similar dose–response relationship. High 2-APB concentrations also produced extremely modest activation of TRPV1. However, we observed no activation of TRPV2 or TRPV4 by 2-APB, whereas TRPV5 is partially inhibited by this compound (Nilius et al., 2001). Thus, 2-APB-evoked activation is not a universal trait among TRPV channels. The rapid onset and reversal of TRPV3 current activation and the activation of TRPV3 by 2-APB in inside-out patches are consistent with a direct mechanism of action. Still, we cannot rule out the presence of cytosolic components in these patches, and therefore cannot distinguish definitively between a direct activation mechanism and one mediated by a distinct regulatory factor (Schindl et al., 2002).

In this study, the single-channel amplitude of TRPV3 currents evoked by heat and 2-APB exhibited a linear I–V relationship, in apparent contradiction to the outward rectification we observe at the whole-cell level and one previous report describing outwardly rectifying single-channel TRPV3 currents (Xu et al., 2002). The basis of these differences is unclear, but may include different recording modes (outside-out vs inside-out patch) or intracellular solution compositions. For example, a fourfold to 100-fold higher intracellular free [Mg$^{2+}$] in the previous study might have produced outward rectification through divalent cation blockade of inward currents, as seen with Ca$^{2+}$ and TRPV4 (Nilius et al., 2004). The difference between single-channel and whole-cell rectification may also be explained by an apparent voltage dependence of channel open probability, especially in the case of heat activation (Fig. 3A).

It is not clear why heat alone is insufficient to activate TRPV3 in the majority of primary keratinocytes. As suggested previously (Chung et al., 2004), this could stem from low levels of TRPV3 expression together with limited agonistic efficacy of heat. Low TRPV3 expression levels might accurately reflect the in vivo con-
dition or be an artifact of keratinocyte culture (Peier et al., 2002b). Alternatively, cultured keratinocytes may lack obligate molecular cofactors for TRPV3 thermal responsiveness. Another possibility is that TRPV3 activity in keratinocytes is masked by phospholipids or other cellular constituents, as demonstrated for TRPV1 (Chuang et al., 2001). Regardless, 2-APB, which acts supra-additively with heat, appears to overcome these limitations by increasing TRPV3 gating or inhibiting its suppression. TRPV3 modulation by endogenous molecules analogous to 2-APB (or other targets of this compound) might dictate TRPV3 in vivo responsiveness to heat or, in some tissues, represent its primary mode of regulation. Tight control over TRPV3 thermal activation apparently differs from that of TRPV4, the heat-evoked activity of which is more readily observed in cultured keratinocytes (Chung et al., 2004). Together with their distinct kinetic properties, such differential regulation may reflect separate functions for these two channels in keratinocyte thermotransduction. Our results also indicate that TRPV3 activation may provide a facile readout for the identification of 2-APB targets. Conversely, as a chemical stimulus, 2-APB represents a potentially powerful tool for the physiological analysis of TRPV3 and the identification of clinically useful TRPV3 antagonists.

References