

A Transient Receptor Potential-Like Channel Mediates Synaptic Transmission in Rod Bipolar Cells

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On bipolar cells are connected to photoreceptors via a sign-inverting synapse. At this synapse, glutamate binds to a metabotropic receptor which couples to the closure of a cation-selective transduction channel. The molecular identity of both the receptor and the G protein are known, but the identity of the transduction channel has remained elusive. Here, we show that the transduction channel in mouse rod bipolar cells, a subtype of On bipolar cell, is likely to be a member of the TRP family of channels. To evoke a transduction current, the metabotropic receptor antagonist LY341495 was applied to the dendrites of cells that were bathed in a solution containing the mGluR6 agonists *l*-AP4 or glutamate. The transduction current was suppressed by ruthenium red and the TRPV1 antagonists capsazepine and SB-366791. Furthermore, focal application of the TRPV1 agonists capsaicin and anandamide evoked a transduction-like current. The capsaicin-evoked and endogenous transduction current displayed prominent outward rectification, a property of the TRPV1 channel. To test the possibility that the transduction channel is TRPV1, we measured rod bipolar cell function in the *TRPV1*^{-/-} mouse. The ERG b-wave, a measure of On bipolar cell function, as well as the transduction current and the response to TRPV1 agonists were normal, arguing against a role for TRPV1. However, ERG measurements from mice lacking TRPM1 receptors, another TRP channel implicated in retinal function, revealed the absence of a b-wave. Our results suggest that a TRP-like channel, possibly TRPM1, is essential for synaptic function in On bipolar cells.

Introduction

Glutamate hyperpolarizes On bipolar cells by closing a cation-selective channel (Shiells et al., 1981; Slaughter and Miller, 1981). The glutamate receptor (Nakajima et al., 1993; Nomura et al., 1994) and the G protein (Vardi et al., 1993; Nawy, 1999; Dhingra et al., 2000) that mediate this response have been identified, but the cation channel has not. Two major families of cation-selective channels are the cyclic nucleotide-gated channels (CNG) (Craven and Zagotta, 2006) and the transient receptor potential (TRP) channels (Ramsey et al., 2006). Previous studies of On bipolar transduction suggested that the cation channel may be a member of the CNG family of channels, based on the observation that cGMP strongly potentiates the current (Nawy and Jahr, 1990; Shiells and Falk, 1990). However, it was later shown that the

channel is unlikely to be gated directly by cGMP, but rather that cGMP has a modulatory role (Nawy, 1999; Snellman and Nawy, 2004).

In the vertebrate retina, pharmacological evidence suggests that a member of the TRP channel family is likely expressed in light-sensitive ganglion cells (Warren et al., 2006; Hartwick et al., 2007; Sekaran et al., 2007). In On bipolar cells, two types of TRP channels have emerged as candidates for the transduction channel. One candidate is TRPV1, which is expressed predominantly in the peripheral nervous system and mediates heat sensation. Both TRPV1 and the On bipolar cell transduction channel are moderately permeable to Ca²⁺ with a Ca²⁺/Na⁺ permeability ratio of 9.6 in TRPV1 channels expressed in oocytes (Caterina et al., 1997) and 4.9 in salamander On bipolar cells (Nawy, 2000). The entry of Ca²⁺ activates a negative feedback pathway leading to desensitization of both the On bipolar cell transduction current (Shiells and Falk, 1999; Nawy, 2000; Berntson et al., 2004; Nawy, 2004) and the response to heat and capsaicin mediated by TRPV1 (Liu and Simon, 1996; Caterina et al., 1997; Koplak et al., 1997; Piper et al., 1999). Here, we present evidence that the transduction channel can be activated by both capsaicin and anandamide, compounds that are thought to be specific agonists for TRPV1. Another candidate channel is the founding member of the family of melastatin-related TRP channels (TRPM1). Recent

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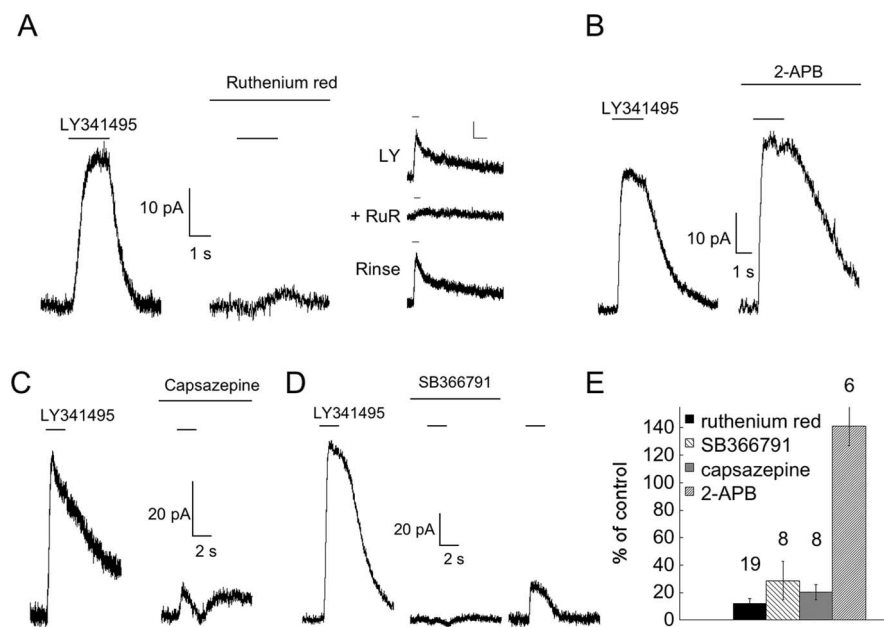


Figure 1. The rod bipolar cell transduction current is blocked by antagonists of TRPV1. **A**, Response to 100 μM LY341495 (delivered via fast-flow apparatus) before (left) and after (center) a 5 min application of 10 μM ruthenium red. Right, Response of another cell to a 1 s puff of LY341495 delivered through a puffer pipette alone (top) or during simultaneous application of ruthenium red from a second puffer pipette (middle). Ruthenium red was applied alone for 10 s before obtaining the middle trace. The inhibition of ruthenium red was readily reversed using this approach (bottom). Calibration: 10 pA, 2 s. **B**, Response to LY341495 before and after a 5 min application of 100 μM 2-APB. **C, D**, Responses to 1 s puffs of LY341495 (100 μM) before and after 5 min bath application of 20 μM capsazepine (**C**) and 20 μM SB366791 (**D**). Responses to LY341495 typically showed partial recovery after removal of antagonists, as shown in the right panel of **D**. Traces in **C** and **D** are from different cells. **E**, Summary of results. The number of cells for each experiment is indicated above each bar.

studies of Appaloosa horses have demonstrated that a dramatic reduction in the expression of mRNA encoding TRPM1 is a possible cause of night blindness and a reduced b-wave in the ERGs (Sandmeyer et al., 2007; Bellone et al., 2008). Both are indicative of a disruption of On bipolar cell function, implying that TRPM1 may play a role in mGluR6 signal transduction. We, therefore, set out to characterize the functional properties of the transduction channel and to further evaluate the possibility that it is composed of TRPV1 or TRPM1 subunits.

Materials and Methods

Preparation of slices. Retinal slices from 4- to 6-week-old C57BL/6 mice (Charles River) and TRPV1 knock-out mice (*Trpv1*^{tm1Jut}; The Jackson Laboratory) were prepared as described previously (Snellman and Nawy, 2004). Briefly, after killing, whole retinas were isolated and placed on a 0.65 μm cellulose acetate/nitrate membrane filter (Millipore), secured with vacuum grease to a glass slide adjacent to the recording chamber. Slices were cut to a thickness of 100 μm using a tissue slicer (Stoelting), transferred to the recording chamber while remaining submerged, and viewed with a Nikon E600FN upright microscope equipped with a water-immersion 40× objective and differential interference contrast optics.

Solutions and drug application. Slices were continuously perfused with Ames media bubbled with 95% O₂/5% CO₂. Picrotoxin (100 μM), strychnine (10 μM), and TPMPA (1,2,5,6-tetrahydropyridin-4-yl methylphosphinic acid; 50 μM) were included in all experiments to block inhibitory conductances. Patch pipettes of resistance 7–9 MΩ were fabricated from borosilicate glass (WPI) using a two-stage vertical puller (Narishige), and filled with a K⁺ gluconate-based solution that also contained 0.5 mM EGTA, 10 mM HEPES, 4 mM ATP, and 1 mM GTP (pH 7.4 by CsOH) and 14 μg/ml Alexa 488 (Invitrogen). In some experiments, the metabotropic receptor antagonist LY341495, or TRP channel reagents, were delivered to the retina from a pipette using positive pressure (2–4 PSI) with a computer-controlled solenoid valve (Picospritzer; Gen-

eral Valve), and the mGluR6 agonist L-AP4 (4 μM) was added to the bath. In other experiments, drugs were applied via a fast-flow apparatus (Snellman and Nawy, 2004), and glutamate was used as an mGluR6 agonist. Drugs and chemicals were purchased from Sigma, with the exceptions of L-AP4 and LY341495 (Tocris Bioscience) and AM251 (Cayman Chemical).

Recording and analysis. Whole-cell recordings were obtained with an Axopatch 1D amplifier (Molecular Devices). Currents were acquired at a sampling rate of 2 kHz with Axograph X software and an Apple G5 computer, low-pass filtered at 50 Hz (Frequency Devices) and digitized with an ITC-18 interface (Heka). Holding potentials were corrected for the liquid junction potential, which was measured to be 10 mV with the standard K⁺ gluconate pipette solution. Recordings were discarded if the series resistance exceeded 20 MΩ. Data were analyzed off-line with Axograph X and Kaleidagraph (Synergy Software). Plots of normalized conductance of the transduction channel versus voltage were fit with a Boltzmann relation of the form $g = (g_{max} - g_{min}) / (1 + \exp((V_m - V_{1/2})/k)) + g_{min}$, where g_{max} is the maximum conductance, g_{min} is the minimum conductance, $V_{1/2}$ is the voltage at which the conductance is half of maximum, and k is the slope factor RT/zF , where z is the valence of the gating charge. Holding potential for all cells was +40 mV, unless indicated otherwise.

ERGs were recorded to flash stimuli presented to the dark-adapted eye from *TRPV1*^{-/-} mice using a previously described procedure (Gregg et al., 2007) and from *TRPM1*^{-/-} mice using a procedure that was generally similar but used a different anesthetic (urethane, 2 g/kg), maximum stimulus duration (5 ms), and sampling rate (10 kHz). The a-wave was measured at 8 ms from the prestimulus baseline, whereas the b-wave was measured from the a-wave trough to the positive peak.

TRPM1^{-/-} mice were generated by Lexicon Genetics (*Trpm1*^{tm1Lex}) and obtained from the European Mouse Mutant Archive. Molecular details of the targeted allele are available at <http://www.emmanet.org/>. The targeted allele deletes 212 bp from exon 3 and all of exons 4 and 5 (accession #AY180104). This will produce a frameshift mutation in the transcript terminating translation at amino acid 79. Although there are several splice variants listed on Ensemble (www.ensembl.org), this deletion would truncate all the splice variants. The genotype of the mice was confirmed by PCR using 1 μM of each primer (LexKo-428-31, GCAT-AGTCCATGGACCTAGC; Neo3a, GCAGCGCATCGCCTTCTATC; trp-82, TGCAGCTTTGATTACATCAT) and Accuprime *Taq* polymerase in Buffer II as described by the manufacturer (Invitrogen), yielding fragments of 319 bp for the wild-type (WT) and 280 bp for the mutant allele.

Results

The rod bipolar cell transduction current is inhibited by TRP antagonists

To test the hypothesis that the transduction channel is a member of the TRP family, we first examined the effects of compounds known to antagonize TRP channels on the rod bipolar cell transduction current. To evoke a transduction current, rod bipolar cells were bathed in either 1 mM glutamate or 4 μM L-AP4 and then exposed to brief applications of the mGluR antagonist LY341495 (100 μM). Blockade of mGluR6 resulted in the opening of the transduction channel which, at positive voltages, generated an outward current (Fig. 1A) (mean amplitude, 30.4 ± 3.0 pA;

$n = 58$). Using this approach, we were able to measure the transduction current for extended periods of time without any significant rundown, as reported previously (Snellman and Nawy, 2004). Application of $10 \mu\text{M}$ ruthenium red, a noncompetitive antagonist of most TRPV and TRPC channels (Clapham, 2007) using either a fast-flow apparatus (see Materials and Methods) (Fig. 1A, left, center panels) or a puffer pipette (Fig. 1A, right panel), reduced the transduction current to an average of $15.3 \pm 2.5\%$ of control (Fig. 1E). When applied via puffer pipette, the effects of ruthenium red were readily reversible. Conversely, application of 2-aminoethoxydiphenyl borate (2-APB), which is an antagonist at many TRPC channels, but an agonist at TRPV channels (Clapham, 2007), potentiated the transduction current (Fig. 1B) to $141 \pm 14.1\%$ of control (Fig. 1E).

This pharmacological profile is consistent with a TRPV-like channel. In an attempt to further narrow this profile, we examined the effects of capsazepine and SB366791, which have been reported to be specific antagonists for TRPV1 (Caterina et al., 1997). Both compounds dramatically reduced the size of the transduction current (Fig. 1C,D), SB366791, to $28.7 \pm 14.1\%$ of control and capsazepine to $20.4 \pm 5.6\%$ of control (Fig. 1E).

TRPV1 agonists evoked a current with properties that are similar to the transduction current

Our results suggest that TRPV1 antagonists are capable of blocking the gating of the transduction channel by the endogenous activator of the channel. We, therefore, tested the possibility that TRPV1 agonists can activate the rod bipolar cell transduction current. Application of $10 \mu\text{M}$ capsaicin, the prototypical TRPV1 agonist (Caterina et al., 1997), elicited a response in every rod bipolar cell that we examined (Fig. 2A) (mean amplitude, $14.8 \pm 1.4 \text{ pA}$; $n = 41$). To examine the specificity of capsaicin, we applied it to Off bipolar cells, which were identified morphologically by dye filling and physiologically by their lack of response to LY341495. Application of capsaicin to Off bipolar cells produced no detectable response ($n = 4$; data not shown). We also recorded from rod bipolar cells in mice that were 8–9 d old. At this age, there was no detectable response to LY341495 or capsaicin ($n = 4$; data not shown), suggesting that the transduction cascade was not yet functionally developed. Finally, the response to capsaicin was completely blocked by capsazepine ($n = 2$) (Fig. 2A).

The endocannabinoid anandamide, another agonist of TRPV1 receptors (Caterina et al., 1997; Jordt and Julius, 2002; van der Stelt et al., 2005), also elicited a response in rod bipolar cells (Fig. 2B) (mean amplitude, $9.6 \pm 1.7 \text{ pA}$; $n = 8$). The response to anandamide was inhibited by capsazepine (34.8% of control; $n = 2$) but was unaffected by the cannabinoid-1 receptor

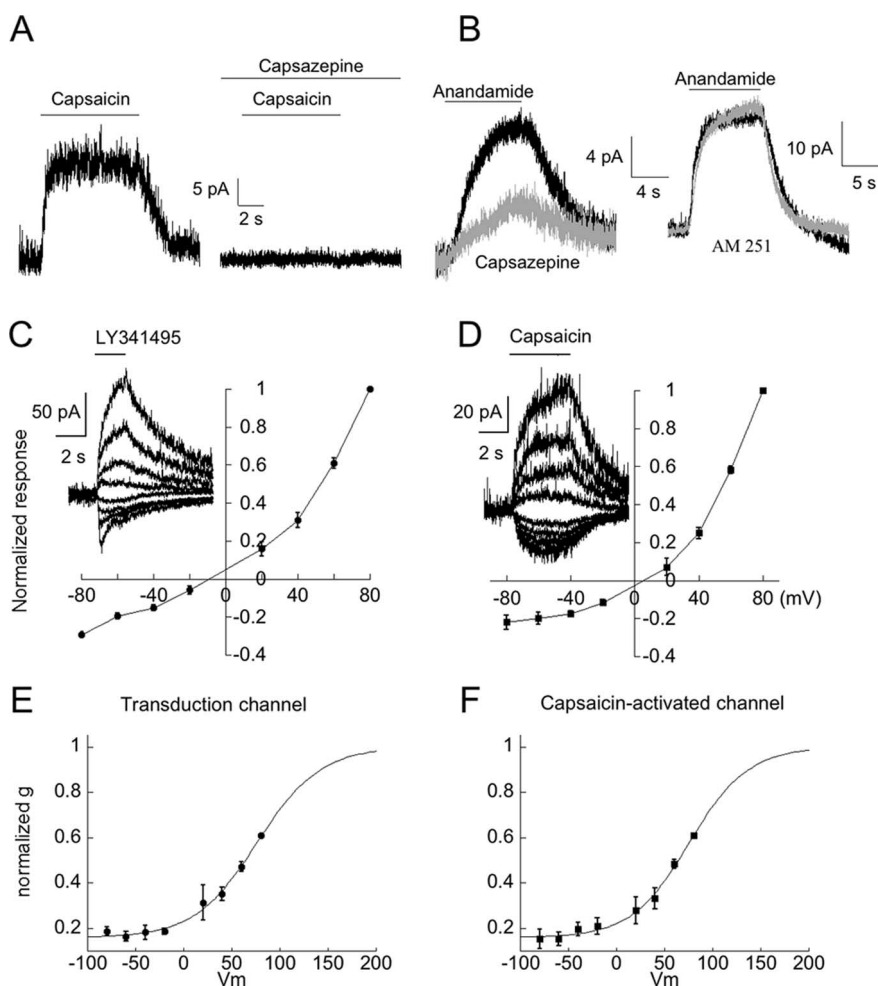


Figure 2. Rod bipolar cells respond to agonists of TRPV1. **A**, Response to a puff of $10 \mu\text{M}$ capsaicin in normal solution (left) and in solution containing $20 \mu\text{M}$ capsazepine (right). **B**, Response to a puff of $50 \mu\text{M}$ anandamide in normal bath solution (black traces, left and right) and solution containing $20 \mu\text{M}$ capsazepine (gray trace, left) or $5 \mu\text{M}$ AM251, a CB1 receptor antagonist (gray trace, right). Left and right panels are from different cells. **C, D**, Summary I - V relations for LY341495 ($n = 7$ cells) and capsazicin ($n = 5$ cells). Peak currents were normalized to the response at $+80 \text{ mV}$ for each cell, and the results were pooled. Inset, Responses to LY341495 or capsazicin obtained from representative cells. Voltage steps were from -80 to $+80 \text{ mV}$ in 20 mV increments. **E, F**, Plots of normalized conductance for the cells of the transduction channel and the capsazicin-gated channel. Lines are the fits to a Boltzmann function (see Materials and Methods). Plots were obtained from the same sets of cells whose I - V relations are summarized in **C** and **D**, using the equation $g = I/(V_m - V_{rev})$, where $V_{rev} = 0 \text{ mV}$, to obtain the conductance for each cell.

antagonist AM251 (105.4% of control; $n = 3$), indicating that it is not attributable to activation of cannabinoid receptors.

To more closely compare the transduction current and the current elicited by capsaicin, we measured the relationship between current and voltage by varying the holding potential from -80 to $+80 \text{ mV}$ in 20 mV increments while applying either capsaicin or LY341495. An example of each is shown in the insets of Fig. 2, C and D. At negative, but not positive voltages, the transduction current often displayed a prominent peak followed by a decay to a plateau, which has been previously shown to be Ca^{2+} dependent (Berntson et al., 2004; Nawy, 2004). To minimize the influence of Ca^{2+} on the I - V relation, we measured the peak current, rather than the steady-state. For each cell, currents were normalized to the amplitude of the current at $+80 \text{ mV}$, and the results were pooled (Fig. 2C,D). The I - V relations for both the native transduction current and the current evoked by capsaicin exhibited strong outward rectification. Furthermore, the mean reversal potential (E_{rev}) for each group were not significantly dif-

ferent (LY341495: $E_{rev} = -6.4 \pm 3.7$ mV, $n = 7$; capsaicin: $E_{rev} = -0.6 \pm 1.0$ mV, $n = 5$; $p > 0.15$, unpaired Student's t test).

By fitting the conductance of the transduction channel (Fig. 2E) with a Boltzmann function, we obtained a charge valence, z , of 0.80 and a $V_{1/2}$ of +76.4 mV. The charge valence is much less than for voltage-gated channels such as the Shaker K^+ channel (Zagotta et al., 1994; Islas and Sigworth, 1999) but very similar to values obtained in TRP channels (Nilius et al., 2005). Fitting the capsaicin-activated conductance yielded similar values, with a z of 0.87 and a $V_{1/2} = +75.9$ mV (Fig. 2F). Thus, both the voltage dependence and the pharmacology of the transduction channel in mouse rod bipolar cells are consistent with the properties of a TRP channel.

Mutual occlusion of capsaicin and mGluR6-generated currents

If the same population of channels is targeted by application of LY341495 and capsaicin, then one compound might be expected to occlude the actions of the other. To test this possibility, we applied LY341495 and capsaicin separately and measured the amplitude of the response to each. Next, we applied the two compounds simultaneously and again measured the response. When the duration of positive pressure application was sufficient to produce a maximal response to each drug, the response to the combination of both drugs was significantly less than predicted, based on linear summation of the responses obtained separately (Fig. 3A,C). The same result was obtained regardless of whether we measured the peak response ($p < 0.01$; $n = 7$) or total charge transfer ($p < 0.01$; $n = 7$) during drug application. This finding is consistent with the idea that both drugs compete for a limited number of channels. If this is the case, then lowering the concentration of each compound should reduce competition for the channel. To test this possibility, we reduced the amount of drug delivered to the rod bipolar cell by shortening the duration of the application (Fig. 3B). Under these conditions, the response to simultaneous delivery of both LY341495 and capsaicin coincided closely with a simple summation model (Fig. 3C). These findings support the idea that mGluR6 and capsaicin operate on the same population of channels.

TRPM1, but not TRPV1, may play a role in mGluR6 transduction

Two candidate channels for playing a role in the mGluR6 transduction pathway are TRPV1 and TRPM1. TRPV1 displays a similar pharmacology to the transduction channel as described above. However, TRPM1 has recently been implicated in transduction in the Appaloosa horse (Sandmeyer et al., 2007; Bellone et al., 2008). To address the possibility that one or both channels are a component of the transduction cascade, we recorded from two types of transgenic mice, one with a targeted deletion of *TRPV1* (Caterina et al., 2000) and the other with a deletion of *TRPM1* (*Trpm1*^{tm1Lex}). The mGluR6 pathway appeared to be unperturbed in the *TRPV1*^{-/-} mouse, as responses to LY341495, capsaicin, and anandamide were all present (Fig. 4A). Further analysis of both the I - V relation and average amplitudes of the TRPV1 agonist responses failed to reveal any differences compared with wild-type animals (Fig. 4B,C). Similarly, the amplitude and kinetics of the b-wave of the ERG were virtually identical in wild-type and *Trpm1*^{-/-} mice (Fig. 4D). Summary amplitudes of the b-wave, which is generated by On bipolar cell activity, and the a-wave which is generated by the activity of photoreceptors, are plotted as a function of light intensity in the right-hand panel of Figure 4D. In contrast, measurements of

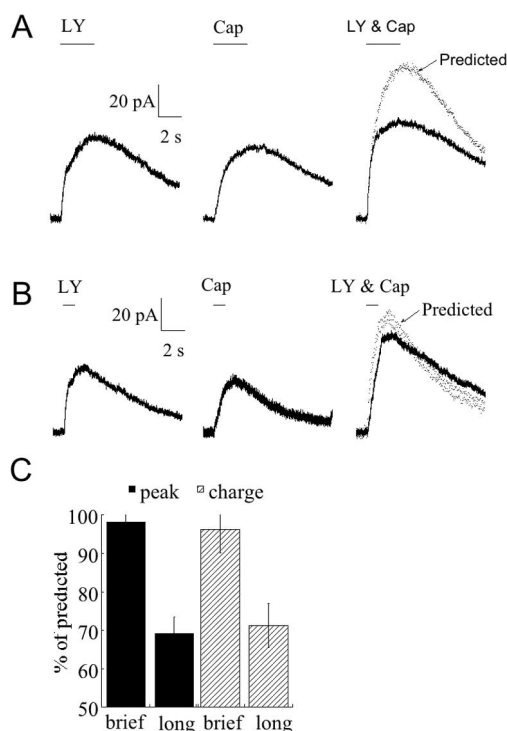


Figure 3. Responses to LY341495 and capsaicin occlude each other. **A**, Responses to 3 s applications of LY341495 and capsaicin, first separately and then together. Also shown in the right panel is the predicted response to simultaneous application based on linear summation. **B**, Similar to **A**, except that the duration of the application was 1 s. Note that for subsaturating application of drugs, the predicted response more closely approximates the response to simultaneous application of LY341495 and capsaicin. Same cell as in **A**. LY, LY341495; Cap, capsaicin. **C**, Comparison of evoked and predicted responses to simultaneous application of LY341495 and capsaicin as a function of puff duration. Long applications were for a duration of 3 s, whereas brief applications were either 1 s or 0.5 s. Charge was obtained by integration of current during the period of drug application. For both the peak response and the total charge transfer, there was no significant difference between the sizes of the predicted and actual responses to short puffs ($p > 0.5$; $n = 7$), but the response to long puffs was significantly smaller than predicted based on summation ($p < 0.01$; $n = 7$).

ERG in the *TRPM1*^{-/-} mouse revealed a complete lack of a b-wave but a normal a-wave, an indication that On bipolar cell function was completely disrupted (Fig. 4E).

Discussion

The identity of the postsynaptic channel that mediates synaptic transmission from photoreceptor to On bipolar cells is currently unknown. Here, we present evidence that the channel is likely to be a member of the family of TRP channels, perhaps TRPM1. The synaptic current is reduced by antagonists of TRP channels and mimicked by TRP channel agonists. Furthermore, the b-wave, which is thought to be generated by the opening of On bipolar cell synaptic channels, is normal in a TRPV1 knock-out mouse but completely eliminated in a mouse lacking functional TRPM1 channels. Our results are consistent with a recent study showing expression of TRPM1 RNA in mouse On bipolar cells (Kim et al., 2008). To date, a physiological characterization of TRPM1 has yet to be reported, and so it is unclear if this TRP channel can be gated by endovanilloids or whether TRPM1 currents rectify as do those of many other TRP channels. Although it is tempting to speculate that the current evoked by endovanilloids in rod bipolar cells is attributable to the opening of TRPM1 channels, confirmation of this hypothesis will require further investigation of the functional properties of TRPM1.

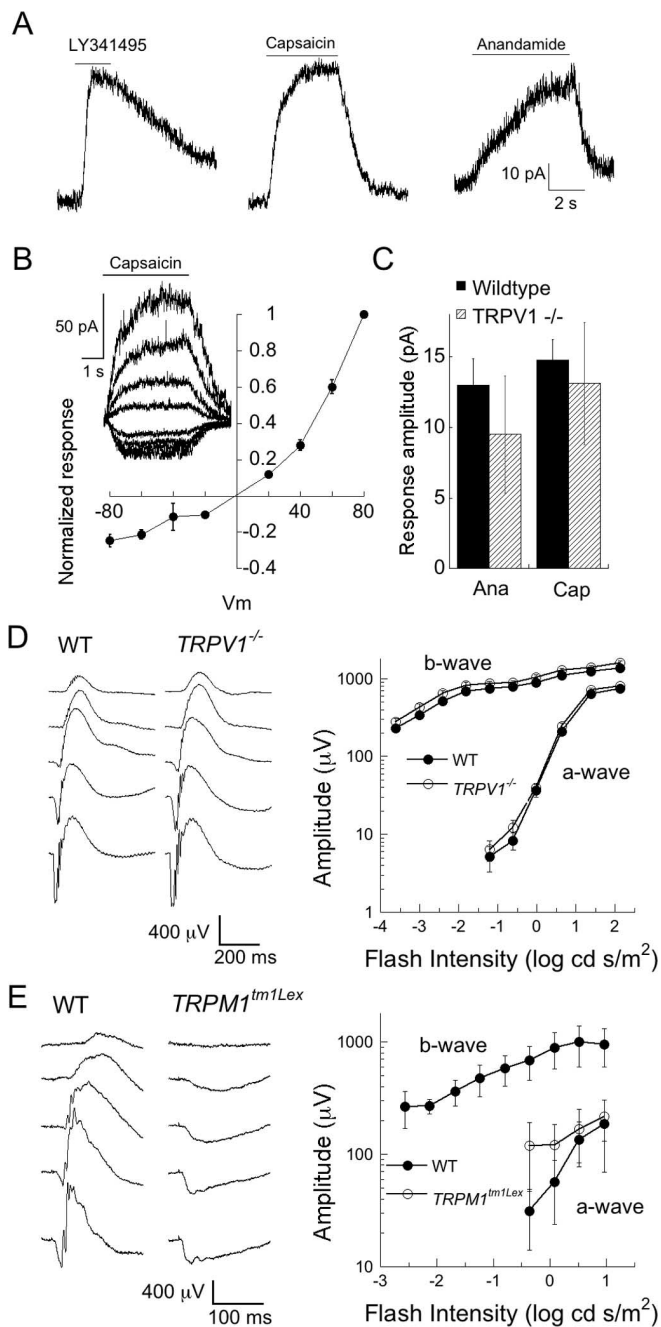


Figure 4. *TRPV1*^{-/-} mice retain the transduction current, TRPV agonist activated currents and ERG b-waves; *TRPM1*^{-/-} mice lack the ERG b-wave. **A**, Examples of the responses to LY341495, capsaicin, and anandamide, indicating that both endogenous and exogenous gating of the transduction channel in the *TRPV1*^{-/-} is preserved. **B**, Summary *I*-*V* relation for capsaicin in *TRPV1*^{-/-} mice (*n* = 6). Inset, Responses to voltage steps from -80 mV to +80 mV in 20 mV increments. **C**, Responses to both anandamide and capsaicin were not significantly different in amplitude between wild-type and *TRPV1*^{-/-} mice. Ana, Anandamide (WT; *n* = 13, *TRPV1*^{-/-}; *n* = 4, *p* > 0.25); Cap, capsaicin (WT; *n* = 41, *TRPV1*^{-/-}; *n* = 15, *p* > 0.50). **D**, Left, Dark-adapted ERGs from a wild-type and a *TRPV1*^{-/-} mouse. Flash intensities are (in log cd s/m²) -3.6, -2.4, -1.2, -0.0, and 1.4. Right, Summary of the b-wave and a-wave amplitudes as a function of light intensity in wild-type (*n* = 8) and *TRPV1*^{-/-} (*n* = 6) mice. **E**, Left, Dark-adapted ERGs from a wild-type and a *TRPM1*^{tm1Lex} mouse. Flash intensities are (in log cd s/m²) -2.6, -1.7, -0.8, 0.1, and 1.0. Right, Summary of the b-wave and a-wave amplitudes as a function of light intensity in wild-type (*n* = 3) and *TRPM1*^{-/-} (*n* = 4) mice. For *TRPM1*^{-/-}, only the a-wave data are plotted, as there was no measurable positive-going response.

Our findings are consistent with the findings of several recent studies of bipolar cell function in Appaloosa horses. In these horses, there is a link between a specific pattern of coat coloration and congenital stationary night blindness (Sandmeyer et al., 2007). Animals with this coloration lack the ERG b-wave, indicating a loss of function of On bipolar cells, although the structure of the retina appears normal (Witzel et al., 1978). Genetic analysis of this phenotype revealed decreased expression of mRNA encoding TRP channel TRPM1 (Bellone et al., 2008). Of course, the loss of On bipolar cell function could potentially result from a number of underlying etiologies other than a mutation in the transduction channel (McCall and Gregg, 2008). Nevertheless, an intriguing possibility, based on the results presented here and previous work on the Appaloosa horse, is that the transduction channel in the dendrites of rod bipolar cells is composed of TRPM1, either as a homomer or in association with other TRP channels.

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