UV light activates a G\(\alpha_{q/11}\)-coupled phototransduction pathway in human melanocytes

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While short exposure to solar ultraviolet radiation (UVR) can elicit increased skin pigmentation, a protective response mediated by epidermal melanocytes, chronic exposure can lead to skin cancer and photoaging. However, the molecular mechanisms that allow human skin to detect and respond to UVR remain incompletely understood. UVR stimulates a retinal-dependent signaling cascade in human melanocytes that requires GTP hydrolysis and phospholipase C\(\beta\) (PLC\(\beta\)) activity. This pathway involves the activation of transient receptor potential A1 (TRPA1) ion channels, an increase in intracellular Ca\(^{2+}\), and an increase in cellular melanin content. Here, we investigated the identity of the G protein and downstream elements of the signaling cascade and found that UVR phototransduction is G\(\alpha_{q/11}\)-dependent. Activation of G\(\alpha_{q/11}\)/PLC\(\beta\) signaling leads to hydrolysis of phosphatidylinositol (4,5)-bisphosphate (PIP\(_2\)) to generate diacylglycerol (DAG) and inositol 1, 4, 5-trisphosphate (IP\(_3\)). We found that PIP\(_2\) regulated TRPA1-mediated photocurrents, and IP\(_3\) stimulated intracellular Ca\(^{2+}\) release. The UVR-elicited Ca\(^{2+}\) response appears to involve both IP\(_3\)-mediated release from intracellular stores and Ca\(^{2+}\) influx through TRPA1 channels, showing the fast rising phase of the former and the slow decay of the latter. We propose that melanocytes use a UVR phototransduction mechanism that involves the activation of a G\(\alpha_{q/11}\)-dependent phosphoinositide cascade, and resembles light phototransduction cascades of the eye.

INTRODUCTION

Sunlight is crucial for life and has many beneficial effects, but, at the same time, the UV radiation (UVR) contained by sunlight is the most common environmental carcinogen (Routaboul et al., 1999; Bennett, 2008). Unlike other mammals that have fur to protect their skin, human skin is constantly exposed to solar UVR (280–400 nm) and is susceptible to its damaging effects, primarily skin cancers and photoaging. Human skin also has a unique protection mechanism against UVR: the presence of melanocytes in the epidermis allows skin to respond to UVR by increasing its pigmentation. Because UVR is omnipresent and is able to interact with human skin, identifying the molecular pathways that allow human skin to detect and elicit an immediate response to UVR is critical for developing new photoprotective methods.

How does human skin detect UVR? UVR consists of photons; photons can activate G protein–coupled opsin receptors (GPCRs) in the eye that elicit cellular responses through the activation of different G proteins and downstream effectors. G\(\alpha_{q/11}\) is used by vertebrate photoreceptors (Fung et al., 1981), whereas G\(\alpha_{q/11}\) mediates Drosophila melanogaster phototransduction (Hardie, 2001) and non–image forming vision in the mammalian retina (Berson et al., 2002; Panda et al., 2005; Yau and Hardie, 2009). Activation of G\(\alpha_{q/11}\) pathways leads to stimulation of phospholipase C\(\beta\) (PLC\(\beta\)), which induces hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP\(_3\)). Changes in the levels of PIP\(_2\), DAG, and IP\(_3\) modulate the activity of many proteins, including transient receptor potential (TRP) ion channels.

We recently characterized a retinal-dependent UVR-sensitive phototransduction pathway in human epidermal melanocytes (HEMs) that is G protein and PLC\(\beta\) dependent and results in the activation of TRP subfamily A1 (TRPA1) channels; activation of this pathway results in a rapid increase in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{ic}\)) and increased cellular melanin content (Wicks et al., 2011; Bellono et al., 2013). In this study we investigated the G protein that mediates this pathway and the downstream molecular events. We found that UVR phototransduction in HEMs is mediated by G\(\alpha_{q/11}\) signaling, and provide evidence for a phosphoinositide cascade.
involving IP₃-mediated intracellular Ca²⁺ release via IP₃ receptors (IP₃R) and PIP₂ regulation of Ca²⁺-permeable TRPA1 ion channels. The two sources of Ca²⁺ have different dynamics and, combined, result in a Ca²⁺ response with a fast rising phase and a slow decay. Our results demonstrate that UVR phototransduction in HEMs activates a Gαₛ/₁₁-dependent signaling pathway similar to well-characterized phototransduction pathways in the eye.

**MATERIALS AND METHODS**

**Reagents**

Cholera toxin (CTX), pertussis toxin (PTX), HG030031, 1-oleoyl-2-acetyl-sn-glycerol (OAG), phosphatidylcholine phospholipase C (PC-PLC; from Clostridium perfringens), polylysine (PolyK, 70–150 kD), heparin, and ionomycin were purchased from Sigma-Aldrich. Endothelin, GPAnt-2, GPAnt-2a, and Xestospongin C (XeC) were from Tocris Bioscience. mSRIR and L9A were from EMD Millipore. DiC8-PIP2 was from Echelon Biosciences. Stocks of all reagents in water, DMSO, or ethanol were stored at −4°C or −20°C until use and diluted to the final concentration to contain ≤15% solvent. For Ca²⁺ imaging experiments, HEMs were preincubated with pharmacological reagents for 3–15 min, with the exception of PTX and CTX, which used 24 h incubations.

**Cell culture**

Primary HEMs isolated from neonatal foreskin were cultured in Medium 254 containing Human Melanocyte Growth Supplement (HMG2; Cascade Biologics/Invitrogen) and 1% penicillin-streptomycin (Invitrogen), and propagated for a limited number of cell divisions (≤15). Vitamin A or retinoid derivatives are not components of either Medium 254 or HMG2. Human embryonic kidney (HEK293) cells were cultured in Dulbecco’s Modified Eagle Medium and F12 nutrient mixture (DMEM-F12; Gibco/Invitrogen) containing 10% fetal bovine serum (Atlanta Biologicals) and 1% penicillin-streptomycin (Invitrogen).

**Molecular biology**

MicroRNAs (miRNAs) were designed and expressed in HEMs using a lentiviral system, as described previously (Wicks et al., 2011). BLOCK-iT miRNA oligos (Invitrogen) were cloned into the pcDNA6.2-GW/EmGF-miR expression vector modified to contain mCherry instead of EmGF to allow for simultaneous fluorescence detection and Fluo-4–based Ca²⁺ imaging. miRNAs were recombined from pcDNA6.2-GW into pDONR221 and pLENT6/V5-DEST vectors (Invitrogen) for lentiviral production. Lentiviral particles containing miRNA were obtained as described previously (Wicks et al., 2011). HA-tagged RGS2 (Missouri S&T cDNA Resource Center) was recombined into pDONR221 and pLENT6/V5-DEST vectors (Invitrogen) for lentiviral production as described previously (Wicks et al., 2011). The mRNA expression level of Gαₛ, Go₁₁, or Go₁₁ in control or targeted miRNA-treated cells was determined ≥7 d after infection using comparative Ct quantitive PCR (qPCR). Total RNA was extracted from infected HEMs using the RNeasy Plus kit (Qiagen) and converted to cDNA using RT-PCR (SuperScript III; Invitrogen). qPCR reactions were prepared according to manufacturer instructions using Power SYBR green. All reactions were done in triplicate and actin was used for normalization.

**Western blots**

Expression of HA-tagged RGS2 in HEMs was confirmed via Western blotting. Cells were homogenized 24 h after infection in ice-cold RIPA buffer (Thermo Fisher Scientific) containing protease inhibitor cocktail (Roche). Samples were agitated at 4°C for 30 min and then centrifuged at 16,000 g for 30 min at 4°C. Protein content was determined using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific). Equal amounts of protein were loaded onto each lane, separated by electrophoresis on NuPAGE Bis-Tris gels (Invitrogen), and transferred to PVDF membranes (Roche). Membranes were blocked at room temperature for 1 h and incubated overnight at 4°C with rat monoclonal anti-HA antibody clone 3F10 (1:500; Roche), followed by 1 h at room temperature with HRP-conjugated goat anti-rat IgG affinity-purified antibody (1:5,000; EMD Millipore). Antibodies were detected using the SuperSignal West Fermo enhanced chemiluminescence system (Thermo Fisher Scientific) and imaged using autoradiography film (Thermo Fisher Scientific).

**Light stimulation**

Ultraviolet light stimulation of cultured HEMs was conducted using a 200 W Hg-Xe arc lamp with converging optics and appropriate filters (Wicks et al., 2011). A dichroic mirror (200–400 nm) was used in combination with 280-nm long pass and a 400-nm short pass filters (Newport). The levels of light lost due to scattering by imaging buffer were negligible. Physiological doses of UVR were applied by varying the duration and/or power of the pulse. A handheld silicon detector was used to measure power (Newport).

**Calcium imaging**

Ca²⁺ imaging was performed as described previously (Wicks et al., 2011; Bellono et al., 2013). Cultured HEMs plated on glass coverslips were incubated for 15 min at room temperature in Ringer’s solution with 2 µM Fluo-4 (Molecular Probes/Invitrogen) and 250 µM sulpnpyrazone (Sigma-Aldrich), followed by dark incubation for 15 min with 12 µM of 9-cis or all-trans retinal (Sigma-Aldrich). Imaging was performed in modified Ringer’s extracellular solution containing (in mM): 150 NaCl, 1.8 CaCl₂, 1.2 MgCl₂, 10 D glucose, 25 HEPES, pH 7.4, and 310 mOsm/liter. Fluorescence images were acquired every 2 s, and 2 µM ionomycin (Sigma-Aldrich) was added at the end of some experiments to elicit a maximal Ca²⁺ response used for normalization.

The fluorescence intensity of individual cells (measured using ±25% of the cell area, Fcell) was quantified using MetaMorph (Molecular Devices) and MATLAB (MathWorks) and plotted in Prism 6 (GraphPad Software). Fcell values were normalized as Fnorm = (Fcell − Fmin)/(Fmax − Fmin), where Fmax is maximal fluorescence with ionomycin, and Fmin is baseline fluorescence averaged from ≥15 data points acquired before light stimulation. Final data values for each dish were obtained by averaging Fnorm values from individual cells. In experiments where ionomycin was not used for normalization, Fluo-4 fluorescence intensities were quantified as ∆F/F₀(t) = (Fcell(t) − Fbaseline)/Fbaseline and averaged as described previously. ∆F/F₀ values were plotted as a function of time and fitted with a single-exponential function in Prism 6 (GraphPad Software) to calculate decay time constants. Ca²⁺ response initial slopes were calculated over the first 30 s after UVR stimulation. Paired Ca²⁺ imaging experiments were used when the dish-to-dish variability was significant. Cells for each of the paired experiments were plated on glass coverslips and treated identically by incubation in the same Fluo-4 and retinal solution, then imaged sequentially in alternating order: control followed by experimental condition or experimental condition followed by control. The averaged fluorescence intensities of cells from one coverslip measured in each condition were plotted as the two experimental values connected by the dotted line. Statistical significance of paired experiments was evaluated using a paired Student’s t test. When pairing is not mentioned, the cells were cultured and treated identically, but we did not strictly alternate control and experimental condition measurements. In such cases, we averaged the
values from all the control and all the experimental conditions and represented them as bar graphs.

**Electrophysiology**

Electrophysiology experiments were performed as described previously (Bellono et al., 2013). All-trans retinal was stored, solubilized, and applied as described previously (Wicks et al., 2011). Experiments were performed under dim-red or infrared illumination. Whole-cell patch clamp recordings were carried out using micropipettes with 3–6 MΩ resistance at room temperature using an EPC 10 amplifier (HEKA) with PatchMaster software (HEKA), filtered at 2.9 kHz and digitized at 20 kHz. Experiments were performed using modified Ringer’s solution (see “Calcium imaging”). Unless stated otherwise, internal pipette solution contained (in mM): 140 CsCl, 1 MgCl₂, 4 MgATP, 10 EGTA, 10 HEPES, pH 7.2, and 290 mOsm/liter. The low EGTA solution used in Fig. S3 contained 20 µM EGTA. UVR-10 EGTA, 10 HEPES, pH 7.2, and 290 mOsm/liter. The low EGTA solution used in Fig. S3 contained 20 µM EGTA. UVR-induced currents were measured using a step protocol consisting of a step from a holding potential of −60 mV to +80 mV immediately before UVR exposure. Current values were calculated by subtracting initial current at +80 mV (Io) from maximal current (Imax) after UVR exposure: IUV = Imax − Io. All recordings were inspected for baseline drift before analysis. In most recordings, the baseline did not drift significantly. If the baseline drift was >20% of the UVR response, cells were excluded. Cell membrane capacitance values were used to calculate current densities. Current–voltage (I–V) relations were established using voltage step protocol from the −60-mV holding potential to voltages between −80 mV and +80 mV in 20-mV increments.

HEMs are very difficult to patch clamp. Our experiments are further complicated by the fact that UV exposure often causes us to lose patches. Because UV photocurrents are small and exhibit strong outward rectification (Bellono et al., 2013), most of our voltage-clamp experiments use positive membrane voltages that are not physiological (+80 mV) in order to increase current amplitude. Although current amplitude is higher at positive membrane voltages, current kinetics are significantly altered compared with recordings carried out at more physiological membrane voltages (Bellono and Oancea, 2013). In addition, positive voltages are not well tolerated by these cells, preventing us from recording and plotting the current over a long period of time for every single trace. We have previously recorded currents over longer periods using voltage pulses (Bellono et al., 2013) and at more negative voltages (Bellono and Oancea, 2013), and showed that the current returns to baseline after the UV pulse. Our success rate, even for the short periods of UV irradiation, is well below 10%. Consistent with the difficulty of these experiments, the large noise is likely caused by the relatively low seal resistance (~1 GΩ) and patch stability.

**Statistical analyses**

Experimental data are presented as mean ± SEM, where n refers to the number of dishes for imaging data or the number of cells for electrophysiology. We calculated p-values by unpaired or paired Student’s t test and considered results significant when P ≤ 0.05.

**Online supplemental material**

Fig. S1 shows that RGS2 expression in HEMs reduces Ca²⁺ responses to endothelin. Fig. S2 shows that HEMs expressing Goα7, Goα11, or Goαq11-targeted miRNA have reduced Ca²⁺ responses to endothelin. Fig. S3 shows that neither IP3-mediated Ca²⁺ release nor DAG activate the UVR photocurrent. Fig. S4 shows selective inhibition of UVR phototransduction signaling components versus ion channels by cellular dialysis. Online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.201311094/DC1.

**RESULTS**

Identification of the Goα subunit that mediates UVR-induced Ca²⁺ responses in HEMs

We have recently shown that exposure to physiological doses of UVR activates a retinal-dependent phototransduction pathway that is mediated by G protein activation (Wicks et al., 2011; Bellono et al., 2013). However, we have yet to identify the type of G protein responsible for initiating the retinal-dependent signaling pathway activated by UVR. HEMs express a variety of GPCRs and their related G proteins, including the melanocortin-1 receptor coupled to Goα (Park et al., 2009) and the Goαq/11-coupled endothelin-1B (ET-1) receptor (Yada et al., 1991; Imokawa et al., 1992).

Because we previously showed that UVR exposure leads to retinal-dependent Ca²⁺ responses (Wicks et al., 2011), we monitored intracellular Ca²⁺ levels using the fluorometric Ca²⁺ indicator Fluo-4 in response to treatments that modulate the function of different G protein subunits. We first tested if UVR-induced Ca²⁺ responses in HEMs were mediated by members of the Goα or Goα,Goq family. CTX treatment inhibits Goα-mediated signaling and PTX inhibits Goα,Goq signaling (Beckman et al., 1974; Malbon et al., 1984). HEMs incubated for 24 h with 500 ng/ml CTX, 500 ng/ml PTX, or vehicle, and stimulated with 150 mJ/cm² UVR showed no difference in the amplitude of retinal-dependent Ca²⁺ responses (Fig. 1, A and B; amplitude of the response: F norm, max = 0.55 ± 0.04 for vehicle, 0.56 ± 0.05 for CTX, 0.52 ± 0.04 for PTX). This result suggests that Ca²⁺ responses elicited by physiological doses of UVR (150 mJ/cm²; Wicks et al., 2011) are not mediated by Goα or Goα,Goq signaling.

The Gβγ subunit of heterotrimeric G proteins can lead to increase in intracellular Ca²⁺ levels ([Ca²⁺]i) by directly activating PLCβ (Park et al., 1993; Wu et al., 1993; Barr et al., 2000). To test if UVR-mediated elevation in [Ca²⁺]i requires activation of Gβγ, we incubated HEMs with the Gβγ-inhibiting peptide mSIRK (10 µM) or its inactive analogue L9A (10 µM; Goubaeva et al., 2003; Malik et al., 2005; Wang and Hatton, 2007) before stimulation with 150 mJ/cm² UVR. No significant difference was measured between UVR-induced Ca²⁺ responses in HEMs treated with mSIRK compared with the inactive analogue L9A (Fig. 1, C and D; F norm,max = 0.45 ± 0.05 for mSIRK vs. 0.43 ± 0.04 for L9A), which suggests Gβγ does not mediate UVR-induced Ca²⁺ responses in HEMs.

We next investigated whether members of the Goαq/11 family, known to promote Ca²⁺ mobilization through PLCβ activation (Smrcka et al., 1991; Taylor et al., 1991), mediate the UVR-induced Ca²⁺ response. To alter Goαq/11 signaling, we expressed RGS2 in HEMs (Heximer et al., 1997; Heximer, 2004; Roy et al., 2006), a member of the regulators of G protein signaling (RGS) family, which inactivates Goαq/11 by promoting the hydrolysis of GTP to...
We found that in HEMs $G_{\alpha_q}$ and $G_{\alpha_{11}}$ are expressed at similarly high levels, whereas $G_{\alpha_{14}}$ and $G_{\alpha_{15}}$ are expressed at lower levels (Fig. 1 G).

**Both $G_{\alpha_q}$ and $G_{\alpha_{11}}$ contribute to the UVR-induced $Ca^{2+}$ response in HEMs**

We investigated the contribution of $G_{\alpha_q}$ versus $G_{\alpha_{11}}$ to UVR signaling using RNA interference. Because $G_{\alpha_q}$ and $G_{\alpha_{11}}$ share a high degree of homology, we designed miRNA targeting either $G_{\alpha_q}$ or $G_{\alpha_{11}}$ individually, or both $G_{\alpha_q}$ and $G_{\alpha_{11}}$ ($G_{\alpha_q/11}$). Expression of $G_{\alpha_q}$-targeted miRNA in HEMs resulted in a significant reduction in the mRNA transcript levels of $G_{\alpha_q}$, but not of $G_{\alpha_{11}}$, relative to control (scrambled) miRNA-expressing cells (Fig. 2 A; $G_{\alpha_q}$ mRNA relative to control = 0.14 ± 0.01, $G_{\alpha_{11}}$ mRNA relative to control = 0.83 ± 0.16). Cells expressing $G_{\alpha_q}$-targeted miRNA had a reduced $Ca^{2+}$ response to 6 nM endothelin when compared with control miRNA-expressing cells, which suggests that
Goα-q-targeted miRNA significantly reduced Goα-q signaling (Fig. S2, A and B). We then measured retinal-dependent Ca2^+ responses elicited by 150 mJ/cm^2 UVR and found that HEMs expressing Goα-q-targeted miRNA had significantly reduced Ca2^+ responses compared with HEMs expressing control miRNA (Fig. 2, B and C; F_{norm,max} = 0.57 ± 0.04 for control miRNA, 0.35 ± 0.03 for Goα-q miRNA).

Expression of Goα-q-targeted miRNA resulted in a significant reduction in the mRNA levels of Goα-11 (Goα-11 mRNA relative to control = 0.12 ± 0.03) and a smaller decrease in the mRNA transcript levels of Goα-q (Fig. 2 D; Goα-q mRNA relative to control = 0.75 ± 0.07). HEMs expressing Goα-11-targeted miRNA had reduced Ca2^+ responses to 6 nM endothelin (Fig. S2, C and D), which suggests that Goα-11 signaling is also reduced. In response to stimulation with 150 mJ/cm^2 UVR, HEMs expressing Goα-q-targeted miRNA had a reduced retinal-dependent Ca2^+ response compared with HEMs expressing control miRNA (F_{norm,max} = 0.61 ± 0.04 for control miRNA, 0.40 ± 0.05 for Goα-11 miRNA; Fig. 2, E and F). Collectively, our data from HEMs expressing Goα-q or Goα-11-targeted

![Figure 2](image-url)
miRNA suggest that both Goq and Gai1 contribute to the UVR-induced retinal-dependent phototransduction pathway in HEMs.

We next examined if decreasing Goq and Gai1 levels simultaneously had a larger effect on UVR-induced Ca2+ responses in HEMs. Expression of Goq11 miRNA that targets both Goq and Gai1 significantly reduced the mRNA levels of Goq and Gai1 relative to control miRNA-expressing cells (Fig. 2 G; Goq mRNA relative to control = 0.24 ± 0.08, Gai1 mRNA relative to control = 0.32 ± 0.08). Stimulation with 6 nM endothelin resulted in reduced Ca2+ responses in HEMs expressing Goq11-targeted compared with control miRNA (Fig. S2 E and Fig. 2 F; Fnorm,max = 0.72 ± 0.04 for control miRNA, 0.44 ± 0.07 for Goq11 miRNA). UVR stimulation of HEMs expressing Goq11-targeted miRNA led to smaller increases in intracellular Ca2+ levels compared with control miRNA-treated cells (Fig. 2 H).

Quantification of these responses showed that the UVR-induced Ca2+ responses were significantly reduced in the presence of Goq11-targeted miRNA compared with control miRNA (Fig. 2, I and J), which suggests that both subunits Goq and Gai1 contribute to retinal-dependent UVR-induced Ca2+ responses in HEMs.

UVR stimulation of HEMs leads to activation of a whole-cell current mediated by TRPA1 ion channels in a retinal and G protein–dependent manner (Bellono et al., 2013). We thus investigated if Goq11 is also required for UVR-induced whole-cell currents. We first tested the effect of Goq11-targeted miRNA on whole-cell currents measured at +80 mV in response to 240 mJ/cm2 UVR and found that expression of control miRNA had no effect on the photocurrents, whereas Goq11-targeted miRNA nearly abolished them (Fig. 3, A and B; IUVR/ctrl miRNA = 4.06 ± 0.29 pA/pF, IUVR/ Goq11 miRNA = 0.28 ± 0.17 pA/pF) at all voltages (Fig. 3 B, inset). The whole-cell patch clamp technique used to measure the photocurrents allowed us to use an alternative method to block Goq11 signaling by dialyzing GPAnt-2a, a Goq11 inhibitory peptide, into HEMs (Mukai et al., 1992). The time-dependent effect of peptide inhibitor dialysis allowed us to measure the UVR-induced current at 2 min after break-in, when the peptide was not effective, and thus the measurement was used as control, and at 10 min of dialysis, when the peptide became effective. This experimental protocol allowed us to compare the effects of the peptide inhibitors in the same cell. When GPAnt-2a was included in...
the pipette solution, the UVR photocurrents were similar to control cells 2 min after break-in, but decreased significantly after 10 min of dialysis (Fig. 3, E and F; I_UVR/GPAnt-2a = 3.98 ± 0.50 pA/pF at 2 min, 0.69 ± 0.08 pA/pF after 10 min of dialysis with GPAnt-2a). As a control we performed a similar experiment using the GPAnt-2 peptide, which inhibits Go_{q/11} signaling (Mukai et al., 1998; Klein et al., 2008), and detected no change in the amplitude of the UVR current after 2 or 10 min of dialysis (Fig. 3, C and D; I_UVR/GPAnt-2 = 3.68 ± 0.49 pA/pF at 2 min, 3.64 ± 0.26 pA/pF after 10 min). These results suggest that UVR-induced photocurrents are dependent on Go_{q/11} signaling.

UVR-activated TRPA1 photocurrents are regulated by PIP_{2} in HEMs

Our results so far indicate that UVR phototransduction leads to activation of Go_{q/11}, which in turn activates PLC_{q}, required both for intracellular Ca^{2+} release (Wicks et al., 2011) and TRPA1 activation (Bellono et al., 2013). Nonetheless, the mechanism by which UVR leads to TRPA1 activation downstream of PLC_{q} remains unknown. Because PLC_{q} hydrolyzes plasma membrane PIP_{2}, generating DAG and the soluble messenger IP_{3}, we reasoned that PLC_{q}-dependent signaling could modulate TRPA1 channel activity in HEMs via IP_{3}, DAG, or PIP_{2}.

We first tested whether IP_{3} or IP_{3}-mediated Ca^{2+} release was sufficient to activate TRPA1 by using a control internal solution, allowing for an increase in [Ca^{2+}]_{ic} (see Materials and methods), internal solutions containing 100 µM IP_{3} to stimulate IP_{3}R_{s}, or 1 mg/ml heparin to block IP_{3}R_{s}, with both treatments occluding subsequent UVR-induced IP_{3}R-mediated Ca^{2+} release. UVR (240 mJ/cm^{2}) exposure after dialysis for 5 min with each of the three solutions elicited retinal-dependent photocurrents with similar amplitudes (Fig. S3, A and B; I_UVR/Ctrl = 4.01 ± 0.33 pA/pF, I_UVR/IP_{3} = 4.04 ± 0.47 pA/pF, I_UVR/heparin = 5.05 ± 0.56 pA/pF), which suggests that UVR-induced activation of whole-cell currents is not mediated by IP_{3} or IP_{3}-mediated Ca^{2+} release.

We next examined the contribution of DAG to retinal-dependent UVR photocurrents. Bath application of the PC-PLC (10 U/ml), which generates DAG in the plasma membrane (Oancea et al., 1998), or the DAG analogue OAG (100 µM), did not elicit a significant current in HEMs, and 5 min of incubation with the respective treatments did not affect the retinal-dependent photocurrents elicited by UVR (Fig. S3, C and D; I_PC-PLC = 0.10 ± 0.10 pA/pF, I_OAG = 0.20 ± 0.09 pA/pF, I_UVR/PC-PLC = 4.31 ± 0.70 pA/pF, I_UVR/OAG = 4.13 ± 0.48 pA/pF). Because increasing DAG levels failed to elicit whole-cell currents and had no effect on UVR photocurrents, we concluded that DAG does not modulate TRPA1 downstream of UVR.

PI_{2} hydrolysis is a key regulator for many ion channels (Suh and Hille, 2005), including TRPA1 (Dai et al., 2007; Karashima et al., 2008; Kim et al., 2008). To test if the presumed decrease in PIP_{2} levels caused by PLC_{q}-mediated hydrolysis affects UVR photocurrents, we attempted to maintain elevated PIP_{2} levels by dialyzing HEMs with the PIP_{2} analogue diC8-PIP_{2} (20 µM; Karashima et al., 2008; Kim et al., 2008). We found that after 5 min of patch pipette dialysis to allow diC8-PIP_{2} to diffuse into cells, UVR stimulation (240 mJ/cm^{2}) elicited significantly smaller photocurrents at all voltages compared with UVR photocurrents measured immediately after break-in (Fig. 4, A, B, and D). This finding suggests that increased PIP_{2} prevents UVR-induced TRPA1 activation and raises the question of whether PIP_{2} hydrolysis is required for the UVR photocurrent.

To test if the dialysis with diC8-PIP_{2} inhibited TRPA1, or another component of the UVR phototransduction cascade, we compared the whole-cell currents elicited by UVR and the TRPA1 agonist cinnamaldehyde (CA) in HEMs dialyzed with diC8-PIP_{2} or GPAnt-2a (Fig. S4). We found that dialysis with both diC8-PIP_{2} and GPAnt-2a inhibited UVR photocurrents, whereas only diC8-PIP_{2} inhibited currents elicited by CA (Fig. S4). These data suggest that GPAnt-2a inhibits an important component of the UVR signaling cascade, Go_{q/11}, while diC8-PIP_{2} directly inhibits TRPA1 activity, which is required for both the UVR- and CA^{2+}-elicited increase in whole-cell current.

To test if a decrease in PIP_{2} levels is also sufficient to cause TRPA1 activation in HEMs, we dialyzed cells with polylysine (polyK; 50 µg/ml), which binds and sequesters PIP_{2} (Lukacs et al., 2007; Klein et al., 2008; Ufret-Vincenty et al., 2011), preventing it from acting on the TRPA1 channels. A subsaturating UVR dose (160 mJ/cm^{2}; Bellono et al., 2013) evoked a submaximal UVR photocurrent in HEMs dialyzed with control internal solution. PolyK alone, when included in the pipette solution and allowed to diffuse into the cell and sequester PIP_{2}, did not lead to an increase in whole-cell current; however, it significantly enhanced UVR photocurrents elicited by the same subsaturating UVR dose (Fig. 4, C and E; I_{PolyK} = 0.07 ± 0.05 pA/pF, I_{UVR/Ctrl} = 2.51 ± 0.07 pA/pF, I_{UVR/PolyK} = 4.90 ± 0.44 pA/pF). The polyK-modulated UVR photocurrent was inhibited by the TRPA1 antagonist HC030031 (HC; 100 µM; Fig. 4, C, right; and Fig. 4 E; I_{UVR/PolyK+HC} = 0.27 ± 0.12 pA/pF), which suggests that the enhanced photocurrent was mediated by TRPA1.

To address the specificity of the phospholipids that modulate the UVR photocurrents, we used recombinant pleckstrin homology (PH) domains from phospholipase C δ1 (PLCδ1-PH) that selectively bind PIP_{2} and from the general receptor for phosphoinositides type 1 (GRP1-PH) that selectively binds PIP_{3} (PIP_{3}; Klein et al., 2008). Dialysis of recombinant PH domains in HEMs will bind and sequester the phosphoinositides, resulting in decreased cellular levels. When PLCδ1-PH was included in the pipette, a significantly enhanced
photocurrent was elicited by a subsaturating UVR dose (160 mJ/cm²), compared with boiled PLCδ1-PH orGRP1-PH (Fig. 4, F and G; I_{PLCδ1-PH} = 3.01 ± 0.36 pA/pF, I_{Boiled PLCδ1-PH} = 1.32 ± 0.26 pA/pF, I_{GRP1-PH} = 1.34 ± 0.24 pA/pF). These results suggest that sequestering PIP₂, but not PIP₃, levels in HEMs leads to increased TRPA1 activity in response to UVR, leading us to hypothesize that UVR-mediated PIP₂ hydrolysis releases the PIP₃-mediated inhibition of TRPA1.

IP₃R and TRPA1 mediate UVR-induced Ca²⁺ responses and regulate Ca²⁺ signaling kinetics

Retinal-dependent UVR-induced Ca²⁺ responses triggered downstream of Gα_{q/11} signaling are mediated by two sources of Ca²⁺: (1) efflux from intracellular thapsigargin-sensitive stores and (2) influx via TRPA1 at the plasma membrane (Bellono et al., 2013). Because Ga_{q/11}/PLCβ signaling generates the second messenger IP₃, we hypothesized that IP₃Rs mediate Ca²⁺ release from intracellular stores. To test this hypothesis, we treated HEMs with the IP₃R antagonist XeC (25 µM; Gafni et al., 1997; Oka et al., 2002) and found that UVR-induced Ca²⁺ responses were significantly reduced (Fig. 5, A and B). To study the contribution of each Ca²⁺ source (IP₃R vs. TRPA1) to the overall response, we measured Ca²⁺ responses in HEMs preincubated with XeC in order to inhibit IP₃R, or HC-030031 (HC; 100 µM) in order to inhibit TRPA1 (Fig. 4 A). Ca²⁺ responses elicited by 240 mJ/cm² UVR were reduced by 71% in the presence of XeC, by ~45% in the presence of HC, and by ~90% in the presence of both antagonists, as compared with vehicle-treated cells (Fig. 4 B; fluorescence increase over baseline: ΔF/F₀ = 3.21 ± 0.27 for vehicle, 0.94 ± 0.16 for XeC, 1.77 ± 0.29 for HC, and 0.34 ± 0.07 for vehicle).

![Figure 4](image-url)

**Figure 4.** PIP₂ regulates UVR-activated TRPA1 currents. (A) The UVR (240 mJ/cm²)-induced whole-cell current of a representative HEM dialyzed with the PIP₂ analogue diC8-PIP₂ and measured at +80 mV immediately after break-in was significantly reduced after 5 min of dialysis to allow diC8-PIP₂ to diffuse into the cell. (B) HEMs dialyzed with diC8-PIP₂ had reduced UVR photocurrent densities at all voltages when stimulated after 5 min of dialysis, compared with immediately after break-in. (C) HEMs exposed to a submaximal UVR dose (160 mJ/cm²) elicited a small but significant increase in whole-cell current at +80 mV (first trace). Including poly-lysine (polyK, 50 mg/ml) in the patch pipette did not alter the baseline current, but significantly potentiated the UVR (160 mJ/cm²)-induced current after 5 min of dialysis (second and third trace from the same representative cell). The augmented UVR-induced current measured in the presence of polyK was abolished by treatment with the TRPA1 antagonist HC-030031 (HC; 100 µM; fourth trace). (D) Dialysis with diC8-PIP₂ reduced mean peak UVR photocurrents by ~93% compared with control. n = 7 cells, P < 0.0001, ±SEM (error bars). (E) PolyK dialysis did not elicit a significant increase in mean current density in the absence of UVR stimulation, but increased retinal-dependent photocurrents induced by 160 mJ/UVR by ~96%. This effect was abolished by HC. n = 6 cells per condition, P < 0.0008, ±SEM (error bars). (F) The UVR photocurrent induced by 160 mJ/cm² UVR in a representative HEM was enhanced after 7 min of dialysis with PLCδ1-PH (60 µM), when compared with boiled PLCδ-PH (60 µM) or GRP1-PH (60 µM). The broken horizontal lines represent the baseline current for each recording. (G) Dialysis of HEMs with PLCδ1-PH (60 µM) enhanced mean peak UVR photocurrents by ~128% compared with cells dialyzed with boiled PLCδ1-PH (60 µM) or GRP1-PH (60 µM). n = 7–8 cells per condition, P < 0.002, ±SEM (error bars).
These results suggest that UVR phototransduction evokes a rise in $[\text{Ca}^{2+}]_c$ via intracellular Ca$^{2+}$ release from IP$_3$R and Ca$^{2+}$ influx through TRPA1 ion channels.

We next sought to distinguish the contribution of each Ca$^{2+}$ source (IP$_3$R and TRPA1) to the biphase nature of the transient UVR-induced Ca$^{2+}$ response. To do that, we measured the initial slope and the decay time constant of Ca$^{2+}$ responses in HEMs treated with XeC or HC, compared with vehicle. The mean initial slope of UVR-induced Ca$^{2+}$ responses (measured during the first 30 s after the beginning of UVR stimulation) was reduced by $\sim76\%$ with XeC treatment and by $\sim35\%$ with HC treatment when compared with vehicle-treated HEMs (Fig. 5, C and D; $\Delta F/s = 0.100 \pm 0.009$ for vehicle, $0.024 \pm 0.003$ for XeC, and $0.064 \pm 0.01$ for HC). These results suggest that both sources contribute to the initial rising phase of the Ca$^{2+}$ response, but IP$_3$-mediated Ca$^{2+}$ release has a significantly greater contribution.

The time constant for the decay phase ($\tau_{off}$) of the UVR-induced Ca$^{2+}$ response was measured by monitoring cellular Ca$^{2+}$ levels for $\geq200$ s after the peak of the response in cells treated with XeC, HC, or vehicle. HEMs treated with XeC had Ca$^{2+}$ responses with mean $\tau_{off}$ values $\sim79\%$ higher than vehicle-treated cells, which suggests that the IP$_3$-mediated response decays on a fast time scale. Interestingly, treatment with HC...
results suggest that IP3R-mediated Ca2+ release is significantly decreased both components of the Ca2+ response to UVR-evoked Ca2+ responses that decayed 38% faster than vehicle-treated cells (Fig. 5, E and F; \( \tau_{\text{off}} = 225.90 \pm 37.44 \text{ s for XeC}, 78.42 \pm 12.68 \text{ s for HC, and } 126.20 \pm 10.61 \text{ s for vehicle} \). Hence, TRPA1 activation extends the duration of the Ca2+ response, whereas IP3R activation reduces the duration. Collectively, our results suggest that IP3R-mediated Ca2+ release is important to ensure a fast rising phase of the response. In contrast, consistent with our previous findings (Bellono and Oancea, 2013; Bellono et al., 2013), TRPA1 activation is slow but persistent, contributing to a prolonged Ca2+ response (Fig. 6 B).

**DISCUSSION**

We have recently discovered that primary human melanocytes are capable of rapidly detecting UVR by first increasing intracellular Ca2+ and later producing more melanin (Wicks et al., 2011). The Ca2+ response is retinal dependent and in part due to calcium release from intracellular stores and in part to calcium influx through TRPA1 ion channels (Bellono et al., 2013). Both components of the response require heterotrimeric G proteins and PLCβ activation (Wicks et al., 2011; Bellono et al., 2013). Here we investigated the identity of the G protein subunit that mediates the response and found that reducing the expression of both Gαq and Gα11, as well as using a peptide that inhibits Gαq/11 signaling, significantly decreased both components of the Ca2+ response (Figs. 2 and 3). These results suggest that the UVR-activated pathway is mediated by Gαq/11, which, in turn, activates PLCβ.

Signal transduction pathways associated with G protein activation often result in modulation of TRP ion channels. For example, in *Drosophila*, light stimulation of rhodopsin results in activation of Gαq/11 and PLCβ, which hydrolyzes PIP2 to produce DAG and IP3, to regulate TRP ion channels (Hardie, 2001). We reasoned that one of these messengers (IP3, DAG, or PIP2) might regulate the UVR-activated TRPA1 photocurrent and tested our hypothesis by exogenously altering the levels of these messengers (Figs. 4 and S3). We found that reagents used to manipulate the levels of IP3 and DAG had no effect on the photocurrent, whereas reagents that affect PIP2 levels did. Allowing diC8-PIP2 to diffuse into the cell blocked the current in response to a UVR dose that evokes a maximal response, which suggests that decreasing PIP2 levels is a necessary step in TRPA1 activation. But is the decrease in PIP2 sufficient to activate TRPA1?

Sequestration of PIP2 using polyK did not elicit a significant current in the absence of UVR, but did potentiate TRPA1 currents evoked by UVR. Furthermore, using PLCδ1-PH to specifically sequester PIP2 enhanced UVR photocurrents, whereas sequestration of PIP2 by GRP1-PH had no effect. These results suggest that a decrease in PIP2 can modulate the photocurrent, but may not be sufficient for TRPA1 activation. Therefore, other messengers or proteins that contribute to the phototransduction pathway could be involved. Our results add to the already controversial role of PIP2 in modulating TRPA1. PIP2 was found to potentiate agonist-activated TRPA1 currents (Karashima et al., 2008), but also to inhibit TRPA1 activity (Dai et al., 2007; Kim et al., 2008). GPCRs that activate Goq/11 (bradykinin, PAR2, and Mrgpr8) can regulate TRPA1, but the mechanism is unclear (Bandell et al., 2004; Dai et al., 2007; Wang et al., 2008; Wilson et al., 2011). It remains to be determined in future experiments whether PIP2 directly interacts with TRPA1 to modulate its activity and what other cellular messengers contribute to TRPA1 activation in response to UVR and to Goq/11-coupled receptors.

Examination of the relative contribution of UVR-induced IP3R- and TRPA1-mediated Ca2+ responses revealed that IP3R-mediated Ca2+ release is rapid, but also declines fast. In contrast, TRPA1-mediated influx is slower to increase intracellular Ca2+, as suggested by the slow time course of the photocurrent activation, and slow to decay, allowing intracellular Ca2+ to remain elevated after UVR exposure, a necessary step for melanin production (Bellono et al., 2013). However, there is a discrepancy between the time course of photocurrents and that of Ca2+ responses. UVR photocurrents recorded under voltage-clamp conditions peak during or shortly after light stimulation, whereas Ca2+ responses measured in intact cells, in which the membrane voltage could
change, peak seconds after irradiation. We recently found that UVR phototransduction depolarizes the plasma membrane of melanocytes to delay TRPA1 inactivation and prolong Ca\textsuperscript{2+} responses (Bellono and Oancea, 2013), a finding consistent with the TRPA1-mediated influx kinetics found in this study. The discrepancy between the time course of the response measured by the two methods is likely to be caused by the significant effects of membrane depolarization on the TRPA1 channel and consequent Ca\textsuperscript{2+} signaling dynamics. Our analyses of Ca\textsuperscript{2+} response dynamics also revealed the possibility of cross-talk between the Ca\textsuperscript{2+} release and influx pathways. Inhibition of IP\textsubscript{3}R resulted in a significantly slower decline of the TRPA1-mediated Ca\textsuperscript{2+} response, which suggests that the IP\textsubscript{3}-mediated response decays considerably faster than the overall response. However, when TRPA1 was inhibited, the decay of the IP\textsubscript{3}-mediated Ca\textsuperscript{2+} responses was only slightly (although significantly) faster, which suggests that Ca\textsuperscript{2+} release may accelerate TRPA1 inactivation.

Based on our data, we propose that UVR exposure of HEMs stimulates a retinal-dependent Go\textsubscript{q/11}-coupled receptor, which activates PLC\textbeta. Active PLC\textbeta hydrolyzes plasma membrane PIP\textsubscript{2} into DAG and IP\textsubscript{3}. Soluble IP\textsubscript{3} binds IP\textsubscript{3}Rs in the ER, resulting in Ca\textsuperscript{2+} release, whereas the decrease in PIP\textsubscript{2} levels modulates TRPA1 activation and Ca\textsuperscript{2+} influx (Fig. 6). Our model for UVR signal transduction in human melanocytes resembles visual phototransduction in Drosophila photoreceptors (Hardie, 2001) and nonvisual phototransduction in the mammalian retina (Berson et al., 2002; Graham et al., 2008). The melanocyte UVR pathway also shares many similarities with a recently described UVR-activated signaling mechanism in Drosophila larvae, which is mediated by Ca\textsuperscript{2+} signals resulting from Go\textsubscript{q/11} and TRPA1 activation (Xiang et al., 2010). The receptor for the Drosophila larvae UVR pathway appears to be a gustatory GPCR (Gr28b); how light can activate such a receptor remains unknown.

One of the remaining questions for the UVR phototransduction cascade in melanocytes is the identity of the receptor. The retinal dependence and G protein involvement suggests the involvement of an opsin GPCR. We previously found that rhodopsin expression contributes to UVR-induced Ca\textsuperscript{2+} responses (Wicks et al., 2011). However, the differences in spectral sensitivity and G protein coupling of rhodopsin and of the UVR pathway suggest that rhodopsin might work in conjunction with a different, possibly unidentified, UVR-sensitive receptor to mediate UVR phototransduction in melanocytes. Future experiments will identify the UVR receptor and determine the molecular mechanism of TRPA1 activation. Understanding this pathway might uncover new photoprotection strategies for human skin, thus lowering the incidence of skin cancer.

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REFERENCES

Bandell, M., G.M. Story, S.W. Hwang, V. Viswanath, S.R. Eid, M.J. Petrus, T.J. Earley, and A. Patapoutian. 2004. Noxious cold ion channel TRPA1 is activated by pungent compounds and bradykinin. Neuron. 41:849–857. http://dx.doi.org/10.1016/S0896-6273(04)00150-3

Barr, A.J., H. Ali, B. Haribabu, R. Snyderman, and A.V. Smrcka. 2009. Identification of a region at the N-terminus of phospholipase C-beta 3 that interacts with G protein beta gamma subunits. Biochemistry. 39:1800–1806. http://dx.doi.org/10.1021/bi992021f

Beckman, B., J. Flores, P.A. Witkam, and G.W. Sharp. 1974. Studies on the mode of action of cholera toxin. Effects on solubilized adenylate cyclase. J. Clin. Invest. 53:1202–1205. http://dx.doi.org/10.1172/JCI101766

Bellono, N.W., and E. Oancea. 2013. UV light phototransduction depolarizes human melanocytes. Channels (Austin). 7:243–248. http://dx.doi.org/10.4161/chan.25322

Bellono, N.W., L.G. Kammel, A.L. Zimmerman, and E. Oancea. 2013. UV light phototransduction activates transient receptor potential A1 ion channels in human melanocytes. Proc. Natl. Acad. Sci. USA. 110:2383–2388. http://dx.doi.org/10.1073/pnas.1215551110

Bennett, D.C. 2008. Ultraviolet wavebands and melanoma initiation. Pigment Cell Melanoma Res. 21:520–524. http://dx.doi.org/10.1111/j.1755-148X.2008.00500.x

Berson, D.M., F.A. Dunn, and M. Takao. 2002. Phototransduction by retinal ganglion cells that set the circadian clock. Science. 295:1070–1073. http://dx.doi.org/10.1126/science.1067262

Dai, Y., S. Wang, M. Tominaga, S. Yamamoto, T. Fukushima, T. Higashi, K. Kobayashi, K. Obata, H. Yamanaka, and K. Noguchi. 2007. Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. J. Clin. Invest. 117:1979–1987. http://dx.doi.org/10.1172/JCI30951

De Vries, L., B. Zheng, T. Fischer, E. Elenko, and M.G. Farquhar. 2000. The regulator of G protein signaling family. Annu. Rev. Pharmacol. Toxicol. 40:235–271. http://dx.doi.org/10.1146/annurev.pharmtox.40.1.235

Fung, B.K., J.B. Hurley, and L. Stryer. 1981. Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc. Natl. Acad. Sci. USA. 78:152-156. http://dx.doi.org/10.1073/pnas.78.1.152

Gafni, J., J.A. Munsch, T.H. Lam, M.C. Catlin, L.G. Costa, T.F. Molinski, and I.N. Pessah. 1997. Nostospongic potentiob membrane permeable blockers of the inositol 1,4,5-trisphosphate receptor.
