Potassium Channel in the Mitochondria of Human Keratinocytes

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The activation of mitochondrial potassium channels induces cytoprotection in various cell types. Hence, the identification of ion channels present in the inner mitochondrial membrane of keratinocytes is important in distinguishing possible protective mechanisms in these cells. In this paper, inner membrane mitochondrial ion channels of the human keratinocyte HaCaT cell line were investigated using a patch-clamp technique. We observed potassium-selective channel activity with a conductance of 83 pS at positive voltages. The I–V curve indicates that the observed channel has rectifying properties. Moreover, the channel activity was inhibited by acidic pH and 1 mM lidocaine. Using reverse transcriptase–PCR, we found an mRNA transcript for the TASK-3 (tandem pore domain acid-sensitive K channels) channel. We observed co-localization of the TASK-3 protein and a mitochondrial marker in the mitochondria of HaCaT cells. Additionally, we showed that TASK-3 knockdown HaCaT cells markedly decreased viability after UVB radiation exposure compared with control cells. In summary, the single-channel activity and properties of a mitochondrial potassium channel in a keratinocyte HaCaT cell line have been described.

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INTRODUCTION

Since the first identification of an ATP-regulated mitochondrial potassium channel (mitoK$_{ATP}$) in the inner membrane of mitochondria (Inoue *et al.*, 1991), several other mitochondrial channels have been described (for a review, see Szewczyk *et al.*, 2009). MitoK$_{ATP}$ channels have been found in the liver, heart, brain, renal, and T-lymphocyte mitochondria (Paucek *et al.*, 1992; Bajgar *et al.*, 2001; Debska *et al.*, 2001; Cancherini *et al.*, 2003; Dahlem *et al.*, 2004). Other potassium channels that have been identified include large-conductance Ca^{2+}-regulated potassium (mitoBK$_{Ca}$) channels in the human glioma cell line (Siemen *et al.*, 1999), intermediate conductance Ca^{2+}-regulated potassium (mitoIK$_{Ca}$) channels in human colon cancer cells, and voltage-gated (mitoKv1.3) channels in human T lymphocytes. Similar potassium channels were also described in the mitochondria of unicellular eukaryotes (Kicinska *et al.*, 2007) and plants (Koszela-Piotrowska *et al.*, 2009).

Recent studies have shown that the activation of mitochondrial ion channels protects cells from death (for a review, see O'Rourke, 2007). The cytoprotective mechanism is most likely based on a slightly decreasing mitochondrial transmembrane potential. Opening of mitochondrial channels has been shown to reduce reactive oxygen species synthesis, cytochrome *c* release, and apoptotic cell death (Busija *et al.*, 2004; Hu *et al.*, 2005; Kulawiak *et al.*, 2008). A cytoprotective function of mitochondrial potassium channel activation has been shown in a cardiac system, where the opening of mitoK$_{ATP}$ channels with drugs known to open potassium channels, such as diazoxide, protected the heart against subsequent ischemic stress (Sato *et al.*, 2000). The mitoK$_{ATP}$ blocker 5-hydroxydecanoate has been reported to block the cytoprotective action of diazoxide (Garlid *et al.*, 1997; Liu *et al.*, 1998). Activation of mitoK$_{ATP}$ channels has also been shown to protect the brain against ischemic and chemical challenge (Wang *et al.*, 2005, 2006). Recently, the ROMK-type potassium channel was identified as a mitoK$_{ATP}$ channel (Foster *et al.*, 2012).

Thus, the cytoprotective effect of mitochondrial channel activation by potassium channel openers is key in the protection of several different cell types against various stresses (Szewczyk *et al.*, 2009). In addition to the cardiac and nervous systems, the protective role of K$_{ATP}$ channel openers has been postulated in skin. Cao *et al.* (2007) studied the role of the K$_{ATP}$ channel in UV-induced skin damage. It has been shown that preconditioning cells with the

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Abbreviations: mitoK$_{ATP}$, ATP-regulated mitochondrial potassium channel; TASK, tandem pore domain acid-sensitive K channels

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K_{ATP} channels openers pinacidil and diazoxide inhibits UV-induced cell death in keratinocytes. However, no strong evidence for the presence of K_{ATP} channels in HaCaT cells has been presented (Cao *et al.*, 2007).

Additionally, several groups have attempted to identify the channels that are present in keratinocytes. Immunofluorescent methods and western blotting have suggested the possibility of the presence of TASK-1, 2 and 3 (tandem pore domain acid-sensitive K+ channels), TREK-1 (TWIK-related K+ channel) and 2, and TRAAK (TWIK-related arachidonic acid-stimulated K+ channel) channels in plasma membrane of these cells and the TASK-3 channel in the mitochondria of keratinocytes (Kang *et al.*, 2007; Rusznak *et al.*, 2008). Nevertheless, until now, there have been no electrophysiological studies conducted at the single-channel level of mitochondrial channels in keratinocytes.

The purpose of this work was to identify the mitochondrial channels that are present in keratinocytes by using a patch-clamp technique to describe the electrophysiological properties of the channel. Next, the identity of the channel was verified by reverse transcriptase–PCR (RT–PCR) and immunofluorescence methods. To test a possible cytoprotective ability of TASK-3 channel, we used UVB irradiation injury model. This is the report, to our knowledge previously unreported, of strong evidence for the presence of the TASK-3 channel in the mitochondria of HaCaT cells obtained using the patch-clamp technique.

RESULTS

We used a patch-clamp technique to evaluate the presence of mitochondrial K^+ channels in HaCaT keratinocyte cells. Previously, this method was successfully used to identify mitochondrial K^+ channels in glioma cells (Siemen *et al.*, 1999), neurons (Bednarczyk *et al.*, 2010), and the liver (Inoue *et al.*, 1991).

Biophysical properties of the mitochondrial channels in HaCaT keratinocyte cells

In patch-clamp experiments using mitoplasts obtained from HaCaT keratinocyte cells (Figure 1a), channel activity was observed ($n=34$). The channel conductance was 83.3 ± 1 pS at positive voltages and 11.9 ± 0.2 pS at negative voltages. Single-channel current traces were recorded at different voltages in symmetric isotonic solutions (150/150 mM KCl). Unitary current traces for representative channel recordings at holding potentials of -90 mV to $+90$ mV are shown in Figure 1b. The channel displays non-linear current–voltage characteristics and rectification properties. Cation selectivity of the measured channel was shown in gradient conditions (450/150 mM KCl), and the measured reversal potential was approximately $+20$ mV, which is close to the value given by the Nernst equation, $+27.7$ mV. Single-channel current–voltage characteristics recorded at different voltages under symmetric and gradient conditions are shown in Figure 1c. The channel activity is voltage dependent, i.e., the probability of channel opening (P_o) increases for positive potentials (Figure 1d). Exemplary amplitude histograms are shown in Figure 1e.

To verify whether the observed channel activity corresponded to a mitoK_{ATP} channel (as the results of the Cao *et al.*, 2007 study on keratinocytes suggested), the influence of 1 mM Mg^{2+} combined with 500 µM ATP on the channel activity was evaluated ($n=3$, data not shown). However, no changes in channel activity were observed after applying the ATP/Mg^{2+} complex, confirming that the channel was not an ATP-regulated mitochondrial channel.

Results from other studies (Kang *et al.*, 2007; Rusznak *et al.*, 2008) have suggested the presence of two-pore domain channels (K_{2P} channels) in HaCaT cells. Thus, in the next step, the effects of K_{2P} channel modulators on the observed mitochondrial channel were tested. K_{2P} channels are blocked by local anesthetics, such as lidocaine (Talley and Bayliss, 2002). Hence, we studied the effect of lidocaine. In our observations, 1 mM lidocaine caused closing of the channel ($n=3$; Figure 2a).

Among K_{2P} channels, there are acid-sensitive channels, such as TASK channels. To determine whether the observed mitochondrial channels are pH modulated, we studied the effect of pH ($n=5$). The channel activity was investigated in isotonic solution at pH 6.2 and compared with data collected at pH 7.2. Figure 2b shows that the current amplitude in isotonic solution at pH 6.2 was zero, which indicates that the channel activity is inhibited at acidic pH. The effect was reversible upon changing the pH to 7.2. Amplitude histograms at control conditions, after the addition of 1 mM lidocaine and in isotonic solution at pH 6.2 are shown in Figure 2c.

To conclude, based on these patch-clamp experiments, the observed mitochondrial channel is acid sensitive and inhibited by lidocaine. This observation suggests that the detected channel belongs to the K_{2P} potassium channel family.

Expression of TASK channels in HaCaT cells

To determine which K_{2P} potassium channels from the TASK-type family are expressed in HaCaT cells, we first performed RT–PCR studies using specific primers for TASK-1, TASK-2, and TASK-3 channels (Table 1). To exclude the possibility of contamination with genomic DNA in RNA isolation, we used a DNase enzyme during RNA preparation. We also performed control reactions without reverse transcriptase ($-$RT). As shown in Figure 3, mRNA transcripts for two channels, TASK-2 and TASK-3, were detected. The RT–PCR products of TASK-3 (370 bp) and TASK-2 (546 bp) corresponded to the expected length for these channels.

Immunofluorescent identification of TASK-2 and TASK-3 channels in HaCaT cells

The presence of mRNA transcripts for TASK-2 and TASK-3 channels does not necessarily demonstrate protein translation. Accordingly, we performed immunostaining experiments with antibodies specific for TASK-3 (Figure 4) and TASK-2 channels (Figure 5). Consistent with the RT–PCR data, immunostaining showed that both channels were expressed in human keratinocytes and that both display cytoplasmic localization. Double-labeling immunofluorescence studies revealed differences between the distributions of the two channels. The TASK-3 channel co-localized with mitochondria (Figure 4). As shown in Figure 4, the granular labeling of the TASK-3 channel closely overlapped with cytochrome *c* oxidase (COX)

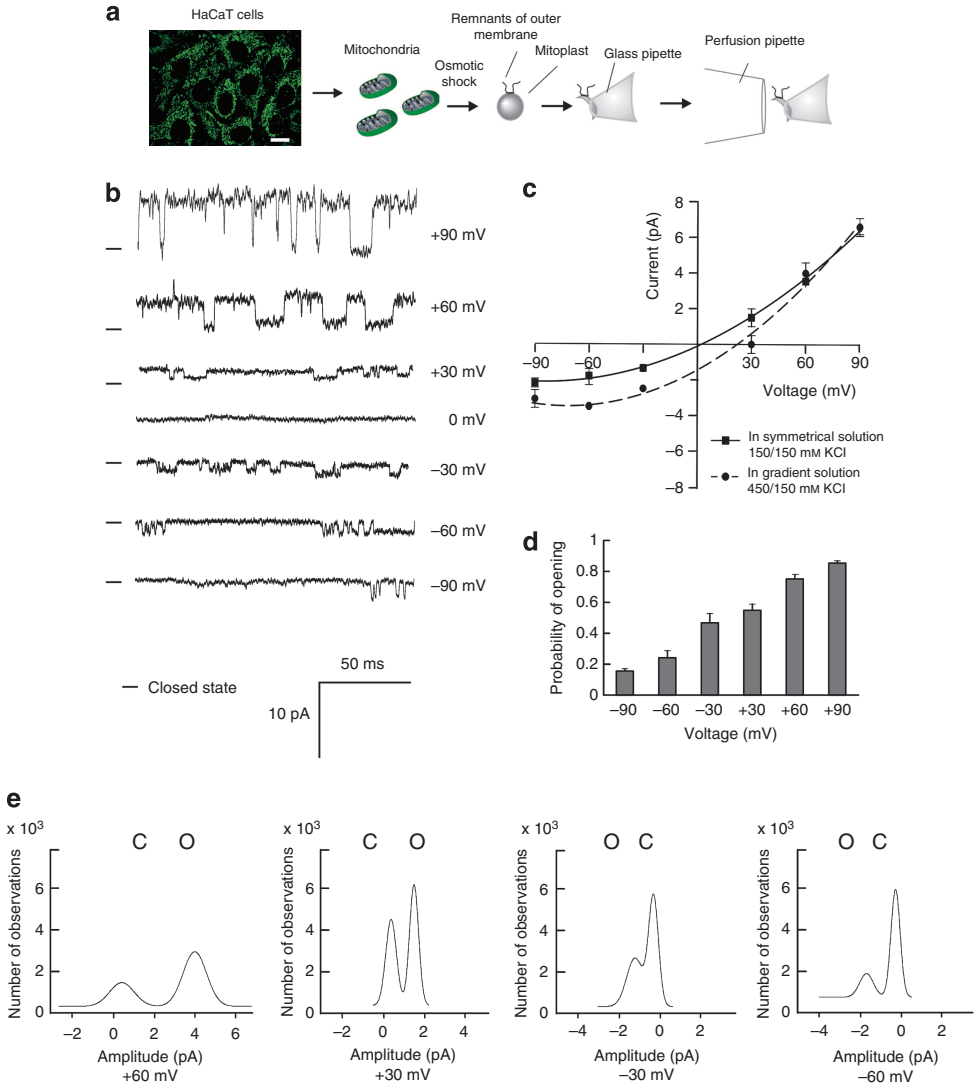

Figure 1. Electrophysiological identification of a potassium channel in mitoplasts isolated from HaCaT keratinocytes. (**a**) Schematic representation of mitoplast (mitochondria without an outer membrane) preparation from rat embryonic hippocampus and the patch-clamp experiments. The right panel shows confocal image of immunolabeling of the mitochondrial marker cytochrome *c* oxidase (COX) subunit VI (green). Bar = 10 µm. The experiments were performed as described in the Materials and Methods section. (**b**) Single-channel recordings at different voltages in symmetric (150/150 mM KCl) isotonic solution; ''–'' indicates the closed state of the channel. (**c**) The current–voltage characteristics of single-channel events in symmetric (150/150 mM KCl, filled squares) isotonic solution and in gradient solution (450/150 mM KCl, filled circles). (**d**) Channel open probability at different voltages. Data points are mean ± SD. (**e**) Exemplary amplitude histograms in the range from −60 to 60 mV. ''O'' indicates open state and ''C'' the closed state of the channel.

IV-positive mitochondria. However, we also observed cytoplasmic TASK-3-positive puncta that were not co-localized with COX IV-positive mitochondria. Therefore, to determine whether the cytoplasmic immunoreactivity is associated with endoplasmic reticulum (ER) compartments, we performed experiments with antibodies against an ER marker—KDEL. Double immunolabeling with an ER marker antibody revealed that the majority of the TASK-3-positive puncta were localized outside the ER compartments (data not shown). However, individual TASK-3-positive puncta with an ER-positive pattern were also occasionally detected. The ER localization of the channel may be due to the vesicular transport from the ER along the secretory pathway to the cell surface or mitochondria. In the case of the TASK-2 channel, mitochondrial co-localization with COX IV-positive mitochondria was not observed (Figure 5).

TASK-3 channel protect against UVB-induced cell death

To assess cytoprotective potential of TASK-3 channel, we used UVB radiation as a model of cytotoxicity. As presented in Figure 6a, UVB-radiated HaCaT cells change their morphology and increase the number of apoptotic cells (see arrows). The effect of UVB on cell death was detectable at 50 mJ cm^{-2} and most obvious at 100 mJ cm^{-2}. These results correlate with the cell viability assay. Data showed in Figure 6b ($n = 3$) demonstrate dose-dependent decrease in cell viability in control untreated cells, cells transfected with pTASK3_shRNA plasmid, and cells transfected with a scramble plasmid

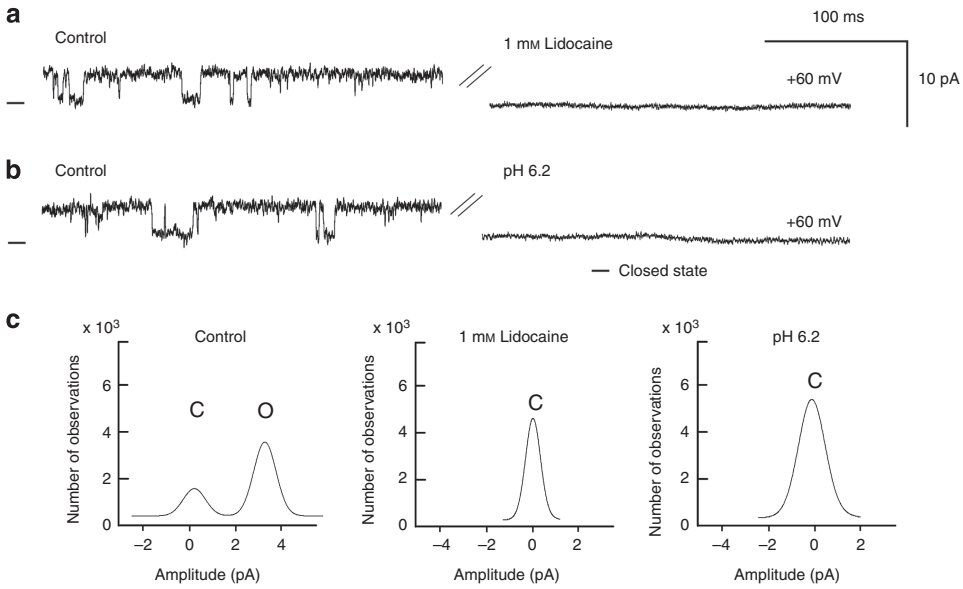

Figure 2. The effect of inhibitors commonly known to modulate tandem pore domain acid-sensitive K channels (TASK)-type channels. (**a**) Single-channel recordings at +60 mV in a symmetric isotonic solution (control) and after adding an isotonic solution with 1 mM lidocaine. (**b**) Single-channel recordings at +60 mV in a symmetric isotonic solution (control) and after adding an isotonic solution at pH 6.2. ''–'' indicates the closed state of the channel. The results are representative of three independent experiments. (**c**) Amplitude histograms under control conditions, after the addition of 1 mM lidocaine and in isotonic solution at pH 6.2. ''O'' indicates open state and ''C'' the closed state of the channel.

Table 1. Reverse transcriptase–PCR (RT–PCR) primer sequences

Gene	Accession no.	Sequence of: F—forward primer R—reverse primer	Product size (bp)
Homo sapiens potassium channel TASK-1 subfamily K, member 3 (*KCNK3*)	NM_002246.2	F: 5′-GACGATGAAGCGGCAGAAC-3′ R: 5′-ATGACGGTGATGGCGAAGTA-3′	397
Homo sapiens two pore domain potassium channel TASK-2 subfamily K, member 5 (*KCNK5*)	AF084830	F: 5′-ATCAGCGAGGAATGCGAG-3′ R: 5′-GCCAAGCTCAGGACAGATG-3′	546
Homo sapiens two pore domain potassium channel TASK-3 subfamily K, member 9 (*KCNK9*)	AF212829	F: 5′-GAACAGTGAGGATGAGCGG-3′ R: 5′-CTTCCGGCGTTTCATCAG-3′	370
Homo sapiens glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	NM_002046.4	F: 5′-CAAGGTCATCCATGACAACTTTG-3′ R: 5′-GTCCACCACCCTGTTGCTGTAG-3′	496

Abbreviation: TASK, tandem pore domain acid-sensitive K channels.

(pTASK3_Scr). Interestingly, cells without TASK-3 channel are more sensitive to UVB damage than control and transfected with a scramble plasmid. These data demonstrated that the presence of TASK-3 channel can augment cell viability in keratinocytes treated with UVB unlike TASK-3 knockdown cells.

DISCUSSION

The role of mitochondrial ion channel openers in protection against environmental damage of the skin is a relatively new area of investigation. Especially interesting is the issue of the protection of keratinocytes against UV irradiation, as UV irradiation is the main carcinogen inducing skin cancer—the most frequently diagnosed neoplasm in the Caucasian population (Assefa *et al.*, 2005). It has been reported that UV irradiation or bacterial invasion induces the apoptosis of keratinocytes, which is linked to the initiation of mitochondrial pathways for caspase activation (Denning *et al.*, 2002; Nuzzo *et al.*, 2000; Assefa *et al.*, 2005). However, a detailed understanding of the role of mitochondrial ion channels in UV protection requires an identification of ion channels in the mitochondria of keratinocytes.

The identification of ion channels in human keratinocytes has been the subject of investigation by few groups. In plasma membrane of HaCaT keratinocytes, 14 pS nonspecific cation channel and Ca^{2+}-activated 70 pS potassium channel have been found (Mauro *et al.*, 1993, 1997). Patch-clamp studies of the cell membrane allowed observation of other calcium-activated potassium channels: hSK4, hIK1, and large conductance 250 pS channel (Koegel and Alzheimer, 2001; Koegel *et al.*, 2003; Ivanchenko and Markwardt, 2005). Large conductance potassium channel, Ca^{2+}, Mg^{2+}, and ATP-independent was also described (Nguyen and Markwardt, 2002). By means of the RT–PCR method, western blotting and

immunocytochemistry, the presence of six K_{2P} channels in HaCaT cells: TASK-1, 2, and 3, TREK-1 and 2, and TRAAK has been demonstrated (Kang *et al.*, 2007). However, there have been no reports identifying the localization of these channels within the cell.

The possibility of mitochondrial localization of potassium channels in HaCaT keratinocytes have been described by the two groups. Cao *et al.* (2007) confirmed the presence of the Kir 6.1, Kir 6.2, and SUR2 subunits of K_{ATP} channels in HaCaT cells by western blotting. The proposed mitochondrial localization of K_{ATP} channels was based on the finding that the amino-acid structure of the identified subunits differed from that present in the sarcolemmal K_{ATP} channel. However, no other studies confirming this conclusion have been performed. Preconditioning of HaCaT cells with pinacidil and mitochondrial K_{ATP} channel opener diazoxide attenuated UV-induced cell death, and this effect was reversed by mitoK_{ATP} blocker 5-hydroxydecanoate and non-specific K_{ATP} blocker glibenclamide. On that basis, authors suggested that mitoK_{ATP} channel is likely to be present in keratinocytes, but no electrophysiological studies on HaCaT cells mitochondria has been undertaken. Rusznak *et al.* (2008) presented the evidence for the mitochondrial localization of potassium channels in human keratinocytes. They showed that TASK-3 channels are present in mitochondria isolated from HaCaT cells using only immunofluorescence techniques.

The TASK-3 channel belongs to the family of K_{2P} K^+ channels and was first cloned from the rat cerebellum in 2000 (Kim *et al.*, 2000). TASK-3 has been found in many rat tissues such as the aorta, colon, lung, stomach, liver, kidney, spleen, testis, skeletal muscle, dorsal root ganglion neurons, and thalamocortical neurons as well as in several human tissues, particularly in brain tissues (TASK-3 channel was identified in 19 human brain regions) and human melanoma cells (Chapman *et al.*, 2000; Meuth *et al.*, 2003; Pocsai *et al.*, 2006; Jang *et al.*, 2008). TASK-3 has been found to contain four transmembrane segments and two pore-forming regions. Electrophysiological studies of the TASK-3 channel have indicated that the single-channel conductance is 27 pS at −60 mV and 17 pS at +60 mV. The channel opening probability has been found to be higher at more positive potentials, and the channel indicates slight outward rectification under symmetric conditions (Rajan *et al.*, 2000; Ashmole *et al.*, 2009). Human TASK-3 is referred to as a pH sensor, because it is known to be highly sensitive to variations in extracellular pH—acidification inhibits TASK-3 channel activity (Meadows and Randall, 2001). Similar to the TASK-1 channel, TASK-3 is inhibited by local anesthetics, such as lidocaine and bupivicaine (Bayliss *et al.*, 2003).

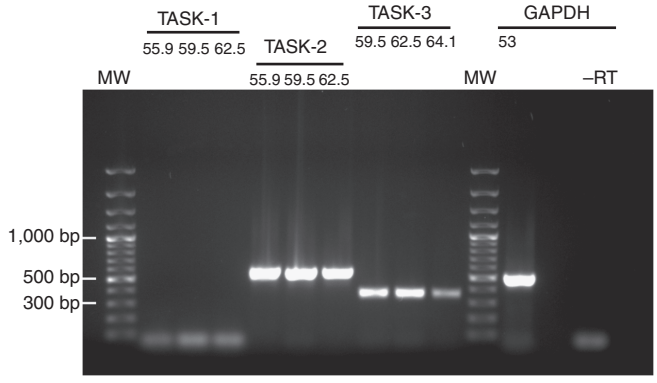

Figure 3. Detection of tandem pore domain acid-sensitive K channels (TASK-1), TASK-2, and TASK-3 potassium channels in HaCaT keratinocytes. PCR products were amplified from 35 cycles in a temperature gradient (marked above the photo). TASK-2 mRNA was detected at 546 bp, and TASK-3 mRNA was detected at 370 bp. The negative control without reverse transcriptase did not show any signal. The results are representative of three experiments. GAPDH, positive control (glyceraldehyde-3-phosphate dehydrogenase); – RT, negative control without reverse transcriptase; MW, molecular weight.

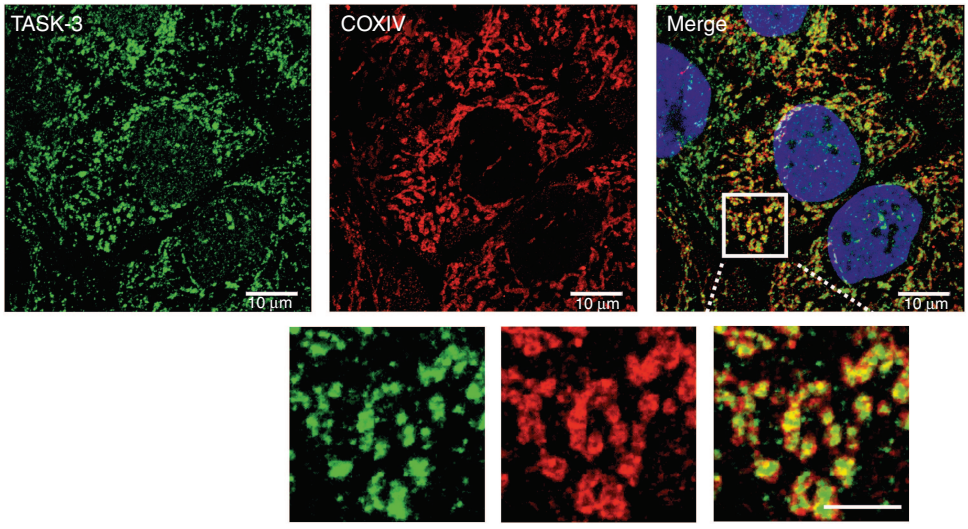

Figure 4. Immunofluorescent localization of tandem pore domain acid-sensitive K channels (TASK-1) channels in human keratinocytes. Confocal image of double-immunolabeling of the TASK-3 channel (green) and mitochondrial marker cytochrome *c* oxidase (COX) subunit IV (red). In the overlay image (green, red, blue) costained with DAPI (4,6-diamidino-2-phenylindole; blue—nuclei), a yellow color was visualized, indicating TASK-3 colocalization with COX-positive mitochondria. The arrows indicate TASK-3-positive mitochondria. Bars = 10 µm and 5 µm.

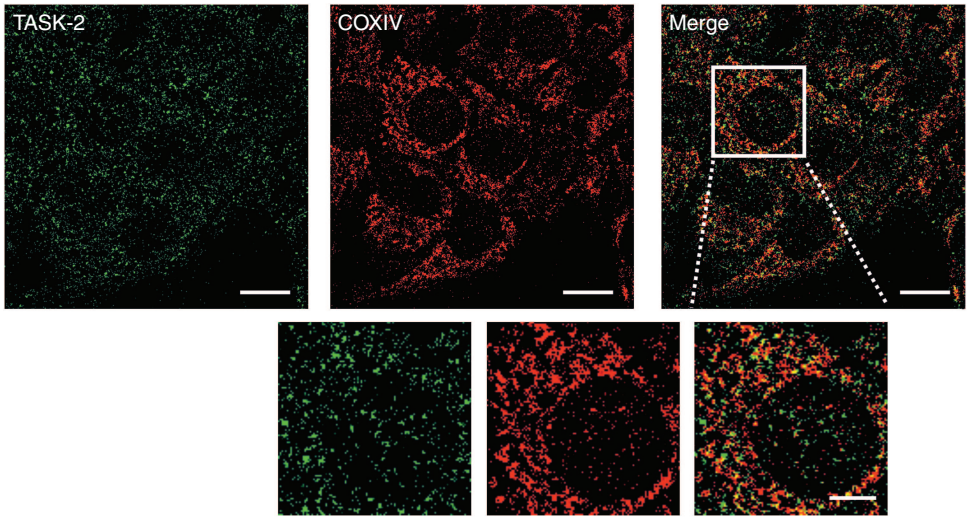

Figure 5. Immunofluorescent localization of tandem pore domain acid-sensitive K channels (TASK-1) channels in human keratinocytes. Confocal image of double-immunolabeling for TASK-2 (green) and mitochondrial marker cytochrome *c* oxidase (COX) subunit IV (red). Bars = 10 μm and 5 μm. The results are representative of three independent experiments.

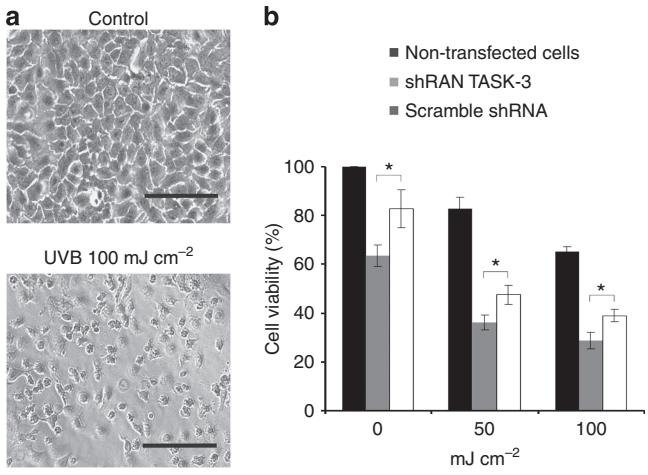

Figure 6. Effect of UV irradiation on keratinocyte cells with or without tandem pore domain acid-sensitive K channels (TASK-1) channel. (**a**) Keratinocyte cells treated with different dosage of UVB irradiation (50, 100, 150 mJ cm^{-2}); bar = 200 μm. (**b**) Viability of keratinocyte cells transfected with pTASK3_shRNA (shRNA TASK-3), pTASK3_Scr (scramble), and non-transfected, untreated cells. Data points are mean ± SD. $P<0.05$.

In this work, we have demonstrated the presence of the TASK-3 channel, to our knowledge previously unreported, in the mitochondrial inner membrane using a patch-clamp technique. In contrast to the work of Kang *et al.* (2007), the RT–PCR results of our studies allowed us to confirm the presence of mRNA transcripts for only two TASK channels in HaCaT cells. No mRNA transcripts for the TASK-1 channel were found. To verify the mitochondrial localization of the TASK-2 and TASK-3 channels, immunofluorescence methods were used. Co-localization of the TASK-3-specific immunopositivity and mitochondrion-specific labeling was observed, demonstrating the presence of TASK-3 channels in mitochondria. However, in the case of the TASK-2 channel, such co-localization was not observed, which suggests the absence of mitochondrial localization of the TASK-2 channel.

Patch-clamp experiments on mitoplasts from keratinocytes allowed us to investigate the mitochondrial inner membrane channel activity. We observed channel activity with a conductance of ~83 pS at positive voltages. The reversal potential in a gradient of 450/150 mM KCl is ~20 mV, which indicates that the channel is potassium selective. The observed channel exhibits rectification, and its P_o value is voltage dependent (the channel opens when a more positive voltage is applied). TASK channels are known to be highly sensitive to pH changes. Accordingly, the influence of this parameter on channel activity was examined. According to our expectations, acidification of the isotonic solution to pH 6.2 caused a decrease in the current amplitude and the channel opening probability. A similar effect was observed after applying 1 mM lidocaine, which also strongly inhibited channel activity. Thus, the biophysical characteristics of the channel are in accordance with TASK-3 channel properties observed at the whole cell level, which allows us to conclude that the observed channel is a mitochondrial TASK-3 (mitoTASK-3) channel. We have also observed other channel activities ($n = 20$). The second type of activity exhibited a conductance of approximately 60 pS and was also inward rectifying. However, its P_o value was not dependent on the applied voltage (data not shown).

We have recently described another potassium channel (sensitive to pH) in embryonic rat hippocampus mitochondria (Kajma and Szewczyk, 2012). Due to a lack of lidocaine inhibition, this channel was determined not to be TASK-3.

After identification of the ion channel in the mitochondria of HaCaT cells, we tested the role of this channel in UVB radiation–induced cell damage. It is well known that biological effects of cells exposure to UV radiation are very complex. Cell responses includes DNA damage, activation of caspases, p53 nuclear transfer (Tendler *et al.*, 2013), mitochondrial

respiratory dysfunction, cytochrome *c* release, increased mitochondrial NO production (Gonzalez Maglio *et al.*, 2005), and loss of mitochondrial transmembrane potential (Takai *et al.*, 2006). It is also known that some of the potassium channels contribute the protection against UV-induced keratinocytes cell damage (Cao *et al.*, 2007). Based on our UVB radiation experiments, we can conclude that knocking down TASK-3 channel causes greater decrease in cell viability that in control cells.

In summary, in this work we have identified mitochondrial TASK-3 channels in the human keratinocyte HaCaT cell line. Mitochondrial localization of the TASK-3 channel was shown by immunofluorescence methods. Electrophysiological studies of mitoTASK-3 were conducted using a patch-clamp technique, which allowed us to obtain the single-channel characteristics of this channel. However, a description of the role of the TASK-3 channel in mitochondrial function requires further investigation.

MATERIALS AND METHODS

HaCaT cell line

HaCaT cells, the immortalized human keratinocyte cell line, were obtained from CLS (Cell Line Service, Eppelheim, Germany). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin at 37 °C.

Isolation of mitochondria from HaCaT cells

The cells were harvested by centrifugation at 800 *g* for 10 minutes, resuspended in 2 ml of mitochondrion preparation solution (250 mM sucrose, 5 mM HEPES, pH = 7.2), and homogenized using a glass homogenizer. To isolate the mitochondria, the homogenate was centrifuged at 9,200 *g* for 10 minutes. The supernatant was decanted, and the pellet was suspended in 1 ml preparation solution. The suspension was centrifuged at 770 *g* for 10 minutes. The supernatant was placed in a new tube and centrifuged at 9,200 *g* for 10 minutes. Pelleted mitochondria were then resuspended in storage solution (150 mM KCl, 10 mM HEPES, pH = 7.2) and centrifuged at 9,200 *g* for 10 minutes. Finally, the mitochondria were resuspended (0.3 ml of storage solution).

Patch-clamp experiments and data analysis

Mitoplasts, mitochondria without the outer membrane, were prepared as previously described (Bednarczyk *et al.*, 2010; Kajma and Szewczyk, 2012). In brief, a sample of keratinocyte mitochondrion suspension was combined with a hypotonic solution (5 mM HEPES, 100 μM $CaCl_2$, pH = 7.2) for 45 seconds to induce swelling and breakage of the outer mitochondrial membrane. Subsequently, a hypertonic solution was added (750 mM KCl, 30 mM HEPES, 100 μM $CaCl_2$, pH = 7.2), which allowed for the restoration of isotonicity. The pipettes were made of borosilicate glass and were pulled by a Flaming/Brown Puller (10–20 Ω resistance). The patch-clamp pipette was filled with an isotonic solution (150 mM KCl, 10 mM HEPES, 100 μM $CaCl_2$, pH = 7.2). The isotonic solution was used as a control solution for all presented data. Modulators of the channel were added as dilutions in the isotonic solution. Test solutions were pumped by a peristaltic pump-driven capillary-pipe system. The gradient solution was composed of 450 mM KCl, 10 mM HEPES and 100 μM $CaCl_2$ at pH = 7.2. For the ground electrode, Ag/AgCl with an agar salt bridge (3 M KCl) was used. The current was recorded using a patch-clamp amplifier (Axopatch 200B, Molecular Devices Corporation, Sunnyvale, CA). The currents were low-pass filtered at 1 kHz and sampled at a frequency of 100 kHz. Recordings were made in the mitoplast-attached single-channel mode.

The channel conductance was calculated from the current–voltage relationship, and the probability of channel opening (P_o) was determined using the single-channel search mode of Clampfit 10 software (Molecular Devices). Amplitude histograms were fitted to experimental data using superimposed Gaussian curves.

RT—PCR analysis

Total RNA was extracted using a Qiagen RNeasy kit (Invitrogen, Paisley, UK) with the use of DNase enzyme (Invitrogen, UK). First, 1.5 μg RNA was reverse transcribed using a RevertAid First-Stand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA). For a 20-μl reverse transcription reaction, 1.5 μg total RNA (in 10 μl diethylpyrocarbonate water) was mixed with 1 μl oligo(dT) primer and then with 4 μl 5-fold concentrated reaction buffer, RiboLock RNase inhibitor, 2 μl 10 mM dNTPs, and 1 μl RevertAid M-MuLV Reverse Transcriptase (200 μ μl^{-1}). Reactions were performed for 60 minutes at 42 °C and terminated by incubating for 5 minutes at 70 °C. Parallel reactions for each RNA sample were run in the absence of RevertAid M-MuLV Reverse Transcriptase to assess for genomic DNA contamination of the RNA sample.

First-strand cDNA was used as a DNA temple for PCR amplification, and PCR was performed with specific primers (Table 1). PCR amplification was performed as follows: initial denaturation at 95 °C for 3 minutes; denaturation at 95 °C for 30 seconds; primer annealing at 59.5 °C (for TASK-1, TASK-2, TASK-3 primers), or 53 °C (for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) primers) for 3 minutes; extension at 72 °C for 1 minute; then 35 cycles of 95 °C for 30 seconds, 54 °C for 30 seconds, and 72 °C for 1 minutes; and a final extension step at 72 °C for 15 minutes.

Immunochemistry

Upon 50–70% confluence, HaCaT cells were fixed for 30 minutes in 4% paraformaldehyde at room temperature and then washed in phosphate-buffered saline (PBS). Cells were permeabilized, and non-specific binding of antibodies was blocked using permeabilization/blocking solution (donkey serum-saponin-bovine serum-albumin solution) containing 5% normal donkey serum (Jackson Immuno Research Laboratories, West Grove, PA), 0.075% saponin (Sigma-Aldrich, St Louis, MO), and 1% BSA (Sigma-Aldrich) diluted in PBS for 30 minutes. Then, cells were incubated in DSB solution containing a mixture of primary antibodies: anti-TASK2 (1:300, Abcam, Cambridge, UK) and anti-TASK3 (1:200, Sigma-Aldrich) together with marker antibodies, such as mitochondrial marker COX IV (1:250, Abcam) and ER marker anti-KDEL (1:300, Abcam). The immunoreaction was visualized using the DSB solution containing a mixture of secondary antibodies (Molecular Probes, Eugene, OR): Alexa-Fluor 488-conjugated donkey anti-mouse antibodies (1:200) and Alexa-Fluor 555-conjugated donkey anti-rabbit antibodies (1:200). Cells were covered with VECTASHIELD mounting medium containing DAPI (4,6-diamidino-2-phenylindole; Vector Laboratories, Burlingame, CA).

UV light apparatus

HaCaT cells were washed twice with PBS and then irradiated in PBS with UVB (312 nm) in range from 50 to 1,000 mJ cm^{-2} without plastic dish lid (BIO-LINK BLX crosslinker, Vilber Lourmat). UVB light intensity was monitored using CX-312 sensor (Vilber Lourmat, Marne-la-Vallee, France). After procedure, PBS was removed, culture medium was added, and the plates were incubated for 24 hours at 37 °C.

Cell viability assay (MTT dye assay)

HaCaT cell culture medium was discarded, and 50 µl of DMEM containing 0.5 mg ml^{-1} of MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well of 96-well plate. After a 2-hours incubation, 50 µl of lysis buffer (5% SDS, 50% N,Ndimethylformamide, 2% acetic acid, and 25 mM HCl) was added to each well, and the plates were incubated for 24 hours at 37 °C. MTT formazan formation was quantified by measuring the absorbance at 570 nm; 650 nm was used as a reference wavelength. The results were normalized to the values of the untreated control cells for each corresponding experiment.

Construction of the plasmids

Target sequences for silencing TASK-3 expression were selected using siRNA selection program (Yuan *et al.*, 2004). Three sequences were selected as silencing targets (Gene bank BC075080):

(1) (711) 5′-UGGCCUUUAGCUUUAUGUAUAUC-3′ (733); (2) (1055) 5′-GAGAUCUCACCAAGCACAUUAAA-3′ (1077); and (3) (292) 5′-CGGCUCCUUCUACUUUGCGAUCA-3′ (314).

Silencing plasmids expressing shRNA were constructed by cloning in plasmid pSilencer 2.1-U6 hygro cleaved with BamHI and HindIII double-stranded oligonucleotides designed according to the vector manual. Recombinant plasmids were verified by dideoxy sequencing. Control plasmid pTASK3_Scr (scramble shRNA) produces shRNA targeting sequence 5′-GCGAAAUCGAAAUACAUAU-3′. Functional testing of the three silencing plasmids revealed first one for the best TASK-3 silencing. This plasmid we named pTASK3_shRNA (shRNA TASK-3). Experiments supporting efficient knockdown of particular proteins in RNAi experiments were made. Mitochondrial fraction of HaCaT cells (control), cells transfected with control scramble plasmid pTASK3_Scr, and silencing plasmid pTASK3_shRNA were processed for western blotting using the antibodies against TASK-3 channel. Experiments revealed that TASK-3 expression is decreased after pTASK3_shRNA transfection (data not shown).

Transfection assay

Transfection assays were performed using Lipofectamine 2000 (Invitrogen, UK) as described by the manufacturer. In brief, lipofectamine-DNA complexes were formed by mixing 0.25 µl of lipofectamine with 0.12 µg of the plasmids: pTASK3_shRNA and pTASK3_Scr (per well). The cells were incubated for 3 hours in transfection medium OptiMEM (Invitrogen). Then, the medium was replaced with fresh culture medium.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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