The potassium channels TASK2 and TREK1 regulate functional differentiation of murine skeletal muscle cells

Ali M. Afzali,1* Tobias Ruck,1* Alexander M. Herrmann,1 Janette Iking,1 Claudia Sommer,2 Christoph Kleinschnitz,2 Corinna Preuße,3 Werner Stenzel,3 Thomas Budde,4 Heinz Wiendl,1 Stefan Bittner,5* and Sven G. Meuth1*

1Department of Neurology, University of Münster, Münster, Germany; 2Department of Neurology, University Hospital of Würzburg, Würzburg, Germany; 3Department of Neuropathology, Charité-Universitätsmedizin, Berlin, Germany; 4Institute of Physiology I, University of Münster, Münster, Germany; and 5Department of Neurology, University Medical Center of the Johannes Gutenberg-University Mainz, Mainz, Germany

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Afzali AM, Ruck T, Herrmann AM, Iking J, Sommer C, Kleinschnitz C, Preuße C, Stenzel W, Budde T, Wiendl H, Bittner S, Meuth SG. The potassium channels TASK2 and TREK1 regulate functional differentiation of murine skeletal muscle cells. Am J Physiol Cell Physiol 311: C583–C595, 2016. First published August 3, 2016; doi:10.1152/ajpcell.00363.2015.—Two-pore domain potassium (K2p) channels influence basic cellular parameters such as resting membrane potential, cellular excitability, or intracellular Ca2+-concentration [Ca2+]i. While the physiological importance of K2p channels in different organ systems (e.g., heart, central nervous system, or immune system) has become increasingly clear over the last decade, their expression profile and functional role in skeletal muscle cells (SKMC) remain largely unknown. The mouse SKMC cell line C2C12, wild-type mouse muscle tissue, and primary mouse muscle cells (PMMs) were analyzed using quantitative PCR, Western blotting, and immunohistochemical stainings as well as functional analysis including patch-clamp measurements and Ca2+ imaging. Mouse SKMC express TWIK-related acid-sensitive K+ channel (TASK) 2, TWIK-related K+ channel (TREK) 1, TREK2, and TWIK-related arachidonic acid stimulated K+ channel (TRAaK). Except TASK2 all mentioned channels were upregulated in vitro during differentiation from myoblasts to myotubes. TASK2 and TREK1 were also functionally expressed and upregulated in PMMs isolated from mouse muscle tissue. Inhibition of TASK2 and TREK1 during differentiation revealed a morphological impairment of myoblast fusion accompanied by a downregulation of maturation markers. TASK2 and TREK1 blockade led to a decreased K+ outward current and a decrease of ACh-dependent Ca2+ influx in C2C12 cells as potential underlying mechanisms. K2p-channel expression was also detected in human muscle tissue by immunohistochemistry pointing towards possible relevance for human muscle cell maturation and function. In conclusion, our findings for the first time demonstrate the functional expression of TASK2 and TREK1 in muscle cells with implications for differentiation processes warranting further investigations in physiologic and pathophysiologic scenarios.

muscle cells; K2p channels; TASK2; TREK1; C2C12; differentiation; myogenesis; patch clamp; calcium imaging

by fusion muscle cells are able to generate a multinucleated syncytium. Muscle cell fusion is an ordered multistep process consisting of “recognition,” “adhesion,” and “membrane fusion” (62). Knudsen and Horwitz (41) firstly described the aggregation and merging of detached chick embryonic myoblasts generating multinucleated cells. By adding chemical agents to the suspension they were able to observe these three developmental stages. During the first stage myoblasts are arranged next to each other, before they start to adhere and aggregate. By adding EDTA and complexes calcium (Ca2+), the aggregation can be revoked, unless the myoblasts have not already adhered completely. This stage of development is characterized by EDTA resistance and sustained trypsin sensitivity. The onset of myoblast fusion is defined by the resistance against both EDTA and trypsin (41). Many molecules have been described to participate in these processes (39, 41, 62). An elevation of the intracellular calcium-concentration intracellular Ca2+ concentration ([Ca2+]i) immediately before myoblast fusion plays a critical role in this process initiating intracellular Ca2+-dependent signaling pathways like the NFATC2 signaling pathway, which ultimately lead to the expression of myogenic regulator factors (MRF). The source of intracellular Ca2+ elevation still remains unclear, although different theories have been proposed over the last years (5, 19). MRFs comprise a group of transcription factors such as MyoD, Myf5, myogenin (MYOG), and MRF4, which trigger gene expression for myoblast differentiation (15, 20, 38, 39). MRFs initiate the expression of muscle specific molecules such as myosin or α-actin providing a molecular basis for contractility (15, 21).

Regulation of [Ca2+]i is closely interrelated with potassium channel activity sustaining the electrochemical driving force for Ca2+ ion entry upon muscle cell activation (5). Two-pore domain (K2p) potassium channels influence basic cellular parameters such as the resting membrane potential or cellular excitability and are essential in the regulation of [Ca2+]i. They are mainly voltage independent and are modulated by changes in pH, lipid metabolites or O2 tension (22, 30, 34, 35, 47). K2p channels have been implicated in various physiological processes like HCO3- transport in the kidney, T-cell effector functions, heart rate modulation, volume regulation of T cells, pain perception, and aldosterone secretion (1, 14, 24, 40, 50,
and cardiomyocytes. TWIK-related acid-sensitive K channel (TASK) 1 and 2 have been shown to respond to vascular pH changes in pulmonary vascular SmMCs and to be downregulated in pulmonary vascular SmMCs in a murine animal model of sepsis (7, 37). TASK1 and 2 as well as TWIK-related K+ channel (TREK) 1 are supposed to respond to enteric inhibitory nerve stimulation or stretch in intestinal SmMCs (59). TREK1 has been demonstrated to mediate uterine quiescence during gestation (16) and to contribute to mechanosensitivity in detrusor SmMCs (44). Concerning cardiomyocytes, previous studies revealed an influence of TASK1 and TREK1 on cardiac electric activity (17). However, the role of K2P channels for skeletal muscle cell (SkMC) differentiation and function has not been investigated so far. Our study investigated functional K2P-channel expression, their influence on [Ca2+]i, as well as their implications for SkMC differentiation.

MATERIALS AND METHODS

Animals. All experiments using animals were performed in accordance with the guidelines of the national institutional animal care and use committee (Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen, LANUV). The project has been approved by the LANUV (84-02.05.20.13.097).

Antibodies and reagents. See Table 1 for a list of primary antibodies used for experiments. The respective isotype controls were purchased from either BD Biosciences (Heidelberg, Germany) or BioLegend (San Diego, CA).

Cell culture of C2C12. The murine muscle cell line C2C12 (ATCC, Manassas, VA) was cultured in uncoated T-25 flasks with muscle growth medium containing 10% fetal calf serum (PAA, Pasching, Austria) in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 1% penicillin and streptomycin (Biochrom, Berlin, Germany). The medium was changed every other day. When cells reached up to 70% confluence, differentiation and suspended in Trizol reagent for RNA isolation. qPCR was run with a specific primer for MYOG (Mm00464194_m1, FAM-labeled; Applied Biosystems) and endogenous control primers for 18sRNA (Hs_439413E; VIC-labeled; Applied Biosystems) following the manufacturer’s protocol.

Western blot. Cell lysis was performed with 2 × 10^6 freshly harvested C2C12 cells in 30 μl lysis buffer [10% n-dodecyl-β-D-maltoside, 1% NP-40, 1 mM sodium monovaradate, 1 mM phenylmethylsulfonylfluoride (PMSF), 50 μM Tris, 10 mM NaF, 10 mM EDTA, and 165 mM NaCl] and 7.5 μl sample buffer (20 μM TRIS, 10% glycerol, 0.05% bromophenol blue and 1% SDS) for 30 min (min) at 4°C. Tissues taken from wild-type mice and one set of C2C12 cells were worked up with T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA). Then, cells were centrifuged for 10 min at 14,000 rpm at 4°C. The supernatant containing the protein fraction was mixed with sample buffer and incubated for 5 min at 99°C. 10% SDS-PAGE was used for protein separation, followed by a transfer to nitrocellulose membranes. In a next step membranes were blocked with 5% dry milk and treated with specific polyclonal rabbit anti-mouse antibodies directed towards TASK2 (55 kDa, 70 kDa glycosylated), TREK1 (45 kDa), TREK2 (55 kDa), or TRAAK (45 kDa; Sigma-Aldrich, St. Louis, MO) overnight at 4°C. To complete the reaction the antibody was incubated with 5% blocking buffer and then incubated with a peroxidase-conjugated secondary antibody. Immunoreactivity was visualized by chemiluminescence (ECL; Amersham Biosciences). For normalization, the membranes were cleared with 2% sodium azide for 1h and incubated with a β-actin antibody (Sigma-Aldrich) for 1 h. Protein quantification was performed with ImageJ software (National Institutes of Health, Bethesda, MD). After protein quantification, the particular protein level of the respective K2P channel in differentiated C2C12 was divided by the protein level of the same respective K2P channel in undifferentiated C2C12 cells. The calculation of the β-actin protein levels was done in the same way. Finally, the ratio of the protein levels for the respective K2P channel was again divided by the ratio of the protein levels for β-actin.

To confirm the specificity of our experiments, we used liver as positive control and bladder as negative control for TASK2 detection as well as lung as positive control and liver as negative control for TREK1 detection as described before (56) in qPCR and Western blotting experiments (data not shown).

Immunohistochemical analysis. Immunohistochemistry (IHC) was performed both on murine and human muscle tissue.

For murine samples, mice were transcardially perfused with PBS and one quadriceps femoris muscle per mouse was dissected and frozen as previously described (49). Normal muscle specimens were obtained from patients who had a muscle biopsy not showing any abnormalities by extensive light microscopic analysis. They had normal creatine kinase levels and no autoantibodies detected in the serum and no signs and symptoms of systemic disease or respiratory...
inflammation. Also, electromyography and electromyography were normal. These patients were biopsied due to unspecific complaints (e.g., fatigue) for which an exclusion diagnosis was performed to rule out mild/subclinical myositis.

Ten-micrometer cryosections were fixed with acetone for 10 min and incubated in goat serum diluted in PBS (1:10) for 30 min at room temperature. In a next step, sections were incubated with antibodies directed towards TASK2 or TREK1, overnight at 4°C. On the next day, Cy3 goat anti-rabbit or anti-mouse horseradish peroxidase (HRP) antibody (Dianova, Hamburg, Germany) was used as secondary antibody. Finally, the sections were mounted with 20 μl of DAB chromogen and 1 ml of DAB substrate (Dako). The reaction was stopped with ddH2O, counterstained with haemalum and covered for analysis with an Olympus BX50 microscope, with the digital camera DP25 and the CELL ®D software (Olympus). Immunohistochemical staining without primary antibodies was used as negative controls.

**Isolation of primary murine myoblasts.** Hindlimbs from neonatal mice (2–5 days of age) were removed and placed in PBS. Muscles were cut in to small pieces and dissociated enzymatically in 2 ml of 1% dispase and collagenase (Sigma-Aldrich, St. Louis, MO) supplemented with 2 mM CaCl2. Suspension was kept on a rotator at 37°C for 45 min. After addition of 10 ml DMEM, the suspension was centrifuged at 1,500 rpm for 5 min. The pellet was resuspended in another 10 ml of DMEM and centrifuged with the same configuration.

The pellet was resuspended in muscle selective medium (SM) consisting of 80% Ham’s F-10 (Irvine Scientific, Santa Ana, CA), 20% FBS supplemented with 2.5 ng/ml basic fibroblast growth factor (bFGF), and 1% penicillin and streptomycin. Subsequently, the suspension was plated on a laminin-coated (10 μg/ml, 2 h at 37°C) cell culture plates. Thereafter, cells were purified using the preplating technique described before (18, 33). After 3–4 h, the supernatant was transferred to a second laminin-coated plate. The first well was filled with SM. Again, after 3–4 h, this procedure was repeated in the same manner with a third laminin-coated well. On the second day of cultivation, medium was removed and changed to a growth medium consisting 40% Ham’s F-10, 40 DMEM, and 20% FBS supplemented with 2.5 ng/ml bFGF and 1% penicillin and streptomycin. After 4–5 days, spindle-shaped myoblasts were visible on the bottom of the third well (see Fig. 2D). Purity was controlled by CD56 expression and was >85%.

**Differentiation index.** C2C12 cells were cultured and differentiated as described above and treated with quinidine (20 μM) or spadin (1 μM). Muscle cell differentiation was monitored for 7 days and numbers of differentiated cells were assessed with an Axio Scope.A1 microscope within a defined surface of 0.28 mm². The index was calculated as myotube count per five different image areas of each culture by two blinded observers. Cell surface area and diameter were measured using ImageJ software.

**Patch-clamp recordings of C2C12 cells.** Whole cell recordings of undifferentiated and differentiated C2C12 cells were performed as previously described for K2P channel measurements on T cells (50). Membrane currents were recorded using a conventional patch-clamp setup equipped with an EPC-10 amplifier (HEKA Elektronik) or the automated patch-clamp platform NPS-16 Patchliner (Nanion Technologies, München, Germany) equipped with HEKA amplifiers and software (HEKA Elektronik). Glass pipettes were prepared from borosilicate glass (GT150T-10; Clark Electromedical Instruments, Pangbourne, UK). The intracellular solution contained 95 mM K-gluc- Ontario, Canada) equipped with HEPES buffer (120 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 30 mM HEPES, 2 mM MgSO4, and 10 mM glucose) containing either 0 mM or 2.5 mM Ca2+ and incubated with 5 μM fura-2-AM and Pluronic F-127 (Molecular Probes, Eugene, OR) for 30 min at 37°C. After a thorough washing, cells were transferred to a 96-well flat bottom plate and incubated for another 5 min at room temperature. Then fluorescence of cells was analyzed using the microplate reader software i-control (Tecan, Männedorf, Switzerland) according to the following protocol: The microplate reader was set to measure light emissions at 340 and 380 nm with a time interval of 3 s. The detector was primed with either 10 μM ionomycin (Molecular Probes) or 10 μM acetylcholine chloride (ACH; Sigma-Aldrich) and the time point for application was set to 90 s after the experiment was started. After a baseline measurement period of 90 s one of the stimuli inducing Ca2+ influx was applied. Subsequently, the Ca2+ response was measured over a period of 180 s. In one set of experiments cells were additionally incubated for 30 min at 37°C with either 20 μM quinidine or 1 μM spadin after fura-2-AM staining. In another set of experiments cells were incubated with the specific blocking reagent against Ca2+-activated K+ channels charybdotoxin (ChiTx; IC50 = 5 nM), a combination of quinidine and spadin or a combination of all three blockers (data not shown).

**Statistical analysis.** If not otherwise stated, the results are presented as mean ± SE. Statistical analysis was performed using a modified Student’s t-test for normally distributed data. A two-way ANOVA with Bonferroni post test was used in the case of multiple comparisons for parametric data. Correlation of two variables was assessed using Spearman’s correlation (rS). For all statistical analyses, P < 0.05 were considered as statistically significant.

**RESULTS**

**Expression patterns of K2P channels change during SkMC differentiation.** A functional expression of K2P potassium channels has not been shown so far in SkMCs. Therefore, we investigated the expression pattern of several members of the K2P-channel family in the murine muscle cell line C2C12. C2C12 cells exhibit typical morphologic features of muscle satellite cells and have the ability to differentiate by fusion processes from myoblasts to myotubes (15); the round or star-shaped myoblasts form a syncytium of elongated or spindle-shaped fibers after differentiation (Fig. 1A). C2C12 cells constitutively express the K2P channels TASK2, TREK1, TREK2, and TRAAK on mRNA and protein level (Fig. 1, B–D). However, we could not detect expression of the closely related K2P channels TASK1, TASK3, and TRESK (Fig. 1B). During differentiation TASK2, TREK1, TREK2, and TRAAK show different changes in expression patterns (Fig. 1, C and
Two-pore domain potassium (K_{2P}) expression in C2C12 cells during differentiation. A: during differentiation of myoblasts to myotubes, C2C12 cells change their cell morphology from round or star-shaped to elongated or spindle-shaped confluent cells. B: quantitative (q)PCR screening of members of the K_{2P}-channel family in C2C12 cells (n = 3). C: qPCR analysis of the relative gene expression of TWIK-related acid-sensitive K^+ channel (TASK) 2, TWIK-related K^+ channel (TREK) 1, TREK2, and TWIK-related arachidonic acid stimulated K^+ channel (TRAAK) from differentiated C2C12 cells normalized to C2C12 undifferentiated myoblasts (n = 7) over time (day 0 to day 8). D: Western blot analysis and densitometric quantification of TASK2, TREK1, TREK2, and TRAAK expression normalized to C2C12 undifferentiated myoblasts (n = 3). β-Actin was used as loading control. All data are shown as mean ± SE. *P < 0.05, determined by either Student’s t-test or Spearman’s correlation test; ns, not significant.
were measured at day 8 and normalized as described in (11, 30) (1.71 ± 0.24-fold at day 8, P < 0.05). TREK1, TREK2, and TRAAK expression showed an increase both on gene and protein levels compared with undifferentiated C2C12 cells (qPCR: TREK1 13.4 ± 4.59-fold, positively correlated with time ᵢ \text{day} 0.95 and ᵢ \text{day} 0.09, TREK2 5.0 ± 2.25-fold, ᵢ \text{day} 0.80 and ᵢ \text{day} 0.64, TRAAK 2.1 ± 0.95 fold, ᵢ \text{day} 0.72 and ᵢ \text{day} 0.52; Western blot: TREK1 7.26 ± 2.04-fold, P < 0.05, TREK2 4.59 ± 0.71-fold, P < 0.05, TRAAK 8 ± 1.27-fold, P < 0.05; all data were measured at day 8 and normalized as described in MATERIALS AND METHODS).

**K₂P channels are expressed in the skeletal muscle of C57BL/6 mice.** In a next step, we evaluated whether TASK2, TREK1, TREK2, and TRAAK are also expressed in native muscle tissue from C57BL/6 mice. Gene expression of aforementioned K₂P channels was detected in quadriceps femoris muscle tissue (Fig. 2A) and immunohistochemical staining revealed a predominant location at the cell membrane (Fig. 2C).

Since only for TASK2 and TREK1 specific blocking agents are available, we focused the following experiments on these channels. Both TASK2 and TREK1 are expressed in skeletal muscle tissue on the 5th day after birth (Fig. 2, B and C). Interestingly, the expression levels on the mRNA and protein levels are higher on day 5 than on day 20 after birth (Fig. 2, B and C; TASK2: 0.24 ± 0.05-fold at day 20 with P < 0.05; TREK1: 0.07 ± 0.02-fold at day 20 with P < 0.05), when skeletal muscle differentiation is more advanced (58, 61). The correlation of a high myogenic propensity and the K₂P-channel expression points towards a potential involvement of TASK2 and TREK1 in skeletal muscle differentiation.

In a next step, we established a protocol for primary murine myoblast (PMM) isolation and cultivated PMMs for differentiation (Fig. 2, D–G). The expression of the differentiation marker MYOG was increased upon differentiation of PMMs (Fig. 2F; MYOG: 2.8 ± 0.17-fold on day 6, P < 0.05). In parallel TASK2 and TREK1 were upregulated on mRNA levels in differentiated PMMs corroborating our findings in ex vivo muscle tissue (Fig. 2G; TASK2: 1.35-fold at day 6 with P < 0.05, TREK1: 1.29-fold at day 6 with P < 0.05).

These findings indicate that TASK2 and TREK1 are also expressed in native muscle tissue as well as primary muscle cells obtained from C57BL/6 mice and their expression is regulated during skeletal muscle differentiation. Therefore, our findings in C2C12 cells can be, with certain limitations, regarded as valid model system for the in vivo situation. Therefore and based on the standardized and controllable experimental accessibility, the C2C12 cell line was used in the following.

**TASK2 and TREK1 influence electrophysiological properties of C2C12 cells.** To assess the functional relevance of K₂P channels in C2C12 cells we investigated the impact of K₂P-channel modulating substances on basic cellular parameters and muscle cell differentiation. Previous studies have suggested quinidine as a specific TASK2 inhibitor (IC⁵₀ = 22 μM) as well as spadin as a specific TREK-1 inhibitor (IC⁵₀ = 70 μM) (13, 45, 48, 51, 52, 56). Whole cell patch-clamp measurements were performed to assess the contribution of TASK2 and TREK1 on the potassium outward current in C2C12 cells.

We measured the membrane capacitance (Cₘ), serial resistance (Rₛ), and the resting membrane potential (Vᵣₑₐₓ) in current-clamp settings to characterize the electrophysiological parameters of undifferentiated and differentiated C2C12 cells (Table 2). Thereafter we used a voltage ramp from −80 to +40 mV over 1,000 ms for current measurements in voltage-clamp mode. Figure 3, A and D, displays representative current traces of undifferentiated and differentiated C2C12 cells with specific features of outward-rectifying potassium currents with a reversal potential of −80 to −90 mV. The rise of the relative protein levels of TASK2 and TREK1 is accompanied by an increase of the K⁺ current upon differentiation (Fig. 3, A and D). Inhibition of both TASK2 and TREK1 reduced the potassium outward current as well as current density of undifferentiated C2C12 cells (Fig. 3, A and D). Compared with control condition, TASK2 blockade by quinidine reduced potassium currents by 35 ± 0.04% in undifferentiated myoblasts (Fig. 3B, left, pA 0.65 ± 0.04-fold, P < 0.05), while TREK1 inhibition by spadin lead to a reduction of 12 ± 0.02% (Fig. 3B, right, pA 0.88 ± 0.02-fold, P < 0.05). Determination of the current density (i.e., current amplitude with respect to cell capacitance) substantiated that our blocker sensitive effects are based on an inhibition of ion channels on the cell surface rather than changing cell size (Fig. 3B; TASK2: 39.16 ± 6.26 vs. 21.02 ± 3.75 pA/pF with P < 0.05; TREK1: 42.62 ± 0.95 vs. 36.54 ± 1.09 pA/pF with P < 0.05; data compared with untreated C2C12 cells). As a consequence of the current decrease the membrane potential was depolarized upon TASK2- and TREK1-inhibition by 13 ± 0.01 and 12 ± 0.02% respectively (Fig. 3C; TASK2: −52.04 ± 1.37 vs. −45.24 ± 1.28 mV with P < 0.05; TREK1: −55.33 ± 1.22 vs. −48.1 ± 1.53 mV with P < 0.05; data compared with untreated C2C12 cells).

In a next step, we aimed at recording K₂P-channel-mediated currents in differentiated C2C12 cells (Fig. 3D). Quinidine and spadin lead to a current reduction of 14 ± 0.005 and 7 ± 0.02%, respectively (Fig. 3E; TASK2: 0.86 ± 0.01-fold with P < 0.05; TREK1: 0.93 ± 0.02-fold with P < 0.05; data compared with untreated C2C12 cells). Effects could further be confirmed by determination of the current density (Fig. 3E; TASK2: 46.816 ± 1.18 vs. 39.92 ± 0.70 pA/pF with P < 0.05 TREK1: 48.97 ± 1.92 vs. 42.22 ± 1.73 pA/pF with P < 0.05; data compared with untreated C2C12 cells). Again, the current reduction by quinidine and spadin depolarized the membrane potential measured in differentiated C2C12 cells by 16 ± 0.01 and 11 ± 0.003% respectively (Fig. 3F; TASK2: −44.27 ± 1.97 mV vs. −37.14 ± 1.43 mV with P < 0.05; TREK1: −48.93 ± 1.59 mV vs. −43.48 ± 1.39 mV with P < 0.05; data compared with untreated C2C12 cells).

With TASK2 and TREK1 we still observed residual K⁺ currents. To identify the responsible K⁺ channels, we performed patch-clamp measurements with Tertiapin Q (TPNQ) and tetraethylammonium (TEA) thereby assessing the activity of inwardly rectifying K⁺ (Kᵢ) and voltage-gated K⁺ (Kᵥ) channels in C2C12 myoblasts, respectively. While TPNQ had no effect, TEA led to a 30% reduction of the K⁺ current (data not shown). Since the presence of gluconate and 3 mM BAPTA indirectly ruled out activity of Ca²⁺-activated K⁺ channels, we assume that the current traces shown in Fig. 3, A, and D are mediated by Kᵢ.
Fig. 2. Functional K\textsubscript{2P} expression in native murine muscle tissue and primary muscle cells. 

A: qPCR analysis of the gene expression of TASK2, TREK1, TREK2, and TRAAK in native murine muscle tissue (n = 3). 

B: qPCR analysis of the gene expression of TASK2 and TREK1 in the muscle tissue of 5- and 20-day-old wild-type C57/BL6 mice (n = 3). 

C: representative immunohistochemical stainings of TASK2 and TREK1 in native muscle tissue from 5- and 20-day-old mice. 

D: during differentiation of myoblasts to myotubes, primary mouse muscle cells (PMMs) change their cell morphology from round or star-shaped to elongated or spindle-shaped confluent cells. 

E: purity of the isolation was controlled by flow cytometric analysis using CD56 expression. 

F: qPCR analysis of the gene expression of myogenin (MYOG) in PMMs (n = 3). 

G: qPCR analysis of the gene expression of TASK2 and TREK1 in PMMs. Data are shown as the mean ± SE. *P < 0.05, determined by Student’s t-test; ns, not significant.
and K<sub>v</sub> channels.

**Reduced Ca<sup>2+</sup> influx under TASK2 and TREK1 blockade.** Electrophysiological parameters such as membrane potential are essential for the homeostasis of permeable ions. The difference between the actual and the equilibrium potential shape the driving force for ion currents following \( I = g \times (V_m - E_{ion}) \) (\( g = \) conductance, \( V_m = E_{ion} = \) driving force).

\([Ca^{2+}]_i\), plays an essential role in the regulation of physiological processes in the skeletal muscle such as differentiation, intracellular signaling, or contraction (4, 21, 32) and K<sub>2P</sub> channels have been shown to be critically involved in intracellular Ca<sup>2+</sup> homeostasis (40). Therefore, we used the Ca<sup>2+</sup>-sensitive probe fura-2-AM to assess the role of TASK2 and TREK1 in the regulation of [Ca<sup>2+</sup>]<sub>i</sub> changes upon ACh (10 \( \mu \)M) stimulation. However, Ca<sup>2+</sup> measurement with the aid of dyes like fura-2-AM displays net changes in the [Ca<sup>2+</sup>]<sub>i</sub>, which does not rule out efflux modulation by both inhibitors. Application of the ionophore ionomycin (10 \( \mu \)M) served as a positive control demonstrating the maximum calcium influx (Fig. 4, A and D; mean peak \( F_{340}/F_{380} \) at time point 99 s: undifferentiated 1.51 ± 0.03-fold and differentiated 1.57 ± 0.06-fold). Nominally Ca<sup>2+</sup>-free extracellular solutions were used to distinguish whether the increase of [Ca<sup>2+</sup>]<sub>i</sub> is related to the release from intracellular Ca<sup>2+</sup> stores and/or calcium influx from extracellular space. Previous studies reported that the ACh-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> in C2C12 cells is related to Ca<sup>2+</sup> release from intra- and extracellular sources in a rate of 1 to 4 (36). However, we were not able to detect any changes in our extracellular Ca<sup>2+</sup>-free experimental setup after ACh-application (Fig. 4A) suggesting no measurable intracellular Ca<sup>2+</sup> release. With 2.5 mM extracellular Ca<sup>2+</sup> solution, ACh application increased the [Ca<sup>2+</sup>]<sub>i</sub> in undifferentiated C2C12 cells 1.25 ± 0.05-fold and in differentiated C2C12 cells 1.27 ± 0.01-fold compared with baseline levels (Fig. 4, A and D). Further experiments were done with thapsigargin leading to a higher Ca<sup>2+</sup> response as seen upon ACh application (data not shown).

To assess the impact of K<sub>2P</sub> channels on Ca<sup>2+</sup> influx from the extracellular site, we incubated our cells with quindine (20 \( \mu \)M) or spadin (1 \( \mu \)M) after fura-2-AM staining and before application of ACh. Preincubation with quindine reduced the Ca<sup>2+</sup> influx in undifferentiated C2C12 cells by 11 ± 0.04% and in differentiated cells by 6 ± 0.02% at time point 100 s (Fig. 4, B and D; mean peak \( F_{340}/F_{380} \) undifferentiated: 1.11 ± 0.02 with \( P < 0.05 \) and differentiated 1.19 ± 0.01 with \( P < 0.05 \)). Inhibition of TREK1 by spadin led to a decreased Ca<sup>2+</sup> influx by 15 ± 0.04% in undifferentiated and 11 ± 0.02% in differentiated C2C12 cells (Fig. 4, C and D, undifferentiated: 1.06-fold ± 0.02 with \( P < 0.05 \) and differentiated 1.14-fold ± 0.03 with \( P < 0.05 \)). Preincubation with either one of the two blockers in a Ca<sup>2+</sup>-free solution had no effect on [Ca<sup>2+</sup>]<sub>i</sub> in both undifferentiated as well as differentiated C2C12 cells under ACh stimulation (Fig. 4, B and C). Area under the curve analysis confirmed these findings (Fig. 4E).

Compared with untreated C2C12 myoblasts and myotubes pretreatment with either quindine or spadin reduced the Ca<sup>2+</sup> influx upon ACh injection [Fig. 4E; TASK2: undifferentiated 16.01 ± 2.98 vs. 5.63 ± 1.19 with \( P < 0.05 \); differentiated 15.19 ± 0.72 vs. 9.23 ± 0.20 with \( P < 0.05 \); TREK1: undifferentiated 16.01 ± 2.98 vs. 4.09 ± 1.03 with \( P < 0.05 \); differentiated 15.19 ± 0.72 vs. 7.05 ± 1.99 with \( P < 0.05 \); data compared with untreated C2C12 cells and shown as \( (F_{340}/F_{380}) \times s \)]. To assess the influence of Ca<sup>2+</sup>-activated K<sub>+</sub> channels (K<sub>Ca</sub>) in this experimental setting we ran measurements with ChTx, a specific blocker for K<sub>Ca</sub>, a combination of quindine and spadin and a combination of all three blockers. Pretreatment with either ChTx or the combination of quindine and spadin show comparable reduction of ACh-induced Ca<sup>2+</sup> influx. However, a combination of all three blockers led to a maximum reduction proving that both K<sub>2P</sub> channel groups have significant effects on the Ca<sup>2+</sup> entry (data not shown).

**Pharmacological TASK2 and TREK1 blockade inhibits differentiation of C2C12 cells.** David and Higginbotham firstly described an intracellular Ca<sup>2+</sup> elevation immediately before myoblast fusion (23). Our results display a decrease of ACh-dependent Ca<sup>2+</sup> influx upon TREK1 and TASK2 inhibition in both C2C12 myoblasts and myotubes hinting towards a possible influence on calcium dependent mechanism such as differentiation. By using a horse serum containing cell culture medium we were able to investigate C2C12 differentiation and the role of TREK1 and TASK2. C2C12 myoblasts showed first signs of merging after 1 day and were fully differentiated after 5 to 7 days as described previously (15). TASK2- and TREK1 (Fig. 5A) inhibition led to a delayed onset of myoblast fusion and a decreased number of total differentiated myoblasts compared with untreated C2C12 cells (Fig. 5, A and B, quinidine: \( P < 0.05 \), spadin: \( P < 0.05 \)). A critical point in skeletal muscle fusion is the initiation of MRFs. The transcription factor MYOG, also known as myogenin or myogenic factor 4 (Myf-4), has been defined as an essential factor and as a marker for SkMC fusion (15, 25, 60). RNA was extracted from myotubes at day 7 of differentiation. qPCR experiments revealed a reduced MYOG expression under TASK2 and TREK1 blockade supporting a functional relevance of these K<sub>2P</sub> channels in SkMC differentiation (Fig. 5C, relative MYOG expression at day 7, quinidine: 0.47 ± 0.15 with \( P < 0.05 \), spadin: 0.37 ± 0.09 with \( P < 0.05 \)).

Furthermore, comparing myotube diameters of untreated and treated myotubes, both quindine and spadin showed an inhibitory effect on myotube growth (Fig. 5, A and C, day 6, area: TASK2: 292.87 ± 25.63 vs. 86.29 ± 12.89 \( \mu \)m<sup>2</sup> with \( P < 0.05 \), TREK1: 292.87 ± 25.63 vs. 83.67 ± 9.94 \( \mu \)m<sup>2</sup> with \( P < 0.05 \); diameter: TASK2: 19.44 ± 1.45 vs. 7.48 ± 0.48 \( \mu \)m with \( P < 0.05 \), TREK1: 19.44 ± 1.45 vs. 7.00 ± 0.87 \( \mu \)m with \( P < 0.05 \); data compared with untreated C2C12 cells).

**K<sub>2P</sub> channels are expressed in human SkMC tissue.** To evaluate the transferability of our findings in mouse SkMcs to the human situation we performed histological staining with human muscle biopsy specimens from healthy control subjects. Hematoxylin and eosin stainings (Fig. 6A) showed normal muscle architecture without any evidence of pathology. Immunohistochemistry demonstrated membrane-related expression

<table>
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<th>Table 2. Electrophysiological parameters of C2C12 cells</th>
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<td>Parameters</td>
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<td>Membrane capacitance (C&lt;sub&gt;m&lt;/sub&gt;), pF</td>
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<tr>
<td>Serial resistance (R&lt;sub&gt;s&lt;/sub&gt;), MΩ</td>
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<td>Resting membrane potential (V&lt;sub&gt;rest&lt;/sub&gt;), mV</td>
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Values are means ± SE.
of TASK2, TREK1, TREK2, and TRAAK comparable to the mouse muscle (Fig. 6B).

**DISCUSSION**

Despite ongoing research for many years, our understanding of physiological skeletal muscle myogenesis remains incomplete (20, 38, 39, 61). Ion channels are important regulators of basic cellular functions and have been implicated in the differentiation of SkMCs (35). We here show for the first time a functional role of K₂P channels for membrane potential, potassium conductance, and degree of intracellular Ca²⁺ increase in SkMCs with important implications for muscle cell differentiation. Different K₂P channels were detected in murine muscle cells in vitro and muscle tissue ex vivo, with increased expression levels of TASK2 and TREK1 during myogenesis in early postnatal mice. In vitro myoblast differentiation was associated

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**Fig. 3.** Electrophysiological characterization of K₂P channels in undifferentiated and differentiated C2C12 cells. Electrophysiological characterization of TASK2 and TREK1 currents in C2C12 cells. A ramp from −80 to +40 mV during an interval of 1.000 ms was applied to induce current changes (insets). Representative current traces of either treated or untreated myoblasts (A) and myotubes (D). Quantification of membrane currents and current density of undifferentiated (B) and differentiated (E) C2C12 cells in the presence or absence of specific channel-modulating substances (n = 5). Quantification of membrane potentials measured in undifferentiated (C) and differentiated (F) C2C12 cells. Except representative current graphs, data are shown as mean ± SE. *P < 0.05, determined by Student’s t-test; ns, not significant.
Fig. 4. Fluorometric measurement of intracellular free Ca²⁺ concentration ([Ca²⁺]ᵢ). Undifferentiated and differentiated muscle cells were stained with the Ca²⁺-sensitive dye fura-2-AM. 

A: after the injector was primed with either 10 μM ACh or 10 μM ionomycin, the application was set at 90 s after the beginning of the experiment (n = 3). Ca²⁺-free solutions were used to evaluate the source of [Ca²⁺]ᵢ changes. In one set of experiments, cells were incubated with either 20 μM quinidine (B) or 1 μM spadin (C) after staining (n = 3). Peak Ca²⁺ influx (D) and area under the curve (E) analysis under different test conditions (time point 99 s). All data are shown as mean ± SE. *P < 0.05, determined by either ANOVA (A–C) or Student’s t-test (D); ns, not significant.
Fig. 5. TASK2 and TREK1 inhibition influences C2C12 cell differentiation. C2C12 myoblasts were cocultured with either quinidine (20 μM) or spadin (1 μM) during differentiation. A: representative image sections (0.28 mm²) of C2C12 cells during differentiation on days 0, 3, and 6. Scale bar = 100 μm. B: average number of myotubes counted in an image section (0.28 mm²) on each day of differentiation. C: qPCR analysis of the gene expression of MYOG from differentiated C2C12 myotubes treated with either quinidine (20 μM) or spadin (1 μM) during differentiation normalized to untreated C2C12 myotubes at day 7 (n = 4) and analysis of mean cell surface and mean cell diameter by ImageJ software. All data are shown as mean ± SE. *P < 0.05, determined by two-way ANOVA or Student’s t-test.
with an alteration of K\textsubscript{2P}-channel expression patterns in C2C12 cells as well as in primary muscle cells. Pharmacological inhibition of TASK2 and TREK1 during differentiation led to impaired myoblast fusion accompanied by a downregulation of maturation markers. Although both channel inhibitors quinidine and spadin have proven specificity towards the respective K\textsubscript{2P} channel (45, 48, 51, 52, 56), we cannot rule out off target effects. Especially quinidine is known to influence further K\textsuperscript{+}/H\textsuperscript{+} channel subtypes (e.g., voltage-gated K\textsuperscript{+} channels) and Na\textsuperscript{+} channels. Therefore, we included ChTx, nominal Ca\textsuperscript{2+}-free intracellular solution, TEA, and TPNQ (Ca\textsuperscript{2+}-dependent K\textsuperscript{+}, voltage-dependent, and inwardly rectifying K\textsuperscript{+} channels) in our approach. Primary myoblast cultures isolated from both TASK2- and TREK1-knockout mice (TASK2\textsuperscript{-/-}, TREK1\textsuperscript{-/-}) would help to address this shortcoming.

Moreover, C2C12 myoblasts were still able to undergo differentiation despite the presence of quinidine or spadin, which might be related to still unknown compensatory mechanisms. In agreement with this conclusion TASK2\textsuperscript{-/-} as well as TREK1\textsuperscript{-/-} mice show no obvious impairment of motor function (28, 29).

Our results display a significant contribution of TASK2 and TREK1 to the compound potassium current and an impairment of the influx of extracellular Ca\textsuperscript{2+} into both undifferentiated myoblasts and differentiated myotubes following channel blocking. Different groups have shown that the ion channel repertoire on the membrane of myoblasts and myotubes differs markedly (5, 21). The ion channel repertoire of myoblasts is quite delicate and K\textsubscript{2P} channels seem to be present and electrophysiologically relevant starting from day 0 of differentiation according to our findings. Bernheim and Bader hypothesize that a hyperpolarization of the membrane potential is required during differentiation to cause the essential elevation of the [Ca\textsuperscript{2+}], (5). They propose that the expression of inwardly rectifying potassium channels (K\textsubscript{ir} channels) in fusion-capable human myoblasts is crucial for the activation of voltage-dependent T-type Ca\textsuperscript{2+} channels providing a basis for intracellular Ca\textsuperscript{2+}-dependent signaling pathways (2, 5, 6, 8, 9, 20, 31, 42, 46). They describe that human myoblasts have a membrane potential of around $-8 \pm 1$ mV. Ether-a`-go-go (EAG) as well as inward rectifier (K\textsubscript{ir}) potassium channels are shown to generate hyperpolarization during differentiation close to $-65$ mV and thus towards the potassium equilibrium potential (5, 8, 31, 46). Based on the present expression analysis and electrophysiological data we propose that K\textsubscript{2P} channels are also involved in the maintenance of the resting membrane potential in C2C12 cells and due to our expression studies might also play a role in the human muscle. K\textsubscript{2P} channels are required to establish a driving force for extracellular Ca\textsuperscript{2+} influx triggered by a still unknown mechanism during differentiation. Constantin et al. (19) demonstrated that particularly cholinergic actions rather than voltage-operated pathways are required for the extracellular Ca\textsuperscript{2+} influx upon myocyte fusion. Considering this hypothesis the maintenance of the differentiation in vitro raises the question of the ACh source. Different groups were able to detect ACh and nicotinic AChR activity in in vitro myoblast cultures hinting towards an endogenous autocrine mechanism (3, 27). Since the inhibition
of TASK2 as well as TREK1 leads to an impairment of muscle cell differentiation, our Ca\(^{2+}\) measurement experiments partially corroborate this hypothesis, but they do not exclude the influence of voltage-dependent Ca\(^{2+}\) channels or a modulating effect of both inhibitors on Ca\(^{2+}\) efflux. Apart from this indirect connection between K\(_{2P}\) channels and ACh/ACHR, a direct interaction might exist. TREK and TASK channels have recently been identified as novel immediate targets of muscarinic ACh receptors (MACHr) in thalamocortical relay neurons (10). Muscarinic stimulation is supposed to inactivate TREK1 and TASK channels leading to a depolarization of these neurons; similar interactions might exist in SkMCs. Further investigations on the putative interaction of K\(_{2P}\) channels and voltage-gated Ca\(^{2+}\) channels as well as the intracellular mechanisms related to TASK2- and TREK1-inhibition are required to solve this issue.

The downregulation of MYOG under K\(_{2P}\) channel blockade raises the question how TASK2 and TREK1 are involved in intracellular signaling pathways during differentiation. Xu et al. (64) have identified p38 mitogen-activated protein kinase-, Ca\(^{2+}\)-calmodulin-dependent protein kinase-, and calcineurin-mediated signaling pathways to regulate MYOG expression in C2C12 cells. Different studies have revealed that a number of Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent transcriptional factors are involved in the regulation of muscle cell fusion (38, 39). Our present findings hint at Ca\(^{2+}\) ions as a putative linkage in this context, even though Ca\(^{2+}\)-independent pathways cannot be ruled out at this stage.

The functional expression of K\(_{2P}\) channels on myotubes and murine and human muscle tissue opens up a multitude of potential future experimental routes. Our group’s latest findings on K\(_{2P}\) channels in the context of an experimental animal model of multiple sclerosis implicate immune modulatory properties for TASK1 and TREK1 with significant impact on clinical disease course. In this context, in vitro experiments showed that TASK1 influences T-cell effector functions and TREK1 is involved in the regulation of immune cell migration at the blood brain barrier (12, 13, 50). A recent investigation showed that TASK1 influences T-cell effector functions and clinical disease course. In this context, in vitro experiments model of multiple sclerosis implicate immune modulatory differentiation probably related to electrotonic and Ca\(^{2+}\) maintenance of basic cellular parameters. Our findings indicate an important role of both TASK2 and TREK1 in skeletal muscle differentiation probably related to electrotonic and Ca\(^{2+}\)-dependent cell processes. The functional expression of K\(_{2P}\) channels in SkMC paves the way for further studies in physiological and pathological muscle processes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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