Both TASK-3 and TREK-1 two-pore loop K channels are expressed in H295R cells and modulate their membrane potential and aldosterone secretion

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Both TASK-3 and TREK-1 two-pore loop K channels are expressed in H295R cells and modulate their membrane potential and aldosterone secretion. Am J Physiol Endocrinol Metab 295: E1480–E1486, 2008. First published October 14, 2008; doi:10.1152/ajpendo.90652.2008.—The rate of aldosterone synthesis by adrenal glomerulosa cells relies on the selective permeability of the glomerulosa cell to K⁺ ions. In rodent and bovine adrenal glomerulosa cells, this background potassium current is provided by a two-pore loop potassium (K2P) channel: largely TASK-3 in the rat and TREK-1 in the cow. The nature of the K2P channel in the human adrenal cortex is not known, and we have addressed this issue here using the H295R human adrenal cell line. We show that these cells express mRNA and protein for both TASK-3 and TREK-1 K2P channels. Using a potentiometric dye (FMP), we also show that TASK-3 and TREK-1 channel modulators can affect the membrane potential of H295R cells. Transfecting H295R cells with TASK-3 or TREK-1 dominant-negative mutants (TASK-3 G95E or TREK-1 G144E) produced depolarization of H295R cells and altered K⁺-permeable to K⁺, giving it the characteristics of a K⁺ electrode over a wide range of extracellular K⁺ concentrations (24). Depolarization causes a secondary rise in intracellular Ca²⁺ through a T-type calcium channel, which is thought to be the primary stimulus for increased production and release of aldosterone (19).

The basis for the high resting permeability to K⁺ was not clear until it was reported that rat adrenal glomerulosa cells express TASK-like K⁺ channels (TWIK-like acid sensing K⁺ channels; Refs. 5, 6). These channels are novel members of the two-pore loop subfamily of K⁺ channels named after the first member of the subfamily TWIK-1 (tandem of P domains in a weak inward rectifying K⁺ channel; Ref. 21). They are only distantly related to other K⁺ channels and have a distinctive topology consisting of four transmembrane segments and two potassium channel pore-forming domains (15). Despite their common topology, their amino acid sequences are very divergent outside of the P domains: TASK-1 and TASK-3 have the closest, sharing 62% homology with other members sharing only 30–35%. They also have distinct expression patterns and regulatory mechanisms. TASK-3 (also known as KCNK9 or K₂P9.1), for example, is highly expressed in rat neurones especially the cerebellar granule cells (2), but outside of the central nervous system it is restricted largely to the adrenal cortex and chemoreceptor cells of the carotid body (5, 6, 31). TASK-1 (KCNK3 or K₂P3.1) is also expressed but its functional importance in the rodent adrenal glomerulosa cell is unclear. A TASK-1 knockout mouse does show hyperaldosteronism, but this is not due to depolarization of adrenal cortical cells. In fact, the adrenals of these animals show abnormal zonation and ectopic expression of aldosterone synthase enzyme in a manner reminiscent of glucocorticoid remediable hyperaldosteronism (12). There are also marked species differences in two-pore loop K⁺ (K₂P) expression. The bovine adrenal, for example, does not express TASK-3, and the functions of this channel are instead taken over by the (distantly) related TREK-1 (9; KCNK2 or K₂P2.1). To date, which K₂P channel operates in human adrenal glomerulosa cells is not known.

To investigate the role of TASK-3 and TREK-1 in human cells, we used the adrenocortical cell line NCI-H295R, due to the limited availability of human adrenal tissue to isolate primary adrenal zona glomerulosa cells. The H295R cell line was originally derived from an adrenocortical carcinoma and grows as a monolayer (10). It expresses most of the enzymes participating in steroidogenesis in the adrenal cortex producing mineralocorticoids, glucocorticoids, and adrenal androgens (10, 27). Since H295R cells respond to both ANG II and K⁺ (1, 4, 23), they have been widely used as a model system for aldosterone secretion by human glomerulosa cells (8, 25, 26).

The lack of selective K₂P channel modulators has hampered in vitro pharmacological studies. Therefore, in this study we used a number of channel modulators in conjunction within a dominant-negative approach to knockdown channel function. This approach is based on a point mutation in the selectivity filter of TASK-3 that abolishes channel activity (22). Since K₂P channels homo- and heterodimerize to form functional
channels, the G95E TASK-3 mutant exerts a dominant-negative effect when coexpressed with wild-type (WT) TASK-3, as reported in *Xenopus* oocytes and cerebellar neurons (16, 22). The selectivity filter motif is conserved between TASK and TREK channels, so this dominant-negative approach is also possible with a mutant TREK-1 (G144E; Ref. 16).

We report here for the first time that the H295R cell expresses message and protein for both TASK-3 and TREK-1 K2P channels. Perturbing these channels by expression of TASK-3/TREK-1 selectivity filter mutants or putative activator/inhibitors of these channels produced significant effects on the membrane potential of the H295R cell measured using the voltage-sensitive dye FMP. These changes in membrane potential were accompanied by changes in ANG II and K⁺ evoked aldosterone secretion in these cells. Activation of Gq signaling that inhibits K2P in other cell types also depolarized H295R cells. Taken together, our data suggest that unlike other species the background K current in cultured human adrenal cells is modified by at least two separate K2P channels, TASK-3 and TREK-1.

**MATERIALS AND METHODS**

**RT-PCR and cloning of constructs.** Total RNA was extracted from H295R whole cell lysate using TRIzol reagent (Invitrogen, Paisley, UK). The RNA was treated with DNase I, and Superscript III reverse transcriptase (Invitrogen) was used to generate full-length TASK-3 and TREK-1 (isoform c) using gene-specific primers. Aliquots of the RT reaction were amplified with primers to produce full-length TASK-3 or TREK-1 product that was visualized by electrophoresis on a 1% agarose gel. TASK-3 was cloned into pEGFP-C2 using *EcoRI* and *SalI*, whereas TREK-1 was cloned into pEGFP-C1 using *BglII* and *SalI*. All constructs were verified by sequencing on an ABI 377 gel sequencer with BigDye chemistry before transfection into H295R cells.

**Gq constructs.** The WT α-subunit of the Gα signaling protein Gqα and the GTPase-deficient mutant of Gqα (Q209L) were purchased from the cDNA Resource Center at the University of Missouri-Rolla (http://www.cdna.org). The Gqα (Q209L) mutant is constitutively active through its defective GTPase activity that prevents reassembly into the inactive Gαβγ heterotrimer. The PLC-defective Gqα mutant (Gqα–RTAA) contains an R256T257<double line>→AA mutation introduced into the Q209L mutant and was a generous gift from D. Bayliss (University of Virginia). This double mutant produces a constitutively active but PLC-defective Gqα protein. All versions of Gqα were 1,085-bp long and cloned into pcDNA3.1+ (Invitrogen) at *KpnI* (5′) and *XhoI* (3′) sites. The constructs were verified by sequencing before transfection into H295R cells.

**Site-directed mutagenesis.** Dominant-negative mutations were introduced using QuikChange XL site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). Mutagenic primers were designed to change 95 Gly to Glu in TASK-3 and 144 Gly to Glu in TREK-1. Both mutant DNA constructs were fully sequenced to confirm the introduction of the correct mutated bases.

**Western blotting.** H295R cells were lysed in RIPA buffer containing protease inhibitor cocktail for mammalian cells (Sigma, Poole, UK). The protein concentration of the whole cell lysate was determined by BCA assay (Pierce Biotechnology, Rockford, IL). The samples were then resuspended in Laemmli sample buffer (62.5 mM Tris•HCl pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue) and heated to 95°C before loading onto a 10% SDS gel. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane via semidyry transfer, blocked (5% nonfat dried milk in PBS), and incubated with polyclonal rabbit anti-TASK-3 or anti-TREK-1 (both Alomone, Jerusalem, Israel) at 1:400 dilution (in 5% nonfat dried milk in PBS). The membrane was then incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Amersham, UK) at 1:1,500 dilution (in 5% nonfat dried milk in PBS) before proteins were visualized by ECL Plus reagents (GE Healthcare) according to the manufacturer’s instructions. To confirm the specificity of the bands seen using the Alomone antibodies, we attempted to immunoblot whole HEK cell lysates (as a negative control) and also preabsorbed the antibodies with the manufacturer’s epitope peptides (TASK-3, DDDYQLELVLQSEPHER; TREK-1, DPKSAAQSNKPLSFSTK) before immunoblotting the H295R lysates. Both of these maneuvers produced membranes without identifiable bands on ECL, confirming the specificity of the anti-TASK-3 and anti-TREK-1 antibodies.

**Tissue culture of H295R cells.** Human adrenocortical carcinoma cells (NCI-H295R) were obtained from the American Type Culture Collection (Manassas, VA). The cells were plated out according to American Type Culture Collection instructions and maintained in a 1:1 mixture of DMEM and Ham’s F-12 medium (Sigma). For routine maintenance, the medium was supplemented with 5% FCS and 100× l-glutamine-penicillin-streptomycin solution (PAA Laboratories, Pasching, Austria) to a final concentration of 2 mM l-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere of 95% O₂–5% CO₂. Before experiments involving aldosterone secretion, the medium was changed to DMEM-F-12 supplemented with 5% Nu-Serum (BD Biosciences, Oxford, UK), l-glutamine-penicillin-streptomycin solution, 10 μg/ml insulin, 5.5 μg/ml transferrin, and 5 ng/ml selenium (ITS supplement, Sigma) at least 48 h before the start of the experiment.

**Transient transfection.** Cells were seeded into 24-well plates in standard medium without antibiotics at least 24 h before transfection with either pEGFP-C1 (as a control to monitor transfection efficiency) or the appropriate construct using Lipofectamine 2000 and Opti-MEM I reduced serum medium (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. Green fluorescence was detected by fluorescence microscopy (Olympus IX17 microscope) 24–48 h after transfection. To confirm membrane localization of the K2P channels in preliminary experiments, the cells were co-transfected with DsRed2-Mem (gift from Dr. Regina Preisig-Müller, University of Marburg) in which a plasma membrane marker is fused to DsRed2.

**Aldosterone RIAs.** The aldosterone concentration in the cell culture medium was determined by the Coat-A-Count [¹²⁵I]alderosterone RIA (Diagnostic Products, Los Angeles, CA). In brief, 200 μl of cell culture medium were added to aldosterone antibody coated tubes and incubated with 1 ml of iodinated aldosterone solution before the tubes were counted on a Perkin Elmer Cobra II Auto γ-counter (PerkinElmer, Waltham, MA). Results were normalized by measuring protein content per well by BCA assay (Pierce Biotechnology, Rockford, IL).

**Membrane potential assays using FLIPR system.** Potentiometric experiments were performed using the commercially available FLIPR (Fluorometric Imaging Plate Reader) membrane-potential assay kit from Molecular Devices (Sunnyvale, CA). This FMP dye system (the chemical identity of which is not declared) is optimized for use with the FlexStation plate reader and the SoftMax Pro software (all Molecular Devices). H295R cells were incubated for 30 min with FMP dye according to the manufacturer’s instructions in assay buffer (4 mM KCl, 2.3 mM CaCl₂, 5 mM NaHCO₃, 1 mM MgCl₂, 150 mM NaCl, 5 mM HEPES, and 5.5 mM glucose). The membrane potential assays were performed at room temperature. The channel modulators fluoxetine, fluropirielone, ruthein red, and spermine were all purchased from Sigma and added directly to the assay buffer.

**Data analysis.** Differences between groups were compared using one-way ANOVA with post hoc testing. Figures 1–6 show data as means ± SE unless stated otherwise. The SPSS statistical package (software version 11) was used throughout with significance defined as P < 0.05.
RESULTS

Expression of TASK-3 and TREK-1 mRNA and protein. Total RNA was extracted from H295R cells and subjected to RT-PCR to generate full-length TASK-3 and TREK-1 (isoform b). The bands at 1.1 kb for TASK-3 and 1.25 kb for TREK-1 show that both channel mRNAs are expressed in H295R cells (Fig. 1A). To determine the expression of the channels at the protein level, Western blotting was performed using H295R whole-cell lysate and rabbit anti-TASK-3 and anti-TREK-1 antibodies. Figure 1B shows that protein bands for both TASK-3 and TREK-1 at 50 and 45 kDa, respectively, were detected. RT-PCR was also performed on human adrenal cortex to show mRNA expression of both TASK-3 and TREK-1 in this tissue (Fig. 1C).

Effect of KCl and ANG II on membrane potential of H295R cells. The FMP fluorescence from H295R cells increased after depolarization with KCl or ANG II (Fig. 2). The normalized change in fluorescence (ΔF/F₀) showed the expected log-linear relation to the extracellular concentration of K in keeping with its behavior as a K electrode (Fig. 2). ANG II depolarized the cells within the physiological picomolar range after an initial hyperpolarization. The addition of the selective ANG II type 1 receptor antagonist losartan (1 μM) completely abolished the ANG II-induced depolarization, confirming that it was mediated through this receptor (Fig. 2). Losartan did not affect the initial transient hyperpolarization, confirming that it is not mediated by ANG II type 1 receptor activation. The molecular basis for the hyperpolarizing response to ANG II is not clear, but it has been reported (24) before in clamped rodent glomerulosa cells. At higher concentrations of ANG II (>1 nM), there appeared to be rapid receptor tachyphylaxis in the H295R cell (Fig. 2).

Effect of K2P channel modulators on baseline and depolarization-induced fluorescence of H295R cells. A number of putative K2P channel modulators have been used in previous studies (11, 14, 17, 20), although their specificity is limited. Here, we used the TREK-1 inhibitors fluoxetine (10 μM) and fluspirilene (1 μM), as well as the TASK-3 inhibitors ruthenium red (10 μM) and spermine (200 μM), to investigate their effects on membrane potential of H295R cells. All inhibitors were added to the assay buffer, and cells were incubated for 30 min before measurements with the FMP dye. Halothane, a volatile anesthetic has been found to activate K2P channels and was used at a concentration of 500 μM added immediately before measurement of fluorescence with FMP dye. Both fluoxetine and fluspirilene depolarized the cells, as indicated by an increase of baseline FMP fluorescence compared with control cells (Table 1). When the cells were further depolarized by addition of 14 mM KCl, this produced a greatly reduced fluorescence response in cells treated with fluoxetine and fluspirilene. Ruthenium red also caused an increase in baseline fluorescence with the expected reduced response to KCl on further depolarization. Spermine on the other hand did not produce any significant changes in baseline fluorescence or response to K⁺. Halothane significantly decreased baseline fluorescence, suggesting hyperpolarization and the response to KCl increased as expected.

Effect of transfection with TASK-3 and TREK-1 constructs on membrane potential and aldosterone secretion of H295R cells. To explore the role of TASK-3 and TREK-1 in the H295R cell, we selectively targeted each channel by transfection with constructs that expressed either the WT and GE mutants of TASK-3 or TREK-1. The effect of these interventions on FMP fluorescence is shown in Fig. 3. Expression of either K2P channel produced the same pattern: the WT channel hyperpolarized the cell and the dominant-negative GE mutant produced a small but significant depolarization (Fig. 3). Given the role of membrane potential in regulating aldosterone secretion in the glomerulosa cell, we also measured aldosterone secretion in these transfected H295R cells. The effect on basal and secretion stimulated with either ANG II (1 nM) or KCl (14 mM) is shown in Fig. 4. Basal aldosterone secretion was not
significantly affected, but the effect of both ANG II and KCl was blunted by the GE mutants and enhanced by expression of WT channels. This is in keeping with their effects on basal membrane potential.

**Effect of G_{q} on membrane potential and aldosterone release by H295R cells.** Because K2P channels are frequently inhibited by activation of the G_{q} signaling pathway in many cells, we investigated whether constitutive G_{q} expression could affect membrane potential and aldosterone release in the untransfected H295R cell. Hence, we transfected H295R cells with constructs expressing either WT, constitutively active G_{q}-QL (a GTPase-deficient mutant), or a double mutant of G_{q}-QL that lacks PLC activation (RTAA). Only transfection with the G_{q}-QL mutant produced significant depolarization of the H295R cell with a 6% increase in relative fluorescence units (Fig. 5). We then went on to measure basal and ANG II-evoked aldosterone release in the transfected H295R cells expressing G_{q} constructs (Fig. 6). Transfection with G_{q}-QL resulted in more than an eightfold rise of baseline aldosterone release. Stimulation of the cells with ANG II (1 nM) produced a further increase of ~40% in aldosterone release. Transfection with either WT or the RTAA G_{q} mutant had no effect on basal or ANG II induced aldosterone release in keeping with their lack of effect on membrane potential.

**DISCUSSION**

In rodent and bovine glomerulosa cells, the background leak current appears to be controlled in large part through a single K2P channel: TASK-3 and TREK-1, respectively. Our work shows that in the human H295R cell both of these K2P channels are expressed and probably functionally active. Hence, the membrane potential of the H295R can be perturbed by drugs that are known modulators of these K2P channels as well as by the expression of specific dominant-negative mutant forms of the channel subunits. The drugs we used (Table 1) have low selectivity for TASK-3 vs. TREK-1 channels, which prompted us to use of a molecular approach to target them selectively. The TASK-3 and TREK-1 channels are sufficiently different structurally that they do not heterodimerize, although the region of the selectivity filter is highly conserved. In fact, *Xenopus* oocytes even expressed at 100-fold excess the TASK-3 and TREK-1 channels are sufficiently different from each other. Hence, the effects of

Table 1. Effect of various K2P channel activators and inhibitors on the baseline FMP fluorescence and its response to KCl-induced depolarization

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect: activates (+) or inhibits (-)</th>
<th>Concentration, mM</th>
<th>ΔBaseline RFU</th>
<th>Change ΔF/F_0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>TREK1 (-)</td>
<td>10</td>
<td>↑ 39±5*</td>
<td>↓ 83±7*</td>
</tr>
<tr>
<td>Fluspirilene</td>
<td>TREK1 (-)</td>
<td>1</td>
<td>↑ 13±5*</td>
<td>↓ 31±11*</td>
</tr>
<tr>
<td>Halothane</td>
<td>TREK1 (+), TASK3 (+)</td>
<td>500</td>
<td>↓ 10±3*</td>
<td>↑ 23±1*</td>
</tr>
<tr>
<td>Ruthenium red</td>
<td>TASK3 (-)</td>
<td>1</td>
<td>↑ 19±4*</td>
<td>↓ 11±10</td>
</tr>
<tr>
<td>Spermine</td>
<td>TASK3 (-)</td>
<td>200</td>
<td>↑ 3.9±2.1</td>
<td>↓ 4.9±1.4</td>
</tr>
</tbody>
</table>

Changes shown are means ± SE for n = 4–6. K2P, two-pore loop potassium; ΔF/F_0, normalized change in fluorescence; RFU, relative fluorescence units. *P < 0.05, significant differences vs. control.
the GE mutants we report here in H295R cells are likely to reflect channel specific K2P knockdown.

Voltage-sensitive dyes, such as DiSC3, have been used to measure membrane potential in rat adrenal glomerulosa cells (13, 29), but this required cell suspensions and was cumbersome to perform. The proprietary dye used in this study (FMP) has better response characteristics than DiSC3 and in combination with the Flexstation allows measurement of the membrane in cultured cells without the need to detach them. In our hands, ANG II and KCl, which both depolarize rat and bovine glomerulosa cells, produced an easily detectable increase in FMP dye fluorescence. The FMP dye has also been used to successfully probe TASK-3 channel behavior in cultured embryonic fibroblast cells (22). This together with the response of the FMP signal to manipulation of TASK-3/TREK-1 function suggests that FMP provides a reliable indicator of the H295R membrane potential.

Transfecting the GE selectivity filter mutants produced a relatively small depolarization compared with the hyperpolarization seen with expression of the WT channel subunits. This deserves some comment, as it was not explained by differences in transfection efficiency. The subunits were expressed as green fusion proteins that could be easily monitored, and transfection efficiencies were routinely in the range of 60–80% for all of the K2P constructs. It may reflect slow native K2P channel turnover, because the GE mutants must dimerize...
with native K2P WT channel subunits to exert their effect. In contrast, over-expressed WT K2P subunits will dimerize even in the absence of native WT subunits and are presumably rapidly trafficked to the cell surface. The membrane potential of the H295R cell membrane as predicted by the Goldman-Hodgkin-Katz equation is also related in a nonlinear way to the $K^+$ conductance. Hence, even large changes in resting $K^+$ permeability may produce relatively small perturbations in the membrane potential, especially if the baseline $K^+$ permeability is large compared with $Na^+$ and $Cl^-$. By the same token, if transfection with the WT and GE mutants produces quantitatively the same but opposite effects on K2P channel density at the cell surface, this would not necessarily produce equal but opposite changes in membrane potential. We have not looked extensively at later time points after transfection or the effects of expressing combinations of the mutants. Hence, if the GE constructs continue to be expressed it is likely that the degree of depolarization would increase with time.

The K2P channels are inhibited by $G_{oq}$ coupled receptor activation in many cell lines, especially those of the central nervous system origin (18). Our results here with the transfection of the $G_{oq}$-QL mutant suggest that a similar mechanism may operate for K2P channels in H295R cells. Exactly how the $G_{oq}$ signaling protein inhibits TASK and TREK channels is not clear cut and probably varies with cell type. Some authors (28) have suggested that the secondary depletion of membrane PIP2 by $G_{oq}$ is the key step. However, PKC activation and direct phosphorylation of the COOH terminus of TASK channels have been highlighted by others (30). It is also reported that in some cells the G protein subunit is able to interact directly with TASK channels (3). In our hands, the transfection with constitutively active double $G_{oq}$ RTAA mutant that lacks PLC activity cannot mimic the effect of the $G_{oq}$-QL mutant, suggesting that PLC is necessary for its effects on membrane potential in the H295R cell.

The GE mutants despite producing depolarization of H295R cells did not affect basal aldosterone production. This is probably because of the relatively small reduction in the membrane potential produced. The change in FMP fluorescence was certainly small compared with either ANG II or the KCl concentrations used to depolarize H295R cells (Fig. 1). The $G_{oq}$-QL transfected cells did show larger changes in relative fluorescence units, presumably representing a larger depolarization. The elevated basal aldosterone secretion from these cells suggests that depolarization may in fact have exceeded the threshold for activation of aldosterone release in these cells. Of course, the constitutively active $G_{oq}$ may have triggered secretion by a pathway independent of any effect on the membrane potential. However, both the WT and the PLC-deficient $G_{oq}$ (RTAA) did not affect FMP fluorescence or the basal release of aldosterone, suggesting that aldosterone production does occur in parallel with depolarization per se. It is also pertinent that in double knockout TASK mice the elevation of plasma aldosterone is accompanied by substantial depolarization (~20 mV) of isolated glomerulosa cells from these mice (7).

Assuming the H295R is a robust model of the glomerulosa cell, then both K2P channels would be predicted to contribute to the human glomerulosa cell background leak current. We cannot of course exclude other K2P channels as being involved such as TASK-1, but the evidence to date that they regulate even rodent background current is lacking. Our finding that human adrenal cortex expresses both TASK-3 and TREK-1 (message (Fig. 1C) suggests that our findings in H295R cells may also be applicable to adrenal glomerulosa cells in vivo. Further work is ongoing but limited by the availability of fresh human adrenal tissue. Nevertheless, our work reported here identifies TASK-3 and TREK-1 as important regulators of the resting membrane potential of the cultured H295R cell that is widely used as a model of the human adrenal cortical cell.

REFERENCES


