Combined Antisense and Pharmacological Approaches Implicate hTASK as an Airway O₂ Sensing K⁺ Channel*

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Neuroepithelial bodies act as airway oxygen sensors. The lung carcinoma line H146 is an established model for neuroepithelial body cells. Although O₂ sensing in both cells is via NADPH oxidase H₂O₂/free radical production and acute hypoxia promotes K⁺ channel closure and cell depolarization, the identity of the K⁺ channel is still controversial. However, recent data point toward the involvement of a member of the tandem P domain family of K⁺ channels. Reverse transcription-polymerase chain reaction screening indicates that all known channels other than hTWIK1 and hTRAAK are expressed in H146 cells. Our detailed pharmacological characterization of the O₂-sensitive K⁺ current described herein is compatible with the involvement of hTASK1 or hTASK3 (pH dependence, tetraethylammonium and dithiothreitol insensitivity, blockade by arachidonic acid, and halothane activation). Furthermore, we have used antisense oligodeoxynucleotides directed against hTASK1 and hTASK3 to suppress almost completely the hTASK1 protein and show that these cells no longer respond to acute hypoxia; this behavior was not mirrored in liposome-only or missense-treated cells. Finally, we have used Zn²⁺ treatment as a maneuver able to discriminate between these two homologues of hTASK and show that the most likely candidate channel for O₂ sensing in these cells is hTASK3.

Oxygen-sensitive K⁺ channels are found in numerous tissues, where they regulate cellular function in the face of reduced or increased pO₂ (1, 2). One such tissue is the airway of the lung, where neuroepithelial bodies (NEBs) are believed to be involved in optimizing ventilation-perfusion matching via local vasoconstriction and ascending input to medullary respiratory centers (3). At the cellular level, NEBs have been suggested that in native neonatal rabbit NEB cells, Kv3.3 may be involved in O₂ transduction (10). This channel has also been identified in small cell lung carcinoma cell lines (including H146) at the mRNA level. Kv3.3 expressed in Xenopus oocytes has been shown to be sensitive to a downstream product of NADPH oxidase activity, H₂O₂ (10). However, this channel has not been shown to influence resting membrane potential, and other cell types (e.g. carotid body glomus cells) are known to possess multiple O₂-sensitive K⁺ channels (12, 13). Therefore, although Kv3.3 may indeed be an O₂-sensitive K⁺ channel, there are a number of lines of evidence (physiological, molecular, pharmacological and comparative) to suggest that hypoxia-evoked depolarization, and hence the physiologically more important response, may be underpinned by closure of a member of the tandem P domain K⁺ channel family (Kᵥ₃.₃), namely the acid-sensitive TASK gene product. These lines of evidence include 1) our earlier observation of differential amplification of mRNA encoding hTASK1 (over hTWIK1) from H146 cells by RT-PCR (7); 2) the observation that rTASK1 mRNA has been detected by in situ hybridization and implicated in O₂ signal transduction in another important O₂-sensing tissue, the carotid body (12); 3) hypoxia-evoked cellular depolarization in H146 cells is almost insensitive to the broad spectrum K⁺ channel blocker tetraethylammonium (TEA) at concentrations well above those documented to inhibit most voltage-sensitive K⁺ channels, including Kv3.3 (8); and 4) O₂-sensitive K⁺ currents of H146 cells (as well as hypoxia-evoked cell depolarization in this cell model), isolated NEB cells, and NEB cells in lung slice are 4-aminopyridine-insensitive but completely blocked by quinidine (7). Because Kᵥ₃.₃ channels are known to be active at (and so be major determinants of) resting membrane potential (14), they are attractive candidate channels that could link to NADPH oxidase in both NEBs (5) and small cell lung carcinoma cells (6). However, our previous suggestion that hTASK was the O₂-sensitive channel in H146 cells can be criticized on the basis of our initial failure to demonstrate pH sensitivity of the K⁺ current (7). In our initial studies, we conducted all pH dependence experiments in the presence of Cd²⁺ in order to remove any Ca²⁺-activated K⁺ current.
contamination from the whole cell conductance recordings. However, interpretation of these data is confounded by the suggestion that there is competition between Cd$^{2+}$ and H$^+$ when binding to a number of different channel types, including Cl$^-$ (15) and K$^+$ channels (16). To investigate this possibility in H146 cells, we have revisited the pH sensitivity of their whole-cell currents and demonstrated that in the absence of external Cd$^{2+}$, the O$_2$-sensitive K$^+$ current is indeed depressed by lowering pH, consistent with TASK contributing significantly to O$_2$ sensing in these cells.

To date, many investigations have concentrated on cloning and characterizing tandem P-domain channels in recombinant systems (see e.g. Refs. 14 and 17–20), and limited headway (12, 21) has been made in demonstrating directly the involvement of this emerging class of ion channels in cell physiological function of tissues or cells. To this end, and to test directly the hypothesis that hTASK is an O$_2$-sensitive K$^+$ channel in this model cell line (and, by inference, in NEB cells), we have used RT-PCR to screen for K2P channel mRNA and employed antisense oligodeoxynucleotides as a method by which to reduce/block hTASK transcription/translation. These data, in combination with a sequential pharmacological approach using compounds that can discriminate between different members of the K$_{2p}$ class of channels specifically, and other K$^+$ channels in general, indicate hTASK3 is the O$_2$-sensitive K$^+$ channel in this model for airway chemoreceptors and represents a significant advance in our understanding of the molecular basis of ion channel O$_2$ sensing.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The small cell lung carcinoma cell line, H146, was purchased from American Tissue Type Cell Collection (Manassas, VA) and was maintained in culture using the same regimen as previously described (6).

**RT-PCR Screening for mRNA Encoding Human K$_{2p}$ Channels**—Total RNA was extracted from pelleted H146 cells using the RNeasy Mini Kit (Qiagen, Crawley, West Sussex, United Kingdom) and treated with RQ-1 RNase-free DNase (1 units $\mu$g$^{-1}$ RNA; Promega, Southampton, Hampshire, United Kingdom) to remove genomic DNA contamination, before reextraction using the RNeasy mini kit. The yield, purity, and integrity of the RNA was verified by spectrophotometry at 260/280 nm, followed by electrophoresis on 1% agarose, and high quality RNA was then stored in aqueous solution at $-80 \degree C$.

Reverse transcription was performed on 1-$\mu$g aliquots of RNA using the reverse transcription system A3500 (Promega), comprising avian myeloblastosis virus reverse transcriptase and oligo(dT) (15) primers (42 $\degree C, 15$ min). The resulting cDNA was amplified by PCR using a panel of oligonucleotide primer pairs (described in Fig. 2B) designed against the published sequences of the human homologues of TASK1 (GenBank accession number AF006823), TASK2 (AF084830), TASK3 (AC007869), TREK1 (AF004711), TREK2 (XM012342), TWIK1 (U90065), TWIK2 (AF117708), and TRAAK (XM006543). Amplification of 1 $\mu$l of cDNA (equivalent to 160 ng of reverse-transcribed RNA) was performed using...
a Hybaid Express thermal cycler (Ashford, Middlesex, United Kingdom), in a volume of 50 μl, containing 1 μl of Advantage-GC2 polymerase (Promega), under optimized conditions. The hot-start PCR protocol was 94 °C for 1 min, X °C for 1 min, and 72 °C for 1 min for 35 cycles with a final extension period of 10 min at 72 °C. Optimized annealing temperatures (X °C) were gene-specific and are shown in Fig. 2B. Products were sequenced on 2% agarose gels and visualized with ethidium bromide/UV transillumination. Sequencing of all amplicons was carried out by dye terminator PCR with an ABI PRISM automated sequencer (School of Biology, University of Leeds, United Kingdom) and allowed verification of the identity of each PCR product.

Antisense and Missense Oligodeoxynucleotide Design and Application—H146 cells were transfected with either 1) LipofectAMINE only, 2) 5'-FITC-labeled, phosphothioate-modified antisense (sequence 5'-ggctgccgctcttcg-3'; Genosys Biotechnologies, Pampisford, Cambridge, United Kingdom) directed across the translation start site of human TASK1 and TASK3, or 3) 5'-FITC-labeled, phosphothioate-modified missense (sequence 5'-cgccgctcttcg-3'; Genosys Biotechnologies), which consisted of the same bases as employed in the antisense probe but in “random” order (see Fig. 2C). We confirmed, by PCR and sequencing, that the first 115 base pairs of H146 cell hTASK1 and the first 125 base pairs of hTASK3 open reading frames were 100% identical to the published sequences. This allowed the design of an antisense oligodeoxynucleotide probe that spanned the atg start codon of both sequences (nucleotides 123–126 of TASK1 and 94–97 of TASK3), a region of the gene known to be a useful target functionally for antisense maneuvers in other systems (see Ref. 22 for a review of technical considerations). TASK1 and TASK3 exhibit high sequence identity that was impossible. Therefore, the probe that we employed in this study did not distinguish between TASK1 and TASK3; thus, a functional approach was combined with this molecular abrogation technique for final channel identification. The missense sequence did not recognize any known sequences available in GenBank™.

Cells were seeded in six-well plates at a density of 2 × 10⁵ cells/well in 0.8 ml of serum-free RPMI 1640 medium (Life Technologies, Inc.). The oligodeoxynucleotides were diluted in 0.1 ml of serum-free RPMI 1640 medium and mixed with 0.1 ml of 6% (v/v) LipofectAMINE (Life Technologies, Inc.) in serum-free RPMI 1640 medium. The resulting oligodeoxynucleotide and cationic lipid mixture was incubated at room temperature for 30 min to allow formation of DNA-liposome complexes, which were then added (0.2 ml) to the cell suspension, mixed gently, and incubated in a humidified atmosphere of 5%CO₂/95% air at 37 °C for 4 h. Following incubation, 4 ml of complete RPMI 1640 medium (8) was added to each well. Cells were then cultured as normal for up to 5 days. The concentrations and time course of transfection were followed by measuring cellular/nuclear FITC fluorescence incorporation and were shown to be optimal at 1 μM and 4–5 days, respectively (data not shown).

Four to 5 days after lipofection, H146 cells (approximately 5 × 10⁶ cells) were cytospun onto glass poly-L-lysine-coated microscope slides for 5 min at 1200 rpm. Cells were heat-fixed by placing the slides on a hot plate for 10 s and then fixed in 10% neutral-buffered formalin for 5 min at 37 °C. The remaining procedures were carried out at room temperature. Cells were permeabilized by incubation with 0.5% Triton X-100 in PBS for 15 min, refixed in 10% formalin for 5 min, washed in PBS for 10 min, and then incubated with blocking solution (10% v/v fetal calf serum, 0.1% (w/v) bovine serum albumin and 0.01% (w/v) NaN₃, in PBS) for 1 h. Cells were then incubated overnight in 1:500 dilution of rabbit anti-hTASK1 antibody (Alomone Labs, Jerusalem, Israel) in blocking solution. Following the antibody incubation, cells were washed three times in PBS and incubated in TRITC-labeled anti-rabbit antiserum for 3 h. Finally, cells were washed an additional three times in PBS prior to mounting and viewing. Specificity of immunoreactivity was confirmed by an antigen preadsorption step using the peptide to which the antibody was originally raised. The preadsorption step employed 1.2 μg of peptide per 1 ml of diluted antibody and was

![Figure 2](http://www.jbc.org/Downloaded from http://www.jbc.org/)}
solution was Na embedded in a 3M KCl/3% agarose bridge in order to minimize junction the appropriate pH (6.0 or 7.4) using NaOH, and the bath electrode was NaOH. In the pH dependence experiments, solutions were brought to

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\text{A (n = 6), arachidonic acid blockade of hypoxic inhibition (B) (n = 9), and dithiothreitol insensitivity of the hypoxic suppression of K}\text{' currents (C) (n = 6). Application of hypoxic perfusate and pharmacological agents is indicated by horizontal bars.}
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Fig. 3. Pharmacological manipulation of K\text{' currents in H146 cells—Mean (± S.E.), normalized time series plots of K\text{' current amplitudes evoked at 0 mV by the ramp protocol (shown in Fig. 1A) demonstrating transient halothane reversibility of hypoxic inhibition (A) (n = 6), arachidonic acid blockade of hypoxic inhibition (B) (n = 9), and dithiothreitol insensitivity of the hypoxic suppression of K\text{' currents (C) (n = 6). Application of hypoxic perfusate and pharmacological agents is indicated by horizontal bars.}

Electrophysiology—Unless stated otherwise, all chemicals for whole-cell patch-clamp were of the highest grade available and were purchased from Sigma. Pipette solution was K\text{'-rich and contained 10 mM NaCl, 117 mM KCl, 2 mM MgSO\text{4}, 10 mM HEPES, 11 mM EGTA, 1 mM CaCl\text{2}, 2 mM Na\text{2}ATP, pH 7.2, with KOH; free [Ca\text{2+}] = 27 nm. Bath solution was Na\text{'-rich and contained 135 mM NaCl, 5 mM KCl, 1.2 mM MgCl\text{2}, 5 mM HEPES, 2.5 mM CaCl\text{2}, 10 mM \text{n-glucose, pH 7.4, with NaOH. In the pH dependence experiments, solutions were brought to the appropriate pH (6.0 or 7.4) using NaOH, and the bath electrode was embedded in a 3 M KCl/3% agarose bridge in order to minimize junction potentials; these were routinely measured and never exceeded 0.3 mV for the maneuvers presented here. All tubing was gas-impermeant (Tygon tubing, BDH, Atherstone, Berkshire, United Kingdom). Normoxic solutions were equilibrated with room air. Solutions were made hypoxic, where appropriate, by bubbling with N\text{2} (q) for at least 20 min prior to perfusion of cells. This procedure produced no shift in pH. Solution flow rate was approximately 5–7 ml/min\text{'}. pO\text{2} was measured (at the cell) using a polarized (~800 mV), calibrated carbon fiber electrode (23); for the experiments reported herein, the pO\text{2} values were 150 (normoxia) and 15–25 (hypoxia) mm Hg. Cells were allowed to adhere at 37 °C for at least 1 h to poly-l-lysine-coated glass coverslips before being placed in a perfusion chamber mounted on the stage of either a Nikon TMS or a Olympus CK40 inverted microscope. All experiments were carried out at 22 ± 1 °C. Patch pipettes were manufactured from standard-walled borosilicate glass capillary tubing (Clare Electro-medical Instruments, Reading, Berkshire, United Kingdom) on a two-stage Narishige PP-83 pipette puller (Narishige Scientific Instrument Laboratory, Kasuya, Tokyo, Japan), were heat-polished on a Narishige microforge, and had measured tip resistances of 5–8 MΩ (when filled with K\text{'-rich pipette solution).

Resistive feedback voltage-clamp was achieved using an Axopatch 200 A amplifier (Axon Instruments, Foster City, CA). Voltage protocols were generated and currents were recorded using pClamp 6.05 or pClamp 8 software employing Digidata 1200 or 1310 A/D converters (Axon Instruments). Data were filtered (4-pole Bessel) at 1 kHz and digitized at 5 kHz. Following successful transition to the whole-cell recording mode (24), capacitance transients were compensated for and measured. To evoke ionic currents in H146 cells, a ramp voltage protocol was used (Vh = −70 mV, 0.1 Hz) as shown in Fig. 1A. The magnitude of the steady-state outward currents was measured from the 0 mV step, and current-voltage relationships were constructed from the ramp. Statistical comparisons were made using the paired or unpaired Student\text{'s t test, as appropriate, with p < 0.05 being considered significant.

RESULTS

Hyposia-sensitive Currents: Effects of TEA—Fig. 1A shows exemplar currents elicited in voltage-clamped H146 cells during the ramp voltage protocol (shown below) before and during reduction in bath pO\text{2} from 150 mm Hg to between 15 and 25 mm Hg. This response is consistent with our earlier observations of hypoxic K\text{' current suppression in this cell line (6–8). Sensitivity to blockade of currents by the broad spectrum K\text{' channel inhibitor, TEA, is a maneuver capable of discriminating between specific K\text{' channels. For instance, members of the shaw K\text{' channel family (which includes Kv3.3) are highly sensitive to the actions of TEA and characteristically are blocked maximally by 1 mM (25, 26), whereas TASK channels are essentially unaffected at this concentration (14, 27–29). We have previously shown that hypoxic depolarization in current-clamped H146 cells is unaffected by 10 mM TEA (8) and extend this observation to cells under voltage-clamp. 1 mM TEA caused 30.9 ± 7.2% (n = 10) inhibition of the total K\text{' current (consistent with the concentration-response data that we have previously reported (8)). In the continuing presence of 1 mM TEA, hypoxic depression of the K\text{' current was not significantly different from that in the absence of inhibitor (Fig. 1, A and B). This is quantified in the correlation plot of Fig. 1C, where the mean hypoxia-sensitive currents were 20.1 ± 5.8 pA in control solution and 15.9 ± 5.0 pA in 1 mM TEA (p > 0.15, n = 10). This observation essentially discounts the possibility that the TEA-sensitive Kv3.3 is a significant O2-sensitive K\text{' channel under these experimental circumstances in this cell line. Relative insensitivity to TEA (shown here) and 4-aminopyridine (shown in Ref. 7) and activity at resting membrane potential (shown in Ref. 8) are all hallmarks of K2P channels; therefore, before further characterization of the current was attempted, RT-PCR screening for expression of mRNAs encoding all human K2P channels thus far cloned (with the exception of very recently identified TASK4 (29), TASK5 KT3.3 (accession numbers AF336342 and AP257061, respectively) and THK (30)) was conducted.

K\text{p Channel mRNA Expression in H146 Cells—Employing primer pairs (Fig. 2B) directed against the published sequences of human K\text{p} channels (TWIK1 and 2, TASK1, 2 and 3, TREK1 and 2, and TRAAK), only hTWIK1 and hTRAAK were not amplified from RQ1-treated, reverse-transcribed H146 cell mRNAs (Fig. 2A). This confirms our earlier observation of hTASK1 expression and extends the number of amplifiable K\text{p} channels in this cell line to six. Verification of the PCR protocols for TRAAK and TWIK1 was demonstrated by the positive control lanes using uncleaned (therefore, genomic DNA-
Fig. 4. Acidosis suppresses hypoxic inhibition of K\(^+\) currents in H146 cells. A, exemplar ramp-current traces recorded in the same cell under normoxic (−150 mm Hg) and hypoxic (15−25 mm Hg) conditions at pH\(_7.4\) and pH\(_6.0\), as indicated to the right of the records. Voltage protocol used to evoke these currents was as shown in Fig. 1A, B, exemplar time series plot of a cell under normoxic (−150 mm Hg) and hypoxic (15−25 mm Hg) conditions at pH\(_7.4\) and at pH\(_6.0\). Each point represents the current amplitude measured at 0 mV (taken from consecutive ramp depolarizations at 0.1 Hz, and the conditions were altered as indicated by the horizontal bars. C, a plot showing the correlation between the magnitude of the hypoxic response at pH 7.4 (x axis) and at pH 6.0 (y axis). Each data point (solid circles) was taken from paired experiments exemplified in B. The dotted lines indicate the no-effect and 50% inhibition levels; the latter represent the mean effect (solid square, with S.E. bars, taken from the six cells studied).

contaminated) reverse-transcribed mRNA (see Fig. 2A, TWIK\(_1\)genomic and TRAAKgenomic). All products were confirmed by sequencing.

Halothane, Arachidonic Acid, and DTT as Tools for Discriminating between K\(_{\mathrm{p}}\) Channel Members—To date, two members of the K\(_{\mathrm{p}}\) channels (TREK and TASK) have been shown to be activated by volatile anesthetics (31) and such activation may be a hallmark response of this channel family. Fig. 3A shows that during exposure of cells to hypoxia, 1 mM halothane was able to rescue the K\(^+\) currents from hypoxic inhibition (n = 7 cells), albeit transiently. The time course of current enhancement by halothane is reminiscent of the effect of another potent activator of K\(^+\) currents in H146 cells, namely H\(_2\)O\(_2\) (6). In order to refine the evidence for involvement of specific K\(_{\mathrm{p}}\) channels, we employed two further pharmacological maneuvers. First, the effects of arachidonic acid were investigated. Arachidonic acid stimulates TREK1, TREK2, TASK2, and TRAAK; is without effect on TWIK1; and inhibits TASK1 and TASK3 (see Ref. 32 for a recent review). The data presented in Fig. 3B show clearly that bath application of 20 \(\mu\)M arachidonic acid causes profound whole-cell current inhibition (of 59.9 ± 7.5%, n = 6) and completely abolishes the hypoxia-induced depression of currents normally observed in these cells.

All K\(_{\mathrm{p}}\) channels except TASKs have a conserved cysteine in the P1-M2 extracellular linker (32), and it has been firmly established (at least for TWIK) that functional dimerization through the disulfide bridge formation at this residue is imperative for full channel activity (18). It follows, therefore, that treatment of cells with a potent reducing agent should perturb the function of all K\(_{\mathrm{p}}\) channels except that of TASKs. Fig. 3C demonstrates that although 1 mM DTT causes partial inhibition of the whole-cell K\(^+\) current, the hypoxia-evoked current suppression is not significantly altered (hypoxic inhibition = 26.7 ± 6.2% in the absence of DTT versus 31.3 ± 3.5% in the presence of DTT; n = 6, p > 0.5).

Proton Sensitivity Revisited: The Effect of External pH on O\(_2\) Sensing—The evidence presented above further reinforces our earlier suggestion that TASK is an O\(_2\)-sensitive K\(^+\) channel in these cells. However, our previous failure to demonstrate pH sensitivity of the K\(^+\) current may have been due, as in other channel types, to competition between Cd\(^{2+}\) and H\(^+\) at the pH-sensitive domain. The data in Fig. 4 address this possibility and show that, indeed, in the absence of external Cd\(^{2+}\), K\(^+\) currents are suppressed in pH 6.0 by 17.5 ± 5.8% (Fig. 6, A and B). Importantly, there was a high correlation between the pH-sensitive current and the O\(_2\)-sensitive current (Fig. 6C), suggesting that they are one and the same; at pH 7.4 the O\(_2\)-sensitive K\(^+\) current was 50.5 ± 15.6 pA, whereas lowering pH to 6.0 resulted in an 50% inhibition of the O\(_2\)-sensitive current to 25.7 ± 6.6 pA (Fig. 4C, n = 6, p < 0.05). Because the proton IC\(_{50}\) of TASK3 is equivalent to pH values of between 6 and 6.5 (see, e.g. Ref. 33), this degree of inhibition is completely consistent with TASK3 as the O\(_2\)-sensitive current. In contrast, all investigators report that TASK1 is inhibited by 85–100% at pH 6.0 (14, 34–36).
Antisense Transfection and Protein Knock-down: Fluorescence and Immunocytochemistry—In order to verify and quantitate transfection efficiency of the oligodeoxynucleotides and specific protein knock-down, dual fluorescence studies were carried out. These showed that FITC-labeled antisense and missense oligodeoxynucleotides were incorporated with high efficiency. The efficiency of probe incorporation and protein knock-down was assessed through co-localization of probe and protein. Dual fluorescence images demonstrated that only antisense transfection resulted in specific hTASK-1 protein knock-down. The scale bar represents 40 μm and applies to all panels.
efficiency. Morphometry on random low power fields (see Fig. 5A for exemplar fields) from two separate transfections revealed that missense incorporation was 100% (118 of 118; Fig. 5A, panel e), whereas antisense incorporation was found in 53% (76 of 87; Fig. 5A, panel i) of cells. Fig. 5A, panel a, shows that LipofectAMINE-only-treated cells had low FITC autofluorescence. The TRITC fluorescence micrographs show that hTASK-1 protein was detectable in the majority of cells treated only with LipofectAMINE (Fig. 5A, panel b) or missense probe (89%, 105 of 118; Fig. 5A, panel f). Most striking, however, was the dramatic reduction of TRITC fluorescence (hTASK1 antibody) in cells treated with antisense incorporation (18%, 16 of 87 cells showing detectable fluorescence; Fig. 5A, panel f). This is an underestimation of the knock-down effect of antisense probe because in the dual fluorescence micrographs (exemplified in Fig. 5A, panels c, g, and k), it is clear that almost all cells that demonstrated successful transfection were TRITC-negative. That is, only 6% (5 of 87) of antisense-treated cells demonstrated dual labeling compared with 89% of missense-treated cells. Finally, the specificity of the hTASK-1 antibody employed in these studies is supported by the almost complete lack of fluorescence when the antibody was applied after preadsorption with the epitope against which it was raised (Fig. 5A, panels d, h, and l). These observations from low power micrographs are more clearly illustrated in the high power images of Fig. 5B. In this exemplar series of images, it is apparent that cells successfully transfected with missense probe are still hTASK1-positive (Fig. 5B, panels d–f), whereas those that had been successfully antisense-transfected are all hTASK1-negative (Fig. 5B, panels g–i).

Response of K⁺ Currents to Acute Hypoxia following Successful Antisense Transfection and Protein Knock-down—Capacitance measured in cells following 4–5-day antisense treatment was not significantly different (p > 0.1) from those following LipofectAMINE-only treatment or missense treatment (LipofectAMINE, 4.32 ± 0.36 pF, n = 11; antisense, 4.00 ± 0.32 pF, n = 12; missense, 3.92 ± 0.28, n = 5). In cells treated for 4–5 days with LipofectAMINE-only or missense probe, hypoxia elicited an inhibitory effect that was similar to that exemplified in Figs. 1–4 (Fig. 6, A, B, and D). In this series of experiments, a pO₂ of 15–25 mm Hg reversibly reduced mean outward K⁺ current amplitudes (measured at 0 mV from the step depolarization; see Fig. 1) in LipofectAMINE-treated cells from 130.3 ± 30.8 to 102.5 ± 23.5 pA (n = 11), a significant (p < 0.005) reduction of 22.2 ± 3.3% (Fig. 6, A and D). In missense-treated cells, there was a similar hypoxic inhibition from 100.4 ± 12.0 to 82.2 ± 11.2 pA (p < 0.005, n = 5), corresponding to a reduction of 19.5 ± 4.2% (Fig. 6, B and D). However, in antisense-treated cells, hypoxic inhibition was almost completely absent, with currents being reduced from 89.6 ± 19.6 to 88.6 ± 19.6 pA, a nonsignificant decrease (p > 0.3, n = 12) of less than 1.7 ± 2.5% (Fig. 4, C and D). Fig. 7A–C, plots typical current-voltage relationships derived from the ramp voltage-clamp protocol (see Fig. 1). Fig. 7D shows the calculated hypoxia-sensitive current ((current in control + current after wash-out)/2) – current in hypoxia). For LipofectAMINE-only-treated cells (Fig. 7A) and missense-treated cells (Fig. 7B), hypoxic inhibition was apparent at voltages positive to −40 mV (see Fig. 7D). In stark contrast, hypoxia had no effect on currents at any test potential in antisense-treated cells (Fig. 7, C and D).

Zn²⁺ as Final Discriminator—The data presented thus far cannot conclusively discriminate between hTASK1 and...
In the final series of experiments, following molecular confirmation of the involvement of one or both of these human K<sub>2P</sub> channels, we employed 100 μM Zn<sup>2+</sup> as a method of distinguishing between hTASK1 and hTASK3. Zn<sup>2+</sup> blocks both channels, but because the concentration-response curves of the two recombinant channels are an order of magnitude apart, 100 μM would be expected to cause significant inhibition of the hTASK1 current (37) while not significantly affecting hTASK3 (33). Fig. 8 shows that although 100 μM Zn<sup>2+</sup> blocked about 20% of the K<sup>+</sup> current (Fig. 8, A and B), it was an ineffective inhibitor of the hypoxic K<sup>+</sup> current suppression because hypoxia evoked a 23.1 ± 6.2% inhibition in the absence of Zn<sup>2+</sup> and 28.1 ± 9.1% in the absence of Zn<sup>2+</sup> (Fig. 8C), thus suggesting that hTASK3 is the more likely of the K<sup>+</sup> channels to be O<sub>2</sub>-sensitive in these cells.

**DISCUSSION**

The data presented herein demonstrate directly the molecular identity of the oxygen-sensitive ion channel in the chemosensing cell line H146. Relative TEA insensitivity (Fig. 1) coupled to an ability to influence resting membrane potential (8) is a characteristic exclusive to K<sub>2P</sub> channels, and we have demonstrated the mRNA encoding six members of this K<sup>+</sup> channel family in these chemosensing cells (Fig. 2). Activation by halothane (Fig. 3A), pH dependence (Fig. 4), and insensitivity to DTT (Fig. 3C) effectively rule out the involvement of all K<sub>2P</sub> channels other than the subfamily TASK (32) and blockade by arachidonic acid (Fig. 3B) points firmly toward hTASK1 or hTASK3 as the O<sub>2</sub>-sensitive channel in H146 cells. Although in other systems there have been significant steps taken to identify the O<sub>2</sub>-sensitive channel in that cell/tissue (12, 38), direct evidence that disrupting channel transcription/translation leads to loss of loss of oxygen sensitivity has not, until now, been presented. Here, we have shown that antisense knockdown of hTASK1 (and presumably hTASK3) results in a complete lack of O<sub>2</sub> sensing in this model cell line. This effect is selective, because missense treatment was without effect, dis-
rupting neither channel expression nor functional \(O_2\) sensitivity. This is the clearest evidence to date that hTASKs are of central importance in the chemotransductive pathway linking environmental \(O_2\) levels to membrane potential, \(Ca^{2+}\) influx, and hence neurosecretion in this model NEB cell line. In this regard, our findings compare well with recent, albeit indirect, evidence in the carotid body arterial chemoreceptor glomus cell, where TASK1 has been shown to be present and where the electrophysiology suggests that TASK1 underlies a component of the oxygen-sensitive \(K^+\) current (12). This class of voltage-insensitive \(K^+\) channel, which exerts important influences on membrane potential and so on electrical excitability, is therefore of fundamental importance in \(O_2\) sensing cells in general and, owing to its widespread distribution (14), is likely to be similarly important in a plethora of other cell types.

Our choice of missense and antisense probes, which was based on our PCR and gene sequence information, would appear to be appropriate. This assumption is based on the fluorescence data presented in Fig. 5 and corroborated by the electrophysiological data in Figs. 6 and 7. Thus, we have demonstrated that treatment of cells with an 18-mer antisense probe directed across the \(atg\) start codon of hTASK1 and hTASK3 results in robust, almost complete knockout of hTASK1 protein expression, which is mirrored by loss of \(O_2\) sensitivity. That this effect is specifically due to down-regulation of antisense-mediated protein transcription/translation is evidenced by the inability of the missense probe to affect either immunoreactivity or \(O_2\) sensitivity. These data also highlight two important technical issues. First, because LipofectAMINE-only-treated cells behaved in a manner comparable to both missense-treated (Figs. 6 and 7) and untreated H146 cells, it appears that in this system it may be more expedient to employ untreated cells as a suitable control. Second, optimized treatment with small oligodeoxynucleotides using LipofectAMINE is both nontoxic and an extraordinarily efficient procedure for ion channel knock-down/knockout (see Fig. 5). Interestingly, protein knock-down with the antisense probe was successful in 89% of cells (Fig. 5). This finding corresponds well with the hypoxic responses in these cells (Fig. 6). Although the mean hypoxic inhibition was negligible (approximately 2%), 1 of the 12 cells tested (and included in our analyses) responded in a manner comparable to LipofectAMINE or missense-treated cells.

Experimentally, it is unfortunate that hTASK1 and hTASK3 show such high sequence identity. Thus, design of an antisense probe that could distinguish between these two channels and that could be directed across the translation start site was impossible. Furthermore, there is no antibody available to hTASK3. Therefore, we assumed that hTASK1 immunoreactivity after hTASK1/hTASK3 antisense treatment would reflect protein knock-down of both of these channels. This assumption appears to hold true; Fig. 8 shows directly that the hypoxic inhibition was negligible (approximately 2%), 1 of the 12 cells tested (and included in our analyses) showed 50% inhibition of the \(O_2\)-sensitive \(K^+\) current, strongly suggesting that the underlying channel resembles TASK3 rather than TASK1.

Although we have accumulated a pharmacological profile for the \(O_2\)-sensitive \(K^+\) channel in H146 cells that suggests it to be hTASK3, there is a minor discrepancy in the degree of blockade by 100 \(\mu M\) quinidine that we have previously reported (79% (7)). As with the considerations of \(pH\) and the \(Zn^{2+}\) regulation outlined above, there is little convergence between laboratories or species on the effect of quinidine on hTASK. However, it is important to note that hTASK1 is blocked by less than 20% (100 \(\mu M\) quinidine (14)), whereas the same concentration evokes greater inhibition of hTASK3 (42% (28) and \(rTASk3\) (37% (33)), i.e. quinidine is a more effective blocker of TASK3 than TASK1. Our previous data show 100 \(\mu M\) quinidine to inhibit by 79% (7), a value more consistent with TASK3 than TASK1. It is important to note that our data cannot rule out the possibility that the \(O_2\)-sensitive current is carried by a heterodimer of hTASK1 and hTASK3, a possibility that may explain some of the mixed pharmacology reported here and previously. Also worthy of consideration is the possible presence of TASK5 (KT3.3), because the sequence for this putative channel shows significant homology to TASK1 and TASK3. However, our antisense probe only shares 12 of the 18 bases, a mismatch unlikely to promote good annealing. Furthermore, there are no functional data available on this candidate member of the TASK family.

In conclusion, although we have no direct evidence that the same channel underlies the hypoxic response in native NEB cells, the available comparative pharmacology suggests this to be the case. \(O_2\)-sensitive currents in both NEB and H146 cells are 4-aminopyridine-insensitive, weakly TEA-sensitive, blockable by quinidine, and have a significant calcium-independent component (7, 8, 39, 41). Thus, the present study provides direct functional evidence, through the use of pharmacology and an antisense knock-down/knockout approach, that hTASK channels are oxygen-sensitive in a native cellular system. Furthermore, hTASK1 and hTASK3 are clear candidate channels in the chemotransduction pathway in other oxygen sensing tissues, and it is tempting to speculate that their expression in diverse tissues may represent a unifying oxygen-sensitive effector protein family.

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