Human hair follicles contain two forms of ATP-sensitive potassium channels, only one of which is sensitive to minoxidil

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ABSTRACT **Hair disorders cause psychological distress but are generally poorly controlled; more effective treatments are required. Despite the long-standing use of minoxidil for balding, its mechanism is unclear; suggestions include action on vasculature or follicle cells. Similar drugs also stimulate hair, implicating ATP-sensitive potassium (K_{ATP}) channels. To investigate whether K_{ATP} channels are present in human follicles, we used organ culture, molecular biological, and immunohistological approaches. Minoxidil and tolbutamide, a K_{ATP} channel blocker, opposed each other's effects on the growing phase (anagen) of scalp follicles cultured in media with and without insulin. Reverse transcriptase-polymerase chain reaction identified K_{ATP} channel component gene expression including regulatory sulfonylurea receptors (SUR) SUR1 and SUR2B but not SUR2A and pore-forming subunits (Kir) Kir6.1 and Kir6.2. When hair bulb tissues were examined separately, epithelial matrix expressed SUR1 and Kir6.2, whereas both dermal papilla and sheath exhibited SUR2B and Kir6.1. Immunohistochemistry demonstrated similar protein distributions. Thus, human follicles respond biologically to K_{ATP} channel regulators in culture and express genes and proteins for two K_{ATP} channels, Kir6.2/SUR1 and Kir6.1/SUR2B; minoxidil only stimulates SUR2 channels. These findings indicate that human follicular dermal papillae contain K_{ATP} channels that can respond to minoxidil and that tolbutamide may suppress hair growth clinically; novel drugs designed specifically for these channels could treat hair disorders.—Shorter, K., Farjo, N. P., Picksley, S. M., Randall, V. A. Human hair follicles contain two forms of ATP-sensitive potassium channels, only one of which is sensitive to minoxidil. *FASEB J.* 22, 1725–1736 (2008)**

Key Words: alopecia · hirsutism · tolbutamide · sulfonylurea receptors SUR1 and SUR2B

HUMAN HAIR PLAYS IMPORTANT, often underappreciated, roles in social and sexual communication (1). Hair removal generally has strong depersonalizing roles, *e.g.*, ritual shaving of prisoners and monks, whereas long uncut hair has positive connotations, such as strength for Samson in the Bible. Consequently, balding, hirsutism (male hair pattern in women), and drug-stimulated abnormal hair growth cause psychological distress and reduced quality of life (2, 3). Even men with common baldness who had never sought medical help report negative effects (2). Unfortunately, our understanding of how hair follicles function is still limited and hair loss conditions, such as androgenetic alopecia or the putative autoimmune disease, alopecia areata (4), are currently poorly controlled (5). The most widely used treatment, minoxidil, only promotes hair growth in less than one-third of individuals with androgenetic alopecia (6, 7). Originally minoxidil was developed as an oral antihypertensive drug, but its frequent side effect of excessive hair growth proved to be unacceptable because of the importance of hair in social communication and led to its remarketing as a topically applied treatment for hair loss (Regaine or Rogaine). The mechanism of action of minoxidil is unclear, despite its use for >20 yr; suggestions include stimulation of skin vasculature to increase blood flow or actions within the follicle itself (8). Greater understanding of how minoxidil works should lead to more effective, tailored treatments.

Minoxidil stimulates small vellus follicles, producing tiny, virtually colorless hairs to produce longer, more visible hairs (8). Throughout life follicles pass through repeated cycles of growth (anagen), regression (catagen), and rest (telogen), during which the follicle replaces the hair with a similar one or one that differs in size and/or color, in line with changes such as sexual development or season (1). The marked variations in hair length seen on the human body reflect differences in the anagen period; anagen ranges from 2 to 5 yr for long scalp hair to a few weeks for short finger hairs, whereas the growth rate is very similar everywhere (9, 10). In androgenetic alopecia, androgens inhibit scalp follicles producing normal visible terminal hairs, causing them to miniaturize and produce vellus hairs with a much shorter anagen period (11). Systemic minoxidil causes hypertrichosis including increased hair growth

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doi: 10.1096/fj.07-099424

on the forehead, eyebrow thickening, and diffuse growth on the back and limbs in about 3 months in 60–80% of adults (12, 13), whereas topical minoxidil treatment for androgenetic alopecia causes a rapid increase in hair weight (14) and hair density (15). These results suggest that minoxidil is recruiting telogen follicles into anagen and then producing a longer hair by increasing the length of the actively growing phase of the anagen hair cycle.

Minoxidil, or rather its active metabolite minoxidil sulfate (16), diazoxide, and pinacidil reduce blood pressure by opening K_{ATP} channels in vascular smooth muscle, causing the musculature to relax (17, 18). Because minoxidil and some other, but not all, K_{ATP} channel-opening drugs, including diazoxide (19) and pinacidil (20), can cause unwanted hair growth despite their diverse structures, opening of K_{ATP} channels is probably part of the action of minoxidil, although whether the target is the vasculature or the follicle's cells is unclear. Topical minoxidil increased scalp blood flow in balding men when applied at 5% (21) but not 3% doses (22) and increased fenestrations in rat follicular capillaries (23), implicating actions *via* the vasculature. However, clinical effects are seen with even 2% minoxidil (6) and not all vasodilators stimulate hair growth, so follicular responses seem more probable. K_{ATP} channels play key physiological roles in many tissues with significant clinical implications (24–26), but whether they are present in the follicle and, if so, what role(s) they may have is unknown.

K_{ATP} channels are protein channels that control the flow of potassium ions across cell membranes in many tissues, *e.g.*, heart (24, 26, 27). Recent research has yielded much information about the structure and function of these channels. Physiologically they are regulated by intracellular levels of nucleotides, *e.g.*, ATP and ADP, thereby linking the electrical activity of a cell's membrane to its metabolism, controlling diverse cellular functions such as pancreatic insulin secretion or providing protection against heart ischemia (25–27). A diverse group of compounds can also bind to K_{ATP} channels causing them to open or close; drugs used therapeutically include sulfonylureas, *e.g.*, glibenclamide and tolbutamide, which close pancreatic channels to help control type 2 diabetes, and nicorandil, a K_{ATP} channel opener used for angina pectoris (25–27).

K_{ATP} channels consist of eight subunits arranged in two rings (**Fig. 1**). The inner ring of four inwardly rectifying K^+ channel (Kir6.X) subunits forms the pore through which potassium ions pass, whereas the outer ring comprises four regulatory sulfonylurea receptor (SUR) subunits that can alter channel activity in response to intracellular nucleotides or extracellular drugs (26). Both subunit classes are necessary to form a functional channel (28, 29). The pore subunit, Kir6.X, is found in two forms, Kir6.1 and Kir6.2, which combine with a sulfonylurea subunit. These include two isoforms of SUR, SUR1 and SUR2; SUR2 also has two splice variants, SUR2A and SUR2B (24, 26). The subunit combinations present in specific tissues vary, *e.g.*,

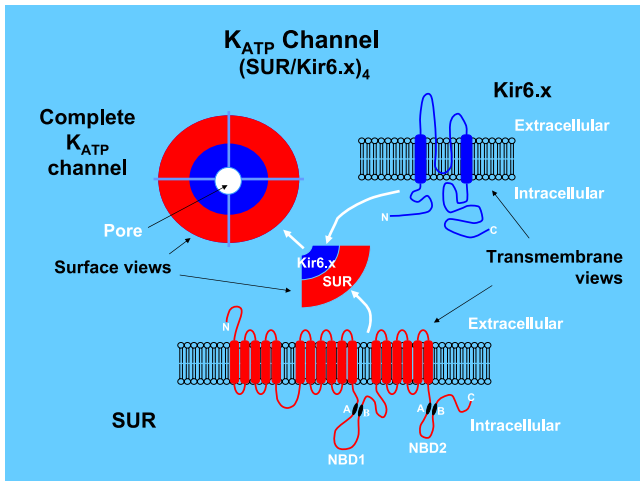

Figure 1. Diagrammatic representation of K_{ATP} channel structure. K_{ATP} channels regulate the transport of potassium ions (K^+) through cell membranes. They are formed by the combination of two types of subunit, the pore-forming inwardly rectifying subunit (Kir6.X) and the regulatory SUR. Transport of K^+ *via* the Kir6.X pore is controlled externally *via* potassium channel regulators binding to SUR or by Mg-ATP *etc.* binding to the nucleotide binding sites (NBD1 and NBD2) on the internal surface (28). Each channel consists of an outer ring of four (SUR) receptor subunits and an inner ring of four pore forming subunits, Kir6.X (Complete channel, surface views). Each subunit is a transmembrane protein (Transmembrane views). Adapted from Davies *et al.* (30).

pancreatic β cells and neurons have Kir6.2/SUR1 channels, whereas cardiac muscle has Kir6.2/SUR2A channels, nonvascular smooth muscle has Kir6.2/SUR2B channels, and vascular smooth muscle has Kir6.1/SUR2B channels (25–27). These differences mean that drugs have varying abilities to affect K_{ATP} channels in different tissues depending on their type of sulfonylurea receptor (25, 27). Therefore, elucidating what forms, if any, are present in human follicles could not only improve our understanding of hair follicle function but also facilitate the development of novel therapies better adapted to promote hair growth or more precisely focused on other tissues, avoiding unacceptable hair growth side effects.

One reason that the role of potassium channels and minoxidil is still unclear is the confusing *in vitro* results to date. Studies assessing the effects of minoxidil on cultured follicles and cells from several species have produced variable, often conflicting, results (8, 30). Initial experiments with neonatal mouse whisker follicles showed stimulation with minoxidil and some other K_{ATP} channel openers (31), but channel blockers, tolbutamide and glyburide, did not prevent this stimulation (32). Unfortunately, earlier experiments were often confused by adding serum that inhibits follicle growth (33) and/or streptomycin, which can interfere with the action of minoxidil (34). Using serum- and streptomycin-free conditions, we recently showed that minoxidil and diazoxide stimulated cultured isolated deer hair follicle growth and that K_{ATP} channel inhibitors, tolbutamide and glibenclamide, blocked their

effects (30). This finding strongly supports a mechanism *via* direct effects of minoxidil on K_{ATP} channels within follicles themselves, as cultured follicles have no vascular supply.

The effects of minoxidil on human follicles *in vitro* are less clear-cut. Minoxidil at low concentrations stimulated human follicles, an effect attributed to attenuation of the serum inhibitory effect, whereas high concentrations caused inhibition (33). Recently a carefully conducted large study involving follicles cultured without serum or streptomycin detected no effect of minoxidil (35). In contrast, small studies reported that minoxidil increased follicle growth (36, 37) or [^{3}H]thymidine incorporation, indicating cell division (38). Investigations involving cultured components of human follicles are also contradictory. Both follicular dermal papilla cells (36, 39, 40) and epidermal keratinocytes (41) responded to minoxidil *in vitro*, but potassium channels were not detected in either dermal papilla or outer root sheath cells (follicular keratinocytes) using patch-clamp techniques to investigate potassium ion transport across their membranes (42).

With the aims of establishing whether or not human hair follicles contain K_{ATP} channels, we used organ culture, molecular biological, and immunohistological approaches to investigate scalp follicles from nonbalding regions of healthy individuals. Initially, we investigated whether K_{ATP} channel regulators could alter hair follicle cycle activity in culture by observing follicle bulbs carefully every day to see any effects on the length of anagen, as this seems to be the main way in which minoxidil alters hair size. Human follicles were cultured for the first time in the presence of the K_{ATP} channel blocker, tolbutamide, to see if this would inhibit hair follicles and/or oppose any effects of minoxidil. Because Han *et al.* (36) reported increased growth in cultured follicles from three young men, rather than the older donors frequently studied, using high concentrations of minoxidil, we cultured individual anagen scalp hair follicles from young adults in serum- and streptomycin-free medium with similar high concentrations of minoxidil. These concentrations are believed to represent those reaching the follicle *in vivo* after topical treatment with 5% minoxidil (35, 36). Because phenol red (phenolsulfonphthalein), the pH indicator regularly added to standard culture media, may interfere with agents that modulate potassium channels (A. G. Messenger and M. P. Birch, personal communication, 1999) and commonly contains a number of lipophilic impurities generated during its synthesis (43), the follicular response to potassium channel modulators was also examined in parallel experiments in the absence of phenol red.

We also investigated the expression of genes for the various subunits of K_{ATP} channels using reverse transcriptase (RT) -polymerase chain reaction (PCR) to determine whether K_{ATP} channel genes are expressed in human follicles. The mRNA coding for genes actively being expressed in the lower part of individually isolated scalp anagen hair follicles from healthy younger adults was translated into cDNA before amplification by PCR using specific primers to identify genes for K_{ATP} channel subunits. RT-PCR allows detection of genes expressed at low levels in small samples of isolated hair follicles owing to extensive amplification.

To identify which genes were expressed in individual parts of the hair bulb, the regulatory dermal papilla, the epithelial hair matrix, which divides to form the hair and inner root sheath, and the dermal, or connective, tissue sheath, which surrounds the follicle, were isolated by microdissection and analyzed by RT-PCR. To confirm the presence of the actual proteins and check their location in the hair bulb, immunohistochemistry was also carried out on frozen scalp sections.

MATERIALS AND METHODS

Skin samples

Human scalp skin from nonbalding areas was obtained from healthy individuals undergoing elective cosmetic surgery operations; appropriate ethical committee approval was obtained, and donors provided written consent. For the organ culture investigations donors were young adults (1 woman and 12 men aged between 21 and 40 yr). Samples were collected into sterile tubes containing basic culture medium: William's E medium containing 10 μg/ml phenol red (Sigma-Aldrich Ltd., Dorset, UK) supplemented with 10 μg/ml insulin (Sigma-Aldrich Ltd.), 10 ng/ml hydrocortisone (Sigma-Aldrich Ltd.), 2 mM L-glutamine (Life Technologies, Inc., Paisley, UK), and 10 U/ml penicillin (Sigma-Aldrich Ltd.). They were transported on ice and stored at 4°C until hair follicles were isolated within 24 h of removal.

For the molecular biological investigations skin samples collected from occipital and parietal regions of three men (aged 26, 33, and 41 yr) and two women (aged 52 and 65 yr) were placed individually into sterile tubes containing the RNA stabilization solution, RNAlater (Sigma-Aldrich Ltd.) to inhibit RNases. They were transported on ice and kept at 4°C overnight to allow tissue penetration by RNAlater.

Isolation of hair follicles and hair bulb components

Human hair follicles in the growing phase, anagen, were microdissected individually from each skin sample under a Leica MZ8 dissecting microscope using sterile equipment and plasticware. Each sample was transferred to a Petri dish containing sterile PBS (Oxoid, Hampshire, UK) for organ culture or RNAlater at 4°C for further molecular biological studies. The sample was cut at the level of the dermal-subcutaneous fat interface using a scalpel blade. Intact anagen follicles were then gently pulled from the subcutaneous fat using fine forceps, taking care not to damage them. Isolated follicles were pooled into a fresh dish of cold PBS or RNAlater, and each follicle was gently cleaned of any attached dermis or subcutaneous fat using 27.5-gauge sterile syringe needles. Special care was taken to ensure that the follicles were not damaged during isolation as undamaged follicles are essential for successful culture (44). To localize the gene expression in the hair bulb, bulb components, the epithelial matrix, dermal sheath, and dermal papilla, were microdissected from 120 follicles from each individual before separate total RNA isolation. Components from three individuals were processed separately.

Hair follicle organ culture

Isolated human anagen hair follicles were carefully transferred to an individual well of a 24-well plate (Corning Glassworks, Corning, NY, USA) containing 1 ml of William's E medium supplemented as described below. At least six hair follicles were cultured in each type of medium for each individual. Follicles were maintained free-floating at 37°C in an atmosphere of 5% CO_2 and 95% air in a humidified incubator. Medium was changed every 3 days, taking care not to damage the follicles.

Media preparation

Basic culture medium (see Skin Samples) was supplemented with either minoxidil (1 mM; dissolved into a stock solution of William's E medium; Sigma-Aldrich Ltd.), tolbutamide [1 mM; dissolved into a stock solution of dimethyl sulfoxide (DMSO); Sigma-Aldrich Ltd.] or both minoxidil (1 mM) and tolbutamide (1 mM). Stock solutions of minoxidil and tolbutamide were dissolved using a sonicating water bath (Dawe Instruments Ltd., Middlesex, UK). Control medium was prepared without minoxidil or tolbutamide supplements, but the vehicle (0.001% DMSO) was added to each medium. All culture media were sterile-filtered (0.2 μm; Sarstedt, Nümbrecht, Germany) before use.

To investigate any effect of factors in the phenol red, human hair follicles were also grown in the presence of potassium channel modulators in phenol red-free William's E medium (Sigma-Aldrich Ltd.). The supplements added to the phenol red-free William's E media for these experiments were prepared as for the normal media described above. To investigate whether growing human hair follicles in a more basic medium would enable minoxidil to have a stimulatory effect, follicles were also cultured in basic culture medium without insulin and hydrocortisone.

Assessment of hair follicle growth in culture

Hair follicles were assessed for morphology of the follicle bulb every 24 h for 10 days, using a Leitz Labovert inverted microscope (Leitz Labovert FS, Wetzlar, Germany). Hair follicles that had not grown after 3 days were classed as nonviable and excluded. Follicles were photographed daily using a Nikon Coolpix 4500 digital camera (Nikon, Tokyo, Japan). The mean percentage of follicles in anagen per person for each treatment was determined before calculation of the sample mean. Data from each experimental group were analyzed for normal distribution using the Kolmogorov-Smirnov test. The effect of the different treatments on the percentage of follicles in anagen with time in culture was analyzed by a two-factor, within-subjects analysis of variance using the SPSS statistical analysis program (SPSS Inc., Chicago, IL, USA). If the sample means of the different experimental groups differed significantly ($P<0.05$), selected experimental group means were compared using a Student's paired *t* test with Sidak's correction for multiple comparisons. The effect of phenol red and minoxidil in the absence of insulin and hydrocortisone on the numbers of follicles in anagen was compared at day 9 using an unpaired Student's *t* test.

Molecular biological investigations

RNA preparation

Total RNA was isolated from 50 anagen terminal follicles from each individual immediately after microdissection; no telogen follicles were used. Total RNA was extracted in an area cleaned before use with 70% (v/v) ethanol and RNase Zap solution (Sigma-Aldrich Ltd.) to prevent RNase contamination, using a GenElute Mammalian Total RNA kit (Sigma-Aldrich Ltd.) following the manufacturer's instructions. The RNA quality of each sample was checked by gel electrophoresis on a 1.5% agarose gel before further purification to isolate poly(A^+)RNA (*i.e.*, mRNA) as the pigment present in hair follicles can interfere with the RT-PCR process. A GenElute mRNA Miniprep kit (Sigma-Aldrich Ltd.) was used following the manufacturer's instructions.

To remove any contaminating genomic DNA the poly(A^+)RNA samples were treated with DNase I (Invitrogen Ltd., Paisley, UK). Poly(A^+)RNA (8 μl) was mixed with 1 μl of 10× DNase reaction buffer [200 mM Tris-HCl (pH 8.4), 20 mM $MgCl_2$, and 500 mM KCI] and 1 U of DNase I amplification grade for 15 min. The DNase was inactivated by incubation with 2.5 mM EDTA at 65°C for 10 min. Samples were either placed on ice for immediate cDNA synthesis or stored at −20°C.

RT-PCR

RT-PCR was used to investigate the expression of mRNA in the K_{ATP} channel subunits (Kir6.1, Kir6.2, SUR1, SUR2A, and SUR2B) in anagen hair follicles from five individuals. DNase-treated poly(A^+)RNA was converted to cDNA using the avian myeloblastosis virus (AMV) reverse transcription system (Promega, Southampton, UK) with 1 mM concentrations of each dNTP (Promega), oligo(dT) primer (25 μg/ml), 2 μl of 5× reaction buffer [250 mM Tris-HCl (pH 8.3), 250 mM KCI, 50 mM $MgCl_2$, 2.5 mM spermidine, and 50 mM dithiothreitol], recombinant RNasin ribonuclease inhibitor (1 U/μl), and AMV RT (0.9 U/μl), made up to 10 μl with nuclease-free water. After the DNase-treated poly(A^+)RNA (10 μl) was added, the tubes were incubated in a PCR Sprint thermal cycler (Thermo Hybaid, Ashford, UK) at 42°C for 1 h to synthesize cDNA, before the RT was denatured at 99°C for 5 min, and the samples were cooled at 4°C for 5 min. The cDNA was aliquoted into 10-μl cDNA portions for storage at −20°C.

PCR amplification was performed using 3 μl of cDNA in a 50-μl reaction volume containing 0.5 μM concentrations of forward and reverse primers (Sigma-Genosys Ltd., Pamisford, UK), 200 μM concentrations of each dNTP (Promega), 5 μl of 10× reaction buffer [200 mM Tris-HCI (pH 8.4), and 500 mM KCI; Invitrogen Ltd.], 1.5 μM (Kir6.1 and Kir6.2) or 2.5 mM (β-actin, SUR1, and SUR2 reactors) $MgCl_2$ depending on primer set (Invitrogen Ltd.), and 2.5 U of recombinant *Taq* DNA polymerase (Invitrogen Ltd.). A negative control in which cDNA was replaced with nuclease-free water was run in parallel with each PCR reaction. The primers used for β-actin were modified from ref. 45 or were as used previously by other workers for SUR1 (46), SUR2A and SUR2B (47), Kir6.1 (48), and Kir6.2 (48). The following primers were used: β-actin (GenBank accession number NM_001101) forward (5′-ATCTGGCACCACACCTTCT-ACAATGAGCTGCG-3′) and reverse (5′-CTCATACTCCTG-CTTGCTGATCCACATCTGC-3′); SUR1 (AF087138) forward (5′-CGATGCCATCATCACAGAAG-3′) and reverse (5′-CTGAGCAGCTTCTCTGGCTT3′); SUR2A and SUR2B (NM_005691) forward (5′-GCTGAAGAATATGGTCAAATCTC-3′) and reverse (5′-TGGAGTGTCATATTCTAAAATA-3′); Kir6.1 (NM_004982) forward (5′-CATCTTTACCATGTCCT-TCC-3) and reverse (5′-GTGAGCCTGAGCTGTTTTCA-3′); and Kir6.2 (D50582) forward (5′-GCTTTGTGTCCAAGAAAGG-3′) and reverse (5′-CCAAAGCCAATAGTCACTTG-3′).

Each reaction mix was covered with 30 μl of mineral oil (Sigma-Aldrich Ltd.) to prevent evaporation during PCR thermocycling. Initial denaturation of cDNA at 95°C for 5

min was followed by 35 cycles of PCR amplification. Cycling conditions were as follows: for β-actin, 35 cycles of 95°C for 1 min and annealing at 55°C for 1 min and 72°C for 1 min (45); for SUR1, 94°C for 1 min, 55°C for 2 min, and 72°C for 3 min (46); for SUR2A and SUR2B, 96°C for 15 s, 55°C for 30 s, and 72°C for 78 s for 35 cycles (47); and for Kir6.1 and Kir6.2, 35 cycles of 95°C for 20 s, 52°C for 45 s, and 72°C for 1 min (48). A final extension period of 10 or 11 min at 72°C completed the thermocycling before cooling at 4°C. The PCR products were analyzed by gel electrophoresis on a 1.5% Tris-acetate-EDTA (TAE) agarose gel (Invitrogen Ltd.) containing 0.25 μg/ml ethidium bromide run in TAE buffer containing 0.5 μg/ml ethidium bromide at 100 V. PCR gene products were visualized and photographed using the UVitec gel documentation system (UVitec Limited, Cambridge, UK) at 312 nm wavelength using the DNA ladder to estimate gene product size.

Sequencing of PCR products

To confirm the identity of the base pair products from the PCR reactions, the PCR process was repeated with thin-walled PCR tubes (VWR International Ltd., Poole, UK) using the thermocycler with the hot lid so that mineral oil, which may interfere with the sequencing, could be omitted. The products were separated on a low-melting point agarose gel (Invitrogen Ltd.), excised, and purified using the MinElute Gel Extraction kit (Qiagen, Crawley, UK) following the manufacturer's instructions and sequenced by Geneblitz (Sunderland, UK). Sequencing results were compared with the known published sequences using the National Center for Biotechnology Information BLAST program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

Immunohistochemistry

Immunohistochemistry was performed to confirm protein expression and to localize K_{ATP} channel subunits in the hair bulb. Five-micrometer cryosections of human occipital scalp were mounted on poly-L-lysine-coated slides. The sections were air-dried at room temperature for 1 h and subsequently fixed in ice-cold acetone for 10 min followed by rehydration in PBS for 10 min. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide in methanol for 30 min followed by a brief rinse in PBS. Potential nonspecific binding was blocked using 5% normal mouse serum (Sigma-Aldridge Ltd.) in PBS for 20 min. Sections were incubated with the appropriate primary polyclonal goat antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 18 h in a moist chamber to avoid dehydration. Antibodies were diluted in 1.5% normal mouse serum in PBS: SUR1 1:5 (sc-5789), SUR2A 1:5 (sc-32461), SUR2B 1:5 (sc-5793), Kir6.1 1:5 (sc-11225), and Kir6.2 1:5 (sc-11228).

Slides were rinsed twice in PBS for 20 min to remove any unbound primary antibody before incubation with a mouse monoclonal anti-goat biotin-conjugated secondary antibody (Sigma-Aldrich Ltd.) diluted 1:20 with 5% normal mouse serum in PBS for 30 min. Sections were washed twice in PBS before application of ExtraAvidin horseradish peroxidase (Sigma-Aldridge Ltd.) diluted 1:20 with PBS for 30 min. After two further PBS rinses, antibody binding was visualized by the addition of the peroxidase substrate, 3-amino-9-ethylcarbazole (Vector Laboratories Ltd., Peterborough, UK). Chromogen formation was observed under a light microscope, and the reaction was stopped by placing the slides in distilled water when sufficient color had developed. Potential nonspecific binding due to the secondary antibody was checked by replacement of the primary antibodies with PBS. Sections were counterstained in Mayer's hematoxylin, blued in Scott's tap water, and rinsed in tap water before being mounted with Aquamount (VWR International, Leicester, UK). The resulting staining was examined and photographed using a Nikon Eclipse 80i light microscope with a Nikon ACT-2U photographic system.

RESULTS

Potassium channel regulators affected human hair follicle growth in organ culture

Human hair follicles were able to synthesize new hair in culture, which increased regularly in length (**Fig. 2*A***). After 2 days there was a gradual decline in the numbers of hair follicles remaining in anagen to ~70% at day 9 (**Fig. 3**). When anagen was ending, follicles showed catagen-like changes in the hair bulb region with pigmentation ceasing and the hair fiber moving upward, losing contact with the dermal papilla, which became rounded up into a ball of cells (Fig. 2*B*). Minoxidil had no effect on this aspect of hair growth, whereas the K_{ATP} channel closer, tolbutamide, significantly decreased the number of growing follicles ($P<0.01$); only ~45% remained growing after 9 days of culture. However, when minoxidil and tolbutamide were incubated together, there was no inhibition of anagen (Fig. 3).

Phenol red in the media had no effect on human hair follicle responses to K_{ATP} channel regulators

When follicles were grown in medium without phenol red for 9 days, they continued to grow, but the rate of growth was reduced; they reached only ~1.25 mm in length in 9 days, compared with almost 2 mm in normal medium (Fig. 2*C*). However, both media types maintained the same proportions of follicles in anagen (**Fig. 4*A***). Importantly, if the percentages of growing follicles were compared when grown in the presence of K_{ATP} channel regulators, the pattern remained the same in the absence of phenol red (Fig. 4*B*); tolbutamide again inhibited the number of follicles in anagen in the absence of phenol red ($P=0.01$), and coincubation with minoxidil was still able to overcome this inhibition.

Minoxidil prolongs anagen in insulin- and hydrocortisone-free medium

When follicles were cultured in the absence of insulin and hydrocortisone, they entered a catagen-like state very rapidly (**Fig. 5**). However, the addition of minoxidil (1 mM) to this medium increased the numbers of follicles in anagen, returning them to only slightly lower levels than those cultured in normal medium (compare Figs. 3 and 5). A lower concentration of minoxidil (10 μM) caused less stimulation. Tolbutamide (1 mM) had no effect on its own, but prevented any stimulation when combined with 1 mM minoxidil.

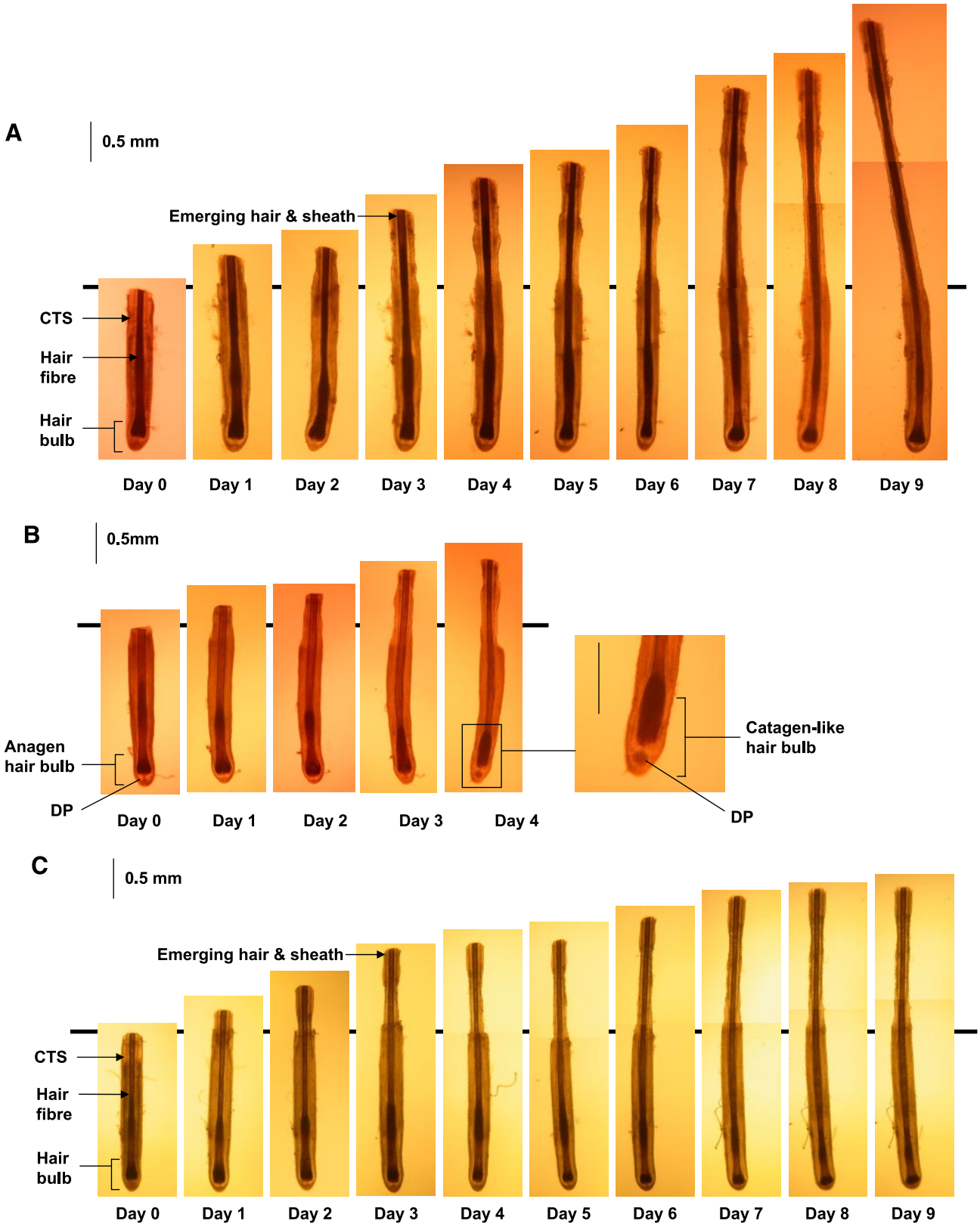

Figure 2. Sequential photomicrographs of human hair follicles growing *in vitro*. *A*) A scalp follicle cultured in normal medium. Photographs taken every 24 h show synthesis of new hair fiber and outer and inner root sheaths, but not the connective tissue sheath (CTS). Hair follicles increase regularly in length over 9 days, and most maintain the follicle bulb anagen morphology. *B*) A scalp hair follicle cultured with tolbutamide. Tolbutamide (1 mM) increased catagen-like changes in hair bulb morphology during culture (compare with *A*). By day 4, pigmentation has ceased; the base of the hair fiber appears to be retracting up the follicle, leaving behind a ball of dermal papilla (DP) cells. *C*) Photographs taken every 24 h of scalp anagen follicles cultured without phenol red in the medium show they increased in length regularly, but more slowly, without phenol red, but maintained their bulb morphology (compare with *A, B*). Scale bars = 0.5 mm.

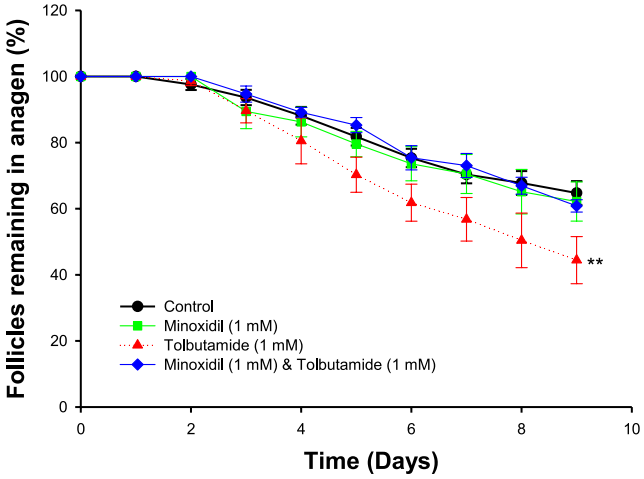

Figure 3. Tolbutamide shortened anagen in cultured human hair follicles, an effect blocked by minoxidil. Anagen follicles were assessed daily for changes in morphology while cultured in basic culture medium containing phenol red and either vehicle alone (control), minoxidil (1 mM), tolbutamide (1 mM), or both minoxidil and tolbutamide. The number of follicles remaining in anagen gradually declined (mean±SE of 7 individuals; at least 6 follicles were examined per person per condition). Tolbutamide significantly decreased the number of anagen follicles; $^{**}P < 0.01$. Minoxidil alone had no effect, but when combined with tolbutamide, it abolished the inhibitory effects of tolbutamide; $P < 0.01$.

Hair follicles express the genes for several K_{ATP} channel subunits

Total and poly(A^+)RNA were successfully extracted from microdissected scalp follicles, and the quality of each individual's cDNA was confirmed by PCR with primers for β-actin, a highly expressed cytoskeletal protein, producing bands of the expected size, 838 bp (data not shown). Because K_{ATP} channels cannot function without Kir6.X subunits to form the pore, the expressions of Kir6.1 and Kir6.2 genes were examined. The predicted sized bands of 336 and 301 bp, respectively (48), were seen after electrophoresis in all samples (**Fig. 6**), indicating gene expression of pore components for at least two channel types. If K_{ATP} channels were present in human follicles, a SUR2 type, which could respond to minoxidil, would be expected (27). Therefore, SUR2 genes were examined using a single pair of primers that would identify both SUR2A and 2B, distinguishing between their expression, as these are different splice variants from one gene (47). A band for SUR2B, at 312 bp, but not for SUR2A, was seen in all samples (Fig. 6). The ability of these primers to also identify SUR2A was confirmed using deer skeletal muscle (data not shown). To determine whether SUR1 receptors were also present, cDNA samples were amplified with primers specific for SUR1 (46), giving bands at 291 bp (Fig. 6). PCRs and agarose gel electrophoresis were repeated using specialized conditions for sequencing; these verified all genes against their relevant human sequences in GenBank.

Human hair bulb tissues express the genes for different K_{ATP} channels

It was difficult and time consuming to isolate the various hair follicle bulb components by microdissection, but sufficient good-quality cDNA was obtained when 120 follicles were used from each individual. The lowest yields came from the dermal papillae. The dermal papilla and dermal sheath samples all expressed SUR2B and Kir6.1, but not SUR 1, SUR2A, or Kir6.2 (**Table 1**). In contrast, the hair matrix samples expressed SUR1 and Kir6.2 but not SUR2A SUR2B, or Kir6.1 (Table 1).

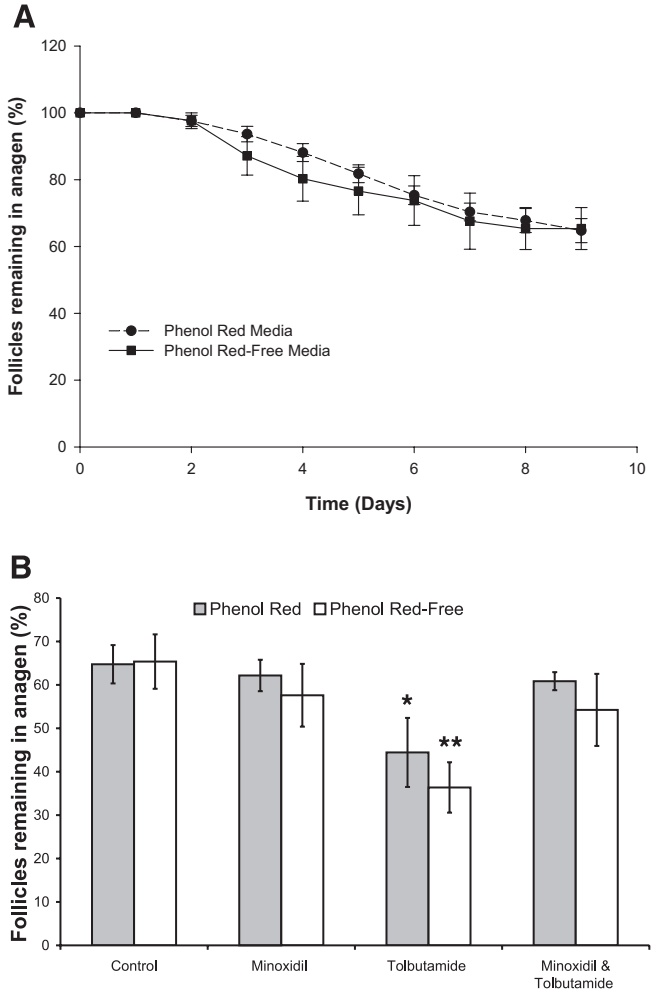

Figure 4. Phenol red in the culture media increased hair follicle growth but had no effect on follicle responses to K_{ATP} channel modulators. *A*) Phenol red in the medium had no effect on the ability of the follicles to maintain anagen. Hair follicles were cultured for 9 days in medium with or without supplementation with phenol red. Results are means ± SE; $n = 7$ follicles with phenol red, $n = 5$ follicles without phenol red; at least 6 follicles were examined per subject per treatment. *B*) K_{ATP} channel regulators had similar effects in both conditions. Tolbutamide reduced the percentage of follicles in anagen significantly when cultured with phenol red in the medium ($^{*}P<0.05$) or without ($^{**}P<0.01$). Values are means ± SE at day 9; $n = 7$ follicles with phenol red, $n = 5$ without phenol red; at least 6 follicles were examined per subject per treatment.

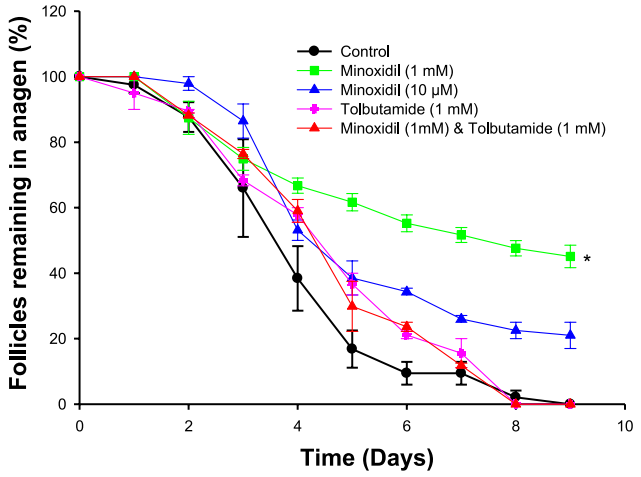

Figure 5. Minoxidil increased the number of follicles maintaining anagen in insulin-free medium, an effect blocked by tolbutamide. Hair follicles in insulin- and hydrocortisone-free medium rapidly entered catagen. However, the presence of minoxidil (1 mM) maintained the levels of anagen to almost those in standard insulin and hydrocortisone-containing medium (*$P<0.05$); 10 μM minoxidil had a lesser effect. Tolbutamide (1 mM) alone had no effect but blocked the stimulation caused by minoxidil (1 mM) when both were given together. Results are means ± SE of 4 experiments each involving 12 follicles for control medium and minoxidil (1 mM) and of 2 experiments for minoxidil (10 μM), tolbutamide, or tolbutamide and minoxidil.

Immunohistochemical localization of K_{ATP} channel subunits in human hair follicle bulbs

SUR2B protein expression was seen in the dermal papilla and dermal sheath but not in the epithelial cells of the hair bulb matrix (**Fig. 7*D***); strong staining was seen around the basement membrane surrounding the dermal papilla and weaker staining in the cells of the dermal papilla and dermal sheath. No similar staining was seen at the dermal-epidermal junction (Fig. 7). In contrast, there was no staining in the dermal papilla or dermal sheath with the antibody to SUR1, but this antibody did stain the hair matrix epithelial cells (Fig. 7*B*). The antibody to SUR2A did not stain any area of the hair bulb or the epidermis (Fig. 7*C*).

Kir6.1 expression was distributed very similarly to that of SUR2B, with strong expression in the papilla basement membrane and weaker staining of dermal papilla and dermal sheath cells; there was no staining in the matrix (Fig. 7*E*). The antibody to Kir6.2 stained the matrix cells but not the dermal papilla or dermal sheath (Fig. 7*F*). No similar staining was seen in the dermal-epidermal junction with either Kir6.1 or Kir6.2 antibodies (Fig. 7).

DISCUSSION

These results are very exciting. They demonstrate a biologically relevant shortening of anagen by a K_{ATP} channel blocker, tolbutamide, in isolated human hair follicles in organ culture, which is opposed by the K_{ATP} channel opener, minoxidil. In addition, they confirm that isolated human hair follicles from both sexes express the genes for both components of K_{ATP} channels, the SURs, and the pore-forming Kir6.X subunits. SUR2B and Kir6.1 gene and protein expression are localized in the dermal papilla and dermal sheath and those for SUR1 and Kir6.2 are localized in the hair matrix. Together these results indicate functional K_{ATP} channels within human hair follicles themselves with Kir6.1/SUR2B channels in the dermal papilla and dermal sheath and Kir6.2/SUR1 channels in the hair matrix.

Hair follicles respond to K_{ATP} channel regulators in organ culture

The effects of K_{ATP} channel modulators on human hair follicles *in vitro* were investigated by examining and photographing follicles daily to observe changes in hair bulb morphology that indicate the end of anagen (Fig. 2*B*). Human follicles were grown floating freely in media under carefully controlled conditions based on those established by Philpott and colleagues (33, 49), but serum and streptomycin were omitted as these have detrimental effects on follicle growth (50) or may

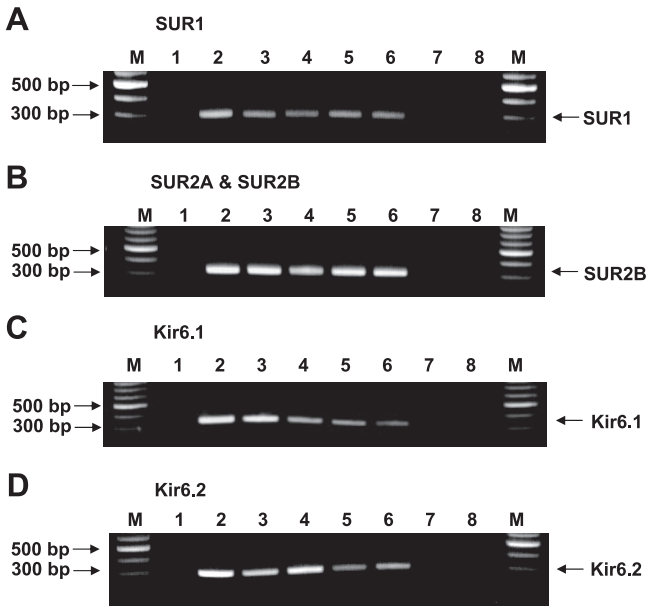

Figure 6. Human anagen hair follicles expressed genes for the sulfonylurea receptors SUR1 and SUR2B, but not SUR2A, and both forms of the pore-forming subunits of K_{ATP} channels, Kir6.1 and Kir6.2. Agarose gel electrophoresis of PCR products (30 μl) of hair follicle cDNAs prepared from 50 follicles from each of 5 different individuals showed bands corresponding to SUR1 at 291 bp (*A*), to SUR2B at 312 bp but not at 451 bp for SUR2A (*B*), to Kir6.1 at 336 bp (*C*) and with Kir6.2 at 301 bp (*D*). To each gel was added 10 μl of DNA ladder. Sequencing of the gene products confirmed each gene's identity. Lane M, 100-bp DNA ladder; lanes 1 and 8, blank; lanes 2–6, PCR products from 5 individuals' cDNAs; lane 7, negative control, no cDNA.

TABLE 1. *Investigations of K_{ATP} channel subunit gene expression in hair bulb components demonstrated the presence of two different types of K_{ATP} channels*

Gene	Dermal papilla	Dermal sheath	Matrix
SUR1	×	×	√
SUR2A	×	×	×
SUR2B	√	√	×
Kir6.1	√	√	×
Kir6.2	×	×	√

Hair follicle components were microdissected, and their expression of potassium channel subunit genes was examined by RT-PCR. Analysis was performed on the dermal papilla, the dermal sheath, and the hair follicle matrix microdissected from 120 follicles from each of 3 individuals separately. Components from all three individuals gave the same pattern of expression. √, expressed; ×, not expressed.

interfere with minoxidil action (34). Possible interference with the effects of K_{ATP} channel regulators by phenol red was investigated by parallel experiments in the absence of this routinely used pH indicator, but the percentage of follicles in anagen under the K_{ATP} channel modulators remained the same (Fig. 4). Presumably, the stimulation of follicular growth in phenol red media observed here and in red deer studies (30) is due to impurities in the phenol red; substances reported have shown estrogenic, cytotoxic, and cellular Na^+ and K^+ homeostasis effects (reviewed in ref. 30). However, phenol red had no effect on the percentage of follicles in anagen nor the responses to either of the K_{ATP} channel modulators used here; similarly, growth rates of human follicles in response to minoxidil (35) or deer follicles in response to minoxidil and tolbutamide (30) were not altered by phenol red. It seems that further investigations into the effects of K_{ATP} channel regulators can be appropriately performed in normal phenol red medium. However, the need for improved hair growth promoters means that identifying the stimulating impurities in phenol red merits further investigation.

Tolbutamide inhibited anagen in the presence and absence of phenol red; *i.e.*, the K_{ATP} channel blocker caused an *in vitro* reduction of anagen length in a total of 11 young (aged 40 yr or younger) adults (Figs. 3 and 4). If this effect was transcribed to the *in vivo* situation, it would result in a shorter hair being formed. Of interest, tolbutamide has been reported to cause human hair loss (51) and was patented in 2007 (US patent 7160921, issued on September 2, 2007) for use as a hair removal agent. The effect of tolbutamide was also prevented in both conditions by simultaneous administration of minoxidil, a K_{ATP} channel opener, indicating an action *via* K_{ATP} channels within the follicle. Minoxidil would be predicted to prolong anagen in cultured follicles from its effect of increasing anagen *in vivo*, but no effect on anagen was seen over 9 days. However, the ability of minoxidil to prolong anagen against the shortening caused by the potassium channel blocker, tolbutamide, does reflect *in vivo* actions of K_{ATP} channel openers in altering anagen length. The conditions used here were designed to replicate those of Han *et al.* (36) who reported increased length with

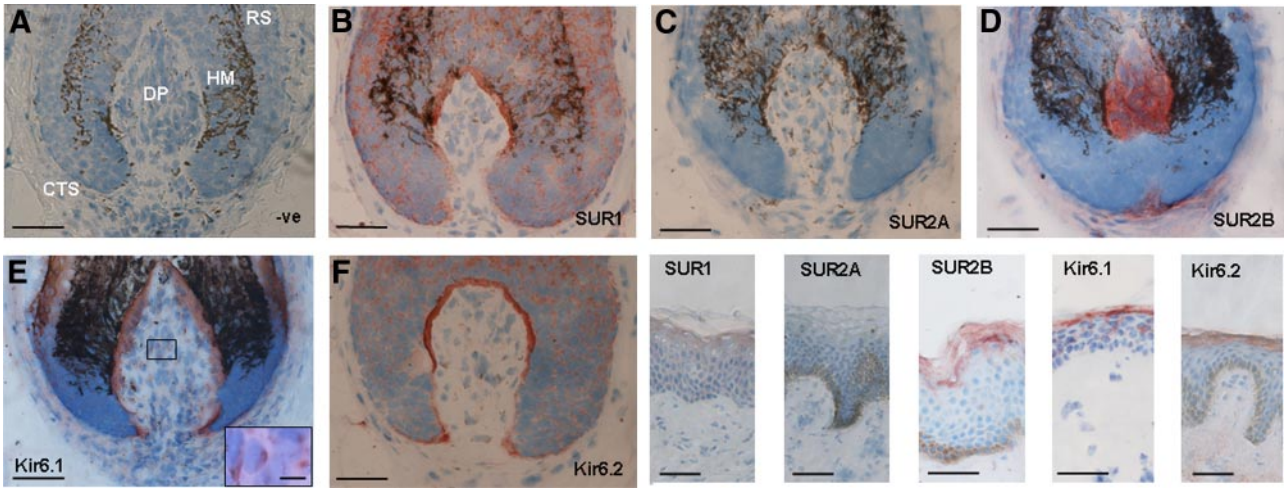

Figure 7. Immunolocalization of K_{ATP} channel subunits in the human hair follicle bulb. Immunohistochemical analysis of normal scalp cryosections localized K_{ATP} channel subunits SUR1, SUR 2B, Kir6.1, and Kir6.2 in the hair bulb (*B–F*), but not in the epidermis (lower row). No staining occurred in the hair bulb with the antibody to SUR2A (*C*) nor in negative control when the primary antibody was omitted (*A*). Normal dark pigment (melanin) is visible in the hair bulb; bulb components labeled are as follows: DP, dermal papilla; CTS, connective tissue, or dermal, sheath; HM, hair matrix; RS, developing root sheaths. SUR1 expression was only present in the matrix, with no staining evident in the dermal sheath or papilla (*B*). SUR2B was expressed in the dermal papilla and dermal sheath, but not the epithelial cells of the hair matrix (*D*); staining was particularly strong in the basement membrane surrounding the dermal papilla but was not seen in the basement membrane between the dermis and epidermis. The pattern of Kir6.1 staining paralleled that of SUR2B (*E*). In contrast, Kir6.2 was expressed in the matrix and not the dermal papilla or connective tissue sheath (*F*). Note the presence of melanin in the basal layers of some epidermal samples. Red stain on the upper layer of the epidermis is nonspecific, associated with dead, fully keratinized, shedding layers. Red: positive staining; blue: hematoxylin counterstain. Scale bars = 50 μm (standard views); 10 μm (high-power views).

100 μM and 1 mM, but not 10 μM, in follicles from three young men. Our observations do not support age or dosage as being responsible for this unusual positive response to minoxidil *in vitro*. It is possible that the large scalp follicles normally used for culture studies have little spare capacity to respond to minoxidil in such a supportive medium. This group also recently showed that when occipital follicles from two young men were allocated into four groups depending on their *in vivo* growth rate, only those growing fastest *in vivo* showed a significant increase in length when incubated with minoxidil (37). They suggested that faster growing follicles indicated a different earlier substage of anagen, which would be more sensitive to minoxidil.

Although minoxidil had no effect on anagen length on its own, the inhibitory effect of the K_{ATP} channel blocker, tolbutamide, and its neutralizing by minoxidil suggest that K_{ATP} channels in human follicles were fully open in these standard human follicle culture conditions (33). These differ from the more basic conditions without insulin or hydrocortisone used for deer follicles, in which K_{ATP} channel regulators had the expected stimulatory and inhibitory effects (30). The early demonstration of increased DNA synthesis in human hair follicles with minoxidil sulfate also involved a more basic serum-free medium without supplementation with insulin or hydrocortisone (38). Insulin was originally added to the medium to prevent cultured follicles from entering a catagen-like state (52), *i.e.*, to prolong anagen, the effect that minoxidil has *in vivo*. When follicles were cultured here in insulin- and hydrocortisone-free medium, control follicles rapidly entered a catagen-like state (Fig. 5) as reported earlier (52). However, the addition of minoxidil (1 mM) prolonged the anagen period, replicating the effect on hair follicles when minoxidil is used *in vivo* in the clinical situation (8); a smaller amount of minoxidil (10 μM) had only a reduced stimulatory effect (Fig. 5). Tolbutamide had no effect on its own, but blocked stimulation by minoxidil (1 mM) when they were used in combination (Fig. 5). Insulin has various effects on cells, which differ depending on the cell type but generally include increased uptake of energy-providing molecules such as glucose and promotion of their incorporation into storage molecules. In addition, insulin activates membrane Na^+,K^+ ATPases, which increase the movement of sodium ions out of and potassium ions into the cell. Such changes may cause K_{ATP} channels to open, preventing minoxidil from exerting any effect on the follicles *via* the K_{ATP} channel mechanism in the standard medium.

The counteracting of the stimulatory effect of minoxidil by tolbutamide in insulin-free medium and prevention by minoxidil of the stimulation by tolbutamide in medium containing insulin strongly suggest that both substances are acting *via* K_{ATP} channels in these situations, rather than by any other route that may be possible at the high concentrations used. Tolbutamide has a higher affinity for SUR1 K_{ATP} channels and would block any such channels at this concentration. However, tolbutamide also has a lower affinity to SUR2B channels which, if present, should also be closed at the concentrations used in these experiments (53–55). Because minoxidil only acts *via* SUR2 channels, these results suggest actions *via* SUR2 channels in organ culture.

Molecular biological and immunohistological demonstration of two types of K_{ATP} channels in human hair follicles

The molecular biological results complement the organ culture observations by confirming that isolated human hair follicles from both sexes express the genes for K_{ATP} channels, including Kir6.1 and Kir6.2 pore-forming units and the sulfonylurea SUR2B subunits (Fig. 6), which would respond to minoxidil (27). The analysis of the hair bulb components and the immunohistochemical localization confirm the presence of two different types of K_{ATP} channel in the hair follicle bulb with different distributions. The location of Kir6.1/SUR2B channels in the regulatory dermal papilla concurs (Table 1; Fig. 7) with an earlier investigation of cultured follicular dermal papilla cells, which demonstrated SUR2B expression but did not include investigation of Kir6.X subunits (56). This combination is physiologically relevant as SUR2B is seen combined with both Kir6.1 and Kir6.2 in other tissues (46) and importantly means that the key regulatory dermal papilla cells, the appropriate cells to be influenced to cause the follicular changes seen clinically, could respond to minoxidil (8, 12–15). Dermal papilla cells have also responded to minoxidil *in vitro* (36, 39, 40).

Even more interesting is the detection of SUR1 gene and protein expression in the hair matrix cells (Table 1; Fig. 7). This finding concurs with our observations that the novel drug NNC 55-0118, a selective opener of Kir6.2/SUR1 potassium channels (57, 58), stimulated deer hair follicle growth *in vitro*, an effect inhibited by the potassium channel blocker, tolbutamide (30). The combination of Kir6.2 and SUR1 detected in the matrix (Table 1) is also physiologically relevant; Kir6.1/SUR1 channels have not been described in other tissues (25–27). The detection of two types of K_{ATP} channels with SUR2B and SUR1 sulfonylurea receptors in the follicle parallels the finding of both SUR1 and SUR2B K_{ATP} channels in pig urethra (59). All of these approaches indicate that potassium channel regulators can work directly on K_{ATP} channels in the follicle independently of any possible vascular effects, in parallel to our cultured deer follicle experiments (30) and some human studies (36, 38). Importantly, the detection of different genes in the various dissected components (Table 1), which concur with the protein expression (Fig. 7) strongly supports the cleanness of the microdissections. The similar patterns in the dermal papilla and dermal sheath fit with the ability of the lower dermal sheath to replace the dermal papilla and induce new hair follicles (60, 61).

Clinical relevance

These results have significant implications for future therapeutic developments as well as for our understanding of hair follicle function. K_{ATP} channels in the follicle will have a physiological role, presumably normally regulated by changes in nucleotide levels such as ATP. The strong matching expression of both SUR2B and Kir6.1 associated with the dermal papilla-matrix junction but not the dermal-epidermal junction suggests that K_{ATP} channels may play a role in regulating transfer of messages between the dermal papilla and matrix. The absence of SUR2A gene expression means that selective drugs could be developed that would activate SUR2A K_{ATP} channels, such as those in cardiac muscle (25) without any possibility of hair growth side effects. Unfortunately, the detection of SUR1 channels suggests, as do our earlier deer follicle studies (30), that at least some drugs that stimulate SUR1 receptors for pancreatic use would also promote hair growth as a side effect. However, these results raise an important prospect of novel pharmaceuticals acting *via* SUR1 K_{ATP} channels being developed to stimulate hair growth, an area in which there is currently great demand for more effective therapies. These may be able to be used in conjunction with therapies such as minoxidil acting *via* the SUR2B channels to promote better hair growth as the drugs would be acting on different parts of the hair bulb.

The focus on K_{ATP} channel regulators to date with regard to the hair follicle has centered on openers to stimulate hair growth for hair loss disorders (5). However, inhibiting excessive hair growth such as hirsutism or even removal of normal hair growth would also be helpful. Our results suggest that K_{ATP} channel blockers, such as tolbutamide, may be able to be harnessed to reduce hair growth, ideally in a topical formulation. Our knowledge of K_{ATP} channels is at an early stage and our understanding of the mechanisms involved in nucleotide binding or channel regulation is still very limited (24, 26); future structural insights should extend clinical therapeutics even further to facilitate or avoid hair growth as appropriate. FJ

The authors gratefully acknowledge a Research Studentship from the School of Life Sciences at the University of Bradford, which supported K.S. for this research.

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Received for publication October 3, 2007.
Accepted for publication December 13, 2007.