Potassium Channel Conductance as a Control Mechanism in Hair Follicles

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The opening of intracellular potassium channels is a common mechanism of action for a set of anti-hypertensive drugs that includes the hair-growth–inducing agent minoxidil. Recent work suggests potassium channel openers (PCOs) also influence hair growth. Correlative studies demonstrate that a series of PCOs including minoxidil, pinacidil, P-1075, an active pinacidil analog, RP-49,356, cromakalim, and nicorandil maintain hair growth in cultured vibrissa follicles. Studies using balding stumptail macaques verify that minoxidil, P-1075, and cromakalim but not RP-49,356 stimulate hair growth. The definition of potassium channels and documentation of drug effects on these channels is classically done using electrophysiologic techniques. Such studies require the identification and isolation of target cells. Both these are among the unsolved problems in the area of hair biology.

Interest in and knowledge about potassium channels and their role in controlling tissue-specific function has expanded greatly in the past few years as illustrated by the recent series of review papers on the subject [1–3]. Much of the recent interest is stimulated by the discovery of potassium channel opening as a mechanism of action for drugs that relax vascular smooth muscle. Two older drugs, diazoxide and minoxidil, have been shown to be potassium channel openers (PCOs), and a new and diverging set of compounds including both cromakalim and pinacidil also open potassium channels. Studies showing that minoxidil alters the function of follicles clinically and in experimental systems suggest that regulation of hair growth is a potassium channel–controlled process. Evidence to support this hypothesis is reviewed in this paper.

Potassium channel biology is a widely diverse field that has an impact on many aspects of physiology. More than 15 different types of potassium channels have been identified in various tissues [3,4]. These channels are classified into four subtypes: voltage dependent, calcium dependent, receptor coupled, and miscellaneous. Specific classes are defined by the magnitude of the electrical conductance of the channel, the types of pharmacologic agents that block the channel, and the physiologic properties of the channel. Minoxidil and other PCOs of interest in hair growth are thought to work through ATP-sensitive K+ channels that belong in the miscellaneous category of potassium channels. There is, however, disagreement over which channels are sensitive to PCOs [5,6]. The majority of research on PCOs has been done in vascular smooth muscle with fewer studies on bladder, uterus, gastrointestinal, airway, skeletal smooth muscle, and neuron. The applicability of these results to non-excitatory tissues such as hair follicles has yet to be assessed.

The PCOs are a rapidly expanding category of drugs in medicinal chemistry [4,7]. Figure 1 illustrates some of the variety in chemical structures among drugs of this class. Minoxidil is unique among these because it is the only prodrug. The active metabolite, a sulfate, is formed through the action of sulfotransferase enzymes in platelets, liver, and hair follicles [8,9]†. Minoxidil sulfate is a PCO in vascular smooth muscle [3,10]. In vitro, micromolar doses of PCOs cause relaxation of various vascular tissues by opening intracellular potassium channels that hyperpolarize the cells, reducing the intracellular free calcium levels and thus reducing muscle tone. PCOs stimulate efflux of K+ that can be measured by release of 42K+ or 86Rb+. These actions are inhibited by sulfonylureas and other drugs that are known to specifically block ATP-sensitive potassium channels. Electrophysiologic studies have directly demonstrated opening of potassium channels in target tissues [11].

PCO EFFECTS ON HAIR GROWTH

The evidence that PCOs are active on hair growth is correlative. In humans three PCOs have been reported to affect hair growth. Minoxidil was reported to induce hypertrichosis during early clinical trials as an antihypertensive [12]. These side effects were characterized by increasingly visual facial hair, thickening of eyebrows, and diffuse hair growth across the upper back and limbs. Systemic minoxidil induced hypertrichosis in 80–100% of adults [13]. Clinical trials using topical minoxidil demonstrate increased scalp hair in about 39% of treated balding men. Oral diazoxide causes hypertrophia.

Figure 1. Structures of selected potassium channel openers.

Hypertrichosis in most hypoglycemic children and about 1% of adults, and induces some scalp hair in 25% of the balding patients [13–15]. Systemic pinacidil induces hypertrichosis in 2–13% of patients [15]. We are not aware of any topical hair growth trials using pinacidil.

We have demonstrated that selected PCOs induce hair growth in cultured mouse vibrissa follicles [16]. Our previous studies showed that minoxidil sulfate, the active metabolite, maintains proliferation and differentiation of the hair epithelium that forms hair shaft that minoxidil sulfate, the active metabolite, maintains proliferation and differentiation of the hair epithelium that forms hair shaft [17,18]. Drug effects were determined by measuring incorporation of $^{35}$S-cysteine in follicles cultured with drug for 3 d. Cysteine incorporation was stimulated by minoxidil (1 mM), pinacidil (1 mM), a more potent pinacidil analog P-1075 (0.01 mM), cromakalim (0.13 and 0.025 mM), nicorandil (0.25 and 0.5 mM), and RP-49,356 (6 mM) but not diazoxide (0.1 or 0.01 mM). This complement other work showing that PCOs including diazoxide stimulate proliferation of cells in cultured vibrissae and skin keratinocytes. Table I summarizes the in vitro results. These data support the idea that PCOs have direct effects on follicles independent of any effects on vascular smooth muscle and follicular blood supply.

The third set of evidence correlating PCOs with hair growth comes from studies with stumptailed macaques. Monkeys of both sexes display androgen-dependent bald frontal following sexual maturity. Histologic studies show that the follicles of the balding scalp are reduced in length and arrested in early anagen similar to balding human follicles [19]. Topical treatment with either minoxidil or diazoxide causes regrowth of hair on the balding scalp [20,21]. We tested the effects of minoxidil and three other PCOs by weighing the amount of hair grown on a defined scalp area [16]. Using once-a-day treatment, 250 µl applied 5 d per week to a 1-inch-square area of frontal scalp, we found a dose response with 100 and 250 mM minoxidil over a 20-week test period. Both P-1075 (100 and 250 mM) and cromakalim (100 mM) also induced hair growth, although RP-49,356 was not effective. The stumptail data is also summarized in Table I.

Although these correlative data clearly suggest that potassium channel opening plays an important role in the regulation of hair follicles, other supportive studies are required for the complete acceptance of that hypothesis. These include electrophysiology, direct measurement of $K^+$ flux with labeled ions, and use of pharmacologic approaches. Further studies will also be required to identify the specific potassium channel(s) involved. We chose to do ion flux and pharmacologic experiments in whole follicles to avoid the problem of specific target cells.

STUDIES WITH POTASSIUM CHANNEL BLOCKERS

Using the pharmacologic approach we tested whether potassium channel blockers inhibit minoxidil-induced stimulation of follicles in vitro. The rationale for these experiments was that because minoxidil is a PCO it should be possible to inhibit its action with specific blockers. Because potassium channel blockers have varying specificity for different channel types, the data from the blocking experiments would also be useful in identifying the specific channels in the follicles with which minoxidil interacts. We used tetraethylammonium chloride (TEA), a potent blocker of both calcium and voltage-sensitive potassium channels but also a weak blocker of other potassium channels; procaine, similar to TEA in its action on potassium channels but more potent; glyburide, a sulfonylurea that is a potent blocker of ATP-sensitive potassium channels; tolbutamide, a less potent sulfonylurea; and tetrethylammonium chloride (TEA), as a control for TEA without any channel effects.

We used standard procedures for culturing neonatal mouse vibrissa follicles with a 1 mM stimulatory dose of minoxidil and measured drug effects by counting the incorporation of $^{35}$S-cysteine in the newly formed hair shafts at the end of the experiment [17]. Groups of follicles were pretreated for 30 min with control medium or blockers and then maintained in media with the minoxidil blockers plus minoxidil, blockers alone, or media control for the remainder of the 3-d culture period. This design allowed us to determine if the blockers eliminated minoxidil stimulation or if the blockers had toxic effects of their own. Cysteine incorporation below control levels, abnormal morphology, or lack of structural changes during the 3-d culture period were signs of direct toxicity by the blockers. Results of these experiments showed none of the blockers inhibited minoxidil stimulation at doses that were not by themselves toxic. Specifically, TEA was toxic at 50 and 30 mM and did not inhibit minoxidil stimulation at 1 or 1 mM. Procaine was toxic at 1 mM but ineffective at 0.1 and 0.01 mM. Likewise, glyburide showed toxic effects at 0.5 mM with no effects at 0.05, 0.005, or 0.0005 mM. Tolbutamide was insoluble above 0.5 mM and had no effects at that or the next two lower log concentrations.

There are several explanations for the failure of this panel of potassium channel blockers to effect minoxidil stimulation of follicles. First, it is difficult to distinguish inhibitory from toxic effects. If potassium channels are an integral part of the systems that regulate growth and differentiation we might expect that these agents

![Figure 1. Structures of selected potassium channel openers.](image)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Clinical Hypertrichosis</th>
<th>Stimulates Cultured Follicles</th>
<th>Macaque Hair Growth</th>
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<td>Minoxidil</td>
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<td>Pinacidil</td>
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<td>P-1075</td>
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<td>Cromakalim</td>
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<td>Diazoxide</td>
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<td>RP-49,356</td>
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<td>Nicorandil</td>
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Table I. Hair-Growth Effects of Potassium Channel Openers in Humans and Two Model Systems

would by themselves cause effects we labeled as toxic. Alternatively, the experimental conditions may not be optimal for detecting inhibitory effects. The pre-treatment time may have been insufficient. We could not measure the metabolism and be sure the blockers were present at suitable levels during the 3-d culture period. Cysteine uptake may not be sensitive enough to measure partial inhibition. Finally, minoxidil, although a PCO, may have other pharmacologic effects. Clearly, blockers must be examined using more sensitive and rapid endpoints. The effects of blockers on the action of other PCOs must also be tested. Ultimately, pharmacology will prove to be a powerful method for examining the effects of the potassium channels in follicles.

MEASUREMENT OF ION EFFLUX IN HAIR FOLLICLES

We used labeled ions to directly measure drug-induced channel opening in follicles. Measuring $^{86}\text{Rb}^+$ flux is a commonly used technique for monitoring opening of potassium channels that has been widely exploited with other types of tissues [3]. This technique is effective because $\text{Rb}^+$ ions have a similar though possibly lesser specificity for potassium channels than $\text{K}^+$ but are easier to work with than $\text{K}^+$ [22]. The methodology involves incubating tissues with media containing $^{86}\text{Rb}^+$ to load the intracellular compartment then, after washing, the flux of $\text{Rb}^+$ into the media reflects endogenous opening of the potassium channels. PCOs enhance opening of the channels so more $\text{Rb}^+$ is released into the medium.

We examined the potential of selected PCOs to open potassium channels in hair follicles by measuring the amount of $\text{Rb}^+$ released from labeled tissues following perfusion with drug-containing medium. Freshly isolated follicles or cells were labeled in filter chambers with media containing $30 \mu\text{Ci/ml of }^{86}\text{Rb}^+$. Preliminary experiments showed that the 1-h labeling times were as effective as longer times. Temperatures were maintained at 37°C by submerging the chambers and the tubing in a water bath. DMEM media was pumped through the chambers at flow rates between 1 and 3 ml/min. In each experiment, excess label was rinsed from the chambers for periods of 15–30 min, then drug-containing or control media was perfused for 10–20 min, and finally control medium was perfused for an additional 15–30 min. The perfusate was collected in tubes using fraction collectors and counted in a gamma counter. Both whole vibrissa follicles and cells from an immortalized cell line were used in these experiments. Intact follicles from neonatal mice were harvested and loaded 20–25 per chamber. Butch, the immortalized hair epithelial line cloned in our laboratory, was produced from ker-CAT mice. This transgenic line contains the promoter for a hair-specific ultrahigh sulfur keratin-related protein linked to the chloramphenicol acetyl transferase (CAT) enzyme [23]. To produce this line, follicles were dissected, the epithelial cells dispersed, and primary explants were grown on irradiated 3T3 cell feeder layers. The cells were immortalized using an E1A construct expressing both the oncogene and G148 resistance. Transfected cells were selected for G148 resistance, ring cloned, and shown to express CAT activity, which confirmed their hair epithelial origin. For the $\text{Rb}^+$ experiments the butch cells were trypsinized, rinsed, counted, and placed in chambers at different densities for each experiment ($1 \times 10^4$, $0.5 \times 10^4$, or $1 \times 10^4$ cells). The cells were allowed to recover for 3–4 h before the efflux experiment began. The passage number of the cells used in these experiments ranged between 4 and 8. The medium used in these studies was Dulbecco's modified Eagle medium supplemented with 25 mM N-2-hydroxyethylpiperazine-N'-d-ethanesulfonic acid, insulin, transferrin, selenium, glutamine, and gentamicin.

Repeated experiments with whole vibrissa follicles demonstrated that pinacidil induced significant increases in $^{86}\text{Rb}^+$ efflux (Fig 2). $\text{Rb}^+$ flux during perfusion with control medium may lum follow an exponential decay curve although active drugs induced a more rapid efflux of the label. A significant release of $\text{Rb}^+$ was observed following treatment with 2 mM pinacidil. The release of radiolabel increased starting about 6 min after drug treatment and remained elevated for about 10 min. No effects were seen following perfusion with 0.2 mM pinacidil or control medium. Similar experiments showed that RP-49,356 at a dosage of 2 mM caused a significant efflux of $^{86}\text{Rb}^+$, whereas lower doses were ineffective. Duplicate experiments with minoxidil indicated that this drug had no effects on $\text{Rb}^+$ release at doses of 5 or 0.5 mM. Results with minoxidil sulfate were also negative; doses of 0.7 and 0.001 mM were tested in two experiments. Solubility limits prevented testing either of these drugs at higher doses. Effects of pinacidil on hair epithelial cells were tested in three experiments. Pinacidil at 2 mM significantly stimulated $\text{Rb}^+$ flux in these experiments whereas lower doses were ineffective (Fig 3). No other drugs were tested with these cells.

These experiments suggest that pinacidil and RP-49,356 open potassium channels in vibrissa follicles, thus complementing our in vitro and in vivo hair-growth studies [16]. The flux studies show that pinacidil was more potent than RP-49,356, confirming the potency relationship from our cultured vibrissa studies. Similarly, our hair growth studies in balding macaques showed that P-1075 was effective whereas RP-49,356 was not. Other studies show that pinacidil and other PCOs relax vascular smooth muscle in vitro and stimulate $\text{Rb}^+$ release although at lower doses than were effective in either our hair growth or $\text{Rb}^+$ studies [3]. Part of the dose discrepancy may be due to the large amounts of follicular connective tissue through which the drugs had to transverse. Other possibilities include differences in tissue specificity and the relatively small amounts of potential PCO target tissues in follicles.

The failure of both minoxidil and minoxidil sulfate to induce $\text{Rb}^+$ release from whole vibrissa was not unexpected. Minoxidil sulfate has been reported to induce increased flux of $\text{K}^+$ but not $^{86}\text{Rb}^+$ in...
POTASSIUM CHANNELS REGULATE HAIR FOLLICLES

Figure 3. Induction of $^{86}$Rb$^+$ release from cells of a hair epithelial line treated with 2 mM pinacidil compared to control Dulbecco's modified Eagle's medium. Each plot represents a group of 1 X 10$^6$ cells at passage 8. Bar, a 10-min treatment perfusion.

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H. **Eagle**'s medium. Each plot represents a group of 1 X 10$^6$ cells at passage 8. Bar, a 10-min treatment perfusion.

FUTURE STUDIES ON POTASSIUM CHANNELS IN HAIR TARGET CELLS

Electrophysiologic techniques should be useful in evaluating the importance of K$^+$ channels in various follicle cells. This is a standard method for identification and characterization of ion channels. It can also be used in conjunction with selected channel blockers and PCOs to address questions about differential effects of drug on different channel subtypes. The key to using electrophysiology in follicular tissue is isolating functional target tissues from follicles. Isolation of specific tissues from follicles is the rate-limiting step in many areas of hair biology. The size and complexity of follicles make tissue isolation difficult; follicles contain multiple cell types including dermal, epidermal, vascular, nervous, and immune tissues. Because it is clear that specific dermal-epidermal interactions are critical, good markers for both cell typing and organ-specific function are needed. Retaining organ-specific function in cultured cells is a problem in hair biology as in other areas. Because cultured cells are not usually in conditions that maintain organ-specific function it is difficult to maintain the specificity of these cells. Loss of CAT activity in the hair epithelial line with time is an example of that problem as we experienced it in our laboratory. The problem of target cells is being attacked with new methods as other work presented at this conference shows.

Binding studies are a possible way of localizing potassium-sensi-

tive target cells and examining the characteristics of the channels and channel-ligand interactions. Although radiolabeled PCOs have been synthesized and binding studies attempted, we are aware of only one report of successful binding studies with these agents [26]. High-affinity binding of labeled P-1075 to rat aorta is displacable with most of the PCOs listed in Fig 1. Comparison of binding affinity and the effective dose required for Rb$^+$ efflux showed excellent correlations for all the PCOs except minoxidil sulfate. We have done extensive experimentation with minoxidil and minoxidil sulfate attempting to localize binding of those drugs in hair follicles. Although these drugs clearly affect hair follicles we have not been successful in identifying specific target cells for these drugs [18,29,30]. Binding has been reported with glyburide receptors, binding proteins have been purified, and binding has been mapped in brain using autoradiography [31,32]. Although there is no evidence to suggest that the glyburide receptor is a potassium channel, the glyburide and PCO binding work provides encouragement that similar studies can be successfully completed with hair follicles.

Cloning of potassium channels offers a potentially powerful approach for attacking the problem of potassium channels in hair follicles. The first potassium channel cloned was the voltage-dependent channel from the Drosophila shaker mutant [33]. Homology cloning has yielded a family of voltage-dependent potassium channels in mammalian tissues and studies defining both the properties of these channels and the functional significance of the protein components have been published [34]. Unfortunately, there are as yet no reports of cloning the ATP-sensitive potassium channel. This channel will eventually be cloned and that event will unleash the full power of molecular biology to attack the problem of PCOs in many target tissues including hair follicles.

In conclusion there are data to support the hypothesis that regulation of potassium channels is an important mechanism in controlling hair follicle function but many questions remain to be addressed. This data includes PCO induction of hypertrichosis in patients, increased hair in balding primate scales, direct effects on cultured follicles, and increased Rb$^+$ efflux in both vibrissae follicles and hair epithelial cells. The problems of identifying the specific PCO-responsive cells, determining which potassium channels are important, and defining the relationship of potassium mechanisms to other control systems remain to be solved. Deciphering these will not be easy because of the complexity of follicular development, hair shaft production, and hair cycling. We do expect progress as more information on potassium channels and their effects on intra-cellular communication is reported in other organ systems. This information, plus expanded pharmacologic tools in the form of more specific drugs and cloning of PCO-sensitive channels will fuel interest in the area. Exploration of the potassium channel hypothesis should provide another area of renewed excitement in hair research.

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