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PRINCIPAL INVESTIGATOR: Lynne A. Fieber, Ph.D.

CONTRACTING ORGANIZATION: The University of Miami
Miami, Florida 33149-1098

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6. AUTHOR(S)
Lynne A. Fieber, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Miami
Miami, Florida 33149-1098

E-Mail: Lfieber@rsmas.miami.edu/

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13. ABSTRACT (Maximum 200 Words)

The K current types of human cultures Schwann cells (SC) formed a continuum from normal SC to malignant tumor SC, with A type K current found in quiescent normal SC never exposed to exogenous growth factors such as glial growth factor (GGF, human recombinant heregulin) and in SC from the dermal neurofibroma, and with non-inactivating K current found in the more malignant tumor cultures. Blocking the non-incativating K current blocked proliferation. A manuscript based on the findings form the second year of the ggrant was published (Fieber et al 2003). We discovered a component of tumor K current called Ca^{2+}-activated K current. We believe this is the current component that induces proliferation in NF1 Schwann cells (SC). Thus our experimental objectives during the third and fourth years of the project were to identify Ca^{2+}-activated K Current s is SC from our different NF1 cultures and to test the effects on cell proliferation of blocking these currents. A specific blocker of this subtype of Ca^{2+}- activated K current blocked 21-35% of the current of NF1-derived SC. One proliferating normal SC culture showed no inhibition of proliferation by the drug. We believe that the Ca^{2+}-activated K current of NF1 SC is a worthwhile focus of future experiments on the electrophysiological characteristics of NF1 SC.

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INTRODUCTION

Ion channels are membrane proteins that mediate electrical communication between cells of the nervous system and are vital to nervous system function. There are many kinds of K channels; we are studying those that open in response to depolarizations of the cell membrane in Schwann cells (SC). The purpose of our study has been to examine the inter-relationships between K channels, Ras, and neurofibromin in SC proliferation. We now recognize that SC have different K currents when they are proliferating vs. when they are quiescent. The goal of the research on this award has been to distinguish the K currents functionally expressed in dividing NF1 SC from those in normal, dividing SC, to divide K currents of SC into those characteristic of proliferating cells and those characteristic of tumor cells, and to test the effects of pharmacological blockers of the channels and other drugs on inhibition of NF1 SC proliferation. K channels play prominent roles in controlling proliferation and differentiation in a variety of inexcitable cell types (Knutson et al 1997; Liu et al 1998), and have been implicated in the growth of tumor cells (Rane 1999; Stringer et al 2001). K channels are receiving increasing attention as a therapeutic target in controlling cell division in oncogenic syndromes (Pardo et al 1999). It is with these studies in mind that we proceeded to studies of Ca^{2+}-activated K currents in normal and NF1 tumor SC.

BODY

The following is an accounting of the Technical Objectives 1, 2 and 3 from the original Statement of Work.

Technical Objective 1: Pharmacologically block tumored K channels in primary cultures of neurofibroma- and neurofibrosarcoma-derived SC to demonstrate that block of K channels inhibits proliferation. This objective has been fulfilled in full. Early on, we settled on the BrdU assay to measure proliferation, rather than flow cytometry and H^3 thymidine assays (this change was approved). Block of total whole cell K current inhibiting proliferation of NF1 SC was described for 11 cultures in Fieber et al (2003). The effects on proliferation of block of a specific component of K current, Ca^{2+}-activated K current inhibiting proliferation was demonstrated in the past year in 3 NF1 cultures. The results of these studies are summarized separately below.

Summary of appended manuscript (Fieber et al. 2003)

Quiescent normal SC never exposed to exogenous growth factors such as glial growth factor (GGF, human recombinant heregulin) studied soon after dissociation had K currents with the same characteristics as those reported in Fieber (1998). These were **A type**, blocked by 4-aminopyridine. 47% of quiescent normal SC had A type currents. Other quiescent normal SC had no outward currents. Many normal SC do not have recorded currents *in vitro* (Fieber 1998).

The hallmark outward K current of tumor-derived SC cultures was a **non-inactivating K conductance** blocked by tetraethylammonium. The non-inactivating K current was present in all malignant peripheral nerve sheath tumor (MPNST) SC and in plexiform neurofibroma SC. We recorded from a variety of NF1 tumors that ranged in severity from benign dermal neurofibroma to MNPST, and found variation in the size and frequency of observation of non-inactivating outward K current in different tumor cultures. These differences were significantly different such

that the benign tumors showed more similarity to normal SC cultures and malignant tumors showed least similarity to normal SC cultures.

The K current types formed a continuum from normal SC to malignant tumor SC, with A type K current found in normal SC and SC from the dermal neurofibroma, and non-inactivating K current found in the more malignant tumor cultures (Table 1). The frequency of occurrence in the different SC cultures of these different K current types was significantly different as assessed by a G test ($p \leq 0.01$). Normal SC cultures had significantly more cells with A type current than cultures of MPNST and the plexiform neurofibroma, and, conversely, MPNST and plexiform neurofibroma cultures had significantly more SC with non-inactivating current than did normal cultures. In addition, the plexiform neurofibroma culture had significantly more cells with non-inactivating current than the dermal neurofibroma culture.

Table 1. Summary of outward K currents in normal and NF1 SC.

SC Culture Type	Predominant Outward K current(s)	Significant Trends
Normal	A type	non-inactivating increases ↓ ↑ A type increases
Dermal neurofibroma	A type, biphasic	
Plexiform neurofibroma	Biphasic, non-inactivating	
MPNST	non-inactivating	

The classical blocker of non-inactivating K currents is tetraethylammonium ion (TEA).TEA analogs inhibited tumored SC proliferation ≥75%. These data suggest a direct role of K channels in SC proliferation in NF1, and thus offer a clear-cut rationale for pursuing studies on K channels in SC proliferation in NF1.

Normal SC maintained in culture medium with exogenous growth factors proliferated with doubling times of 38-80 hrs. Their K currents were usually biphasic K currents, in other words, they retain their A type current, and in addition, they have a non-inactivating K current (which may be induced by the growth factors). Proliferation of normal SC in growth factors also was blocked by TEA analogs, however, average K current amplitude was not significantly decreased by 40 hrs' exposure to TEA analogs. This is because A type current is not as susceptible to block by chronic exposure to TEA analogs as non-inactivating currents. Because the non-inactivating K current was small relative to the A type component in these cells, the blockade of non-inactivating K current did not significantly alter the average K channel current. This provides additional evidence that the non-inactivating K rather than the A type K current is the component associated with proliferation in human SC.

TEA analogs depolarized resting potentials (RP) by >50% of their control values. However, depolarization is probably not the cause of inhibition of proliferation by these agents because 10 mM added KCl reduced the RP of MPNST cells by an average of 23 mV while it had no effect on proliferation rates or K current amplitude.

The only part of aim 1 whose results proved inconclusive was the measurement of intracellular cAMP; we could not detect reliable or interpretable amounts of cAMP in our

cultures using a commercial kit (Sigma). Thus we were unable to test a link between proliferation and elevated intracellular cAMP levels in tumored cells.

Technical Objective 2: Directly disrupt Ras in tumored cells with Ras antibody Y13-259 and farnesyl transferase inhibitors and test if K channels and cAMP levels return to levels characteristic of normal SC. Test if proliferation is inhibited.

This aim was fulfilled in full with regard to the FTI. We learned that FTI did block K channels and proliferation in a plexiform neurofibroma (Fieber et al 2003). We also learned that in other NF1 tumors tested, FTI blocked proliferation without significantly affecting K channels. There are 3 forms of Ras found in mammalian cells, H-Ras, K-Ras, and N-Ras, and while the former is activated by farnesylation, the latter 2 are at least partially activated by GGTase-1 (Mahgoub et al 1999). Therefore it is imperative to use inhibitors of both kinds of post-translational modification of Ras to fully block its effects, and our future work will do this. These planned experiments will improve on our previous experiments that did not conclusively show a link between K channels and Ras, possibly because we used only FTI.

Due to the mixed effects of FTI on K channels and proliferation, we decided not to pursue experiments with the more delicate and harder to use Y13-2559 because we hypothesized results using this reagent would be difficult to interpret. As mentioned above, we could not detect reliable or interpretable amounts of cAMP in our cultures. Thus we were unable to test a link between inhibition of Ras and elevated intracellular cAMP levels in tumored cells.

Technical Objective 3: Compare effects on SC phenotype of elevating cAMP in normal SC and in SC from normal human peripheral nerve tissue from an NF1 patient ("normal-appearing NF1 SC"). Examine K channel profile electrophysiologically and by use of K channel antibodies on untreated and CPTcAMP-treated SC to demonstrate what shift in K channels is occurring with CPTcAMP treatment when neurofibromin is normal vs. when it may be downregulated (normal-appearing NF1 SC). In untreated normal SC and normal-appearing NF1 SC, monitor and compare proliferation and cAMP levels in comparison to normal and tumored SC studied in other aims.

The pharmacological tools used to elevate intracellular cAMP (N^6,2'-O-dibutyryl cAMP (dibutyryl cAMP), 1 mM; 8-(4-chlorophenylthio) cAMP (CPTcAMP), 0.1 mM; forskolin, 200 μM) had variable effects in the different SC cultures. These agents uniformly enhanced proliferation in normal SC cultures 36-50%. Meanwhile, outward K current amplitudes in normal cultures were either not affected (2 cultures) or dramatically increased (5 fold, 1 culture). The effects of these agents on NF1 derived cultures were also mixed: dbcAMP cut proliferation rates by half (1 neurofibrosarcoma culture), while the effects of forskolin were even more dramatic, reducing proliferation to 1/8 of the control rate (1 neurofibrosarcoma culture). Meanwhile, CPTcAMP had no effect on proliferation (1 neurofibrosarcoma culture). The effect of dbcAMP treatment of neurofibromsarcoma cell lines on outward K currents was to increase their amplitude 10 fold, while in a cutaneous neurofibroma culture K current amplitude was increased nearly 3 fold. CPTcAMP had no effect on K current amplitude in a SC culture from a normal nerve of an NF1 patient.

GGF (10 nM recombinant heregulin B1) had the expected effect on normal SC cultures. It increased proliferation ~25%, and increased K current amplitude 0-40% in different cultures. When combined with agents that elevated cAMP directly (CPTcAMP; 1 μM), K currents doubled in amplitude.

Our overall impression of these results is that basal cAMP levels vary in different cultures, and contributing to the basal level with phamacological intervention can either dramatically increase proliferation and K current amplitude (we speculate this happens when basal cAMP levels are low) or have little or no effect (perhaps if basal cAMP levels are already elevated). A reliable means of assessing basal and augmented levels of cAMP is critical to this assessment, which we were never able to do reliably.

Due to the failure of cAMP studies to provide much useful information, we discarded the idea of K channel antibodies, also a part of Objective 3. As an alternative, we have been pursuing studies on a component of outward K current we believe plays a role in proliferation of NF1 SC. The results of these studies are summarized below.

Ca^{2+}-activated K current component in the non-inactivating current of human SC in vitro

We used pharmacological tools to look for Ca^{2+} -activated K currents in normal and NF1 SC in electrophysiological experiments. We used the Ca^{2} -activated K channel blockers charybdotoxin (ChTX) and clotrimazole, with internal Ca^{2+} concentrations appropriate to permit the activation of Ca^{2+} -activated K channels (100 nM). We concentrated our effort in these preliminary experiments on a particular MPNST cell line that has relatively uniform currents from cell to cell, on SC from a plexiform neurofibroma, and on a normal SC culture that was grown in GGF to stimulate proliferation. ChTX (2.5 nM; = K_i for human pancreatic cell derived maxiK channels expressed in *Xenopus* oocytes; Ishii et al 1997) blocked an average of 37% of non-inactivating K currents in MPNST derived SC (n=4; data not shown), indicating that a portion of the non-inactivating K current in these tumor SC is a maxiK or intermediate conductance (IK) Ca^{2+}-activated K current.

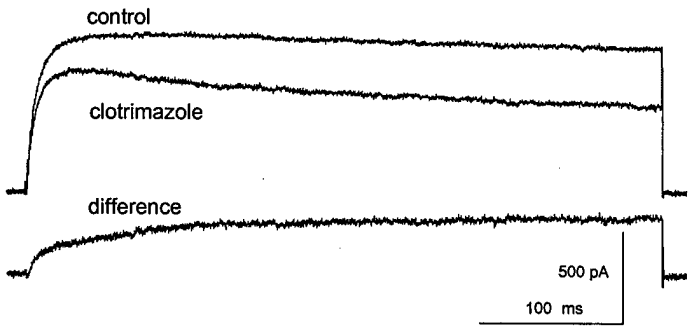


Fig. 1. Cultured MPNST-derived SC with predominantly non-inactivating K current before and after bath exposure to clotrimazole (100 nM). The offset trace is the difference current. Average block in 11 cells from this culture was 35 ± 17%.

Clotrimazole, a specific blocker of IK (100 nM; K_i of IK in human T-lymphocytes = 70 nM; Ghanshani et al 2000) blocked an average 35 ± 17% of the current of MPNST cells (n=11; Fig. 1). The current component blocked by clotrimazole was a non-inactivating current in both normal (Fig. 2) and tumor SC. But whereas in tumor SC clotrimazole blocked a portion of total non-inactivating current, in normal SC, the current blocked by clotrimazole corresponded, 1:1, to the non-inactivating current common in proliferating normal SC. Recall from the studies with TEA analogs that chronic block of the non-inactivating current of these normal SC stopped their proliferation.

65 hrs chronic treatment with clotrimazole depolarized the resting potential of MPNST cells ~15 mV, consistent with its block of a portion of the K current. When recordings were made from MPNST cells chronically exposed to clotrimazole, then the drug was washed off, a proportion of current apparently blocked by clotrimazole immediately became unblocked (~12 sec; Fig. 2). The current component appearing after wash-off was non-inactivating. It was a proportion of total current (35.5 ± 12%; n=4) approximately equal to that blocked in control cells by bath application of clotrimazole. This result suggests that the effects of 65 hrs clotrimazole exposure are specific to block of a portion of non-inactivating K current. This is relevant in light of reports that clotrimazole inhibits cytochrome P450 (Brugnara 2001). Cell health appeared unaffected by 65 hrs in clotrimazole; cells were easily patch clamped and had non-zero resting potentials.

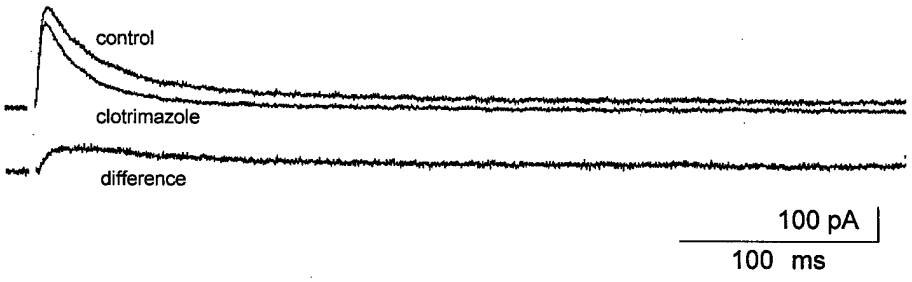


Fig. 2. Normal SC with predominantly A type K current before and after l ath exposure to clotrimazole (acute; 100 nM). The offset trace is the difference current of ontrol current minus current in clotrimazole, and represents the clotrimazole-sensitive current.

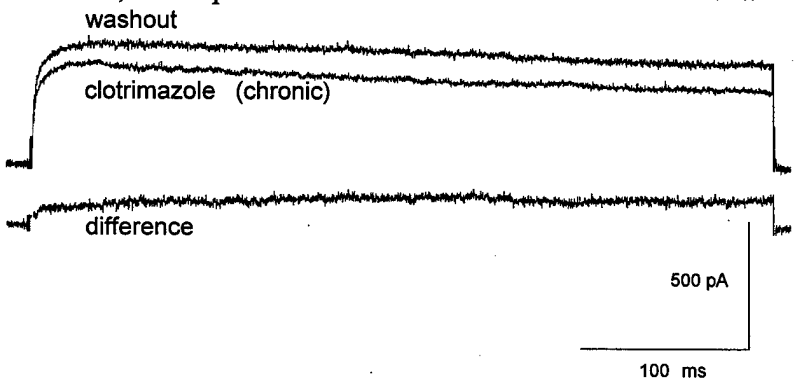
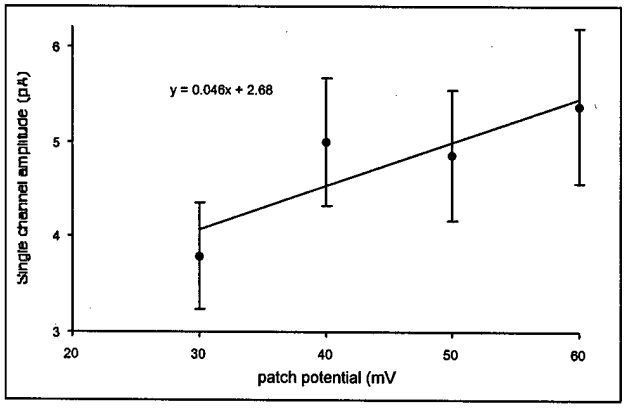


Fig. 3. Current ı a cultured MPNST-derived SC that had been exposed tc clotrimazole for 65 hrs (chronic; 100 nM), washout of the drug, and the difference current, representing the clotrimazole-sensitive current.

Single K channels were recorded from inside-out membrane patches of plexiform neurofibroma SC in which the bath perfused the intracellular face of the channels in a 140 mM KCl solution that had a free [Ca^{2+}] of ~100 nM. These channels opened in bursts separated by quiescent periods and had a unit conductance of 46 pS; both of these properties are typical of IK channels (see Table 1; Fig. 4A). The channels were susceptible to block by 100 nM clotrimazole applied to the cytoplasmic face of the channels (Fig. 4B). Clotrimazole caused a shortening of the length of open channel bursts and lengthening of the quiescent periods between bursts that is characteristic of block of IK channels (Dunn 1998).

A.



B.

Fig. 4. Single IK channels at +60 mV in an excised patch from a plexiform neurofibroma SC. A. Plot of membrane voltage vs. average unitary channel amplitude (± SD). Linear regression fit of the line shows a single channel slope conductance of 46 pS. B. Channel open behavior on a compressed time scale before and after the addition of clotrimazole (100 nM) to the bathing solution. Scale bar: 50 ms, 5 pA.

Clotrimazole blocks proliferation in NF1 SC

It was noted by others that clotrimazole (100 nM) inhibited proliferation of activated human T-lymphocytes 25% after 48 hrs via its effects on IK (Ghanshani et al 2000); greater inhibition was achieved at higher doses (IC_{50}=320 nM). We tested clotrimazole on proliferation of NF1 and normal proliferating cultures for 76-100 hrs. Clotrimazole inhibited proliferation 14% in 1 MPNST cell line and 67% in a plexiform neurofibroma at 200 nM. A different MPNST cell line was inhibited 32% in 300 nM clotrimazole. Removing clotrimazole from cultures after 48 hrs restored their proliferation rate to control levels. Proliferation in 2 normal cultures stimulated with GGF was inhibited an average of 57% at 200 nM. Clotrimazole-exposed cells had typical morphology and appeared non-vacuolated. These electrophysiology and BrdU results suggests that IK may be generally involved in proliferation of cultured SC, and support the idea that clotrimazole and ChTx-sensitive K currents are a worthwhile focus for experiments on inhibition of proliferation mediated by K channels in NF1.

KEY RESEARCH ACCOMPLISHMENTS

1. Ion channel phenotypes of normal, neurofibroma-derived and neurofibrosarcoma-derived SC found to represent a continuum from A type currents in quiescent normal SC, to large tumored K type, in neurofibrosarcoma SC. Blocking tumored K current with K channel antagonists blocks proliferation, implicating this current, or a component of it, in the proliferative capability of SC.

2. Ca^{2+} -activated K currents identified in proliferating normal SC and in tumor SC.

3. Demonstrated that blocking tumor Ca^{2+}-activated K current with clotrimazole inhibits NF1 SC proliferation.

REPORTABLE OUTCOMES

1. Submitted application for U.S. Army Medical Research and Materiel Command National Neurofibromatosis Program grant, July 2001. No award was made

2. Due to her research success and the opportunities for mentoring afforded by this award, support for PhD student Diana González was included in a campus-wide application to the Sloan Foundation to support minority graduate student education at our campus of the University of Miami, 8-01. This application resulted in an award to the university.

3. The P. I. submitted application for NIH R21 February 2002. Learned June 2002, that no award would be made.

4. The P. I. attended the NNFF Annual Meeting Aspen, Colorado, June 2002. Poster presentation: K channel blockers inhibit proliferation in NF1 Schwann cells, by Lynne A. Fieber, Diana M. González, Margaret R. Wallace, and David Muir.

5. The P. I. submitted an abstract for a presentation at the Society for Neuroscience meeting Nov. 3-7, 2002: K channel blockers inhibit proliferation in NF1 Schwann cells, by Lynne A. Fieber, Diana M. González, Margaret R. Wallace, and David Muir.

6. Former PhD student Diana González submitted an internship report including all the SC proliferation data she collected while supported by this award as fulfillment of part of the requirements for the master of arts in biological oceanography, June 2002. Ms. González is currently enrolled at veterinary school at the University of Florida.

7. Submitted application for U.S. Army Medical Research and Materiel Command National Neurofibromatosis Program grant, September 2002. No award was made.

8. The P. I. submitted application for NIH R01 October 1, 2002. No award was made.

9. The manuscript, entitled: Delayed rectifier K currents in NF1 Schwann cells: pharmacological block inhibits proliferation, by Lynne A. Fieber, Diana M. González,

Margaret R. Wallace, and David Muir was published in Neurobiology of Disease, 13:136-46, 2003.

10. Ms. Karen Bates hired September-December 2002 as technician working on proliferation studies.

11. Ms. Sarah Alter hired June-03 as research associate working on proliferation studies.

12. The P. I. submitted application for DAMD award August, 2003.

13. The P. I. attended the Aquatic Animal Models for Human Disease meeting held 9/29-10/2/03 at the ATCC in Manassas, VA. Poster presentation: "Ion channels in cancer: Translation from fish to human studies", by Lynne A. Fieber, Sarah Alter, Karen Bates.

BIBLIOGRAPHY OF PUBLISHED WORKS AND ABSTRACTS RESULTING FROM THIS AWARD

Fieber, L. A., D. M. González, M. R. Wallace, and D. Muir. (2003) Delayed rectifier K currents in NF1 Schwann cells. Pharmacological block inhibits proliferation. Neurobiol. Dis.13: 136-146.

Ionic currents in normal and neurofibromatosis type 1-affected human Schwann cells
Fieber, L. A., D. M. González, M. R. Wallace, and D. Muir
The NNFF International Consortium for the Molecular and Cell Biology of NF1 and NF2, Aspen, Colorado, June 2000

K channel blockers inhibit proliferation in NF1 Schwann cells
Fieber, L. A., D. M. González, M. R. Wallace, and D. Muir
The NNFF International Consortium for the Molecular and Cell Biology of NF1 and NF2, Aspen, Colorado, June 2002

K channel blockers inhibit proliferation in NF1 Schwann cells
Fieber, L. A., D. M. González, M. R. Wallace, and D. Muir
Society for Neuroscience Annual Meeting, Orlando, Florida, October 2002

Poster presentation: "Ion channels in cancer: Translation from fish to human studies", by Lynne A. Fieber, Sarah Alter, Karen Bates. Aquatic Animal Models for Human Disease meeting held 9/29-10/2/03 at the ATCC in Manassas, VA.

Invited Talks

January 12, 1998 Miami Project to Cure Paralysis Seminar Series, Miami, Florida, "Ion currents in Schwann cells from fish and humans in the disease neurofibromatosis"

Jan. 8, 2000 Florida Regional Chapter of the National Neurofibromatosis Foundation, Orlando, Florida, "A new view of the Schwann cell in understanding neurofibromas"

May 4, 2002 Transport 2002, A symposium in honor of the 70th birthday of Dr. Jose Zadunaisky, Miami, Florida, "K currents in Schwann cells from neurofibromatosis: from pisces to people"

October 18, 2002 University of Miami Neuroscience Program Annual Retreat, Islamorada, Florida, "Electrophysiological changes in damselfish and human cancers"

PERSONNEL INVOLVED IN THIS RESEARCH

1. Lynne A. Fieber, PhD, Principal Investigator
2. Diana González, graduate student
3. Karen Bates, technician
4. Sarah Alter, research associate

CONCLUSIONS

1. The ion channel phenotypes of normal, neurofibroma-derived and neurofibrosarcoma-derived SC represent a continuum from A type currents, characteristic of quiescent normal SC, to large tumored K type, characteristic of neurofibrosarcoma SC. Some aspects of the K current profile of all these SC types may be dependent on whether or not the SC is proliferating, while others may be dependent on the neurofibromin status of the cells.

2. Blocking tumored K current with K channel antagonists blocks proliferation, implicating this current, or a component of it, in the proliferative capability of SC. Blocking tumored K current also depolarizes SC resting potential, but we believe this is secondary to K channel block and not the cause of the inhibition of proliferation.

3. A farnysl transferase inhibitor blocks proliferation, which suggests that Ras has a role in the proliferative capability of normal and NF1 SC in tissue culture. The inhibitor blocks K current in a plexiform neurofibroma. It is probably important to block all 3 kinds of Ras present in SC: H-Ras, K-Ras, and N-Ras. While the former is activated by farnesylation, the latter 2 are at least partially activated by GGTase-1. Thus farnysl transferase inhibitor may not block all Ras within SC.

4. The identification of a non-inactivating ChTx- and clotrimazole-sensitive Ca^{2+}-activated K current(s) in proliferating NF1 SC, and a similar current, at lower density, in 1 normal, proliferating SC culture suggests that Ca^{2+}-activated K currents have a connection to SC proliferation. Accordingly, block of the Ca^{2+}-activated K current with clotrimazole inhibited proliferation of tumor SC.

5. As a result of these observations and our review of the literature implicating this current in instigating proliferation in other cell types, we have refined our hypothesis regarding

proliferation in NF1 SC. We believe the key consequence of Ras and neurofibromin-induced signaling leading to proliferation of SC in NF1 is increased expression of K channels, which induces proliferation. We predict that other downstream targets of the Ras pathway are not sufficient to drive proliferation on their own. We predict that this Ca^{2+}-activated K current is a worthwhile focus of therapeutic efforts to treat NF1 tumors.

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APPENDIX

Fieber, L. A., D. M. González, M. R. Wallace, and D. Muir. (2003) Delayed rectifier K currents in NF1 Schwann cells. Pharmacological block inhibits proliferation. Neurobiol. Dis.13: 136-146.

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Delayed rectifier K currents in NF1 Schwann cells Pharmacological block inhibits proliferation

Lynne A. Fieber,[a,*] Diana M. González,[b] Margaret R. Wallace,[b] and David Muir[c]

[a] *Division of Marine Biology and Fisheries, University of Miami Rosenstiel School, Miami, FL, USA*
[b] *Department of Molecular Genetics and Microbiology, University of Florida College of Medicine, Gainesville, FL, USA*
[c] *Department of Pediatrics (Neurology Division), University of Florida College of Medicine, Gainesville, FL, USA*

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Abstract

K^+ (K) currents are related to the proliferation of many cell types and have a relationship to second messenger pathways implicated in regulation of the cell cycle in development and certain disease states. We examined the role of K currents in Schwann cells (SC) cultured from tumors that arise in the human disease neurofibromatosis type 1 (NF1). Comparisons were made between whole cell voltage clamp recordings from normal human SC cultures and from neurofibroma cultures and malignant peripheral nerve sheath tumor (MPNST) cell lines. The outward K currents of normal and tumor cells could be divided into three types based on pharmacology and macroscopic inactivation: (1) "A type" current blocked by 4-aminopyridine, (2) delayed rectifier (DR) current blocked by tetraethylammonium, and (3) biphasic current consisting of a combination of these two current types. The DR K current was present in MPNST- and neurofibroma-derived SC, but not in quiescent, nondividing, normal SC. DR currents were largest in MPNST-derived SC (50 pA/pF vs. 2.1–4.9 pA/pF in dividing and quiescent normal SC). Normal SC cultures had significantly more cells with A type current than cultures of MPNST and the plexiform neurofibroma. Conversely, MPNST and plexiform neurofibroma cultures had significantly more SC with DR current than did normal cultures, and these DR currents were significantly larger. In addition, the plexiform neurofibroma culture had significantly more cells with DR current than the dermal neurofibroma culture. K currents in SC from normal NF1 SC cultures had current abundances similar to GGF-exposed normal SC and the plexiform neurofibroma. We have established a link between DR K current blockade via TEA analogs and inhibition of proliferation of NF1 SC in vitro. In addition, a farnysyl transferase inhibitor (FTI), a blocker of Ras activation, blocked cell proliferation without blocking K currents in all cultures except a plexiform neurofibroma, suggesting that regulation of proliferation in neoplastic and normal SC in vitro is complex.
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Introduction

Although the human neurofibromatosis type 1 (NF1) gene has been cloned (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990) and its protein product, neurofibromin, identified (Xu et al., 1990), the function of neurofibromin and its role in tumorigenesis remains enigmatic. Neurofibromin is a normal constituent of the cell cytoplasm, with significant expression after embryogenesis limited to cells of neuroectodermal origin (Daston et al., 1992a, 1992b), such as neurons and Schwann cells (SC). While the NF1 gene appears to function as a tumor suppressor gene, contributing to inactivation of the cellular proto-oncogene *ras*, the role of neurofibromin in cell proliferation appears complex and has been difficult to dissect in SC. For example, the critical amount of neurofibromin for normal cell function is unknown. In addition, increased Ras activity leads to SC differentiation, not proliferation (Kim et al., 1995). These observations have led to consideration of additional intracellular elements contributing to the NF1 cellular phenotype besides neurofibromin and Ras. Experiments to explore the function of neurofibromin and its

* Corresponding author. University of Miami RSMAS-MBF, 4600 Rickenbacker Cswy., Miami, FL 33149, USA. Fax: +1-305-361-4600.
E-mail address: lfieber@rsmas.miami.edu (L.A. Fieber).

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modulation in animal models have resulted in several physiological studies implicating the intracellular signaling molecules cyclic AMP (cAMP) and protein kinase C in defining the phenotype of NF1-deficient cells (Guo et al., 1997; Kim et al., 1997a, 1997b; Ratner et al., 1998). In two studies, second messengers were found to control K channel function. In *Drosophila* (Guo et al., 1997), where neurofibromin appears to be strongly associated with the function of adenylyl cyclase, a muscle K current dependent on intact adenylyl cyclase and Ras signaling was nonfunctional in NF1-deficient files, implying that the absence of neurofibromin interfered with normal adenylyl cyclase activity. The link between neurofibromin and adenylyl cyclase was corroborated in mouse neurofibromin-deficient SC, which have increased K current density compared to SC from wild-type controls (Xu et al., 2002). Inhibitors of protein kinase A significantly decreased the K channel density of mouse neurofibromin-deficient SC. Inhibition of Ras with a dominant-negative Ras had a potentiating effect on K current density.

While the ultimate goal of NF1 research is to find appropriate therapies, considerable research remains to be done to understand the cellular physiology of this disease. An important component of SC physiology, which has remained relatively unknown in NF1, is the electrophysiology of affected cells and the relationship of ion currents to development and maintenance of the NF1 SC phenotype. Recent studies have demonstrated that K currents are different in normal human SC and in SC derived from tumors in the diseases NF1 (Fieber, 1998) and NF2 (Kamleiter et al., 1998; Rosenbaum et al., 2000).

Voltage-gated K currents constitute the main conductances found in SC, although SC also have Na^+ currents and, in mouse, Ca^{2+} currents (Amedee et al., 1991). The K currents of SC are (1) inward-rectifier (IR) K current that conducts K^+ out of the cell in response to hyperpolarizing voltage stimuli, (2) time inactivating, A-type current, an outward K current that is blocked preferentially by 4-aminopyridine (4-AP), and a complex, composite current called delayed rectifier (DR) current, which is an outward conductance that is blocked by tetraethylammonium (TEA). DR currents are composed of many different molecular entities that can be present alone or in various combinations to produce the observed current properties. DR channels of SC are represented by the four families Shaker, Shaw, Shal, and Shab and include homo- and heteromultimers of many channel subunits including Kv1.1, Kv1.2, Kv1.5, Kv2.1, Kv3.1, and Kv3.2 (Sobko et al., 1998).

In the present study we examined the relationship between a K channel current, which may be unique to SC derived from tumors, and SC proliferation. Evidence suggests that these K currents are directly related to the proliferative capacity of many cell types and have a relationship to well-characterized second messenger pathways that play a role in the cell cycle during development and in certain disease states (Rane, 1999). By studying the K currents of NF1 SC and their relationship to proliferation, we hope to provide insights about the tumorigenic process in NF1.

Materials and methods

Cell culture

Normal cauda equina from human organ donors were obtained with full legal consent from the family of the donor by the University of Miami Organ Procurement Team. The cauda equina were harvested within 1 h of aortic clamping and stored in Belzer's cold storage solution at 4°C for ≤3 days prior to placement in culture (Levi et al., 1995).

Fresh (never cryopreserved) primary cultures of normal SC obtained from 4 donors constituted the "PW" series of cell cultures, generously provided by Dr. Patrick Wood. Nerve fascicles were freed of connective tissue and superficial blood vessels, cut into 2-mm-long explants, and the explants placed in 35-mm dishes containing D culture medium consisting of Dulbecco's modified Eagle's medium (DMEM; Life Sciences Technologies, Grand Island, NY), 10% heat inactivated fetal bovine serum with 50 U/ml penicillin, and 0.05 mg/ml streptomycin. In 3 of the 4 PW normal SC cultures, the D medium was supplemented with 1 μM forskolin and 10 nM recombinant human heregulin (rh-heregulin, also termed glial growth factor (GGF)); Casella et al., 1996) to stimulate SC proliferation. No growth factors were added to the fourth PW culture (PW1). After 2 weeks, dissociated SC were obtained by 18 h of enzymatic digestion of the explant in 0.25% dispase (Boehringer Mannheim Biochemicals, Indianapolis, IN), 0.05% collagenase (Worthington Biochemical Corporation, Freehold NJ), and 15% fetal bovine serum (Hyclone, Logan, UT) in D medium. The SC were plated on collagen-coated 35-mm dishes and maintained at 37°C in a 6% CO_2 atmosphere. Cultures prepared in this way have been shown to contain 92% pure SC by S-100 staining (Casella et al., 1996). The normal culture not exposed to GGF and forskolin was purified by successive platings for short periods on tissue culture plastic dishes before plating on collagen coated dishes. This culture was approximately 90% SC, and was studied 10 days after dissociation. One to 10 days before use in experiments, normal cultures were transferred to normal culture medium at 37°C in a 5% CO_2 atmosphere. Normal culture medium consisted of 85% DMEM (with high glucose and pyruvate), 15% fetal bovine serum, and 50 U/ml penicillin, 0.05 mg/ml streptomycin, and 2 mM glutamine (all from Life Sciences Technologies), without forskolin or GGF.

The "DM" neurofibroma and malignant peripheral nerve sheath tumor (MPNST) cultures, and DM normal SC cultures were obtained from human donors and cultured according to Muir et al. (2001) to enrich for SC, similarly to the procedure described above for PW SC cultures. The MPNST SC line T265 was obtained as frozen stock from

Dr. George De Vries (Badache and De Vries, 1998). All MPNST lines were derived from patients with NF1. DM9 and DM10 SC cultures were derived from the normal nerves of different NF1 patients, either from limb amputation or post mortem. The nerves from which these cultures were derived were not associated with tumors, nor did histology show neoplastic elements. We refer to these cultures as "normal SC cultured from NF1 patients." Prior to electrophysiological recordings and proliferation studies, DM and T265 cultures were plated from cryopreserved stocks. All of the subcultures and tumor cells lines described in this section were maintained in normal culture medium described above, on poly-L-lysine- and laminin-coated 75-mm^2 plastic tissue-culture dishes. In this medium, tumor cells divided rapidly and became confluent within days of subculture. Subcultures were plated onto poly-L-lysine- and laminin-coated 35-mm Falcon dishes. Poly-L-lysine and laminin were obtained from Sigma.

Electrophysiological methods and analysis

Whole cell currents were recorded using the whole cell variation of the patch clamp technique with patch pipettes of 0.5–2 MΩ. Electrophysiological experiments were performed on isolated cells in sparsely seeded cultures that were not confluent. Recordings were limited to bipolar cells in cultures known to contain other cell types besides SC. The intracellular solution contained (mM): 150 KCl, 1 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 $CaCl_2$, 5 $MgCl_2$, 5 Na_2ATP, and 40 *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES)-KOH, pH 7.0. The calculated intracellular free Ca^{2+} concentration was ~40 pM (Owen, 1976). Normal extracellular solution (ECS) consisted of (mM): 170 NaCl, 3 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES-NaOH, pH 7.25. 4-Aminopyridine solution (4-AP) was made by adding 5 mM 4-AP (Sigma) to ECS and readjusting the pH to 7.25; 50 mM tetraethylammonium (TEA) solution was made by adding TEACl (Sigma) to ECS with a decreased NaCl content. The cells were continuously perfused with extracellular solutions via 1-μL pipettes attached to gravity-dispensed solution reservoirs.

Current amplitudes were directly comparable even though the size of cells varied, because the currents (in picoamperes, pA) were normalized to the cell's capacitance (in picofarads, pF).

Currents were recorded at room temperature (19–22°C) with an Axopatch 200A or 200B using the PClamp 6 programs (Axon Instruments, Foster City, CA) and a 400 MHz PC with a Digidata 1200 A-D converter (Axon Instruments). Electrophysiological data were 4-pole low-pass Bessel filtered and digitized at 10 kHz. The capacitive current cancellation method of Howe and Ritchie (1990) with 80% series resistance compensation was used, with voltage drops resulting from uncompensated capacitance (<3 mV) not subtracted from the data records. In preliminary experiments no differences in SC currents present were observed when the chloride channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; 100 μM) was included in the ECS. Thus, it was assumed that chloride channels did not contribute substantially to the outward currents observed.

Neurofibromin assays

Neurofibromin Western immunoblotting was performed as in Muir et al. (2001), where the neurofibromin status of the DM cultures used in this study was first reported. The dermal and plexiform neurofibroma cultures and the MPNST cultures were neurofibromin-negative, except for 1 MPNST (DM6) that had a variant (and possibly nonfunctional) form. Full-length neurofibromin was undetectable in two SC cultures derived from the normal nerves of different NF1 patients. Normal cultures were either determined to be neurofibromin positive, or were not tested but assumed neurofibromin positive. The neurofibromin-negative status of MPNST culture T275 was reported by Badache et al. (1998).

Proliferation assays

SC were plated at 12,000 per 35-mm dish and five fields per 35-mm dish in three replicate dishes were counted at 200× magnification. Doubling times were determined by dividing the average of cell counts at $t_{0+(54\text{ to }72)h}$ by average cell counts obtained at t_0, where t_0 = 24 h after plating, then normalizing to 2. The bromodeoxyuridine (BrdU) immunoassay (Zymed) was used to assess proliferation of SC in culture. SC cultures were exposed to BrdU for 5–6 h. Immunolabeling for proliferating cell nuclear antigen (PCNA) was used as a proxy for cell division in selected cells in which K currents were previously recorded. PCNA indicates cells that were in the G2 phase of the cell cycle at the time they were fixed. K currents were recorded with lucifer yellow (1 mg/ml; Sigma-Aldrich) in the patch pipette, then the cultures were fixed and the PCNA assay (Zymed) was run. Cells were identified by means of lucifer yellow fluorescence, then the PCNA status of these identified cells was assessed under phase-contrast optics.

The effects of several reagents on proliferation and K currents were studied. TEA analogs tetrapentylammonium (TPeA; 50 μM) and tetrahexylammonium (THeA; 5 μM; Sigma-Aldrich) were used to block K currents. A farnesyl transferase inhibitor (FTI; FPT Inhibitor III, Calbiochem; 10 and 50 μM; Kim et al., 1997b) was used to block Ras. Cells plated on 18- or 25-mm round glass coverslips for proliferation assays and onto the bottoms of 35-mm dishes for electrophysiology were exposed for 48–72 h to these individual reagents. To determine the number of stained cells and total cell counts, five fields of cells per coverslip were counted in three replicate dishes exposed to one of

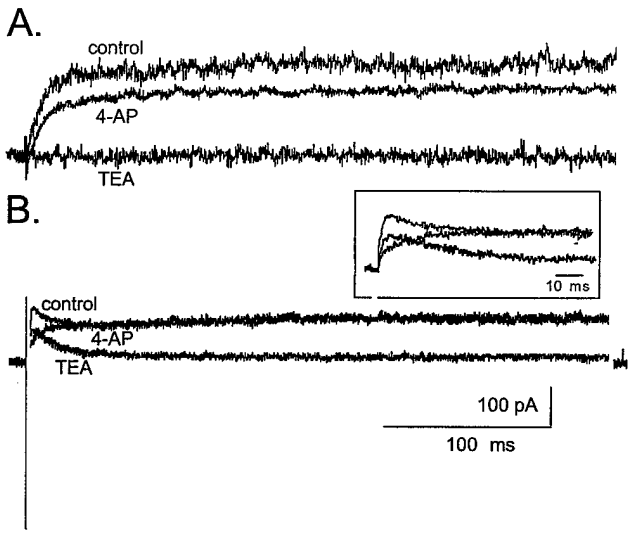

Fig. 1. K currents in tumor Schwann cells (SC). (A) Pharmacological experiments at 20 mV from a holding potential of −70 mV in an MPNST cell to identify the K currents as delayed rectifier (DR) currents, preferentially blocked by tetraethylammonium (TEA). This cell has a small current component blocked also by 4-aminopyridine (4-AP). Traces show control currents and currents after bath application of TEA, then after washout and subsequent bath application of 4-AP. MPNST, malignant peripheral nerve sheath tumor. (B) Pharmacological experiments at 20 mV in a plexiform neurofibroma SC to identify the biphasic K currents. This cell has a DR component blocked only by TEA, and an A type component blocked only by 4-AP. The insert shows the initial phase of these currents on an expanded scale with the TEA-exposed trace as a heavy line.

these agents, in addition to unexposed control cultures and cultures to which no primary antibody or BrdU was added.

Statistical analysis

Data were analyzed for frequency of occurrence using the *G* test (Sokal and Rohlf, 1995), and for average differences using Student's *t* test (Datadesk for the Macintosh, Ithaca, NY).

Results

Overview

Results from assays for cell proliferation and electrophysiology presented in the Tables and Figures were obtained on early passages of normal and NF1 cell cultures, except for MPNST T265 where the passage number was high. Cultures were maintained for extended periods only to assess the durability of proliferation rates with time away from GGF or forskolin. All cell cultures were assumed to remain SC enriched for the brief time they were kept in culture to produce electrophysiological and proliferation results. This assumption was supported by morphological observations on the cell cultures. In addition, data were acquired from cells with clearly distinguishable SC morphology.

While we have classified these tumors in individual categories according to the histological type of neurofibroma, because these are human tumors and not samples from inbred animals, considerable variation was expected in tumor composition and histology. In some tumor cultures, all SC had the same currents. In other cultures there was some degree of cell-to-cell variation. Some cells had more than one K current type. When two K current types co-occurred in a single cell, they produced a biphasic current that could be dissected using component-specific blockers (see Fig. 1B). Thus, we characterized the distribution of the currents both as components of a biphasic current and as "pure" currents.

Normal and neurofibroma-derived SC currents

Studies on NF1 cell cultures and cell lines confirm the findings of Fieber (1998) in NF1 MPNST cell lines. The most conspicuous difference between normal and tumor-derived SC K currents in culture was that tumor cells functionally expressed DR currents (Fig. 1A), while quiescent, normal SC had either no outward K currents or only A type currents (Fig. 2). Na^+ currents and inward rectifying K^+ currents were occasionally observed in recordings from both normal and tumor-derived cultured SC, as in Fieber (1998).

SC currents and proliferation status

Quiescent normal SC never exposed to GGF (PW1) studied soon after dissociation of the nerve (Levi et al.,

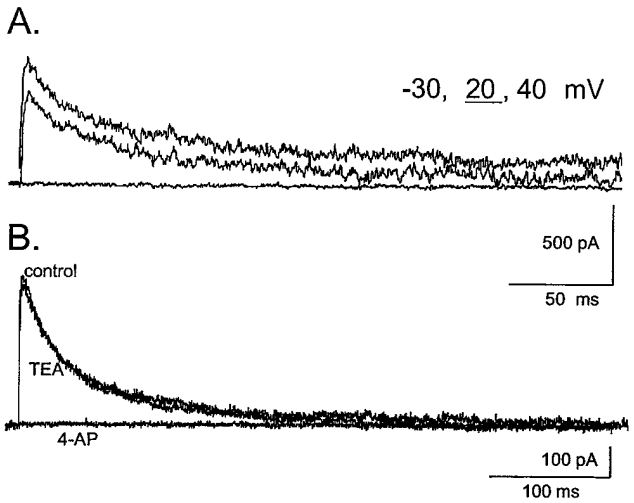

Fig. 2. K currents in normal Schwann cells (SC). (A) Family of K currents to the indicated test potentials from a holding potential of −70 mV. (B) Pharmacological experiments at 20 mV to identify the K currents as A type preferentially blocked by 4-aminopyridine (4-AP). Traces show control currents and currents after bath application of tetraethylammonium (TEA), then after washout and subsequent bath application of 4-AP.

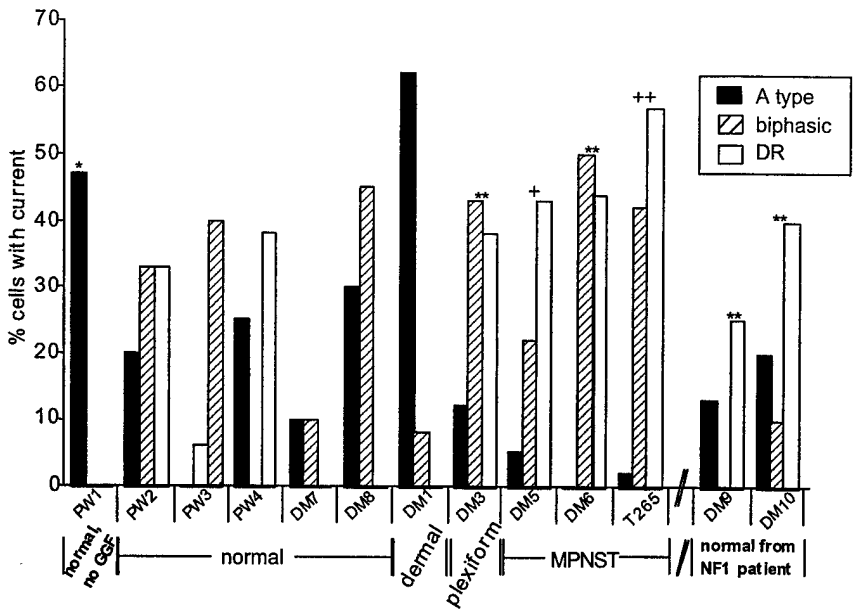

Fig. 3. K current abundances by type. Normal culture PW1 was purified without glial growth factor (GGF) or forskolin, while all other cultures were exposed to GGF during their purification and in some cases prior to electrophysiological experiments. *A type current abundance significantly different from A currents of MPNSTs and DM8, G test, $P \leq 0.05$. **, ***, $^{+}$, $^{++}$DR plus biphasic current abundances significantly different from these current abundances in: **Normal culture PW1 and dermal neurofibroma (DM1), $P \leq 0.05$. Current abundances in DM9 and DM10 cultures combined for this analysis. $^{+}$Normal culture PW1 and dermal (DM1) and plexiform (DM3) neurofibromas, $P \leq 0.05$. $^{++}$Normal cultures PW1 and DM7, $P \leq 0.05$. MPNST, malignant peripheral nerve sheath tumor; DR, delayed rectifier.

1995) had small amplitude outward K currents of the transient, A type, blocked by 4-AP (Fig. 2A and B; Fieber, 1998). Forty-seven percent of quiescent normal SC had A type currents averaging 4.84 ± 0.54 pA/pF (Figs. 3 and 4). Other quiescent normal SC had no outward currents, although some had IR K currents or Na^{+} currents. Many

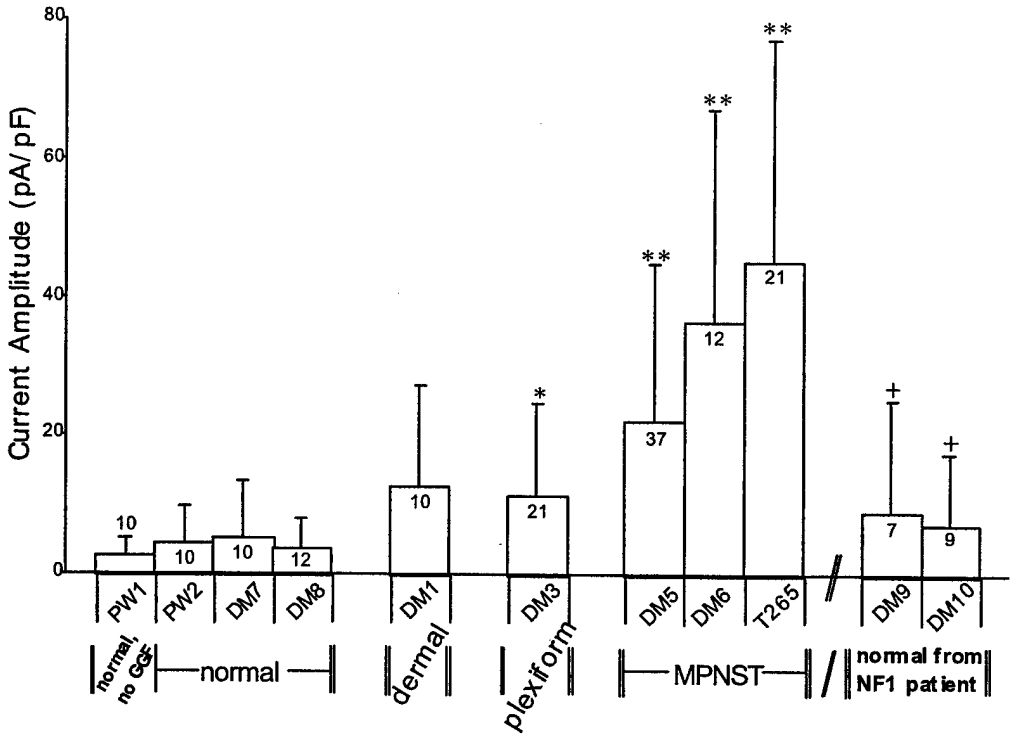

Fig. 4. Maximum current amplitudes without selection for current type, +SD. Numbers above or inside bars are sample size. *Significantly different from MPNSTs and the plexiform neurofibroma culture DM8, t test, $P \leq 0.05$. **Significantly different from all normal cultures, and from dermal and plexiform neurofibromas, $P \leq 0.05$. $^{+}$Significantly different from MPNSTs, $P \leq 0.05$. MPNST, malignant peripheral nerve sheath tumor.

Table 1
Summary of outward K currents in normal and NF1 SC[a]

SC culture type	Predominant outward K current(s)	Significant trends
Normal	A type	Noninactivating increases ↓ ↑ A type increases
Dermal neurofibroma	A type, biphasic	
Plexiform neurofibroma	Biphasic, noninactivating	
MPNST	Noninactivating	

[a] SC, Schwann cells; MPNST, malignant peripheral nerve sheath tumor.

normal SC do not have recorded currents in vitro (Fieber, 1998).

Normal SC cultures maintained in GGF + forskolin exhibited doubling times of 38–87 h and had A type and biphasic currents, plus DR currents of small amplitude (2.1–3.0 pA/pF; Figs. 3 and 4). They occasionally had Na^+ currents.

Normal SC 6 days after withdrawal of GGF proliferated in culture in 15% serum on laminin-coated dishes, with doubling times of 38–87 h. K currents of dividing normal SC were A type or DR currents (1.4–12.2 pA/pF), or biphasic K currents composed of A type and DR currents, respectively (Figs. 3 and 4). Other currents, such as Na^+ currents, were much rarer.

The SC of all tumor cultures plated on laminin divided in culture medium containing 15% serum but no GGF. Doubling times in the neurofibroma culture and the MPNST cell lines after 1 week to 7 months in culture were ~50 h.

The hallmark K current of tumor-derived SC cultures was the DR current that was blocked by TEA (Fig. 1A). The DR K current was present in ~50% of all MPNST SC and in 38% of plexiform neurofibroma SC (Fig. 3). A type, biphasic, and Na^+ currents were also observed, as were, rarely, IR currents. The mean amplitudes of DR K currents of different tumor SC cultures ranged at 10–50 pA/pF, with the largest DR currents occurring in MPNSTs (Fig. 4). Details of the K currents of NF1 tumor cultures are presented below.

K currents in all the *MPNST* cell lines were classical, DR currents (Fig. 1A). Different current profiles were seen in the dermal and plexiform neurofibromas. Some SC from the plexiform neurofibroma had DR or biphasic currents (Figs. 1B and 3). All K currents in cells derived from a dermal neurofibroma resembled those of proliferating normal SC, with A type or biphasic K currents, but no pure tumor DR currents.

Differences in current abundance shown for the A type and DR type (including biphasic) currents are shown in Fig. 3. Normal SC culture PW1, never exposed to growth factors, had significantly more cells with A type current than cultures of MPNST and the plexiform neurofibroma, and, conversely, MPNST and plexiform neurofibroma cultures had significantly more SC with DR currents than did normal culture PW1. In addition, the plexiform neurofibroma culture had significantly more cells with DR current than the dermal neurofibroma culture. K currents in SC from normal NF1 SC cultures, though variable within a culture, had current abundances similar to GGF-exposed normal SC and the plexiform neurofibroma.

Fig. 4 shows the average maximum current amplitude in each culture, without selection for current type. Current amplitude was an additional important difference between the DR currents of normal SC and those of tumor SC. In normal, dividing SC the K currents present, whether DR, A type, or biphasic, were smaller in amplitude than in any tumor cell culture. Mean K current amplitudes of normal SC cultures were significantly different than mean current amplitudes of all MPNST cell cultures and the plexiform neurofibroma culture. K current amplitudes in the dermal neurofibroma were not significantly different from those in any normal SC culture.

Normal SC cultures from NF1 patients

Two SC cultures derived from the normal nerves of different NF1 patients had a doubling time of 63 h after 14 days withdrawn from GGF, and their K current abundances were like normal proliferating SC (Fig. 3). As a pooled sample, these normal NF1 SC cultures from NF1 patients had significantly more DR currents than both the normal culture never exposed to GGF and the dermal neurofibroma, but were not different from any MPNST culture, the plexiform neurofibroma, or proliferating normal cultures withdrawn from GGF. In addition, the amplitude of the DR current of the pooled sample of normal SC cultures from NF1 patients was significantly different from that of MPNST cultures but not the proliferating normal cultures (Fig. 4).

The K current types formed a continuum from normal SC to malignant tumor SC, with A type K current found in normal SC and SC from the dermal neurofibroma, and noninactivating K current found in the more malignant tumor cultures (Table 1).

TEA analogs block SC proliferation and SC K currents

The significant differences in abundance and amplitude of DR currents between normal and tumor-derived SC suggested experiments to investigate a possible relationship between DR currents and proliferation in NF1, as found in NF2 (Rosenbaum et al., 2000), and other proliferating cells (Knutson et al., 1997; Liu et al., 1998). The classical blocker

Table 2
Effect of TEA analogs on cell proliferation and electrophysiological parameters in normal, MPNST- and neurofibroma-derived SC[a]

Tissue source	Name	TEA analogs[b]		
		% inhibition of proliferation[c]	Change in K amplitude	Decrease in RP (mV)
Normal nerve	DM7	100 ± 0%	ND	ND
	PW4	ND	22% decrease[g]	ND
MPNST	DM5	100 ± 0.03%	73% decrease[d]	24[e]
	DM6	96.5 ± 2.6%	97% decrease[e,f]	13[d]
	T265	94 ± 6%	96% decrease[d]	26[e]
Dermal neurofibroma	DM1	71.5 ± 28%	ND	ND
Plexiform neurofibroma	DM3	100 ± 0%	>82% decrease[d]	12[e]
Normal nerve of NF1 patient	DM10	100 ± 0%	ND	ND

[a] TEA, tetraethylammonium; MPNST, malignant peripheral nerve sheath tumor; SC, Schwann cells; ND, not determined; THeA, tetrahexylammonium; TPeA, tetrapenthylammonium.
[b] 5 μM THeA and 50 μM TPeA treatments were approximately equal in their effects; therefore, experiments using either were pooled.
[c] Mean ± standard error.
[d] Significantly different from control (*t* test, $P < 0.05$).
[e] Significantly different from control (*t* test, $P < 0.01$).
[f] THeA only.
[g] Not a significant decrease.

of DR currents is TEA. We exposed SC cultures to two analogs of TEA that do not appear to be toxic to cells: TPeA (50 μM) and THeA (5 μM; Wilson and Chiu, 1993). After 54 h, cell proliferation was assayed. In 3 MPNST cell lines, 2 neurofibroma SC cultures, and 1 normal, dividing SC culture, these agents completely or almost completely inhibited tumor SC proliferation (Table 2). We verified that THeA and TPeA, also blocked K currents in tumor cells at the same concentrations that blocked proliferation, either severely reducing or abolishing recorded whole cell K currents compared to matched controls: (same cell passage; Table 2). The current block washed out within minutes after 24-h exposure to TEA analogs, confirming that THeA and TPeA blocked currents by the conventional manner of a channel plug rather than by causing downregulation of channel expression. Cells exposed to TEA analogs for up to 48 h appeared morphologically similar to controls, with a membrane sufficiently intact for electrophysiological experiments. After 72 h in 5 μM THeA, membrane seals were more difficult to obtain.

The control resting potential (RP) of SC in different MPNST- and neurofibroma-derived SC cultures averaged −26 to −44 mV. TEA analogs applied for >40 h depolarized RPs by >50% of their control values (Table 2). The reduced RPs observed in TPeA and THeA were significantly different from controls. Within 1 h of washout of TEA analogs (and relief of DR channels from block), RPs were not significantly different from matched controls (data not shown).

Proliferation and K currents were monitored in the cell line T265 after 54-h incubation in normal culture medium with 10 mM added KCl to test the idea that the inhibition of proliferation was a secondary consequence of the change in RP caused by TEA analogs. This procedure for reducing RP should have little effect on voltage-gated K channels. Although 10 mM added KCl reduced the RP of T265 cells by an average of 23 mV, the same amount of depolarization caused by TEA analogs in this cell line, it was without effect on proliferation rates or K current amplitude ($n = 8$; data not shown).

Proliferation in a normal SC culture also was blocked by TEA analogs; however, average K channel amplitude was not significantly different after 40-h exposure to these agents (Table 2). A type current is not as susceptible to block by chronic exposure to TEA analogs as DR currents. Because the DR current component was small relative to the A type component in these cells (Fig. 2B), the blockade of DR current of normal SC did not significantly alter the average K channel current.

Effects of blocking ras on proliferation and currents

Neurofibromin inactivates Ras, thereby acting as a tumor suppressor. The effects of blocking Ras on functional expression of different K channel-types were studied via application of a membrane-permeant farnesyl transferase inhibitor (FTI) to tumor and proliferating normal SC.

FTI was added to the normal culture medium of cells for 2 days before BrdU proliferation assays and/or electrophysiological experiments in tumor and normal SC cultures and one normal NF1 SC culture. Controls were passage- and time-matched cultures. In all MPNST, neurofibroma, and normal cultures tested, FTI inhibited proliferation (Table 3). FTI did not revert the tumor cells to a normal morphological phenotype (Yan et al., 1995). 48 hr FTI-exposed cultures had cell counts comparable to t_0 cell counts.

Although significant depolarizations of the RP occurred in some FTI-exposed MPNST-derived SC cultures, the size of the K currents was not significantly different for any tumor SC exposed to FTI except the plexiform neurofi-

Table 3
Effect of farnesyl transferase inhibitor (FTI) on cell proliferation in normal, MPNST-, and neurofibroma-derived SC[a]

Tissue source	Name	FTI	
		10 μM (% inhibition of proliferation)[b]	50 μM (% inhibition of proliferation)[b]
Normal nerve	DM7	87.5 ± 0.9%	100 ± 0%
	PW2	86 ± 0%	ND
MPNST:	DM5	58 ± 0%	94 ± 6%
	DM6	64 ± 19%	99 ± 0.5%
	T265	2 ± 0%	69 ± 0%
Plexiform neurofibroma	DM3	81 ± 0%	100 ± 0%[c]
Normal nerve of NF1 patient	DM10	95 ± 0%	ND

[a] MPNST, malignant peripheral nerve sheath tumor; SC, Schwann cells; ND, not determined.
[b] Mean ± standard error.
[c] Inhibition by 50 μM FTI resulted in 85% decrease in K current amplitude.

broma. FTI did not cause obvious changes in the percent composition of DR, A type, and biphasic currents.

Characterizing K currents of dividing vs. resting cells

Despite significant differences in current abundance and amplitude between cultures, both normal and tumor SC cultures showed interculture variation in K currents, which may have some relationship to the cell cycle. To identify changes in K currents that correlate with changes in the cell cycle, we made electrophysiological recordings from cells, fixed them, then examined whether these specific cells expressed PCNA at the time the recordings were made. Experiments were performed on the plexiform neurofibroma SC and a normal SC culture that was proliferating. The PCNA status of 9 plexiform neurofibroma cells and 10 normal SC was evaluated after locating the cells via staining with lucifer yellow that had been injected through the recording pipette. When the cells from both cultures were grouped together most PCNA-negative (nonproliferating) cells had A type or biphasic K currents. Most PCNA-positive SC had DR currents (Table 4). The frequency of pure DR currents had a significant correlation with a positive PCNA status ($P = 0.05$; G test).

Discussion

These results extend a previous report (Fieber, 1998) describing DR K currents in SC of MPNST cells lines to low-passage-number subcultures of neurofibromas and MPNSTs. DR currents were characteristic of tumor cultures while A type currents were more frequently observed in quiescent normal SC. The variation of K current types in dermal and plexiform neurofibroma cultures, and the similarities of some of their K currents to those present in normal SC raises the possibility that SC ion channels from at least some types of neurofibromas are not physiologically abnormal or that the ion channel phenotypes of normal, neurofibroma-derived, and MPNST-derived SC represent a continuum. Molecular characterization of the channel types present would elucidate this; however, because SC DR currents arise from channels consisting of complex heteromultimers of at least eight known subunits whose arrangement is unknown (Sobko et al., 1998), this is a challenging task with uncertain likelihood of success. The channel differences are as likely to occur in the abundance of subunit types present as in their presence or absence, whereas only the latter can be assessed using the two available techniques of subtype-specific antibodies or analysis of transcripts corresponding to specific subunits.

Table 4
K currents in pooled neurofibroma and normal SC as a function of proliferation status[a]

Proliferation status	% cells with A type or biphasic current	% cells with DR current
Proliferating	11	21
Nonproliferating	57	11

[a] SC, Schwann cells.

Normal SC cultures subcultured in the presence of mitogens such as GGF and forskolin failed to eventually revert to a quiescent state and maintained high proliferation rates weeks after removal from these substances. Sustained cell proliferation has been observed previously in SC stimulated with growth factors (Langford et al., 1988), and attributed to a permanent effect of growth factors on the cell cycle of SC, or an induced predisposition of SC to divide in response to factors present in serum or that SC produce. In our study, serum or laminin might have acted to promote sustained cell proliferation, because these cultures were plated on laminin-coated dishes, and laminin has been found to be mitogenic for SC (McGarvey et al., 1984; Muir et al., 1989).

The SC of the neurofibroma-derived cultures and all MPNST-derived cultures used in this study continue to divide and maintain their S-100-positive status for many weeks after withdrawal of GGF and forskolin (Muir et al., 2001). This is characteristic of many cells derived from

tumors, and it focuses interest on normal cultures derived from NF1 patients. If these SC are growth factor independent, they provide an important intermediate category of SC. The normal cultures derived from NF1 patients proliferated when plated on laminin in contrast to many other neurofibroma-derived SC cultures that remained quiescent under these culture conditions (Muir et al., 2001).

K channels play prominent roles in controlling proliferation and differentiation in a variety of inexcitable cell types (Knutson et al., 1997; Liu et al., 1998), and have been implicated in the growth of tumor cells (Rane, 1999; Stringer et al., 2001). Studies of cell cycle control mechanisms have demonstrated that the K currents characteristic of proliferating cells are involved in induction of proliferation, rather than being a byproduct of this process (Nilius et al., 1993; Jones et al., 1995). This suggests that K channels generally are involved in one of the signaling process associated with cell division.

The close relationship between K channels and SC proliferation has been well described in other systems. Studies of K currents of SC in animal models have demonstrated that large outward DR K currents are characteristic of proliferating SC of the embryonic nervous system (Konishi, 1990; Chiu and Wilson, 1989; Pappas and Ritchie, 1998). Pharmacological block of the DR K current of proliferating SC results in suppression of proliferation (Chiu and Wilson, 1989; Wilson and Chiu, 1993; Pappas and Ritchie, 1998).

We now extend these findings of proliferation associated with development to postembryonic human SC. TEA analogs blocked human SC DR K channels and also blocked proliferation of SC, whether the SC were cultured from tumors or normal nerves. In normal SC, the blockade of DR current did not significantly alter the average K channel current. This provides additional evidence that the DR rather than the A type current is the component associated with proliferation in human SC. These data suggest that DR channels have a role in proliferation of both normal and NF1 SC in vitro and that the SC K channel profile observed may depend in part on the proliferation status of the cells. This latter idea is supported by the results of our lucifer yellow experiments, in which DR currents were positively correlated with cells in G2 phase. These data corroborate those of Rosenbaum et al. (2000) on NF2 schwannoma-derived SC in vitro in which the IC_{50} for inhibition of proliferation by quinidine, which blocks K channels in NF2 SC, was 25 μM. Thus, it appears that K channels have a role in the control of proliferation in postembryonic human SC.

Neurofibromin-positive, proliferating normal SC had, at most, small DR currents while DR currents were common in four cultures derived from plexiform neurofibroma or MPNST. These results suggest that there is a fundamental difference in K currents of NF1 tumor SC that may not be related specifically to proliferation, but instead to their derivation from NF1 tumors. Such a role would be expected to coincide with an absence of the tumor suppressor protein neurofibromin. Accordingly, the plexiform neurofibroma-derived culture and two MPNST-derived cultures lacked full-length neurofibromin and the other MPNST assessed for neurofibromin expression had a variant (and possibly nonfunctional) form. However, no pure DR K currents were found in a fourth neurofibromin-negative culture (the dermal neurofibroma). Thus, it is important to consider the alternative hypothesis that it is not total DR K current size that is associated with the absence of neurofibromin, but rather a particular component of DR current. Pharmacological tools that identify other components of DR K currents could be used to address this hypothesis.

In addition to TEA analogs, an FTI also blocked proliferation in normal and tumor cells, but it did not affect DR currents of most cultures (the exception was a plexiform neurofibroma culture). In NF1, neurofibromin levels in tumor-derived SC are abnormally reduced or absent, theoretically freeing Ras from persistent inactivation by this tumor suppressor (Rutkowski et al., 2000; Muir et al., 2001). Accordingly, Ras-GTP levels are high in MPNST cell lines (DeClue et al., 1992). Drugs such as FTI inhibit Ras proteins in tumor SC by preventing a specific posttranslational modification that promotes their association with the plasma membrane, which is essential to their activation (Lowy and Willumsen, 1993). Thus FTI, while not restoring normal neurofibromin levels, inactivates Ras. In a plexiform neurofibroma, our results suggest that inhibition of proliferation is related to inhibition of both Ras and of DR channels. But this is clearly not the case in any MPNST cell line or in normal SC. In these latter cells, FTI-induced inhibition of proliferation occurs without affecting K channels. One interpretation for this observation is that some Ras proteins act on the cell cycle independently from K channels. Since not all cellular Ras is activated by farnesylation, FTI may knock out sufficient Ras to inhibit proliferation, but not all Ras within SC, specifically sparing those Ras proteins that are linked to K channels. In addition, FTI may affect other proteins besides Ras (Gibbs, 2000), some of which, like ERK-1 and 2, may play a role in the cell cycle (Lewis et al., 1998) but may not be linked to K channels. Finally, K channel modulation by Ras-activated pathways is probably sufficiently complex that blocking Ras alone may not revert the electrophysiological characteristics of tumor cells to normal. Furthermore, there may be significant differences in the control of the cell cycle by Ras proteins and K channels in different NF1 tumor types (DeClue et al., 1992). Additional studies of proliferation and K channel block in NF1 tumor SC are required with specific inhibitors of other Ras proteins as well as inhibitors of other intracellular messengers implicated in the Ras-initiated cascade such as Raf, Ras-mitogen activated protein kinase (MAPK) and MAPK kinase (MEK).

Although TEA analogs significantly depolarized SC RP, depolarization is probably not the cause of inhibition of proliferation by these agents. The RP of a cell is determined primarily by the difference in K^+ concentration on either side of the membrane, as maintained by the different routes

for K^+ entry, such as ion channels, the Na^+/K^+ ATPase, and leak conductances. Kodal et al. (2000) proposed an explanation for how K channel block can cause depolarization. When IR K channels are present at very low density, the opening or closure of only a few DR K channels can cause large (tens of mV) fluctuations of the RP. Few active IR channels cause the cells to become depolarized, which drives open DR K channels. DR channel opening hyperpolarizes the RP again, but if the DR channels are blocked, the RP remains depolarized.

Few studies have addressed the physiological aspects of the altered relationship between neurons and glia that occurs in NF1 tumorigenesis. Among the symptoms of NF1 are assumed electrophysiological changes in the function of the central nervous system that lead to learning and motor deficits (Eldridge et al., 1989; Pensak et al., 1989). These CNS alterations are inarguably complex. Yet a new study has demonstrated that broad manipulations to lower Ras levels in a mouse model of NF1 reverse learning deficits by blocking inhibitory potentials mediated by GABA-activated ion channels (Costa et al., 2002). Therefore, the relationships between ion channels of the nervous system, and Ras, neurofibromin, or other second messengers such as MEK and MAPK are an appropriate focus of studies to understand the different changes occurring in NF1, including tumorigenesis.

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