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The Fibroblast Intermediate Conductance K_{Ca} Channel, FIK, as a Prototype for the Cell Growth Regulatory Function of the IK Channel Family

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Abstract. The fibroblast intermediate conductance, calcium-activated potassium channel (FIK) is proposed here as a functional prototype for other IK channels which to date have undefined physiologic actions. FIK pharmacology in the 10T1/2-MRF4 myogenic fibroblast cell line was determined: to define the relationship of FIK to other IKs; to confirm a physiologic role for FIK; and, thus develop a hypothesis about IK channel family function. Whole cell patch-clamp electrophysiology was used to determine $K_{0.5}$ values for FIK block by the structurally related peptides charybdotoxin (ChTX) (7 nM) and iberiotoxin (IbTX) (536 nM), and a new unrelated FIK inhibitor, *Stichodactyla* toxin (StK) (85 nM). Peptide pharmacology for FIK was consistent with that of recently cloned IKs. ChTX and StK inhibited bFGF stimulated 10T1/2-MRF4 cell proliferation with dose-dependencies consistent with their FIK blocking actions. ChTX, StK, and IbTX also evoked MRF4-dependent transcription as measured by muscle acetylcholine receptor channel functional expression; but they did not evoke subsequent multinucleated fiber formation or myosin heavy chain expression, suggesting a role for FIK in early, rather than late, myogenic events. Thus despite structural differences, ChTX, IbTX, and StK have common effects on cell growth and differentiation reflecting their common FIK blocking action. We suggest that a major function of the IK channel family is to regulate cell growth.

Key words: Calcium-activated potassium channel — Cell growth — Myogenesis — MRF4

Introduction

IK channels are voltage-independent potassium channels that are activated by submicromolar concentrations of intracellular calcium, and exhibit mild inward rectification, giving single channel conductances ranging from 10–20 to 30–40 pS at positive and negative potentials, respectively [4, 10, 16, 27]. Pharmacologically, IK channels have been characterized by their sensitivity to block by the scorpion toxin peptides, ChTX and IbTX, and resistance to block by the bee venom, apamin, an inhibitor of the small conductance (SK) calcium-activated potassium channels. Native IK channels are expressed in nonexcitable, mitogenically active cell types, including human T-lymphocytes [16, 17], endothelial cells [1], and a variety of fibroblast cell lines [4, 11, 20, 22–24]. However, based on physiological and pharmacological evidence, these channels are apparently absent from electrically excitable tissues. Recently, it has been shown that certain features of native IK channels are shared by two IK channels cloned from human pancreatic (hIK1) and lymph node libraries (hKCa4) [12, 18]. Similar to native IK, the cloned IK channels are activated by submicromolar concentrations of intracellular calcium, are sensitive to ChTX and IbTX block, insensitive to apamin, and have single channel conductances of 11–15 pS and 33–40 pS at positive and negative voltages, respectively [12, 13, 18]. The mRNA transcripts of the cloned hIK1 and hKCa4 channels are expressed in activated T-cells as well as in mitogenically active tissues isolated from colon, placenta, and thymus; but, as for native IK channels these transcripts are absent from excitable tissues (brain, skeletal muscle, and heart) [12, 18]. In contrast to other members of the K_{Ca} channel superfamily, including SK and the large conductance (BK) channels, the physiological role of the cloned IK channels remains undefined.

This lab has previously characterized a fibroblast IK (FIK) channel [10] with a pharmacology and electrophysiology similar to cloned IKs [12–14, 18]. We originally used the term SK for this channel [10], rather than FIK, because its conductance (17/35 pS at 60/−60 mV)

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was close to that of the well-known neuronal small-conductance, calcium-activated potassium channels (SK, *ca.* 14 pS), and because genetic evidence did not exist for a distinct IK channel family. However, based on our present pharmacological results, along with previous physiological data, it is now clear that the fibroblast IK channel belongs to the new IK channel class described above. Therefore, we now identify the fibroblast calcium activated potassium channel as FIK rather than SK.

The FIK channel is upregulated in response to mitogenic activation of the Ras/ERK signaling pathway [11, 20, 23, 24]. In addition, ChTX block of FIK inhibits mitogen-stimulated proliferation, suggesting an important role of FIK in cell growth control. We have previously shown in 10T1/2–MRF4 cells that in the presence of Ras/ERK activators typified by bFGF, FIK is upregulated, and MRF4-dependent myogenic gene expression is suppressed [8, 20]. 10T1/2-MRF4 is a multipotent, fibroblast cell line ectopically overexpressing the muscle-specific, basic-helix-loop-helix regulatory transcription factor, MRF4 [25]. Upon bFGF withdrawal, FIK is downregulated and the MRF4-dependent myogenic program is induced, including the expression of acetylcholine (ACh) receptor channels, a classic index of myogenic differentiation. Finally, in bFGF stimulated 10T1/2-MRF4 cells, ChTX block of FIK inhibits proliferation and induces MRF4-dependent muscle specific gene expression identical to mitogen withdrawn cells. Thus, we have proposed that bFGF suppression of MRF4 induced myogenesis is dependent on FIK, and that this suppressive action of the channel is corollary to its positive mitogenic function.

The presence of IK channel transcripts in mitogenically active tissues, and their absence from terminally differentiated tissues, suggests that a general function of IK channels may be to regulate mitogenic cell growth, although this idea has not been directly tested. The FIK channel plays a well demonstrated role in fibroblast cell growth control [11, 20, 23, 24], and therefore could serve as the prototype native channel to address the importance of the cloned IK channels in controlling proliferation and transcriptional activation. This study supports the use of FIK as an IK prototype by showing new pharmacological evidence that FIK and cloned IK channels are related. This new pharmacology was also used to further strengthen the connection between FIK activity and the regulation of transcription via MRF4. These results strongly suggest that other IK channel family members may function as physiological targets for cell signaling events that regulate cell growth and differentiation.

Materials and Methods

Cell Culture and Preparation

Experiments were carried out using 10T1/2 cells constitutively overexpressing a rat MRF4 cDNA (10T1/2-MRF4; provided by Dr. S.F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN). Stock 10T1/2-MRF4 cultures were grown on gelatin-coated dishes and maintained as described previously [20]. For electrophysiology cells were seeded onto gelatin-coated 35-mm dishes and grown to confluence in basal medium, Eagle/15% FBS, and then switched to low glucose DMEM/2% HS with or without 20 ng/ml bFGF and channel blockers for 24 hr. For growth experiments cells were plated at 5 to 10 × 10^4/well (24 well plates) in low glucose DMEM/2% HS plus bFGF with or without channel blockers. Cells were treated and counted daily using a hemocytometer and Trypan blue exclusion was used to insure counts of only viable cells. All cultures were maintained in a humidified, 5% CO_2 atmosphere at 37°C.

Immunocytochemistry

Cells were grown as described above (for electrophysiology) for 48 hr and then rinsed in cold phosphate buffered saline (PBS) (in mM): 150 NaCl, 1.9 NaH_2PO_4:H_2O, and 8.1 Na_2HPO_4, pH 7.3. Following the washes cells were fixed with a 90% methanol solution for 15 min at room temperature. Fixed cells were rinsed with PBS and then permeabilized by incubation with 0.1% Triton X-100 in PBS and 5% HS for 20 min at room temperature. Cells were then incubated with the anti-myosin mouse monoclonal antibody MF-20 for 1–5 hr, and then treated with a biotinylated anti-mouse IgG secondary antibody for 45 min, followed by incubation with Streptavidin, Horse Radish Peroxidase (HRP). Immune complexes were visualized using a 3,3′-diaminobenzidine (DAB) and 30% H_2O_2 solution, and photographed under bright field. All antibody incubations were carried out at room temperature, 22–25°C.

Solutions and Reagents

The standard bath solution used for recording whole-cell FIK channel and ACh receptor channel currents contained (in mM): 138 NaCl, 9 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 10 HEPES. The patch pipettes solution for these recordings contained (in mM): 150 KCl, 1 $MgCl_2$, 10 HEPES, and 0.1 EGTA. For whole-cell FIK channel recordings a pipette solution containing (in mM): 150 KCl, 1 $MgCl_2$, 10 HEPES, and 0.1 EGTA, and 10 μM free Ca^{2+} adjusted by addition of $CaCl_2$, was also used [11, 20]. A23187 (Sigma), ChTX, IbTX, StK (BACHEM Bioscience), and NS1619 (Research Biochemicals International) were stored as frozen stocks and aliquots of each were diluted to final concentrations on the day of use. Application of compounds to cells was accomplished via pressure ejection of solution from blunt-tipped pipettes. All solutions were at pH 7.3, and experiments were carried out at room temperature, 22–25°C. For immunocytochemistry experiments MF-20 (provided by Dr. S.F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN), biotinylated anti-mouse IgG, and streptavidin HRP (Vector Labs) were stored at 4°C. DAB and Triton X-100 (Sigma) were stored at room temperature.

Electrophysiology

Patch-clamp apparatus, techniques, and cell preparation for recordings were as described previously [20]. Whole-cell FIK currents were measured during voltage steps to 0 mV (from −70 mV), and intracellular calcium levels were increased either by extracellular application of A23187 (1 μM), or by increasing the free calcium concentration in the patch pipette solution to 10 μM. Previous studies in 10T1/2-MRF4, and other fibroblast cell lines, have shown that calcium-activated currents recorded at 0 mV are due entirely to FIK channel activity [10, 11, 20, 22]. Whole-cell ACh receptor currents were recorded at −70 mV in

response to extracellular application of 100 μM ACh. The analogue compensation circuitry of the patch-clamp amplifier was used to estimate whole-cell capacitances (expressed in picofarads, pF). Whole-cell currents were normalized to cell capacitance, an indirect measure of membrane area, and expressed as a current density in picoamperes/picofarads (pA/pF). Dose-response curves for ChTX, IbTX, and StK were fit with the Hill equation: % *FIK current block* = $100/[1 + (K_{0.5}/C)^n]$, where $K_{0.5}$ is the concentration of toxin determined to achieve half-maximal block, and C is the toxin concentration. A single binding site was assumed in deriving the curves. All statistical results are given as the mean ± SEM. Significant differences in current densities in response to various growth conditions were assessed by a two-tailed, nonpaired Student's *t*-test at the 0.05 level. Multiple *t*-tests were employed and the critical *P* value per test was adjusted so that an overall error rate of 0.05 was maintained.

Results

Effects of Peptidyl Toxins on FIK Channel Activity and 10T1/2-MRF4 Cell Proliferation

We determined the dose response relationship for FIK block by ChTX, StK, and IbTX. These experiments were carried out to more precisely define the relationship of FIK to other IK channels; and, to test the hypothesis, initially formulated using a single dose of ChTX, that FIK block correlates with inhibition of cell growth. Whole-cell patch clamp recordings were used to determine the percent FIK current block by peptidyl toxins in 24 hr bFGF stimulated 10T1/2-MRF4 cells. The effects of potassium channel-selective scorpion toxin peptides, ChTX and IbTX, and the structurally unrelated marine toxin, StK [3, 26] were evaluated in one of two ways: a population study was performed in which FIK channels were activated by extracellular application of 1 μM A23187, and the FIK current levels were compared with or without toxin in the bath solution; or FIK was activated by elevating free intracellular calcium (10 μM) in the whole-cell patch pipette, and the amount of current block was assessed in response to acute toxin application. There was no measurable difference in FIK current amplitudes measured with either of these two methods. Therefore currents recorded under both conditions were used to report the percent FIK current block in response to ChTX, IbTX, and StK.

The full ChTX dose response curve (Fig. 1*A*) shows the $K_{0.5}$ for FIK block (7 nM) is similar to that reported for cloned IK channels, 2.5 and 10 nM for hIKI and hKCa4, respectively [12, 18], and for a native IK channel in bovine aortic endothelial cells (BAEC, 3.3 nM) [1]. Consistent with the pharmacology of both native BAEC and cloned IK channels, IbTX was a less potent FIK blocking agent ($K_{0.5}$ = 536 nM) than ChTX, despite 70% sequence identity shared by the two toxins [15]. Nonetheless, IbTX may be useful in distinguishing the cell biological effects of FIK from the actions of the more

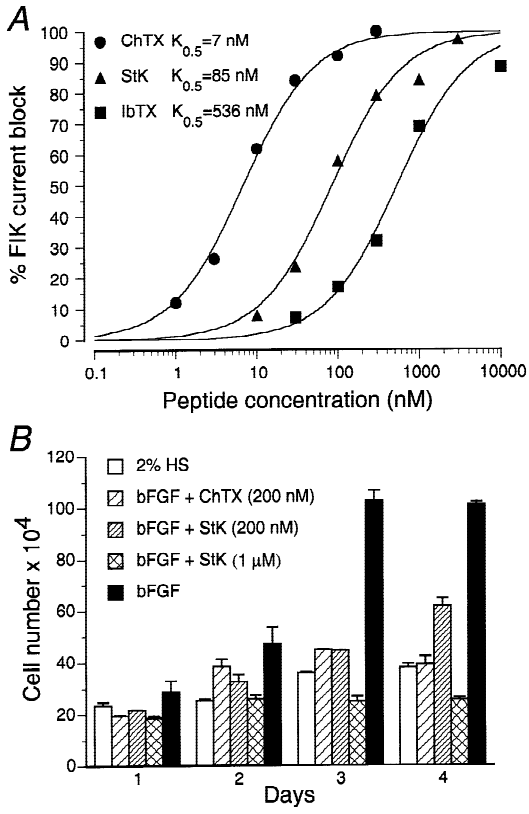

Fig. 1. (*A*) Dose-response curve for ChTX, IbTX, and StK-induced FIK channel block in 10T1/2-MRF4 cells, showing percent FIK channel block as a function of ChTX, IbTX, and StK concentration. Each data point represents the mean percent current block determined from 4–10 whole-cell experiments. The $K_{0.5}$ values were derived by fitting the Hill equation (assuming a single binding site) to the data for each toxin. (*B*) bFGF stimulated 10T1/2-MRF4 cell proliferation is inhibited by chronic treatment with ChTX, and the new FIK channel blocker StK. Results represent data from two identical experiments performed in duplicate. Note that for some data columns error bars are too small to display.

IbTX-sensitive BK channels. The StK toxin is part of a new structural class of potassium channel toxins, isolated from sea anemone, which shares little sequence similarity with other known potassium channel toxins [21]. StK, however, was a more potent FIK blocker than IbTX, with a calculated $K_{0.5}$ value of 85 nM. The full dose-response data shown here are in agreement with the single peptide dose comparisons for the native BAEC IK channel, in which the rank order for IK channel toxin sensitivity is ChTX > StK ⩾ IbTX [1].

ChTX block of FIK inhibits bFGF stimulated 10T1/2-MRF4 cell proliferation, so that these cells grow at a rate similar to mitogen withdrawn cells (2% HS) [20]. We asked if StK also inhibits bFGF-stimulated 10T1/2-MRF4 cell proliferation. Figure 1*B* shows that StK inhibited bFGF-stimulated 10T1/2-MRF4 proliferation in a dose-dependent fashion, with greater inhibition observed

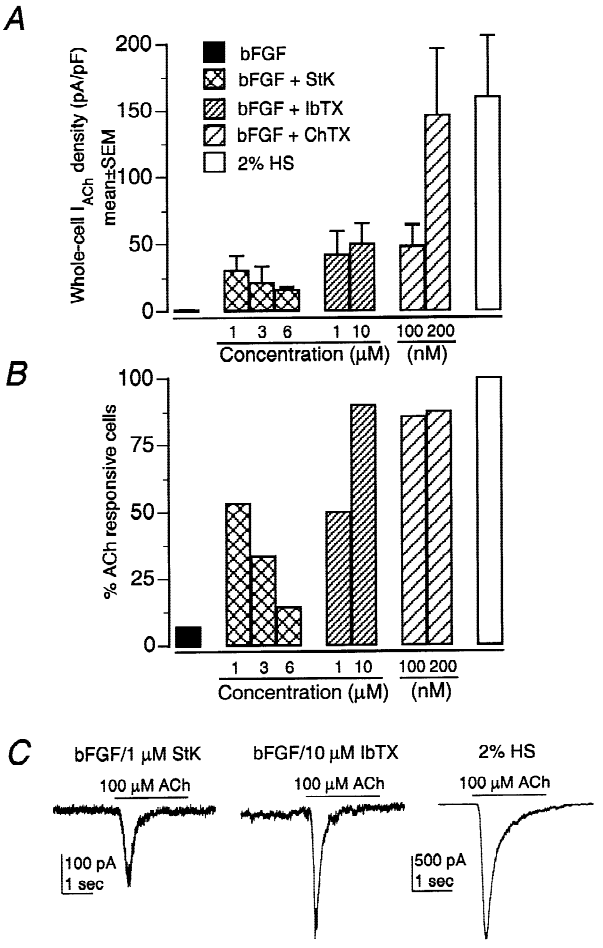

Fig. 2. (*A*) bFGF suppression of ACh receptor channel functional expression is overcome by chronic treatment with FIK channel blockers. Cumulative data for whole-cell ACh current densities from 24 hr bFGF-stimulated 10T1/2-MRF4 cells with or without FIK blockers, StK (1, 3, and 6 μM), IbTX (1 and 10 μM), or ChTX (200 nM). ACh current density for 24 hr mitogen withdrawn (2% HS) cells shown as positive control. Growth factor and toxins were removed prior to ACh receptor recordings. All FIK blocker-induced ACh channel densities were significant larger than the density in cells treated with bFGF alone (*see* Methods). (*B*) Percent ACh responsive cells corresponding to the densities reported in *A*. ACh currents of 10 pA or greater were counted as a response. (*C*) Representative current traces for cumulative data in *A* and *B*.

at 1 μM (approximately 90% FIK block) *vs.* 200 nM (approximately 70% FIK block); 1 μM StK was apparently more effective at inhibiting growth than was 200 nM ChTX. Since both ChTX and StK block FIK and inhibit proliferation, yet are structurally unrelated, their most likely site of antiproliferative action is FIK and not some other cellular process required for growth.

StK and IbTX Treatment Confirms a Role For FIK in Control of MRF4-Driven Transcription

In bFGF-stimulated 10T1/2-MRF4 cells, ChTX block of FIK results in MRF4-driven ACh receptor channel expression identical to that seen under mitogen withdrawal [20]. We asked if StK application to bFGF-treated cells would also result in ACh receptor channel expression. Again, a positive result would suggest that FIK block alone was responsible for StK or ChTX effects on MRF4 mediated transcription. Figure 2*A* shows that pharmacological block of FIK with 1 μM StK results in a modest (30.5 ± 10.9 pA/pF, $n = 39$), but significant increase in ACh receptor channel expression compared to cells treated with bFGF alone (0.6 ± 0.6 pA/pF, $n = 14$ in 2% HS + bFGF). The percentage of ACh responsive cells also increases (Fig. 2*B*). However, at higher StK concentrations (3 and 6 μM), and thus increased (>95%) FIK channel block, smaller increases in ACh receptor channel expression were observed. In the presence of 3 and 6 μM StK, the ACh receptor current densities were 21.1 ± 12.4 pA/pF ($n = 9$), and 15.8 ± 2.3 pA/pF ($n = 7$), respectively. In addition to lower ACh channel current densities, there was also a decrease in the percentage of ACh responsive cells (53, 33, and 14% for 1, 3, and 6 μM StK) (Fig. 2*B*). These observations suggested that in addition to blocking FIK, StK may also inhibit ACh receptor channel function. Therefore, we tested for possible acute StK inhibitory effects on ACh receptor channel activity. For this experiment, whole-cell ACh receptor currents were recorded from 48 hr mitogen withdrawn (differentiated) 10T1/2-MRF4 cells, and 1 μM StK was added to the bath solution at the time of recording. This acute StK application reduced ACh receptor channel currents by 75%. After five complete exchanges of the bath solution over the course of an hour, the ACh currents were re-assayed and remained 60% reduced relative to the control. These data suggest that in addition to blocking FIK, StK directly inhibits ACh receptor channel currents in 10T1/2-MRF4 cells, and reversal of this inhibition is prolonged. Therefore, our estimates of ACh receptor induction in response to chronic StK treatment are compromised by acute ACh receptor block by the toxin. We estimate the induction of ACh receptor channels due to chronic 1 μM StK treatment should be in a higher range (between 76–122 pA/pF) if the acute inhibitory effect of StK on ACh channels could be negated. These densities would coincide with the ACh receptor densities observed for FIK block with 100–200 nM ChTX (Fig. 2*A*). Thus, low ACh current densities (for StK *vs.* ChTX treatment) reflect StK block of ACh receptor channels, and not a failure of StK block of FIK to override negative regulation of MRF4.

For bFGF-stimulated 10T1/2-MRF4 cells grown in the presence of IbTX, the levels of ACh receptor channel expression more closely mimicked that of ChTX-treated cells. In bFGF-stimulated cells treated with 1 and 10 μM IbTX, ACh receptor current densities (41.7 ± 18.2 pA/pF, $n = 4$, and 49.8 ± 15.3 pA/pF, $n = 8$, respectively) were significantly greater than for bFGF alone (*see*

above). Also, at the higher (10 μM) IbTX concentration, thus higher (90–95%) percent FIK current block, the percentage of ACh responsive cells was nearly identical to the percentage of responsive cells grown in bFGF + 200 nM ChTX or 2% HS (Fig. 2*B*). These data indicate that >90% FIK current block is required to completely override bFGF suppression of ACh receptor channel expression, i.e., evoke maximal ACh receptor channel expression equivalent to mitogen withdrawn cells. Also, that bFGF induced negative regulation of MRF4 and thus suppression of MRF4-dependent ACh receptor channel gene expression is a FIK mediated event.

FIK Channel Block Fails to Overcome bFGF-Induced Suppression of Myosin Heavy-Chain Expression and Multinucleated Muscle Fiber Formation

Mitogen withdrawal from 10T1/2-MRF4 cells decreases FIK channel levels coincident with MRF4-dependent muscle gene activation and myogenic differentiation, including ultimately the fusion of uninucleated myoblasts into multinucleated myotubes [8, 20, 25]. The ability of ChTX, IbTX, and StK to evoke increases in ACh receptor channel expression supports the role of FIK as a regulator of MRF4-dependent gene transcription. We also tested whether FIK block with ChTX, IbTX, or StK would evoke MRF4-dependent MHC expression and muscle fiber formation. 10T1/2-MRF4 cells were switched to mitogen-withdrawn (2% HS) or mitogen-stimulated (2% HS + bFGF with or without FIK blocker present) growth conditions for 48 hr, and then assayed for the presence of MHC (Fig. 3). In all cases myosin staining was compared to that of mitogen-withdrawn (2% HS) cells, for which MHC staining level was set to a value of 100. Although ChTX induces ACh receptor expression in bFGF-treated cells, it failed to induce MHC expression or multinucleated muscle fiber formation. StK and IbTX were similarly ineffective (*data not shown*). Mitogen-withdrawn cells treated with ChTX showed normal multinucleated muscle fiber formation and MHC expression, therefore, ChTX does not directly interfere with morphological differentiation. These results suggest that although ChTX or StK block of FIK activates MRF4-dependent ACh receptor channel expression, the effects of these toxins on muscle-specific gene expression are limited to the activation of early myogenic genes, and thus myogenic events that precede morphological differentiation. The expression of MHC, coupled to muscle fiber formation, represent late events of skeletal myogenesis. The failure of FIK block (by ChTX and StK) to evoke these events, suggests that the FIK channel is necessary but not sufficient to regulate the transition from proliferating myoblasts to terminally differentiated skeletal muscle fibers.

Effects of Nonpeptidyl Potassium Channel Modulators on 10T1/2-MRF4 Cell FIK Current and Growth

The identification of compounds that activate FIK, and thus could augment its activity in the presence of bFGF, would serve as useful tools for potentiating the effects of FIK on cell growth. NS1619 is a benzimidazolone compound that was originally characterized as a maxi K_{Ca} channel activator [6, 9]. It was investigated with the aim of identifying a FIK activator. Instead, external application of 50 μM NS1619 caused an immediate, nearly complete block of whole-cell FIK currents in 10T1/2-MRF4 cells (Fig. 4*A*). The effect of NS1619 on peak whole-cell FIK current amplitude was fully reversible. During the course of our experiments it was reported that 100 μM NS1619 maximally inhibited native BAEC IK channel activity [1], thus the pharmacology of FIK and the BAEC IK channel remain consistent. Figure 4*B* shows that 50 μM NS1619 inhibits cell proliferation in bFGF stimulated 10T1/2-MRF4 cells as effectively as 200 nM ChTX. These data present further evidence for the role of FIK as a positive regulator of mitogenesis.

Chronic NS1619 treatment of bFGF-stimulated cells completely abolished FIK channel currents. Cells grown in the presence of bFGF with either 25, 50, or 100 μM NS1619 (24 or 48 hr), failed to show A23187 evoked FIK currents (*data not shown*). Also, we did not observe ACh receptor currents, indicating that the NS1619 induced loss of FIK channel activity failed to override bFGF suppression of MRF4-dependent ACh receptor channel expression (*data not shown*). Further, fully differentiated 10T1/2-MRF4 cells showed a decline in ACh receptor current density upon chronic exposure to NS1619. The mean ACh channel current density observed in 48 hr mitogen withdrawn (2% HS) cells (142.4 ± 40.7 pA/pF, $n = 22$) decreased in proportion to the length of the NS1619 exposure. Following 5 hr 25 μM NS1619 exposure ACh current density decreased by 57% (61.7 ± 18.2 pA/pF, $n = 6$), and the current density of 12 hr 25 μM NS1619-treated cells decreased by 87% (19.1 ± 7.8 pA/pF, $n = 7$). These results suggest that NS1619 may suppress ongoing ACh channel gene expression, or synthesis or membrane insertion of channel protein in already differentiated cells. They also indicate that chronic NS1619 treatment may have suppressed FIK channel expression in bFGF-treated cells. To further screen for potential FIK channel activators, the effects of other potassium channel openers, namely cromakalin, diazoxide, and minoxidil, were also investigated [19]. None of these compounds exhibited any effects on FIK channel currents (Table).

Discussion

This study has defined the pharmacology of the fibroblast IK channel, FIK, demonstrated that its pharmaco-

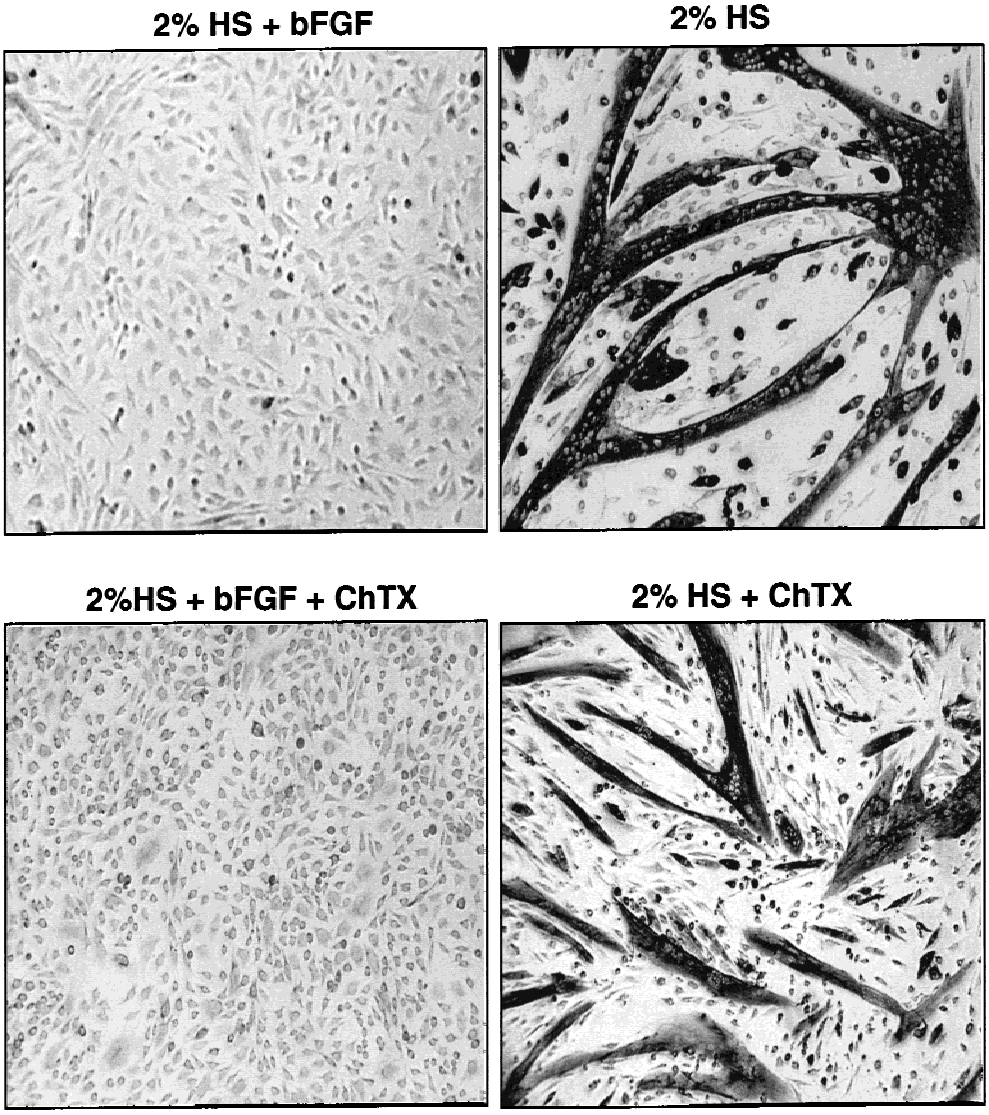

Fig. 3. Chronic ChTX treatment of mitogenically stimulated 10T1/2-MRF4 cells fails to induce myosin heavy chain expression and morphological differentiation. Immunocytochemistry of 48 hr mitogen withdrawn (2% HS) or bFGF-stimulated cells, with or without ChTX 200 nM. The number of myosin positive cells were averaged from six randomly chosen microscopic fields. Cells were photographed under bright-field at 100×.

logical profile is shared by other IK channels, and shown that FIK functions as an important regulator of events that govern cell growth, and differentiation in 10T1/2-MRF4 cells. We have identified new inhibitors of FIK and have shown that these agents also inhibit proliferation, and induce MRF4-dependent ACh receptor channel expression. These results support a biological role of FIK in mitogen-stimulated proliferation and transcriptional regulation. It is proposed that FIK's role in controlling cell growth can serve as a model for understanding the function of the IK channel family, and its selective occurrence in mitogenically active tissues.

The unique pharmacological and cell biological profile of FIK sets this channel apart from other well-characterized members of the potassium channel superfamily, including BK and SK channels. BK and SK channels are widely distributed in excitable tissue, and their activation has been linked to control of neuronal excitability, smooth muscle tone, and spike frequency adaptation [19, 27]. IK channels are apparently absent from excitable tissue based on physiological and expression studies, but are present in mitogenically active cells including T-lymphocytes [16, 17], and mitogen-stimulated fibroblasts [4, 11, 20], where their activity has been correlated with positive proliferative control [23, 24]. Transcripts of the cloned IK channels, hIK1 and hKCa4, are widely expressed in a variety of mitogenically active tissues, including hematopoietic cells [12, 14,

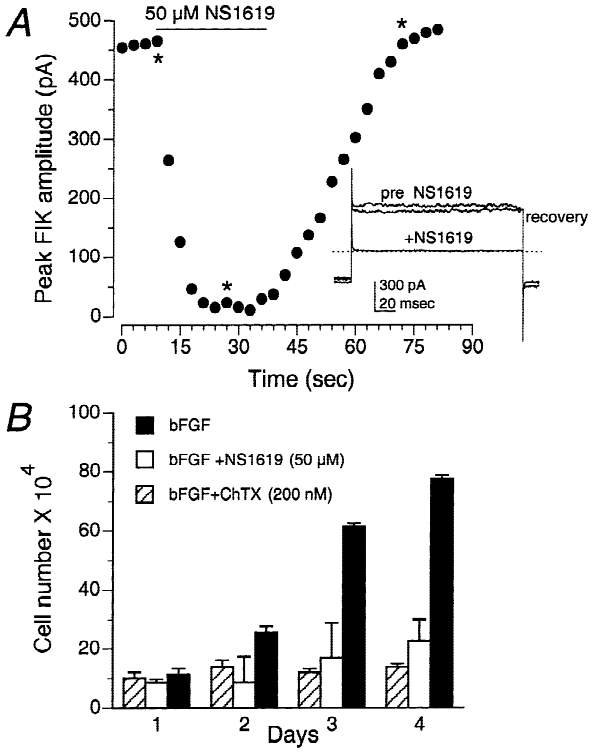

Fig. 4. (*A*) Scatter plot showing NS1619 reduction of peak whole-cell FIK current amplitude. FIK current was activated by inclusion of 10 μM free calcium in the patch pipette. (*Inset*) Asterisks denote the representative FIK current traces. (*B*) Growth experiment showing inhibition of bFGF-stimulated 10T1/2-MRF4 proliferation by 50 μM NS1619. Results represent data from two identical experiments performed in duplicate.

Table. Pharmacology of the FIK channel

Compound	Concentration	% FIK current block	*n*
ChTX	100 nM	>90	5
IbTX	10 μM	>90	7
StK	1 μM	>90	12
Cromakalin	100 μM	N.E.	3
Diazoxide	100 μM	N.E.	8
Minoxidil	30 μM, 60 μM	N.E.	8, 3
NS1619	25 μM, 50 μM	80, 96	4, 4

N.E. indicates no block or potentiation of FIK current.

18]. Furthermore, the cloned IK channels have a pharmacology and electrophysiology very similar to that reported here for FIK.

Like FIK, heterologously expressed IK channels are blocked by ChTX with a $K_{0.5}$ in the low nM range (2.5 and 10 nM) [12, 18]. We found that whole-cell FIK currents are sensitive to StK block, and that IbTX is a less potent FIK blocker. These results are comparable to the pharmacology reported for the BAEC IK channel identified by Cai et al. [1]. The StK toxin is part of a new family of channel toxins with a primary amino acid sequence unrelated to that of the scorpion toxin peptides ChTX and IbTX. Residues Lys22 and Tyr23 in StK are essential for its binding to potassium channels [21, 26]. These amino acid residues are similar to Lys27 and Tyr36 of ChTX in that they are important for channel binding. This suggests that StK may block FIK by interacting with a site on the channel similar to the ChTX binding site. IbTX, a member of the scorpion family of potassium channel toxins, shares a high (70%) sequence identity with ChTX but is a less potent IK blocker. This lower potency of IbTX is shared by other IK channel types, including IK channels from T-lymphocytes and BAEC [1, 16]. IbTX blocks BK channels, at a concentration well below its effective range *vs.* IK [2]. Therefore, IbTX can be used to unambiguously separate the physiological actions of BK channels from those of FIK. These data confirm a peptide pharmacology for FIK that is shared by other IK channel types, but is clearly distinct from BK and SK channel families. More importantly, by analogy to FIK, the cloned IK and other native IK channels may prove to have a similar physiological role also distinct from the other calcium-activated potassium channel types.

This more complete FIK pharmacological profile was used to extend earlier work, which showed that ChTX block of FIK inhibits mitogen stimulated 10T1/2-MRF4 proliferation, coincident with the rapid induction of ACh receptor channel expression via the muscle regulatory transcription factor, MRF4 [20]. From these initial data it was concluded that FIK positively regulates mitogenesis, and suppresses MRF4 dependent transcription of muscle specific genes. A calcium-activated potassium conductance has also been correlated with proliferative stimulation and myogenic suppression in muscle satellite cells, which act as an in vivo reserve of myocyte precursors [7]. The finding reported here that ChTX, StK and IbTX block of FIK promotes the expression of ACh receptor channels, but fails to induce muscle fiber formation or myosin heavy chain (MHC) expression, suggests for the first time that ion channel activity may regulate expression of early myogenic genes. Further, these results suggest there is a definitive breakpoint in the physiological control of early, *vs.* late, myogenic events. Additional analysis of this system may help elucidate the control mechanisms for early myogenic gene expression, specifically for muscle ion channel genes.

The identification of FIK channel activators would provide a means, independent of mitogenic stimuli, by which to increase FIK channel activity and thus stimulate cell growth. A panel of potassium channel activators tested here failed to enhance FIK channel activity. Indeed one, NS1619, unexpectedly blocked FIK channel currents and inhibited mitogen-stimulated cell proliferation. Chronic NS1619 application, however, completely

abolished the expression of FIK and ACh channel currents in bFGF stimulated 10T1/2-MRF4 cells. The reasons for this effect are not immediately obvious, but we speculate that the highly lipophilic nature of this compound may lead to nonspecific toxic effects. The use of NS1619 in electrophysiological studies have been limited to acute modulatory effects of this compound on channel activity [5, 9]. The chronic effects of NS1619 on cell growth require that some caution be observed in the cell biological use of this compound.

The expanded pharmacological profile of the FIK channel developed here serves several purposes. It provides further verification of FIK's role in controlling cell growth by showing that multiple FIK blockers produce the same biological effects, i.e., inhibition of fibroblast proliferation, and, in the 10T1/2-MRF4 cell system, relief of bFGF induced inhibition of MRF4-dependent muscle gene expression. Also, the FIK pharmacology, when compared to that of cloned and other native IK channels, suggests that FIK and these channel types are very closely related. This view has implications for future investigations into the physiological roles of the cloned IK channel members, whose tissue distribution alone suggests they probably do not contribute to regulating electrical excitability as do SK and BK channels. Instead, selective expression of the cloned IK channels in mitogenically active tissues, in conjunction with what has been demonstrated for FIK and fibroblast cell growth, suggests to us that the IK family will prove to be an important modulator of cell growth in a number of tissues. Now that tools are becoming available to perturb IK channel function relatively selectively, it should be possible to determine the mechanistic basis of this channel family's influence on the transcriptional events governing cell growth. The FIK/10T1/2-MRF4 cell system provides a well-defined biological context in which to ask these fundamental questions regarding the IK channel family.

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