Voltage-gated potassium channels regulate the response of retinal growth cones to axon extension and guidance cues

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Abstract

Xenopus retinal ganglion cell growth cones express various voltage-gated potassium (Kv) channels. We showed previously that 4-aminopyridine and tetraethylammonium have different effects on the outward currents of embryonic Xenopus retinal ganglion cells. Therefore, we asked whether these Kv channel inhibitors differentially regulate the response of retinal ganglion cell growth cones to extrinsic cues. First, we tested the role of Kv channels in axon extension mediated by a substrate bound cue and found that 4-aminopyridine blocked, whereas tetraethylammonium enhanced basal extension on laminin. Yet, when the growth cones were stimulated to extend with application of soluble growth factors, both inhibitors resulted in a return to the basal extension rates observed in the presence of laminin alone. Second, we asked if Kv channels modulate the response of retinal ganglion cell growth cones to a guidance cue, the chemorepellent fibroblast growth factor-2. When presented in a gradient to one side of the growth cone, fibroblast growth factor-2 repulsed retinal ganglion cell growth cones in the presence of 4-aminopyridine but not tetraethylammonium. These data argue that tetraethylammonium- and 4-aminopyridine-sensitive Kv channels differ in the manner by which they regulate the response of retinal ganglion cell axons to extension and guidance cues. Non-ratiometric calcium imaging indicated that differences in the ability of tetraethylammonium- and 4-aminopyridine-sensitive Kv channels to regulate calcium activity within the growth cone may underlie their unique modulation of growth cone behaviour.

Introduction

The tip of an extending axon, the growth cone, relies on growth and guidance cues in the environment to successfully innervate its target. How the axon responds to these cues is dependent on the intrinsic state of the growth cone (Song & Poo, 1999; McFarlane, 2000). For instance, altering intracellular cyclic nucleotide or calcium levels can switch a growth cone's response to a guidance cue from one of attraction to repulsion. These levels can be modulated by membrane excitability (Hempel et al., 1996; Ming et al., 2001). In turn, key regulators of membrane excitability are voltage-gated potassium (Kv) channels (Wei et al., 1996; Jan & Jan, 1997). Interestingly, various Kv channels are expressed in the growth cones of Xenopus retinal ganglion cells (RGC) during optic tract development (Pollock et al., 2002). We proposed that Kv channels, through their control of membrane potential, regulate signalling downstream of axon growth and guidance cues (McFarlane, 2000).

Kv channels include four electrically active subfamilies, Kv1–4, which have different pharmacological and biophysical properties (Wei et al., 1996). We found Kv channels expressed in unique patterns in Xenopus RGC growth cones and axons (Pollock et al., 2002; Kv2.2 unpublished observations). Moreover, we demonstrated previously that two classical Kv channel inhibitors, tetraethylammonium (TEA) and 4-aminopyridine (4-AP) when applied to the brain neuroepithelium caused distinct defects in the extension and guidance of RGC axons in vivo (McFarlane & Pollock, 2000). Together these studies suggest that Kv channels are required for RGC axon outgrowth.

Our in vivo study raised certain key issues. First, are Kv channels general modulators that affect the response of growth cones to all extrinsic cues, or are they specific for only a subset? Second, why are multiple Kv channels expressed by RGC growth cones? Possibly, the unique biophysical properties and subcellular localization of Kv channels are important for fine-tuning the response of a growth cone to specific extrinsic cues. If true, blockade of different Kv channel populations would be expected to produce distinct effects on growth cone behaviour. Third, do Kv channels function within the growth cone to modulate the response to guidance cues? In our previous in vivo study, TEA and 4-AP had access to both the RGC growth cones and the neuroepithelial cells over which they extend. Finally, the mechanism underlying Kv channel modulation of growth cone behaviour is unknown. To address these issues we turned to in vitro extension and guidance assays to examine the role of Kv channels in the response of growth cones to various extrinsic signals. In particular, we investigated the role of Kv channels in axon extension stimulated by substrate bound as well as single vs. multiple secreted factors. Further, we investigated the importance of growth cone Kv channels for the directional response of growth cones to FGF-2, a known chemorepellent for Xenopus RGCs (Webber et al., 2003).

Materials and methods

Animals

Embryos were obtained from adult Xenopus laevis (Nasco; Fort Atkinson, WI, USA) by using in vitro fertilization. Female Xenopus laevis were stimulated by injection of 800 international units (IU) chorionic gonadotrophin (Chorulon; Intervet Canada, Whitby, ON,
Canada) and the eggs were collected and fertilized. Embryos were raised in 0.1 × Marc’s Modified Ringers (MMR; 0.1 m NaCl (Sigma, St. Louis, MO, USA), 2 mM KCl (BDH, Toronto ON, Canada), 1 mM MgCl₂ (Sigma), 2 mM CaCl₂ (BDH), 5 mM HEPES (Sigma), pH 7.5) at 14–25 °C. Embryos were staged according to Nieuwkoop & Faber (1994), and anaesthetized for surgery and killed with 0.4 mg/mL tricaine. The animal protocols for these studies were approved by the Faculty of Medicine Animal Care Committee, University of Calgary.

Retinal cell culture
Eye primordia from stage 24 embryos were dissected and cultured following the procedure of Harris & Messersmith (1992).

Explant cultures
Eye buds were plated on polyornithine/laminin-coated (Sigma) coverslips in 35 mm Petri dishes containing culture medium (see below). Eye buds were left to adhere and extend axons for 24 h before use in time-lapse videomicroscopy, calcium imaging and growth cone turning assay experiments.

Dissociated cultures
Eye buds were incubated in low calcium medium for 10 min, mechanically dissociated, plated on coverslips and grown for 1–2 days.

Neurite measurements
To evaluate the significance of Kv channels in neurite outgrowth, the pharmacological Kv channel inhibitors TEA chloride (Sigma) and 4-AP (Sigma) were used. Concentrations of inhibitors were chosen based on previously reported dose-response data (McFarlane & Pollock, 2000), and are comparable to concentrations reported in the literature to block Xenopus Kv channels (Ribera & Nguyen, 1993; Burger & Ribera, 1996; McFarlane & Pollock, 2000; Blaine & Ribera, 2001). Sister cultures were dissociated and incubated in one of several different solutions. 60% L-15 based-medium (Invitrogen, Burlington, ON, Canada) plus 0.1% bovine serum albumin (BSA; Sigma) was used, or was supplemented with 20 ng/mL human recombinant FGF-2 (Invitrogen) or embryo extract prepared from homogenized stage 30–33/34 embryos (as per Harris & Messersmith, 1992). In addition, 30 mM TEA or 3 mM 4-AP was added to these media. The 4-AP solutions were corrected to pH 7.4 with HCl (VWR; Edmonton, AB). After 40 h, cultures were fixed for 1 h in 0.5% glutaraldehyde (EM Science, Gibbstown, NJ, USA) at room temperature. RGCs were identified on the basis of previously reported criteria (Worley & Holt, 1996). The longest unobstructed process was measured by using Axiovision 3.0 software (Carl Zeiss Canada, Toronto, ON, Canada) on digital images taken with a Spot II camera (Diagnostic Instruments, Sterling Heights, MI, USA) connected to a Zeiss Axioplan2 microscope. Statistical analysis was performed using Instat 2.0 (GraphPad Software, San Diego, CA, USA).

Time-lapse videomicroscopy
Recordings were made from RGC growth cones that were extending from retinal explants at a rate >20 μm/h. Data from growth cones that were physically obstructed at any point during the recording period were discarded. An initial outgrowth rate was obtained over 30 min in control medium consisting of 60% L15 medium supplemented with 20 ng/mL FGF-2. Next, half of the bathing solution was changed to achieve final concentrations of either 30 mM TEA or 3 mM 4-AP, while maintaining FGF-2 at a concentration of 20 ng/mL. After 30 min, the solution was washed out three times with 60% L15 medium and replaced with the control solution. Images were obtained by using Scion Image acquisition software (shareware, Scion Corp., Frederick, NJ, USA) with a Zeiss Axiovert 25 inverted microscope and a CCD video camera (Cohu Inc., San Diego, CA, USA). Extension rates in the different conditions were compared with two-tailed, paired t-tests using Instat 2.0 software. In a separate series of experiments, explants were grown for 24 h in Modified Barth’s saline (MBS, 0.7 mM Ca²⁺ Sive et al., 2000), or MBS with lowered calcium concentration (0.07 mM Ca²⁺). Time-lapse videomicroscopy was performed on these growth cones for 30 min before, and after addition of, 3 mM 4-AP.

Growth cone turning assay
A RGC growth cone extending from a retinal explant was exposed to a localized source of FGF-2. The FGF-2 was pressure-ejected out of a micropipette (A-M Systems, Carlborg, WA), at a 45° angle to, and 100 μm away from, the growth cone (Zheng et al., 1994; de la Torre et al., 1997; Webber et al., 2003), by using a Picospritzer III (Parker Instrumentation, Fairfield, NJ, USA) and Grass Stimulator (Grass Product Group, West Warwick, RI, USA). Growth cones actively extending from 1-day-old, stage 24 retinal explant cultures were recorded by using time-lapse videomicroscopy for 30 min. After the creation of an FGF-2 gradient, the growth cones were recorded for another 50–100 min. Growth cones were rejected if they were not motile during a 5-min recording period before the trial. The FGF-2 gradient was produced as described previously (Webber et al., 2003). Previous calculations indicate that a 1 μm opening at the end of a micropipette, located 100 μm from the growth cone, delivers a constant gradient to the growth cone that is approximately 1/1000 the concentration in the pipette (Lohof et al., 1992). In all experiments, the pipette contained 100 μg/mL FGF-2 plus 0.1% BSA, and the bathing solution was either 60% L15 solution (control), or control medium to which either 3 mM 4-AP (pH 7.4) or 30 mM TEA was added. The researcher was blinded to the identity of the bath solution. Images were collected every 30 s by using a Cohu CCD camera and Scion Image acquisition software. Turning angle was measured using Scion Image, and was defined as the angle between the initial direction of growth (over the final 20 μm of growth before application of the gradient) and the position of the growth cone after the recording period (Zheng et al., 1994). Growth cones that had not moved 10 μm during the recording period were removed from the analysis.

Calcium imaging
Intracellular free calcium levels were imaged nonratiometrically, using the acetoxymethyl (AM)-ester of calcium green-1 (CaGD-1, Molecular Probes Eugene, OR). Explant cultures were grown in the presence of 20 ng/mL FGF-2 plus 0.1%BSA in 60% L15 for one day, incubated with 0.1 mM CaGD-1 [1.5 mM stock in 20% pluronic acid (Sigma), diluted with 60% L15] for 30 min, and then washed three times with 60% L15 plus 0.1% BSA. Washing of the cultures minimized background fluorescence and removed FGF-2 from the bath. Explants were left in the dark for approximately 30 min after washing to facilitate hydrolysis of the CaGD-1 AM-ester before imaging. Preparations were excited with 480 ± 20 nm light. Fluorescence emission was measured, in the dark, at a bandwidth centred
close to the peak emission wavelength for CaGD-1 by combining a 505 nm beam splitter with a 535 ± 40 nm bandpass filter (all filters by Chroma Technology, Rockingham, VT). Images were collected using an intensified CCD camera (Stanford Photonics XR-GENIII Ultra-blue; Solamere Technology Group, Salt Lake City, UT, USA) installed on an inverted Axiovert 100 TV microscope (Zeiss) with 64 x or 100 x oil immersion objectives. Data acquisition and analysis were carried out with Axon Imaging workbench version 2.1 (builds 98–109; Axon Instruments, Foster City, CA). Images were acquired at 0.2 Hz for the first 5 min to determine background fluorescence levels, and then at 1 Hz after either 4 mM 4-AP or 30 mM TEA was delivered by a bath perfusion system. Recordings of growth cones were made over 10–50 min, depending on the intensity of the signal and bleaching of the dye. Data-acquisition parameters were set to obtain optimal responses in regions of interest within the growth cone. Imaging growth cones at 1 Hz often resulted in a decline of fluorescence intensity due to bleaching, as is common with CaGD-1.

Results

TEA and 4-AP have differential effects on laminin-dependent extension

We showed previously that single Kv channels have distinct subcellular localizations within Xenopus RGC growth cones (McFarlane & Pollock, 2000; Pollock et al., 2002). To determine whether neurite extension stimulated by various extension cues is affected in a similar fashion by the blockade of different populations of Kv channels, we first treated RGC neurites extending on a laminin substrate with either TEA or 4-AP. The two inhibitors have overlapping but distinct sensitivities for members of the Kv channel family: Xenopus Kv channel subunits expressed heterologously in cells can be selectively insensitive to either 4-AP or TEA (Burger & Ribera, 1996; Kerschbaum et al., 2002); and Xenopus spinal neurons and retinal horizontal cells express Kv channels that are preferentially sensitive to either TEA or 4-AP (Ribera, 1990; Akopian & Witkovsky, 1994). Dissociated retinal cultures from stage 24 embryos were incubated in the presence or absence of a Kv channel inhibitor. TEA and 4-AP had opposite effects on neurite extension on a laminin substrate (Fig. 1). A concentration of 3 mM 4-AP caused a significant decrease in mean neurite length (P < 0.05; ANOVA, Student Newman-Keuls post hoc test) (Fig. 1A), whereas addition of 30 mM TEA stimulated neurite extension by approximately 75% (P < 0.05; ANOVA, Student Newman-Keuls post hoc test; Fig. 1B). It is likely that 4-AP and TEA affected Kv channels in both RGC growth cones and somata. We believe, however, that the effects of TEA and 4-AP on neurite extension are at the level of the growth cone, as Xenopus RGC growth cones can extend and make guidance decisions in the absence of their cell body (Harris et al., 1987; Campbell & Holt, 2001). Moreover, tetrodotoxin, a blocker of sodium-dependent action potentials, had no effect on RGC axon extension in vitro (data not shown), which indicates that neuronal activity in RGC somata does not influence the growth cone.

TEA and 4-AP both inhibit growth factor-stimulated outgrowth in culture

We were interested in whether 4-AP- and TEA-sensitive Kv channels also had opposite roles in extension in response to soluble growth factors. First, we determined whether Kv channel blockade affected growth cones stimulated to extend by the multiple growth factors present in a soluble extract isolated from tadpole stage (stages 30–34) embryos (Harris & Messersmith, 1992). Dissociated stage 24 retinal cultures were grown on laminin in the presence of embryo extract and 30 mM TEA. In similar culture conditions, 4-AP had previously been shown to impair RGC neurite extension (McFarlane & Pollock, 2000). The mean length of neurites in the presence of TEA was significantly shorter than those observed in embryo extract alone [embryo extract, 38.5 ± 70 μm (SEM); embryo extract + 30 mM TEA, 243.5 ± 15 μm (SEM); n = 4 independent experiments, unpaired two-tailed Student’s t-test, P < 0.05]. Thus, growth cones fail to respond to a growth factor mixture in the presence of either TEA or 4-AP.

Fig. 1. 4-AP and TEA differentially regulate RGC neurite outgrowth. Graphs that show mean lengths (μm) of the longest neurite of RGCs developing in dissociated sister retinal cultures, grown for 40 h. RGC growth cones extended on a laminin substrate in the presence or absence of 20 ng/ml human recombinant FGF-2. Either 3 mM 4-AP (A) or 30 mM TEA (B) were added to these cultures at the time of plating. 4-AP and TEA differentially regulate basal RGC neurite outgrowth on laminin, but both inhibit FGF-2-stimulated RGC neurite outgrowth. Error bars indicate SEM, numbers in parentheses indicate the total number of neurites measured, and the numbers above indicate the number of separate experiments analysed. Each experiment was performed on a different batch of embryos, and, for each experimental day, neurites were measured in at least two cultures dishes for each condition (*P < 0.05, **P < 0.01, ***P < 0.001; ANOVA, followed by Student Newman-Keuls post hoc test).
We next asked if similar effects were observed when growth cones were stimulated by a single growth factor, FGF-2, to extend over the baseline rate seen on laminin. Human recombinant FGF-2 (20 ng/mL) was added to the bathing medium of dissociated retinal cultures extending on laminin. These cultures were treated with either 30 mM TEA or 3 mM 4-AP (Fig. 1). As reported previously, FGF-2 stimulated RGC neurite extension above the basal level observed on laminin (McFarlane et al., 1995; Webber et al., 2003). Interestingly, no stimulation was observed when either TEA- or 4-AP-sensitive channels were inhibited. The mean neurite lengths in FGF-2 with either TEA or 4-AP were significantly shorter than cultures with FGF-2 alone. These data argue that growth cone Kv channels are necessary for a growth cone to respond normally to bath-applied FGF-2.

The failure of growth cones to extend longer distances in response to FGF-2 when in the presence of the Kv channel inhibitors, could result from either overall slowed growth, or stalling of the growth cone for long periods. To address this issue, Kv channel inhibitors were applied to RGC growth cones extending in the presence of FGF-2 and laminin, and the effects on growth cone dynamics were observed over a 30-min period with time-lapse videomicroscopy. This time-lapse study would also verify whether acute, as well as chronic, application of inhibitors impaired neurite extension. In these experiments, stage 24 eye primordia were grown as explants on a laminin substrate. The only axons to extend significant distances out of the explant are RGC axons (Worley & Holt, 1996). Isolated growth cones were recorded for 30 min in control L-15 medium containing 20 ng/mL FGF-2. Subsequently, 30 mM TEA or 3 mM 4-AP was added to the bath and outgrowth rate was monitored for another 30 min. After a washout period, the growth cones were recorded for a final 30 min in control solution.

Both inhibitors significantly slowed the rate of growth cone extension when compared to control recordings (Fig. 2). For the TEA experiments, a ‘low-salt’ TEA medium was also used, where the concentration of NaCl was reduced proportionately to correct for the increase in ionic strength resulting from TEA addition. A similar reduction in outgrowth rate was observed with both TEA solutions and the data in Fig. 2A is pooled from both data sets. Of interest, not all axons responded to TEA (7/27). Similarly, the effect of 4-AP application was variable. 4-AP reduced extension rates in 65% (13/20) of growth cones tested, and had no effect on, or increased, the outgrowth rate of the remaining growth cones (Fig. 2B). The effects of both inhibitors were reversible. Therefore, it appears that Kv channels are required to maintain the normal extension rate of most RGC growth cones in response to an FGF-2 stimulus.

**TEA-sensitive Kv channels are important for repulsive turning of RGC growth cones from FGF-2**

Kv channels are necessary for growth factor stimulated RGC axon extension in vitro, therefore we asked whether they also function in guidance decisions. To test this, we used a growth cone turning assay with FGF-2 as the tropic cue, because this molecule was recently shown to repulse *Xenopus* RGC growth cones in vitro (Webber et al., 2003). The FGF-2 gradient was created by pressure ejection from a micropipette positioned 100 μm from the growth cone, and at an angle to the direction of neurite extension. Importantly, we showed previously that a control pipette solution caused no turning response in RGC growth cones (Webber et al., 2003). Medium with and without Kv channel inhibitors was added to the bath, the gradient established, and images of the growth cone were captured every 30 s for a period of 50–100 min.

The two Kv channel inhibitors had differential effects on the ability of an RGC growth cone to sense the FGF-2 as repulsive. In the presence of both the control and 3 mM 4-AP solutions, RGC axons were repulsed by the point source of FGF-2 (Figs 3A–D and 4A–C). The mean turning angle for growth cones in the control bath was not significantly different than that for growth cones extending in the presence of 4-AP (P > 0.05, nonparametric Mann–Whitney test). These results suggest that 4-AP-sensitive channels are not essential for transduction of a repulsive FGF-2 cue. As expected, the mean extension rate of growth cones in 4-AP was less than that observed in control solution (Fig. 4D). In contrast to what we observed in 4-AP, in the presence of 30 mM TEA, RGC growth cones responded in a...
random fashion to FGF-2 (Figs 3E and F. and 5A–C). The mean turning angle for growth cones responding to a point source of FGF-2 in the control bath was significantly different than that measured in the presence of TEA (P < 0.05, nonparametric Mann–Whitney test). Therefore, TEA-sensitive Kv channels appear to be necessary for the repellent effects of FGF-2 on RGC growth cones. As expected, TEA-treated growth cones extended significantly more quickly than control growth cones on a laminin substrate (Fig. 5D).

**TEA and 4-AP have different effects on calcium dynamics in RGC growth cones extending on a laminin substrate**

How might Kv channels modulate a growth cone’s response to extrinsic cues? Intracellular calcium is an important mediator of growth cone behaviour in response to extension and guidance cues (Gomez & Spitzer, 2000; Petersen & Cancela, 2000). Growth cone Kvä channels may regulate calcium influx via voltage-dependent calcium channels (VDCCs), because Kv channels function to modulate the amplitude, frequency and duration of membrane depolarizations (Wei et al., 1996). We asked whether Kv channel inhibition could affect calcium dynamics in a growth cone. We chose to look at growth cones extending on a laminin substrate because on laminin we could ask if differences in the modulation of calcium signalling could explain the opposite effects of TEA and 4-AP on laminin-dependent extension.

A nonratiometric cell-permeant calcium indicator, Calcium Green-1 AM (CaGD-1) was used to follow relative changes in growth cone calcium levels after acute application of TEA or 4-AP. RGCs were grown in explant cultures for 24 h on a laminin substrate. Subsequently, the explants were incubated with 0.1 μM CaGD-1 for 30 min. Growth cones were imaged in the dark for 5 min at 0.2 Hz to obtain a baseline reading of calcium activity. A significant number of growth cones (21/47; 39 growth cones used for analysis) exhibited spontaneous transients during this initial recording period. These transients were more similar, in terms of frequency, to those reported for cultured embryonic and postnatal hamster cortical neurons (Tang et al., 2003), than those observed in Xenopus spinal cord neurons (Gomez & Spitzer, 1999). This likely reflects different biophysical membrane properties of Xenopus spinal cord neuron and RGC growth cones.

Kvä channel inhibitors were applied by bath perfusion, and growth cones were assessed for calcium activity. Fluorescent images of representative growth cones are shown in Fig. 6A and B before and after application of TEA. In RGC growth cones with spontaneous activity, TEA application silenced calcium transients (88%, n = 8; Fig. 6A). Conversely, inactive growth cones (90%, n = 10) were unaffected by TEA application (Fig. 6B). Traces of the relative fluorescence intensity over time for four representative growth cones are shown in Fig. 6C and D. After TEA addition, calcium spiking was terminated in three growth cones (C, lower traces in D), while activity in a relatively quiescent growth cone was unchanged (Fig. 6B and D, upper trace). In contrast, 4-AP generally stimulated calcium transients in growth cones, whether they were initially inactive (53%, n = 15; Fig. 7B and D) or exhibited spontaneous calcium transients (67%, n = 6; Fig. 7A and C, upper traces). In the remaining growth cones, 4-AP had little or no effect (43%, n = 21; Fig. 7C, lower trace).

**Kvä channel inhibition ineffective in low calcium medium**

The calcium imaging experiments suggest that Kv channels regulate growth cone behaviour by their modulation of calcium dynamics within the growth cone. To more directly test this possibility we asked whether Kv channel blockade was still capable of inhibiting laminin-stimulated neurite outgrowth when extracellular calcium was lowered in the bathing medium. Eyes were dissected from stage 24 embryos and grown as explants on a laminin substrate, either in control MBS medium (0.7 mM [Ca²⁺]o) or low calcium MBS medium (0.07 mM [Ca²⁺]o). We used low calcium as opposed to zero calcium medium as we found that RGC growth cones failed to extend when a zero calcium medium was applied either chronically or acutely. The behaviour of growth cones in the control and low calcium culture conditions were recorded by time-lapse videomicroscopy over a 30 minute period, prior to the addition of 3 mM 4-AP. In the control cultures, 3 mM 4-AP significantly reduced the rate of extension of RGC growth cones, however, in the low calcium medium 3 mM 4-AP had no significant effect (Fig. 8). These data support the argument that Kv channels mediate their effects on growth cones through the modulation of calcium dynamics.
Discussion

In this paper, pharmacological inhibitors were used to assess the roles of Kv channels in the response of RGC growth cones to known axon extension and guidance cues. Importantly, we found previously that TEA and 4-AP had distinct inhibitory effects on the outward Kv currents expressed by embryonic Xenopus RGC somata (McFarlane & Pollock, 2000). Here, we showed that Kv channels act within the growth cone to modulate their response to extrinsic cues. Interestingly, however, the effect of Kv channel inhibition depended both on the Kv channel subtypes and the extrinsic signals involved. For instance, TEA- and 4-AP-sensitive Kv channels appear to differ in their modulation of the basal extension of RGC growth cones observed on a laminin substrate. Yet, growth cones failed to respond in their normal fashion to one or more growth factors when either channel type was blocked. These data argue that a given cue’s ability to influence growth cone behaviour depends on the particular Kv channels expressed by the growth cone. The observation that TEA and 4-AP had distinct effects on global growth cone calcium dynamics, and the dependence of 4-AP’s action on the growth cone on extracellular calcium levels, suggest a possible mechanism by which Kv channels regulate the transduction of both extension and guidance information.

Presumably, the distinct involvement of TEA and 4-AP sensitive Kv channels in growth cone behaviour is explained both by differences in their kinetic and voltage-dependent properties, and their distribution within the growth cone (Coetzee et al., 1999; Pollock et al., 2002). We have shown that 4-AP and TEA have distinct effects on the transient and sustained components of the outward Kv currents recorded in RGC somata, and that Kv channels differ in their subcellular localization within the growth cone (McFarlane & Pollock, 2000; Pollock et al., 2002). Given the broad specificity of 4-AP and TEA, and, the fact that there are likely to be more Kv channels in RGC growth cones that we have not as yet identified, we can not ascribe the effects of these two inhibitors to the blockade of particular Kv channels. Thus, in the future it will be important to take molecular and more specific pharmacological approaches to identify those Kv channels important in the distinct forms of modulation revealed by TEA and 4-AP. A genetic approach in mouse is unlikely to be useful in this regard, in that redundancy with other Kv channels, and a neuron’s drive to maintain electrical homeostasis (Davis & Bezprozvanny, 2005).

**Fig. 4.** 4-AP does not affect the response of growth cones to FGF-2. (A and B) Superimposed trajectories of growth cones in the presence of an FGF-2 gradient, extending either in control medium (A) or 3 mM 4-AP (B). The origin represents time = 0 and the y-axis indicates the original direction of growth. The star indicates the location of the micropipette. No significant difference was observed in the mean turning angle (x) for the control and 4-AP baths (P > 0.05, nonparametric Mann–Whitney test). (C) Cumulative histogram of the turning angles of growth cones presented with an FGF-2 gradient either in the presence of control medium or 3 mM 4-AP. The x-axis is the turning angle and the y-axis is the percentage of growth cones with turning angles less than the indicated turning angle. No difference is observed. (D) Extension rates of growth cones over the recording period in either control medium or 3 mM 4-AP (*P < 0.05, unpaired, two-tailed, Student’s t-test).
would likely allow axon outgrowth to occur normally in the absence of a single Kv channel. Indeed, various mice deficient for a given Kv channel show no overt axon extension or guidance phenotype (Smart et al., 1998; Lau et al., 2000; Espinosa et al., 2001). A dominant negative approach, whereby an entire Kv channel subfamily can be functionally eliminated, might prove more effective (Ribera et al., 1996; Blaine & Ribera, 1998).

TEA and 4-AP had opposite effects on basal extension on a laminin substrate, with 4-AP and TEA inhibiting and stimulating neurite outgrowth, respectively. This observation may be explained by differences in their regulation of global calcium dynamics within the growth cone. The frequency of calcium waves within the growth cones of Xenopus spinal cord neurons, and calcium spikes in embryonic hamster cortical neurons has been inversely correlated with neurite outgrowth rates (Gu & Spitzer, 1997; Gomez & Spitzer, 1999; Tang et al., 2003). This is consistent with our data which indicated that overall 4-AP stimulated calcium transients and inhibited neurite extension, whereas TEA silenced calcium transients and promoted neurite extension. It is likely that Kv channels regulate global calcium dynamics by modulating the opening and closing of VDCCs through their regulation of membrane potential (McClellan et al., 1994; Koyano et al., 1996; Wang & Gruenstein, 1997; Falk et al., 1999; Song et al., 2002). The data which shows that 4-AP is ineffective at reducing growth cone extension when extracellular calcium levels are significantly reduced is supportive of this idea. If true, we would predict that TEA- and 4-AP sensitive Kv channel activity would cause the activation and inhibition of VDCCs, respectively (McClellan et al., 1994; Koyano et al., 1996; Lepple-Wienhues et al., 1996; Yao & Kwan, 1999). Such regulation would then impact the patterning of global calcium transients within the growth cone.

Whereas TEA and 4-AP had opposite effects on laminin-dependent neurite outgrowth, stimulation of extension over and above this basal level by one or more growth factors failed to occur in the presence of either drug. These data indicate several roles for Kv channels in axon outgrowth. First, Kv channels modulate the pattern of global calcium transients within the body of the growth cone and determine the basal rate of extension of the growth cone. This is how they function in outgrowth on laminin. Second, Kv channels are necessary for growth cones to respond to secreted growth cues with rapid extension. When Kv
Fig. 6.

Fig. 7.
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channels are blocked, growth cones are impaired in their ability to sense these cues. Finally, the growth cone turning assay data indicate that certain Kv channels are also required for the interpretation of directional cues, a process that is thought to be mediated within the filopodia.

Potentially, regulation of calcium signalling mediates all three processes. As discussed above, Kv channels present in the central region of the growth cone could regulate global calcium transients and the basal rate of extension. Further, 4-AP- and TEA-sensitive Kv channels present within lamellopodia and filopodia could also modulate the calcium signalling downstream of many extension and guidance cues (Henley & Poo, 2004). Indeed, in agreement with observations in cerebellar neurons (Williams et al., 1994), we found that FGF-2 stimulated RGC neurite outgrowth was inhibited by the β-type calcium channel blocker, nimodipine (Atkinson-Leadbeater and Poo, 2004). Alternatively, because we found that 4-AP application inhibited RGC axon extension on laminin, we may have selected for axons that were relatively insensitive to 4-AP in the growth cone turning assay, which depends on actively extending axons.

In the time-lapse and calcium imaging experiments, not all growth cones responded to 4-AP or TEA. The idea that embryonic RGCs are heterogeneous with respect to the Kv channels they express is supported by our previous immunocytochemical and electrophysiological data (McFarlane & Pollock, 2000; Pollock et al., 2002). Such heterogeneity may reflect differences between the intrinsic properties of RGCs even at these early stages, or in the differentiation state of RGCs, which mature in a central to peripheral gradient in the retina (Holt, 1989). Alternatively, the intrinsic state of growth cones might be variable, which could influence whether Kv channels can impact the response of growth cones to extension and guidance cues (McFarlane, 2000).

Our data support a model by which Kv channels act both within the body of the growth cone and the filopodia/lamellopodia to regulate both the basal rate of extension and the response of growth cones to extrinsic cues. Determining the mechanism by which they do so, and the particular Kv channels involved, needs to be addressed. Certainly, it is becoming clear that electrical activity may play a critical role in some systems in the guidance of axons to their targets (Catalano & Shatz, 1998; McCaig et al., 2002; Hanson & Landmesser, 2004). As key regulators of electrical activity, growth cone Kv channels might be responsible for fine-tuning this process.

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Abbreviations

4-AP, 4-aminopyridine; BSA, bovine serum albumin; CaGD-1, acetoxymethyl ester of calcium green-1; FGF-2, fibroblast growth factor-2; Kv, channels, voltage-gated potassium channels; RGC, retinal ganglion cell; TEA, tetraethylammonium; VDCC: voltage-dependent calcium channel.

FIG. 6. TEA silences calcium transients in RGC growth cones extending on laminin. RGC growth cones extending from 1-day-old, stage 24 explant cultures were loaded with the nonratiometric calcium imaging dye CaGD-1. (A and B) Panels show images before and after 30 mM TEA was added to representative RGC growth cones. (C and D) Graphs of traces of the relative fluorescence intensity of four representative growth cones over time. The trace in C and the upper trace in D correspond to the growth cones in A and B (white arrowheads), respectively. Two spontaneously active growth cones, both from the same dish, are also shown in D. In general, TEA inhibits calcium transient activity in growth cones. TEA was added where indicated and was present through the remainder of the recording session. Arrows represent the time points corresponding to the images shown in A and B.

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