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Voltage-Activated K^+ Channels and Membrane Depolarization Regulate Accumulation of the Cyclin-Dependent Kinase Inhibitors $p27^{Kip1}$ and $p21^{CIP1}$ in Glial Progenitor Cells

Cristina A. Ghiani,[1] Xiaoqing Yuan,[1] Alex M. Eisen,[1] Peter L. Knutson,[1] Ronald A. DePinho,[2] Chris J. McBain,[1] and Vittorio Gallo[1]

[1]*Laboratory of Cellular and Molecular Neurophysiology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892-4495, and* [2]*Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115*

Neural cell development is regulated by membrane ion channel activity. We have previously demonstrated that cell membrane depolarization with veratridine or blockage of K^+ channels with tetraethylammonium (TEA) inhibit oligodendrocyte progenitor (OP) proliferation and differentiation (Knutson et al., 1997); however the molecular events involved are largely unknown. Here we show that forskolin (FSK) and its derivative dideoxyforskolin (DFSK) block K^+ channels in OPs and inhibit cell proliferation. The antiproliferative effects of TEA, FSK, DFSK, and veratridine were attributable to OP cell cycle arrest in G1 phase. In fact, (1) cyclin D accumulation in synchronized OP cells was not affected by K^+ channel blockers or veratridine; (2) these agents prevented OP cell proliferation only if present during G1 phase; and (3) G1 blockers, such as rapamycin and deferoxamine, mimicked the anti-proliferative effects of K^+ channel blockers. DFSK also prevented OP differentiation, whereas FSK had no effect. Blockage of K^+ channels and membrane depolarization also caused accumulation of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{CIP1}$ in OP cells. The antiproliferative effects of K^+ channel blockers and veratridine were still present in OP cells isolated from INK4a $^{-/-}$ mice, lacking the cyclin-dependent kinase inhibitors $p16^{INK4a}$ and $p19^{ARF}$. Our results demonstrate that blockage of K^+ channels and cell depolarization induce G1 arrest in the OP cell cycle through a mechanism that may involve $p27^{Kip1}$ and $p21^{CIP1}$ and further support the conclusion that OP cell cycle arrest and differentiation are two uncoupled events.

Key words: oligodendrocyte development; cell cycle; ion channels; G1 arrest; cell proliferation; cyclin D

Functional ligand- and voltage-gated ion channels have been identified in different subpopulations of neural precursor cells (Walton et al., 1993; Patneau et al., 1994; LoTurco et al., 1995; Van den Pol et al., 1995; Gallo et al., 1996; Steinhauser and Gallo, 1996; Bardoul et al., 1997; Sah et al., 1997; Sugioka et al., 1998), leading to the proposal that ion channel activity may regulate their development. Neural cell proliferation (Cone and Cone, 1976; Cone, 1980; Chiu and Wilson, 1989; LoTurco et al., 1995; Gallo et al., 1996; Knutson et al., 1997; Yuan et al., 1998; Ghiani et al., 1999), migration (Behar et al., 1998), and differentiation (Jones and Ribera, 1994; Gu and Spitzer, 1995; Spitzer, 1995; Gallo et al., 1996; Knutson et al., 1997; Yuan et al., 1998) are indeed influenced by activation of ligand- or voltage-gated ion channels.

Voltage-gated K^+ channels regulate cell proliferation in different eukaryotic cell types (Lewis and Cahalan, 1988; Chiu and Wilson, 1989; Puro et al., 1989; Amirogena et al., 1990; Nilius and Droogmans, 1994; Pappas et al., 1994; Gallo et al., 1996; Knutson et al., 1997). Distinct K^+ currents are expressed during the mitotic cell cycle (Day et al., 1993, 1998; Pardo et al., 1998) and during embryonic development (Sontheimer et al., 1989; Wilson and Chiu, 1990; Attali et al., 1997; Knutson et al., 1997; Hallows and Tempel, 1998; Sobko et al., 1998). Glial cells display outward K^+ currents during their proliferative phase, which are down-regulated in quiescent or postmitotic states (Puro et al., 1989; Sontheimer et al., 1989; Barres et al., 1990; Gallo et al., 1996; Knutson et al., 1997; MacFarlane and Sontheimer, 1997). It has been hypothesized that voltage-dependent K^+ channel activity could regulate mitogenesis in the nervous system by maintaining the membrane potential hyperpolarized, a condition necessary for progression through G1 phase restriction points (Wonderlin and Strobl, 1996). Membrane potential-dependent transport of essential metabolic substrates during the cell cycle and/or volume regulation could also play a role (for review, see Wonderlin and Strobl, 1996).

Blockage of K^+ channels in glial cells, including oligodendrocyte progenitors (OPs), strongly inhibits proliferation (Wilson and Chiu, 1990; Pappas et al., 1994; Gallo et al., 1996; Attali et al., 1997; Knutson et al., 1997); however, the molecular mechanism by which K^+ channel activity regulates mitogenesis is still unknown. In the present study, we investigated OP cell cycle regulation by K^+ channel activity and membrane depolarization and analyzed whether (1) blockage of K^+ currents or cell depolarization interfere with a specific phase of the OP cell cycle; and (2) cyclins and cyclin-dependent kinase inhibitors (cdkis) known to regulate cell

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Correspondence should be addressed to Dr. Vittorio Gallo, Laboratory of Cellular and Molecular Neurophysiology, National Institute of Child Health and Human Development, National Institutes of Health, Building 49, Room 5A-78, 49 Convent Drive, Bethesda, MD 20892-4495.

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cycle progression through this phase are affected by ion channel activity or changes in membrane potential. We demonstrate that K^+ channel blockers and depolarizing agents cause G1 arrest in OP cell cycle and accumulation of $p27^{Kip1}$ and $p21^{CIP1}$, two cdkis known to regulate cell proliferation and terminal differentiation in a variety of cell types (Ross, 1996; Casaccia-Bonnefil et al., 1997; Durand et al., 1997; Martin-Castellanos and Moreno, 1997). We also show that the G1 cdkis $p16^{INK4a}$ and $p19^{ARF}$, belonging to a distinct gene family (Quelle et al., 1995) and involved in the regulation of glial cell proliferation (Jen et al., 1994; Schmidt et al., 1994; Holland et al., 1998), are not involved in ion channel-dependent cell cycle arrest in OP cells.

MATERIALS AND METHODS

Materials. Platelet-derived growth factor (PDGF; human, AB, heterodimer form) and basic fibroblast growth factor (bFGF; human) were both from Upstate Biotechnology (Lake Placid, NY). Protease was from Sigma (St. Louis, MO; catalog #P6911). Isoproterenol, veratridine, forskolin, dideoxyforskolin, tetraethylammonium chloride (TEA), kainate, deferoxamine, and nocodazole were all from Sigma. Rapamycin and SKF96365 were from Biomol (Plymouth Meeting, PA). Methyl-[^{3}H]thymidine was from Amersham (Arlington Heights, IL). Anti-cyclin D antibodies (anti-human, polyclonal) were from Upstate Biotechnology. Anti-$p27^{Kip1}$, anti-$p21^{CIP1}$, and anti-$p15^{INK4b}$ were from Santa Cruz Biotechnology (Santa Cruz, CA). All secondary antibodies were from Cappel-Organon Teknika (Durham, NC).

Cell culture. Purified cortical OP cell cultures were prepared as previously described (Gallo and Armstrong, 1995; Gallo et al., 1996) from E20 Sprague Dawley rats. The animals were killed following National Institutes of Health animal welfare guidelines. OP cells were plated onto poly-D-ornithine-coated plates (0.1 mg/ml) and cultured in DMEM-N1 biotin-containing medium. After 2 hr, PDGF (10 ng/ml), bFGF (10 ng/ml), or PDGF plus bFGF (10 ng/ml each) was added to the culture medium. OP cells were cultured for 1–3 d and treated every 24 hr with PDGF and/or bFGF. OP cells were synchronized for 24–48 hr in DMEM-N1 biotin-containing medium and then treated with growth factors (PDGF or bFGF).

OP cells cultured from mice carrying the INK4a deletion (Serrano et al., 1996) were prepared from P1 pups, following the same protocol used for the rat progenitor cells.

Purified rat and mouse OP cells used for immunostaining were grown on glass coverslips precoated with poly-D-ornithine. Previously, we demonstrated that 100% of the rat cells expressed nestin, and >90% of the $nestin^+$ cells were $GD3^+$ or $A2B5^+$. Less than 5% of OP cells were $O4^+$, and $O1^+$ cells were absent in the rat cultures (Gallo and Armstrong, 1995; Gallo et al., 1996).

Immunocytochemical characterization of the cortical $INK4a^{-/-}$ mouse cultures demonstrated that >95% of the cells were OPs, based on the following criteria: (1) positive staining with an antiserum against NG2 proteoglycan (Stallcup and Beasley, 1987; Durand et al., 1998); (2) positive staining with anti-GAP-43 antibodies (Curtis et al., 1991; Fanarraga et al., 1995); (3) nestin expression, as detected with anti-nestin antibodies (Gallo and Armstrong, 1995); (4) small percentage (<5%) of $O4^+$ cells (Fanarraga et., 1995; Gallo and Armstrong, 1995); and (5) bipolar or monopolar morphology (Fanarraga et al., 1995; Gallo and Armstrong, 1995). In agreement with previous reports (Fanarraga et al., 1995; Durand et al., 1998), the majority of the cortical mouse OP cells were not stained with A2B5 or anti-GD3 antibodies. In the $INK4^{-/-}$ mouse cultures, GAP-43 expression was downregulated in the small percentage of $O4^+$ cells present compared with OPs (also see Fanarraga et al., 1995). No $GFAP^+$ cells were detected in the purified mouse $INK4^{-/-}$ OP cells.

Cerebellar organotypic slice cultures and cell dissociation. Cerebellar organotypic slice cultures were prepared and processed as previously described (Yuan et al., 1998). Cerebella were dissected from postnatal day 6 Sprague Dawley rats and sagittally sliced (450 μM) using a McIlwain tissue chopper (Mickle Laboratory Engineering Co. Ltd.). Slices were placed on 0.5 μm LCR sterile membrane filters (Millipore, Bedford, MA) in 24-mm-diameter sterilized sieves (Netwell inserts, 500 μm mesh size; Fisher Scientific, Pittsburgh, PA) and cultured in DMEM-N1 medium containing 10% FBS (HyClone, Logan, UT) in six well plates (Falcon; Becton Dickinson, Franklin Lakes, NJ). Groups of four to six cerebellar slices were placed on each filter and maintained in culture for a total of 48–72 hr. TEA (1–10 mM) was added to the slices for 48 hr. Bromodeoxyuridine (BrdU, 50 μM; Sigma) was added to the slices for the last 24 hr. After 72 hr in culture, cerebellar slices were treated with protease (1.5 mg/ml) for 5 min at 37°C and with trypsin inhibitor (0.65 μg/ml; Sigma) for 5 min at 4°C. Cells were then dissociated by trituration through a Pasteur pipette (35 strokes) and plated on poly-D-ornithine-coated 25-mm-diameter coverslips at a density of 2×10^6 cells per coverslip in 200 μl of DMEM-N1 plus 10% FBS for immunocytochemistry. Cells were stained and analyzed 2 hr after plating.

Cell proliferation assays in culture. Cell proliferation was assayed as previously described (Gallo et al., 1996; Knutson et al., 1997). Purified cortical OP cells were plated in DMEM-N1 biotin-containing medium with 0.5% FBS in 24 multiwell plates at a density of 3×10^4 cells/cm^2. After 2 hr, PDGF and/or bFGF and kainate, forskolin, or dideoxyforskolin were added to the cultures along with methyl-[^{3}H]thymidine (0.5 μCi/ml, 85 Ci/mmol). Unless otherwise stated, cells were lysed after 22 hr, and [^{3}H]thymidine incorporation was measured by precipitation with 10% trichloroacetic acid and scintillation counting.

[^{3}H]thymidine incorporation assays in synchronized OP cells were performed by culturing the cells without growth factors for 24 hr and treating them with either PDGF or bFGF. Veratridine, TEA, dideoxyforskolin, rapamycin, deferoxamine, aphidicolin, SKF96365, and nocodazol were either added together with the growth factors or 6–24 hr after. Twelve hours after the stimulation with growth factors, cells were labeled with [^{3}H]thymidine and harvested 18 hr later to measure [^{3}H]thymidine incorporation.

Immunocytochemistry and counting of cell cultures and dissociated cells. The primary antibodies used were LB1 (Levi et al., 1986; Curtis et al., 1988), NG2 (Stallcup, 1981), A2B5 (Eisenbarth et al., 1979), nestin (Tohyama et al., 1992; Gallo and Armstrong, 1995), O4 (Sommer and Schachner, 1981), O1 (Sommer and Schachner, 1981), and anti-BrdU (Dako, Carpinteria, CA). Double indirect immunofluorescence experiments in rat and mouse cultures were performed as previously described (Gallo and Armstromg, 1995; Gallo et al., 1996; Yuan et al., 1998; Ghiani et al., 1999). For cell counting, 10–20 microscopic fields (Axiophot fluorescence microscope, 40× objective; Zeiss, Thornwood, NY) were counted for each coverslip, and two coverslips for each experiment were analyzed. At least three independent experiments were performed for each antibody, corresponding to a total of several thousands of cells counted (see figure legends). Data are presented as averages ± SEM.

Western blot analysis. OP cells were treated for 48 hr with growth factors and drugs. Cells (2×10^6) were then washed twice and harvested in ice-cold PBS. The cells were resuspended in 100–300 μl of sample buffer [150 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 0.25% Na^+-deoxycholate, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride] and lysed by sonication. The lysate was clarified by centrifugation at $5000 \times g$ for 15 min, and the supernatant was collected. An aliquot was taken for protein determination using the Pierce (Rockford, IL) BCA* protein assay kit, and 20–50 μg of cell extracts were resolved on a 12–15% mini SDS-polyacrylamide gel and transferred to Immobilion polyvinylidene fluoride membranes (Millipore, Marlborough, MA). Blots were blocked with 5% nonfat dry milk in PBS-T (17 mM KH_2PO_4, 50 mM Na_2HPO_4, 1.5 mM NaCl, pH 7.4, and 0.05% Tween 20) for 1 hr at room temperature and then incubated at room temperature for 2 hr in PBS-T and 5% nonfat dry milk containing one of the following antibodies: anti-cyclin D, anti-$p27^{Kip1}$, anti-$p21^{CIP1}$, or anti-$p15^{INK4b}$. Protein bands were detected using the Amersham ECL kit with horseradish peroxidase-conjugated secondary antibodies. Relative intensities of the protein bands were quantified by scanning densitometry (Scanwizard Plug-in; Microtek, Redondo Beach, CA).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assays. Apoptotic cell death was determined by fluorescence microscopy by using the terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end-labeling (TUNEL) assay (Boehringer Mannheim, Indianapolis, IN). Synchronized cultured OP cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and 0.1% sodium citrate (2 min at 4°C), and stained with TUNEL according to manufacturer. Apoptotic OP cells were brightly fluorescent. For cell counting, 10 microscopic fields (Zeiss Axiophot fluorescence microscope, 40× objective) were counted for each coverslip, and two coverslips were analyzed for each experiment. A total of two independent experiments were performed.

Electrophysiology. For electrophysiological experiments, cells were cultured with 10 ng/ml PDGF (proliferating OP cells). Cells were perfused with media of the following composition (in mM): NaCl, 160; $CaCl_2$, 1.5; $MgSO_4$, 1.5; glucose, 10; HEPES, 10; and tetrodotoxin, 0.5–1 μM. Tight-seal (>5 GΩ) whole-cell recordings were made from $LB1^+$ ($GD3^+$) OPs. Careful attention was paid to select only cells with strict bipolar morphology for electrophysiological analysis to ensure that an homogeneous population of cells was studied. Patch electrodes were fabricated from thin-walled borosilicate glass (WPI TW150F-6) and had resistance of 2–6 MΩ when filled with (in mM): K-gluconate, 130; NaCl, 10; Na_2ATP, 2; NaGTP, 0.3; HEPES, 10; and EGTA, 0.6, buffered to pH 7.4 and ~275 mOsm. Cell sealing and breakthrough into whole-cell mode was performed in current-clamp conditions permitting an accurate determination of cell resting membrane potential (Table 1). Unless otherwise stated, cells were then voltage-clamped between −70 and −40 mV and test pulse-delivered to −60 to +70 mV (0.1 Hz, 10 mV increments). Linear leak current and capacitative artifacts were digitally subtracted off-line before analysis using Clampfit (Axon Instruments, Burlingame, CA). Records were filtered at 2 kHz and digitized at 5–10 kHz. The series resistances were calculated from the capacitative current peak (filtered at 20 kHz and digitized at 50 kHz) in a 5–10 mV voltage step and were in the range of 2–18 MΩ (mean, 8.3 ± 0.5 MΩ; $n = 25$). Series resistances were compensated to at least 85%. Cell capacitance was measured from the response to a 5–10 mV voltage step. Current density was calculated by dividing current amplitude by the cell membrane capacitance. Plots of the voltage dependence of current activation were constructed by dividing the peak current by the driving force ($V_{test} - V_r$) where V_{test} was the step depolarizing potential, and V_r was the calculated reversal potential ($Ek = -95$ mV). The activation profiles were fitted with a Boltzmann equation of the form: $g/g_{max} = [1 + \exp(V_{1/2} - V/k)]^{-1}$, where g/g_{max} is the conductance normalized to its maximum value, V is the membrane potential, $V_{1/2}$ is the membrane potential at which the current amplitude is half-maximum, and k is a constant. For the construction of the majority of activation curves, the sum of two independent Boltzmann equations was used. All drugs solutions were added directly to the bath via the perfusion system in known concentrations. All data are expressed as the mean ± SEM

RESULTS

Forskolin and dideoxyforskolin block K^+ currents and inhibit OP cell proliferation

Dividing OP cells display outward K^+ currents that are blocked by TEA, 4-aminopyridine, and quinine (Knutson et al., 1997). Among these agents, only TEA was not toxic to OP cells when tested in long-term cell proliferation assays (Knutson et al., 1997; C. A. Ghiani and V. Gallo, unpublished results). In search of other compounds that would block K^+ currents in OP cells, we analyzed the effects of forskolin (FSK), which is known to cause cAMP-independent blockage of voltage-dependent K^+ channels (Laurenza et al., 1989). At a test pulse of +70 mV, FSK dose-dependently reduced the sustained current amplitude (measured at a time point of 250 msec). At concentrations of 5, 50, and 200 μM, the current amplitude was blocked by 39.9 ± 4.2% ($n = 3$), 65.8 ± 4.5% ($n = 11$), and 92.7 ± 1.5% ($n = 3$), respectively (Fig. 1). Interestingly, FSK increased the rate of inactivation of the sustained current component. In control conditions the sustained current inactivated with a single time constant of 125.8 ± 2.4 msec ($n = 8$). At a concentration of 200 μM FSK, the sustained current component inactivation was best fit with two time constants of 26.2 ± 15.4 and 269.7 ± 68 msec ($n = 3$). This mechanism of block by FSK is similar to that described previously in nudibranch neurons, PC12 cells, and human T cells (Coombs and Thompson, 1987; Hoshi et al., 1988; Krause et al., 1988) and is thought to occur by an open channel blocking mechanism and is cAMP-independent. To test this hypothesis we used dideoxyforskolin (DFSK), the naturally occurring analog of FSK, which does not activate adenylate cyclase (for review, see Laurenza et al., 1989). Addition of DFSK also blocked the sustained current

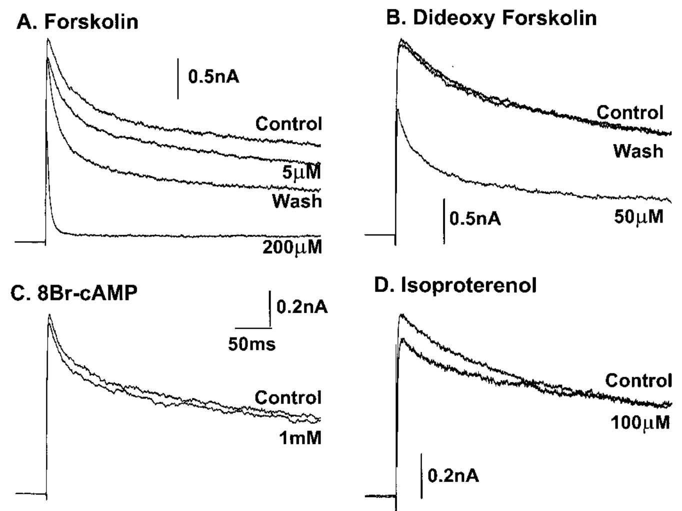

Figure 1. Forskolin reversibly blocks potassium channels and increases the rate of current inactivation by a cAMP-independent mechanism in oligodendrocyte progenitor cells. *A*, Forskolin dose-dependently blocks potassium currents activated at a test pulse of +70 mV. Each *trace* represents the average of 5–8 test pulses. Forskolin, 5 and 200 μM, blocks the outward current by 17 and 94.5%, respectively, at a time point of 250 msec. In addition, the rate of current inactivation is increased in the presence of forskolin. In control conditions, current inactivation could be fit by two time constants of 21 and 148 msec, respectively. In the presence of 200 μM forskolin, the current inactivation was best fit by an exponential with two time constants of 6 and 408 msec, respectively. *B*, Dideoxyforskolin similarly blocked outward currents. At a concentration of 50 μM dideoxyforskolin blocked 65% of the outward current. Like forskolin, dideoxyforskolin increased the rate of current inactivation. In control conditions the outward current inactivation was best fit by a single exponential of 143 msec. In the presence of dideoxyforskolin, the current inactivation was best fit by the sum of two exponentials with time constants of 20 and 129 msec, respectively. *C, D*, In contrast, agents that elevate intracellular cAMP levels, 8-Br-cAMP and the β-adrenergic agonist isoproterenol, blocked only 7 and 3% of the outward current, respectively, and had no effect on the rate of current inactivation.

component. At a concentration of 50 μM, DFSK blocked the sustained current component by 62.2 ± 4.7% ($n = 5$). Similar to FSK, DFSK also increased the rate of current inactivation (Fig. 1). In the presence of DFSK, the sustained current inactivated with two time constants of 32 ± 14 and 152 ± 21.6 msec ($n = 5$) (cf. 129.8 ± 7.5 msec in control). These data suggest that DFSK and FSK act to directly block the sustained current and do not require activation of adenylate cyclase for the blocking mechanism.

To determine whether the activation of adenylate cyclase by forskolin has an additional effect on the sustained current component, we used the analog 8-bromo-cAMP (8-Br-cAMP). Unlike FSK, a maximal concentration of 8-Br-cAMP (1 mM) blocked only 23 ± 1.4% ($n = 4$) of the sustained current component. Furthermore, 8-Br-cAMP was without effect on the time course of current inactivation, suggesting that the primary effect of FSK on the sustained current does not involve activation of adenylate cyclase or an elevation of cAMP levels. Similarly, the β-adrenergic receptor agonist isoproterenol, which also elevates cAMP levels in OP cells (Ghiani et al., 1999), was without effect on the sustained current recorded in these cells. At a concentration 100 μM isoproterenol the sustained current component was 95.9 ± 1.1% of control (Fig. 1).

Previously we have shown that long-term culture (24 and 48 hr) in the presence of the antiproliferative agents isoproterenol and

Table 1. Effects of chronic treatment with antiproliferative agents on K^+ current expression

Treatment	RMP (mV)	C_m (pF)	$V_{1/2}$ (mV) (lower)	k	$V_{1/2}$ (mV) (upper)	k	Current density (pA/pF)[a]
Control[b]	−39.1 ± 2.3	5.0 ± 0.7	−19.6 ± 0.9[e]	4.8	17.5 ± 4.4[e]	15.0	200 ± 24
Forskolin[c]	−41.9 ± 2.6	9.1 ± 0.5*	−13.9 ± 5.5	7.3	20.6 ± 4.5	12.7	75 ± 15*
Dideoxyforskolin[d]	−35.0 ± 1.4	10.1 ± 1.6*	−14.7 ± 1.4	7.0	25.4 ± 7.0	16.0	120 ± 45*

*$p < 0.05$.

A comparison of the electrophysiological properties of control OP cells and cells after chronic treatment with forskolin (50 μM) and dideoxyforskolin (50 μM) for 24–48 hr. After chronic treatment with these agents no changes were observed in the cell resting membrane parameters. Chronic treatment with forskolin and dideoxyforskolin did not significantly alter the voltage-dependent properties of both sustained outward current components. Forskolin and dideoxyforskolin did, however, reduce significantly the current density measured at +70 mV.

[a]Current amplitudes measured at +70 mV.

[b]$n = 25$.

[c]$n = 9$.

[d]$n = 12$.

[e]$n = 30$.

8-Br-cAMP modified the properties of the sustained currents in proliferating OP cells (Ghiani et al., 1999). Both agents caused a rightward shift in the voltage dependence of activation, which was presumably responsible for their antiproliferative behavior. In the present experiments, neither FSK nor DDFSK altered the passive properties of the OP cells (Table 1). Similarly, the voltage-dependent properties of the sustained currents in OP cells were unaffected (Table 1). In contrast, the current density of the sustained current measured at +70 mV was significantly reduced after 48 hr treatment with both FSK and DDFSK.

We have previously demonstrated that agents that block K^+ currents (TEA) or cause cell membrane depolarization (veratridine) inhibit OP cell proliferation and lineage progression (Gallo et al., 1996; Knutson et al., 1997). We therefore tested the effects of FSK and DFSK on OP cell proliferation and development. [^{3}H]Thymidine incorporation was significantly inhibited by culturing OP cells in the presence of either PDGF or bFGF together with increasing concentration of FSK or DFSK (Fig. 2*A,B*). The effect elicited by FSK was concentration-dependent, with an IC_{50} of 21 ± 1 and 22 ± 3 μM ($n = 6$) in PDGF and bFGF, respectively. DFSK displayed an IC_{50} of 30 ± 5 and 27 ± 5 μM in PDGF and bFGF, respectively. The effects of both drugs were reversible within 24 hr (Fig. 2*C,D*). Progenitor cells that were cultured in the presence of PDGF and FSK or DFSK for 24 hr and then [^{3}H]thymidine-pulsed in agent-free medium containing PDGF reentered S phase with a temporal pattern similar to OP cells that were never exposed to FSK or DFSK. This was consistent with the lack of long-term effect of FSK and DFSK on K^+ channel properties (Table 1). Neither FSK nor DFSK significantly affected OP cell viability within the concentration range tested (data not shown).

In conclusion, these results are in agreement with previous studies showing that FSK causes a cAMP-independent blockage of K^+ current in a variety of cell types (Coombs and Thompson, 1987; Hoshi et al., 1988; Castle, 1989; Laurenza et al., 1989; Baxter and Byrne, 1990; Zerr and Feltz, 1994; Herness et al., 1997) and that agents that directly block K^+ channels or modulate K^+ channel activity also have an antiproliferative effect on OP cells (Gallo et al., 1996; Attali et al., 1997; Knutson et al., 1997) .

Effects of forskolin and dideoxyforskolin on OP lineage progression

We tested whether FSK and DFSK reproduced the effects of the specific K^+ channel blocker TEA, which prevented OP lineage progression by reducing the percentage of $O4^+$ preoligodendroblasts (Gallo et al., 1996). As shown in Figure 2*E*, FSK did not affect OP lineage progression under any of the culture conditions tested. However, DFSK significantly decreased the percentage of $O4^+$ cells in OPs cultured under conditions that favored lineage progression (Fig. 2*F*). The lack of effect of FSK on OP lineage progression might be explained by the fact that this agent blocks K^+ channels but is also a direct activator of adenylate cyclase (Laurenza et al., 1989), and it has been shown that an elevation of cAMP levels in OPs promotes cell differentiation (Pleasure et al., 1986; Raible and McMorris, 1989, 1993; Ghiani et al., 1999).

TEA inhibits OP cell proliferation and prevents lineage progression in cerebellar slice cultures

Glial cell proliferation may be differentially regulated in intact tissue than observed in purified cultures. For example, an elevation of intracellular cAMP inhibits Schwann cell proliferation in peripheral rat sciatic nerve segments (Fex Svenningsen and Kanje, 1998), whereas it strongly stimulates mitotic activity in purified cultures (Raff et al., 1978; Stewart et al., 1991). We have, therefore, extended our analysis of proliferation and differentiation of oligodendrocyte lineage cells to a cytoarchitecturally intact system, cerebellar slice cultures. In these slices, OPs and preoligodendroblasts proliferate and differentiate *in vitro* (Yuan et al., 1998). In a previous report, we showed that glutamate receptor agonists inhibit OP proliferation and differentiation in cerebellar slice cultures, and this effect is most likely attributable to an increase in intracellular Na^+ and consequent block of K^+ current (Knutson et al., 1997; Yuan et al., 1998). On this basis, we examined whether OP cell proliferation and differentiation in cerebellar slice cultures was affected by blocking K^+ channels with TEA.

Incubation of cerebellar slices with 1 mM TEA for 24 hr caused a 75% reduction in the percentage of $NG2^+$ OP cells and a 50% decrease in their BrdU incorporation (Fig. 3*A,B*). The K^+ channel blocker also greatly reduced the percentage and BrdU labeling index of $O4^+$ pro-oligodendroblasts (Fig. 3*C,D*). Consistent with these findings, the percentage of $O1^+$ oligodendrocytes was also decreased by ~50% after treatment with TEA (Fig. 3*E*). The effects of TEA were dose-dependent, because the percentage of OP cells and pro-oligodendroblasts was further decreased (80 and 65% decrease, respectively) by 10 mM TEA (data not shown).

In conclusion, blockage of K^+ channels in cells of the oligodendrocyte lineage reduced proliferation and prevented lineage progression also in cerebellar tissue slices, as previously demonstrated with GluR agonists (Yuan et al., 1998).

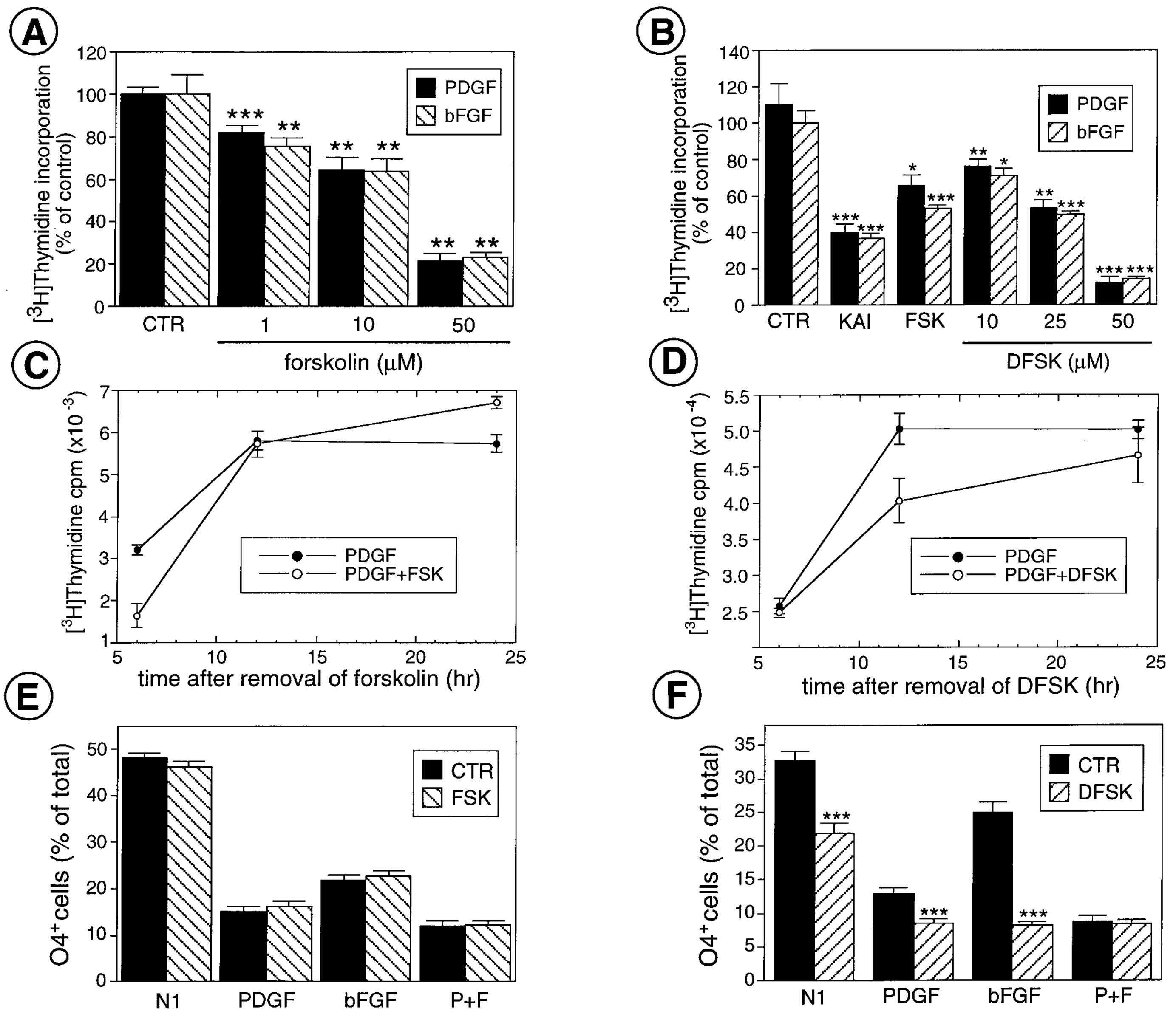

Figure 2. Forskolin and dideoxyforskolin reversibly inhibit oligodendrocyte progenitor cell proliferation. *A, B,* FSK and DFSK inhibit OP cell proliferation at concentrations that also block K^+ currents. Cells were plated in 24 well plates (3×10^4 cells per well). After 2 hr, PDGF or bFGF (both 10 ng/ml) in combination with forskolin or dideoxyforskolin were added to the culture medium along with [^{3}H]thymidine (0.5 μCi/ml). After 22 hr, [^{3}H]thymidine incorporation was measured by trichloroacetic acid precipitation and scintillation counting. In *B*, kainate (*KAI*; 100 μM) was used as a positive control, and forskolin (25 μM) was used for a direct comparison with DFSK. *CTR*, Control, cells cultured in PDGF or bFGF alone. Averages ± SEM obtained from three to six independent experiments run in triplicate are shown. ***$p < 0.0001$; **$p < 0.01$; *$p < 0.05$ compared with respective control (PDGF- or bFGF-cultured cells; Student's *t* test). *C, D,* The antiproliferative effects of forskolin and dideoxyforskolin are reversible. OP cells were cultured in PDGF (10 ng/ml) in the presence or absence of forskolin (25 μM) or dideoxyforskolin (25 μM). After 22 hr, all cells were placed in fresh culture medium without inhibitors containing PDGF (10 mg/ml) and [^{3}H]thymidine (0.5 μCi/ml). OPs were harvested after 6, 12, and 24 hr of inhibitor-free medium, and [^{3}H]thymidine incorporation was determined by trichloroacetic acid precipitation and scintillation counting. Averages ± SEM ($n = 3$) are shown. *E, F,* Treatment with dideoxyforskolin prevents OP lineage progression, whereas forskolin does not affect OP development. Immunohistochemical staining of oligodendrocyte lineage cells with the monoclonal antibody O4. OP cells were purified and cultured on coverslips in DMEM-N1 medium and 0.5% fetal bovine serum with PDGF (10 ng/ml), bFGF (10 ng/ml), or PDGF and bFGF (*P+F*; both 10 ng/ml). Forskolin and dideoxyforskolin (both 50 μM) were added to the cultures 2 hr after plating. After 46 hr, cells were immunostained with O4 and counted. Averages ± SEM obtained from three to five experiments are shown ($n = 30$–50 microscopic fields counted). The total number of cells counted for each culture condition ranged between 1755 and 10,137. ***$p < 0.001$ compared with respective controls (Student's *t* test).

Blockage of K^+ channels and cell depolarization cause G1 arrest in OP cell cycle

We reported that OP cells can be synchronized by culturing them in the absence of growth factors for 48 hr and then adding PDGF (Ghiani et al., 1999). Cyclin D1 expression is very low in purified OP cells cultured without growth factors and upregulated within 6 hr after treatment with PDGF, indicating that OPs reenter G1 phase (Fig. 4). Cyclin D1 expression reached a plateau 18 hr after treatment with the growth factor and displayed a decrease between 18 and 24 hr (Fig. 4; also see Ghiani et al., 1999). Cell

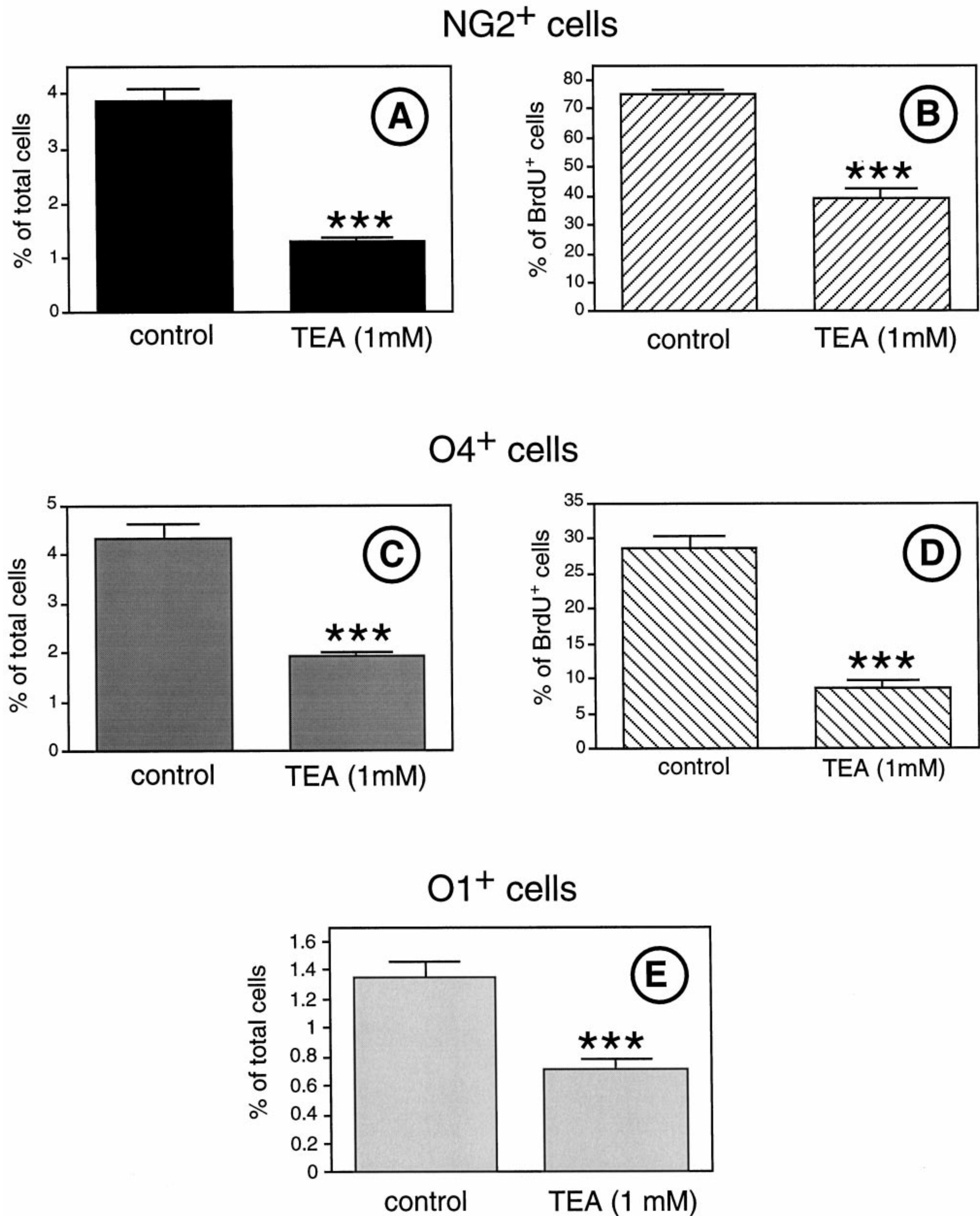

Figure 3. Blockage of K^+ channels inhibits OP proliferation and development in cerebellar slice cultures. Quantitative analysis of cerebellar cells dissociated from P6 rat slice cultures and treated for 48 hr with the K^+ channel blocker TEA (1 mM). Control, no TEA. All tissue slices were treated with BrdU for 24 hr. Cells were dissociated and stained with NG2 and anti-BrdU (*A, B*), O4 and anti-BrdU (*C, D*) or O1 and anti-BrdU (*E*). As previously demonstrated (Yuan et al., 1998), none of the $O1^+$ cells was $BrdU^+$. A total of six coverslips (10 fields per coverslip) were counted for each antibody staining from three independent experiments. Between 25,064 and 47,083 total cells were counted for each culture condition, using phase-contrast imaging. Data represent averages ± SEM ($n = 60$ microscopic fields counted per antibody combination). $^{***}p < 0.0001$ compared with their respective controls (Student's t test).

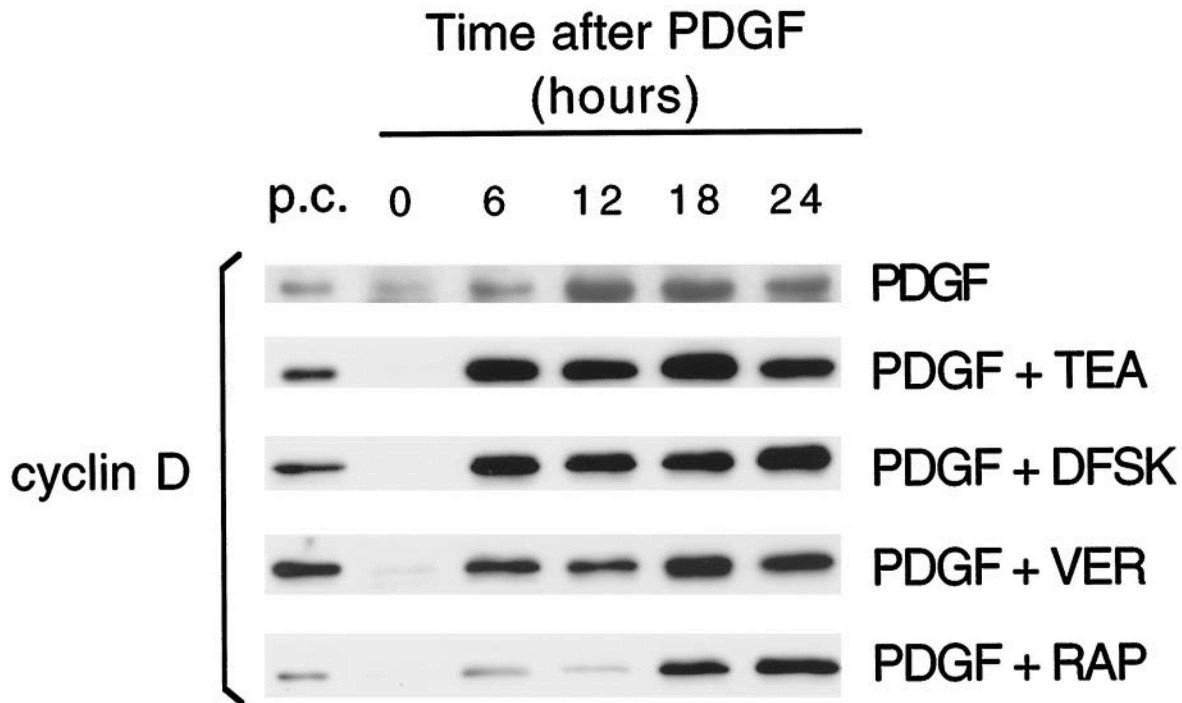

Figure 4. K^+ channel blockers and depolarizing agents do not affect G0–G1 transition in synchronized oligodendrocyte progenitors. Time course of cyclin D expression as determined by Western blot analysis. Oligodendrocyte progenitor cells were synchronized by culturing in the absence of growth factors for 48 hr and treated with PDGF (10 ng/ml) in the presence or in the absence of TEA (10 mM), DFSK (50 μM), veratridine (*VER*; 30 μM), or rapamycin (*RAP*; 25nM). Cells were harvested at different times after re-adding PDGF and the antiproliferative agents (0–24 hr). Cyclin D expression was analyzed with anti-cyclin D polyclonal antibodies that recognized both cyclin D1 and D2. The major band identified by the antibody is cyclin D1 (36 kDa). *p.c.*, Positive control; purified cyclin D1 protein comigrates with the cyclin D1 from OP cells. Twenty micrograms of total proteins were loaded on the gel for each sample.

depolarization with the Na^+ channel opener veratridine or blockage of voltage-dependent K^+ channels with TEA or DFSK did not modify cyclin D1 accumulation (Fig. 4), indicating that OP cell entry into G1 phase was not affected by changes in membrane potential. Consistent with this interpretation, treatment with the G1 blocker rapamycin also did not modify cyclin D1 accumulation in synchronized OP cells (Fig. 4).

To determine whether veratridine, TEA, or DFSK specifically affected a phase of the cell cycle that precedes S phase, these agents were added at different times to synchronized OP cells treated with PDGF. Maximal inhibitory effects were observed when one of the three agents was added to the cultures within 12 hr after the growth factor (Fig. 5*A–C*). The G1 blocker rapamycin displayed a time course of inhibition similar to veratridine, TEA, and DFSK. None of the treatments caused apoptosis in OP cells, as determined by TUNEL assays (data not shown).

A variety of cell cycle inhibitors were tested in synchronized OP cells to compare their effects with those of K^+ channel blockers and of veratridine. All these agents were used in a concentration range that was not toxic to OP cells. Figure 6 shows that, in cells cultured in PDGF or bFGF the G1 and G1–S blockers deferoxamine, rapamycin, and aphidicolin inhibited OP cell cycle progression to the same extent as TEA, whereas the G2–M and M blockers SKF96365 and nocodazol did not affect [^{3}H]thymidine incorporation into OP cells (Fig. 6*A,B*).

Altogether, these results indicate that membrane depolarization causes a block in G1 phase of the OP cell cycle and prevents G1–S transition, as previously demonstrated for GluR activation (Ghiani et al., 1999).

Blockage of K^+ channels and cell depolarization increase $p27^{Kip1}$ and $p21^{CIP1}$ levels

Eukaryotic cell cycle progression is tightly regulated by the activity of several cyclin-dependent kinases (cdks) and cdkis (Ross, 1996). We analyzed the involvement of members of two families of cdkis that are known to act in the G1 phase of the cell cycle. The *INK4* family comprises $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{ARF}$, whereas the Kip/CIP family comprises $p21^{CIP1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (Martin-Castellanos and Moreno, 1997). Induction of one or several of these proteins by antiproliferative signals prevents G1–S transition in the cell cycle (Martin-Castellanos and Moreno, 1997).

The role of $p16^{INK4a}$ and $p19^{ARF}$ was analyzed in OP cells isolated from mice carrying a targeted deletion of the INK4a locus, i.e., deficient for both these gene products (Serrano et al., 1996). The mouse cultures prepared from the knock-out mutants displayed morphological and antigenic properties previously described for wild-type mouse OPs (Stallcup and Beasley, 1987; Fanarraga et al., 1995; Durand et al., 1998; Ghiani et al., 1999). More than 95% of the cells were $NG2^+$, $nestin^+$ OPs (also see Ghiani et al., 1999). [^{3}H]Thymidine incorporation assays in *INK4a* $^{-/-}$ OPs demonstrated that veratridine, FSK, DFSK, and TEA significantly inhibited cell proliferation stimulated by PDGF (Fig. 7*A*) and bFGF (Fig. 7*B*). These results indicate that neither $p16^{INK4a}$ nor $p19^{ARF}$ is necessarily involved in cell cycle arrest induced by membrane depolarization. Expression of the *INK4* cdki $p15^{INK4b}$ in OP cells cultured with PDGF was very low and unmodified by membrane depolarization (data not shown).

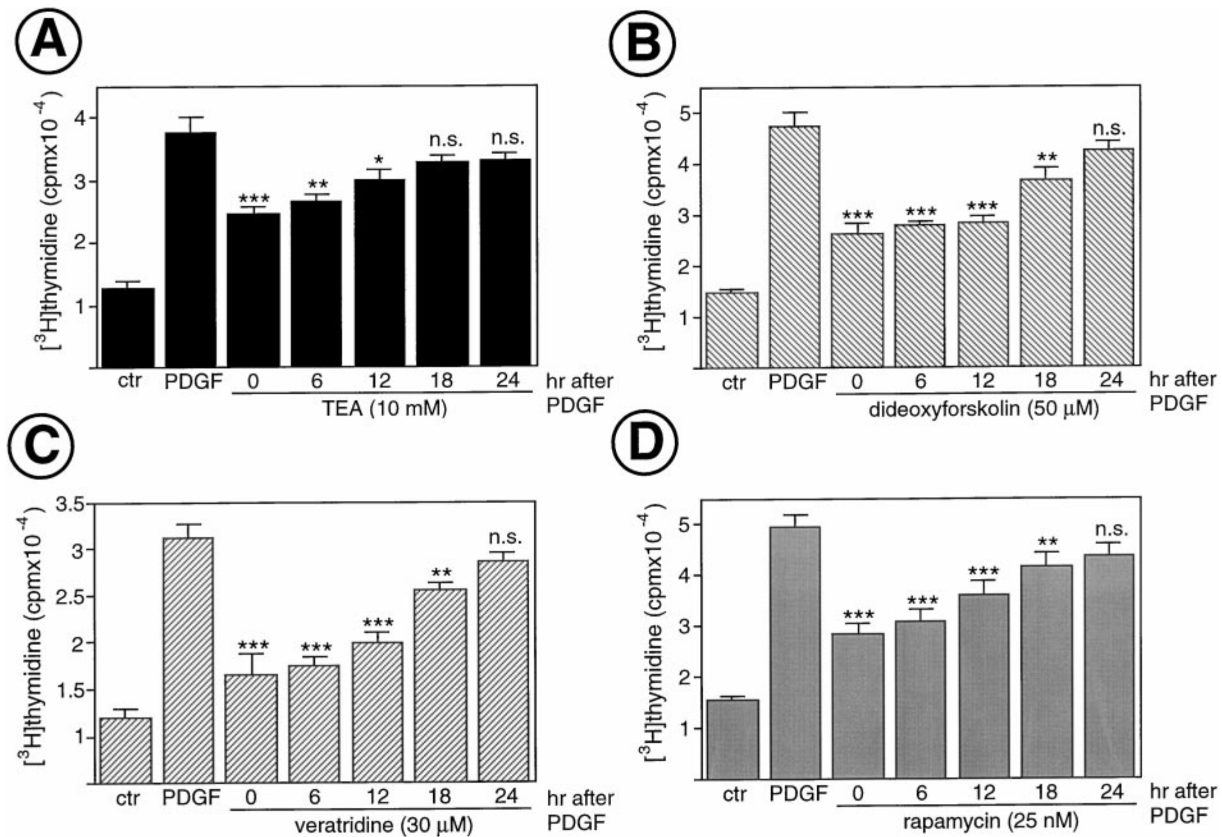

Figure 5. The antiproliferative effect of K^+ channel blockers and depolarizing agents is attributable to G1 arrest in OP cell cycle. TEA, dideoxyforskolin, veratridine, and rapamycin lose their antiproliferative effects as OP cells proceed through the cell cycle. The G1 blocker rapamycin was used as a positive control. [^{3}H]Thymidine incorporation assays were performed on synchronized cells, which were pulsed with PDGF (10 ng/ml). TEA (*A*; 10 mM), dideoxyforskolin (*B*; 50 μM), veratridine (*C*; 30 μM) or rapamycin (*D*; 25nM) was added to the culture medium at the same time as PDGF or 6–24 hr later. [^{3}H]Thymidine was added to the cultures 12 hr after PDGF. OP cells were harvested 30 hr after PDGF addition, and [^{3}H]thymidine incorporation was determined by trichloroacetic acid precipitation and scintillation counting. TEA, veratridine, dideoxyforskolin, and rapamycin prevented OP cells from entering S phase only if added to synchronized cells within 12–18 hr after PDGF. Data represent averages ± SEM of two or three experiments performed in triplicate (n = 6–9 wells). *A*, ***$p < 0.0005$; **$p < 0.005$; *$p < 0.05$ compared with PDGF. *B*, ***$p < 0.0001$; **$p <$ 0.0005 compared with PDGF. *C*, ***$p < 0.005$; **$p < 0.05$. *D*, ***$p < 0.005$; **$p < 0.05$ compared with PDGF. *n.s.*, Not significant.

Accumulation of the cdki $p27^{Kip1}$ has been recently associated with cell cycle arrest in OP cells (Casaccia-Bonnefil et al., 1997; Durand et al., 1997; Ghiani et al., 1999). We therefore analyzed whether blockage of K^+ channels and cell membrane depolarization caused accumulation of $p27^{Kip1}$ in nonsynchronized rat OP cells. After 48 hr of treatment, veratridine, FSK, DFSK, and TEA increased $p27^{Kip1}$ expression twofold to threefold over the levels observed in cells cultured in PDGF alone (Fig. 8). Interestingly, $p27^{Kip1}$ accumulation induced by these agents is similar to that induced by the β-receptor agonist isoproterenol and significantly higher than the constitutive accumulation measured during OP cell differentiation (Ghiani et al., 1999).

An increase in the cdki $p21^{CIP1}$ has also been demonstrated in OP cells during differentiation and under conditions that cause cell cycle arrest (Casaccia-Bonnefil et al., 1997; Ghiani et al., 1999). We therefore analyzed $p21^{CIP1}$ expression in OP cells treated with veratridine and K^+ channel blockers. Figure 9 shows that veratridine, TEA, and DFSK caused a significant 2- to 2.5-fold increase in $p21^{CIP1}$ levels, as measured by Western blot, but FSK was ineffective.

In summary, these results show that both cell membrane depolarization and blockage of voltage-dependent K^+ channels trigger accumulation of two cdkis, $p27^{Kip1}$ and $p21^{CIP1}$, in dividing OP cells.

DISCUSSION

Nerve cell development can be regulated by the timely expression of distinct ionic channels (Cone and Cone, 1976; Cone, 1980; Chiu and Wilson, 1989; Jones and Ribera, 1994; Gu and Spitzer, 1995; LoTurco et al., 1995; Spitzer, 1995; Gallo et al., 1996; Knutson et al., 1997; Behar et al., 1998; Yuan et al., 1998; Ghiani et al., 1999) or by their modulation by growth factors (for review, see Chew and Gallo, 1999). Glial cells express membrane ionic channels with molecular and functional properties that are generally identical to those of neurons (Duffy et al., 1995; Ransom and Orkand, 1996; Sontheimer et al., 1996; Steinhauser and Gallo, 1996; Verkhratsky and Kettenmann, 1996). The function of these channels in glia is largely unknown, although some have been linked to normal or abnormal cell proliferation. Activity of outward K^+ channels is higher in mitotically active Schwann cells, oligodendrocyte progenitors, astrocytes, and retinal glial cells, whereas it is greatly reduced in postmitotic cells (Puro et al., 1989; Sontheimer et al., 1989; Wilson and Chiu, 1990; Pappas et al., 1994; Gallo et al., 1996; Knutson et al., 1997). Interestingly, expression of distinct K^+ currents has been linked to proliferative potential in both reactive astrocytes (MacFarlane and Sontheimer, 1997) and neuroblastoma cells (Arcangeli et al., 1995).

Expression and regulation of K^+ channels in myelinating cells

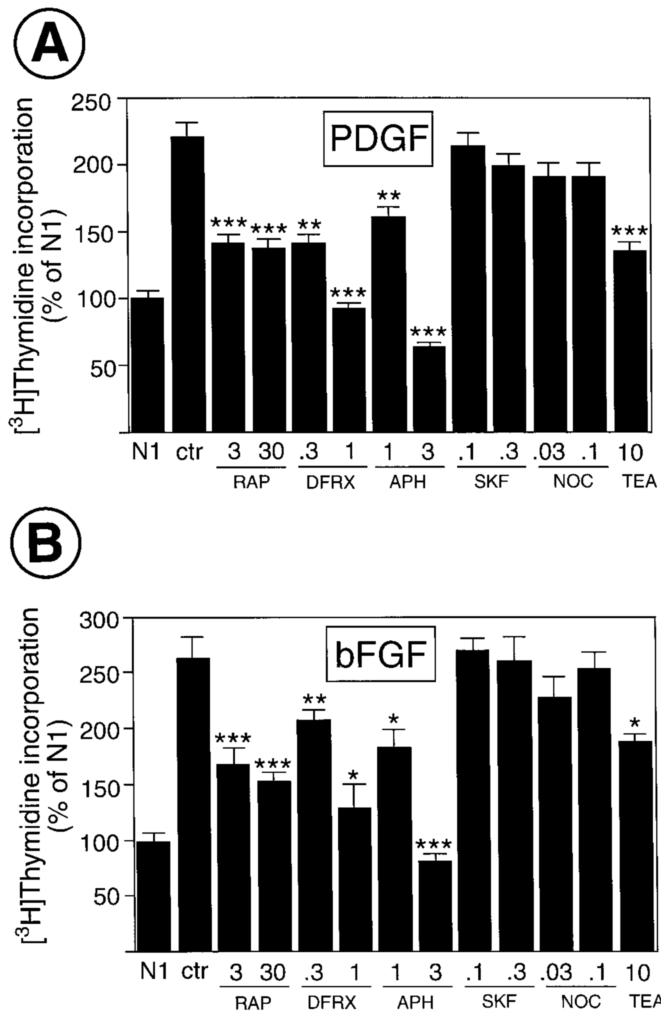

Figure 6. Agents that cause G1 arrest in mitotic cell cycle mimic the effects of K^+ channel blockage on oligodendrocyte progenitor proliferation. [^{3}H]Thymidine incorporation assays were performed in OP cells synchronized by culturing in the absence of growth factors for 24 hr and treated with PDGF (10 ng/ml) or bFGF (10 ng/ml). Rapamycin (*RAP*; 3–30nM), deferoxamine (*DFRX*; 0.3–1 mM), aphidicolin (*APH*; 1–3 μM), SKF96365 (*SKF*; 0.1–0.3 μM), nocodazole (*NOC*; 0.03–0.1 μg/ml), or TEA (10 mM) was added together with [^{3}H]thymidine and the growth factors. The concentrations of antiproliferative agents used were not toxic and did not cause visible morphological changes in OP cells. Cells were harvested 24 hr later to measure thymidine incorporation by trichloroacetic acid precipitation and scintillation counting. *N1*, Cells cultured without growth factors; *ctr*, control, cells cultured in PDGF or bFGF alone. Data represent averages ± SEM of three to five experiments run in triplicate (n = 9–15). *A*, $***p < 0.0001$; $**p < 0.001$ compared with PDGF. *B*, $***p < 0.0001$; $**p < 0.005$; $*p < 0.05$ compared with bFGF.

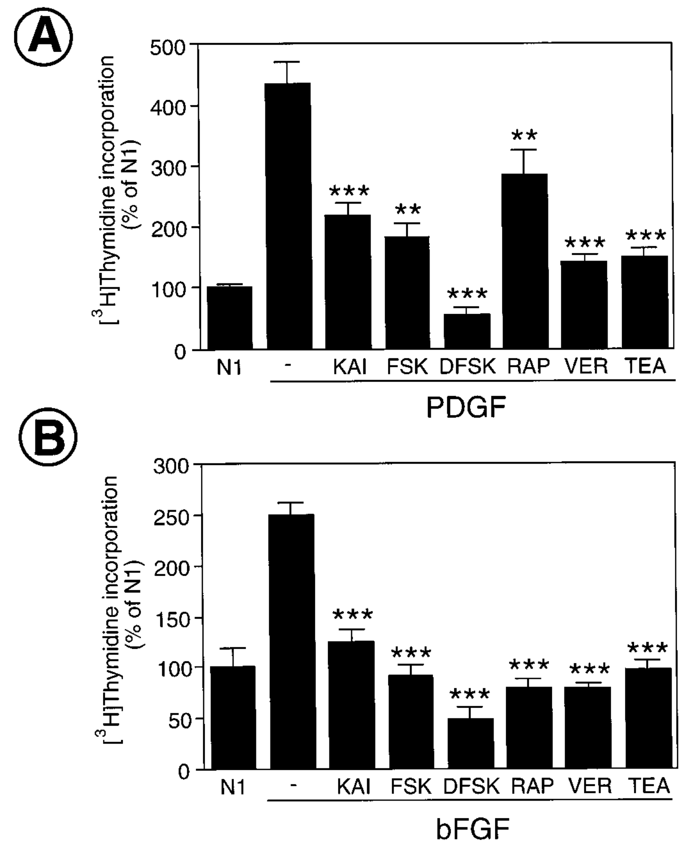

Figure 7. Blockage of K^+ channels or cell depolarization inhibits proliferation of oligodendrocyte progenitor cells purified from INK4$^{-/-}$ mice lacking the p16^{INK4a} and p19^{ARF} genes. [^{3}H]Thymidine incorporation assays in nonsynchronized mouse cells. OP cells were purified from P1 mice and cultured in DMEM-N1 plus 0.5% FBS and PDGF (*A*) or bFGF (*B*). Cells were plated in 24 well plates (3×10^4 cells per well). After 2 hr, PDGF or bFGF (both 10 ng/ml) was added to the culture medium in combination with kainate (*KAI*; 100 μM), TEA (10 mM), FSK (50 μM), DFSK (50 μM), or veratridine (*VER*; 50 μM). Cells were harvested 22 hr later, and [^{3}H]thymidine incorporation was assessed by trichloroacetic acid precipitation and scintillation counting. Cells in N1 medium in the absence of growth factors incorporated 5615 ± 268 cpm/well per 22 hr (average ± SEM; n = 9). Averages of three experiments in triplicate ± SEM are shown. *A*, $***p < 0.0005$; $**p < 0.005$ compared with PDGF. *B*, $***p < 0.0001$ compared with bFGF.

has drawn attention for a variety of reasons. First, in oligodendrocytes and Schwann cells it is possible to identify specific developmental stages associated with a particular K^+ channel phenotype (Sontheimer et al., 1989; Barres et al., 1990; Wilson and Chiu, 1990). Second, K^+ channel activity in myelinating cells is clearly linked to proliferation (Chiu and Wilson, 1989; Gallo et al., 1996; Knutson et al., 1997). Finally, earlier studies have demonstrated that blockage of K^+ channels can affect oligodendrocyte development and myelination (Shrager and Novakovic, 1995).

In the experiments described here, we analyzed the influence of K^+ channel activity and membrane potential on OP cell cycle progression by using nontoxic K^+ channel blockers (TEA, FSK, and DFSK) and the Na^+ channel opener veratridine, which causes OP cell depolarization (Knutson et al., 1997). One of the central findings of this study is that either blockage of K^+ channels or membrane depolarization prevents OP cells from entering S phase by causing G1 arrest. This conclusion is based on observations made in OP cells maintained in G0 phase, as demonstrated by their low levels of expression of the G1 phase marker cyclin D (Fig. 4) and by their low BrdU incorporation index (Ghiani et al., 1999). In G0-arrested OP cells, accumulation of cyclin D stimulated by treatment with the mitogen PDGF was not affected by K^+ channel blockers or veratridine, indicating that OP cells can still progress from G0 to G1 when K^+ channel activity is blocked, or their membrane potential is depolarized. Furthermore, K^+ channel blockers and veratridine prevented OP cell proliferation only if present during G1 phase and did not cause apoptosis. Finally, the G1 blockers rapamycin and deferoxamine mimicked the antiproliferative effects of K^+ channel blockers, whereas the G2–M and M blockers SKF96365 and nocodazol did not affect OP proliferation.

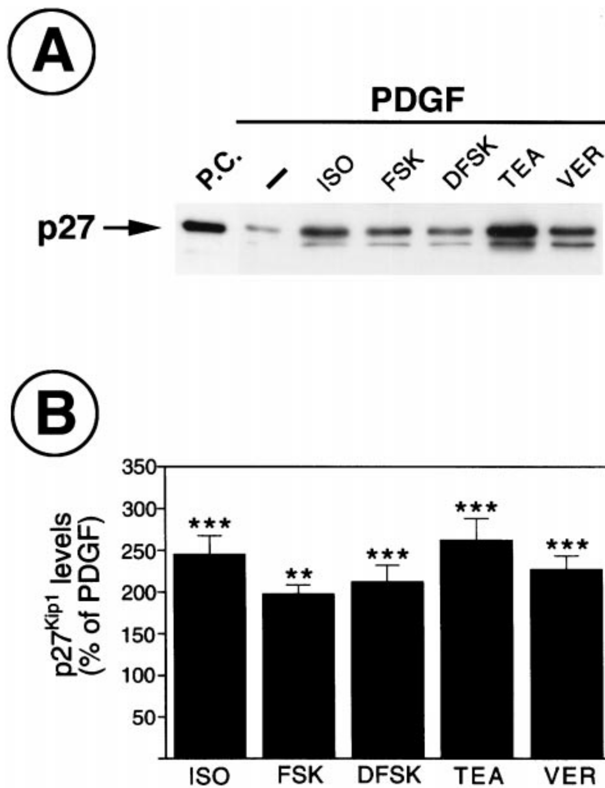

Figure 8. Blockage of K^+ channels or cell membrane depolarization induces accumulation of $p27^{Kip1}$ in oligodendrocyte progenitor cells. Western blot analysis of $p27^{Kip1}$ expression in nonsynchronized oligodendrocyte progenitors. Cells were plated in PDGF (10 ng/ml) in the presence or absence of isoproterenol (*ISO*; 50 μM), FSK (50 μM), DFSK (50 μM), TEA (5 mM), or veratridine (*VER*; 30 μM). Cells were harvested after 48 hr, and 15–20 μg of total protein were loaded on the gel for each sample. Histograms represent relative levels of $p27^{Kip1}$ determined by densitometric analysis of autoradiographs from Western blots. Values are expressed as ratios of cells treated with PDGF alone and are mean ± SEM of three to five separate experiments. *P.C.*, Positive control; purified $p27^{Kip1}$ protein comigrates with the $p27^{Kip1}$ from OP cells. $^{***}p < 0.01$; $^{**}p < 0.05$ compared with PDGF alone (Student's *t* test).

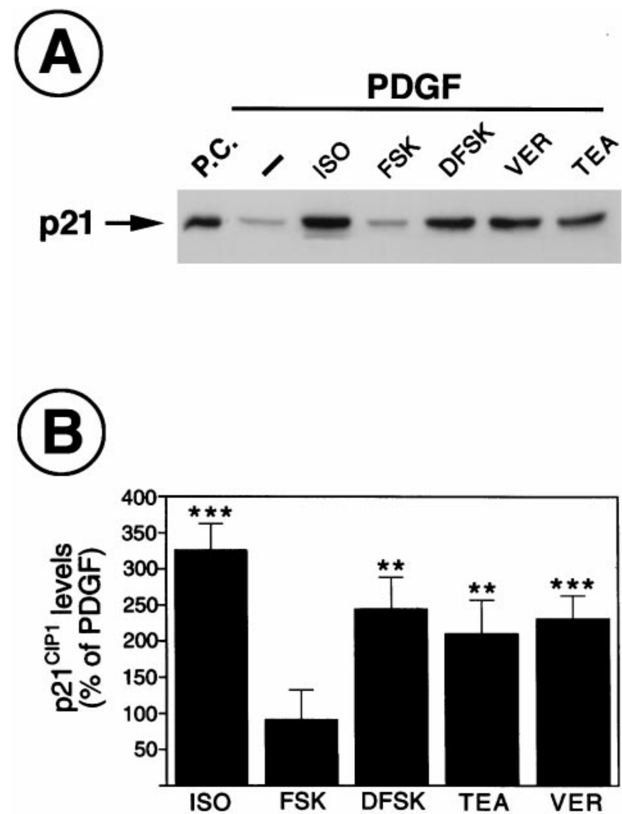

Figure 9. Blockage of K^+ channels or cell membrane depolarization stimulates $p21^{CIP1}$ accumulation in oligodendrocyte progenitor cells. Western blot analysis of $p21^{CIP1}$ expression in nonsynchronized oligodendrocyte progenitors. Cells were plated in PDGF (10 ng/ml) in the presence or in the absence of isoproterenol (*ISO*; 50 μM), FSK (50 μM), DFSK (50 μM), TEA (5 mM), or veratridine (*VER*; 30 μM). Cells were harvested after 48 hr, and 30–40 μg of total protein were loaded on the gel for each sample. Histograms represent relative levels of $p21^{CIP1}$ determined by densitometric analysis of autoradiographs from Western blots. Values are expressed as ratios of cells treated with PDGF alone and are mean ± SEM of three to five separate experiments. *P.C.*, Positive control; purified $p21^{CIP1}$ protein comigrates with the $p21^{CIP1}$ from OP cells. $^{***}p < 0.005$; $^{**}p < 0.05$ compared with PDGF alone (Student's *t* test).

K^+ channel activity regulates G1 progression in the cell cycle of distinct eukaryotic cell types (Deutsch, 1990; Dubois and Rouzaire-Dubois, 1993; Nilius and Droogmans, 1994; Wonderlin and Strobl, 1996). The intricate molecular network of proteins involved in G1–S phase progression comprises two distinct cdki families, INK4 and Kip/CIP (Martin-Castellanos and Moreno, 1997). Of these, $p27^{Kip1}$ is thought to be primarily involved in cell cycle arrest of OP cells. Overexpression of $p27^{Kip1}$ in dividing OP cells after adenoviral infection results in arrest in cell proliferation (Tikoo et al., 1998). During development, $p27^{Kip1}$ is accumulated in mitotically active OP cells to increase to maximal levels in differentiated oligodendrocytes (Casaccia-Bonnefil et al., 1997; Durand et al., 1997), and a significantly higher number of glial cells was detected in the optic nerve of $p27^{Kip1}$-deficient mice (Casaccia-Bonnefil et al., 1997).

We have previously demonstrated that neurotransmitter receptor agonists that cause OP cell cycle arrest also increase $p27^{Kip1}$ expression (Ghiani et al., 1999). We also found that the protein levels of another member of the Kip/CIP family, $p21^{CIP1}$, are increased by the same stimuli, indicating that both $p27^{Kip1}$ and $p21^{CIP1}$ are part of the G1 arrest pathway in mitotically active OPs. In the present study, we show that $p27^{Kip1}$ and $p21^{CIP1}$ accumulation is triggered by blockage of K^+ channels or cell membrane depolarization. These results indicate that changes in membrane potential can activate a pathway involving $p27^{Kip1}$ and $p21^{CIP1}$ similar to the constitutive pathway of cell cycle arrest that occurs during development (Casaccia-Bonnefil et al., 1997; Durand et al., 1997). It can be concluded that fluctuations in K^+ channel activity and membrane potential may play a fundamental role in modifying the levels of cdkis in mitotically active cells of the mammalian CNS.

Cerebellar slice cultures represent a cytoarchitecturally intact system that maintains, at least in part, the complex cellular interactions that occur during neural development (Yuan et al., 1998; Ghiani et al., 1999). In previous studies, we demonstrated that agonists acting at glutamate and β-adrenergic receptors regulate oligodendrocyte development in a similar manner both in cerebellar tissue slices and in purified cultured cells (Yuan et al., 1998: Ghiani et al., 1999). This indicates that the mechanism coupling receptor and channel activity in the membrane with OP cell cycle progression is similar *in situ* and in cultured cells.

In agreement with our previous experiments in purified cul-

tured OPs (Gallo et al., 1996; Knutson et al., 1997), the present study demonstrates that treatment with the K^+ channel blocker TEA decreased the number of OPs and preoligodendroblasts also in cerebellar tissue slices. Consistent with our findings, Shrager and Novakovic (1995) showed that myelination is severely impaired by incubation of spinal cord slice cultures with TEA, whereas neuronal function is unaffected. Our analysis indicates that the reduction in OPs and preoligodendroblasts in cerebellar slices is attributable to inhibition of cell proliferation, as shown by a parallel decrease of the BrdU incorporation index in $NG2^+$ and $O4^+$ cells. These findings are consistent with TEA-induced OP cell cycle arrest in cerebellar slices because of direct blockage of K^+ channels, rather than indirect effects. In agreement with this interpretation, we found that TEA reduced proliferation of purified cerebellar OP cells cultured with PDGF or bFGF to 49.4 ± 6.0 and 51.7 ± 5.1% of controls, respectively ($n = 9$; average ± SEM; three independent experiments).

It can be hypothesized that regulation of cell proliferation in the oligodendrocyte lineage, through modulation of K^+ channels and changes in membrane potential, is also relevant to oligodendrocyte development or regeneration *in vivo*. Neuronal or astrocytic release of cellular factors or ions that may modulate K^+ channel function (for review, see Chew and Gallo, 1999) would also affect OP cell proliferation and development. We have previously demonstrated that K^+ channel function and the K^+ channel phenotype of OP cells can be strongly modulated by environmental cues (Knutson et al., 1997; Ghiani et al., 1999). Retinoic acid and β-adrenergic receptor agonists do not directly block K^+ channels in OP cells but reduce their functional activity, for example, by shifting their voltage dependence of activation after long-term exposure (Knutson et al., 1997; Ghiani et al., 1999). Long-term exposure of OP cells to high $[K^+]_o$ caused an upregulation of inward rectifier K^+ currents, a phenotype observed in postmitotic preoligodendroblasts (Knutson et al., 1997). *In vivo*, during their migratory and proliferative phases, OPs and preoligodendroblasts can become exposed to high concentrations of K^+ ions released from axons during the propagation of action potentials. This would alter K^+ channel activity by changing the K^+ driving force and/or by long-term modifications in the K^+ channel phenotype (Knutson et al., 1997).

In conclusion, we have demonstrated that K^+ channel activity and cell membrane potential play a pivotal role in the regulation of G1–S transition in glial progenitor cells. Our analysis in the present and previous studies (Knutson et al., 1997; Pende et al., 1997: Yuan et al., 1998; Ghiani et al., 1999) demonstrates that the intracellular signal transduction pathways associated with activation of different receptor systems and distinct membrane channels converge on two cdkis, $p27^{Kip1}$ and $p21^{CIP1}$, which regulate proliferation and differentiation in a variety of cell lineages, including oligodendrocytes (Macleod et al., 1995; Parker et al., 1995; Skapek et al., 1995; Casaccia-Bonnefil et al., 1997; Di Cunto et al., 1998; Ghiani et al., 1999). The finding that K^+ channel activity and membrane depolarization modulate OP cell cycle progression in cerebral cortex (Gallo et al., 1996; Knutson et al., 1997), spinal cord (Shrager and Novakovic, 1995), and cerebellum (Yuan et al., 1998) indicates that this is likely to be a general mechanism of control of OP proliferation in the CNS.

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