Multiple Kinase Pathways Regulate Voltage-Dependent Ca^{2+} Influx and Migration in Oligodendrocyte Precursor Cells

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It is becoming increasingly clear that voltage-operated Ca^{2+} channels (VOCCs) play a fundamental role in the development of oligodendrocyte progenitor cells (OPCs). Because direct phosphorylation by different kinases is one of the most important mechanisms involved in VOCC modulation, the aim of this study was to evaluate the participation of serine–threonine kinases and tyrosine kinases (TKs) on Ca^{2+} influx mediated by VOCCs in OPCs. Calcium imaging revealed that OPCs exhibited Ca^{2+} influx after plasma membrane depolarization via L-type VOCCs. Furthermore, VOCC-mediated Ca^{2+} influx declined with OPC differentiation, indicating that VOCCs are developmentally regulated in OPCs. PKC activation significantly increased VOCC activity in OPCs, whereas PKA activation produced the opposite effect. The results also indicated that OPC morphological changes induced by PKC activation were partially mediated by VOCCs. Our data clearly suggest that TKs exert an activating influence on VOCC function in OPCs. Furthermore, using the PDGF response as a model to probe the role of TK receptors (TKr) on OPC Ca^{2+} uptake, we found that TKr activation potentiated Ca^{2+} influx after membrane depolarization. Interestingly, this TKr modulation of VOCCs appeared to be essential for the PDGF enhancement of OPC migration rate, because cell motility was completely blocked by TKr antagonists, as well as VOCC inhibitors, in migration assays. The present study strongly demonstrates that PKC and TKrs enhance Ca^{2+} influx induced by depolarization in OPCs, whereas PKA has an inhibitory effect. These kinases modulate voltage-operated Ca^{2+} uptake in OPCs and participate in the modulation of process extension and migration.

Introduction

It is becoming increasingly clear that expression of Ca^{2+} channels in the oligodendroglial lineage is highly regulated and their activity may be related to different stages of oligodendrocyte (OL) development. Understanding the mechanisms of voltage-dependent Ca^{2+} influx is important because changes in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_{\text{int}}$) are central to many cellular activities. For example, in OL progenitor cells (OPCs), voltage-dependent Ca^{2+} influx plays a key role in several important processes, such as proliferation, apoptosis, and cell migration (Paez et al., 2009b,c). We found recently that increased voltage-dependent Ca^{2+} influx was associated with enhanced OPC motility, and this effect was accompanied by increases in the amplitude of spontaneous somatic Ca^{2+} transients, which appeared to be essential for OPC migration (Paez et al., 2009c).

Voltage-operated Ca^{2+} channels (VOCCs), which are common in neurons and muscle, provide transmembrane Ca^{2+} for transmitter release, contraction, the coupling and integration of synaptic inputs to action potentials, and other intracellular signaling processes. Six types of VOCCs (P/Q, N, L, R, and T) have been classified on the basis of electrophysiological and pharmacological properties (MacVicar, 1984; Akopian et al., 1996; Puro

et al., 1996; Robitaille et al., 1996; Oh, 1997). Immunohistochemical studies have reported the expression of L-, N-, and R-type VOCCs in OLs *in vivo* (Butt, 2006).

The pore of a voltage-gated Ca^{2+} channel is formed by an α subunit, which consists of four homologous domains connected by six transmembrane helices. Gating of this pore is regulated by phosphorylation at multiple cytoplasmic regions on the α subunit, including the N and C terminals, and the loops between each domain. This structure allows for complex interactions between the α subunit and many regulatory protein complexes. The Ca_v1 family of $\alpha1$ subunits conducts L-type Ca^{2+} currents and is regulated primarily by second-messenger-activated protein phosphorylation pathways. The Ca_v2 family of $\alpha1$ subunits conducts N-type, P/Q-type, and R-type Ca^{2+} currents and is regulated primarily by direct interaction with G-proteins and secondarily by protein phosphorylation (Catterall, 2000). The latter regulation is important for electrically active cells, such as neurons. Both L-type channels and T-type channels are regulated through PKC and PKA. Several of the α -subunit isoforms for L-type Ca^{2+} channels contain PKC and PKA phosphorylation sites (Puri et al., 1997).

An emerging body of evidence suggests that VOCCs are also regulated by phosphorylation of tyrosine residues (Strauss et al., 1997; Wijetunge et al., 2002). Several growth factors, such as PDGF and basic FGF (bFGF), activate receptor tyrosine kinases (TKr) and trigger complex intracellular signal transduction pathways, finally leading to cell proliferation and migration in OPCs and other cell types (Taniguchi, 1995). Ca^{2+} entry from extracellular sources is known to play a key role in these events. However, the nature of the Ca^{2+} channels involved and a possible regula-

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tion through direct channel phosphorylations by TKr remains controversial (Wijetunge et al., 2000; Schröder et al., 2004).

The aim of this study was to evaluate the participation of several kinases on the regulation of voltage-operated Ca^{2+} channels in OPCs. $[\text{Ca}^{2+}]_{\text{int}}$ was measured in real time in cultured OPCs and live brain sections, using a spectrofluorometric technique with fura-2 as an intracellular Ca^{2+} indicator. High extracellular K^{+} was used as a depolarization stimulus to activate and open VOCCs, enhancing $[\text{Ca}^{2+}]_{\text{int}}$ in OPCs (Paez et al., 2007, 2009a,c).

Materials and Methods

Primary cultures of cortical oligodendrocytes. Enriched oligodendrocytes were prepared as described by Amur-Umarjee et al. (1993). First, cerebral hemispheres from 1-d-old mice were mechanically dissociated and were plated on poly-D-lysine-coated flasks in DMEM and Ham's F-12 (1:1 v/v) (Invitrogen), containing 100 $\mu\text{g}/\text{ml}$ gentamycin and supplemented with 4 mg/ml anhydrous dextrose, 3.75 mg/ml HEPES buffer, pH 7.4, 2.4 mg/ml sodium bicarbonate, and 10% fetal bovine serum (FBS) (Omega Scientific). After 24 h, the medium was changed and the cells were grown in DMEM/F-12 supplemented with insulin (5 $\mu\text{g}/\text{ml}$), transferrin (50 $\mu\text{g}/\text{ml}$), sodium selenite (30 nM), D-biotin (10 mM), 0.1% BSA (Sigma-Aldrich), 1% horse serum, and 1% FBS (Omega Scientific). After 9 d, OPCs were purified from the mixed glial culture by the differential shaking and adhesion procedure of Suzumura et al. (1984) and allowed to grow on poly-lysine-coated coverslips in defined culture media (Agresti et al., 1996), including PDGF-A chain homodimer (PDGF-AA) (10 ng/ml) and bFGF (10 ng/ml) (Peprotech). OPCs were kept in mitogens (PDGF and bFGF) for 2 d and then induced to differentiate by switching the cells to a mitogen-free medium (mN2) (Oh et al., 2003). mN2 contained the following: DMEM/F-12 supplemented with D-glucose (4.5g/L), insulin (5 $\mu\text{g}/\text{ml}$), human transferrin (50 $\mu\text{g}/\text{ml}$), sodium selenite (30 nM), L-3,3,5-triiodothyronine (15 nM), D-biotin (10 mM), hydrocortisone (10 nM), 0.1% BSA, 1% horse serum, and 1% FBS.

Slice preparation. Calcium imaging acquisitions of green fluorescent protein (GFP)-labeled living OPCs were performed on coronal slices at postnatal day 4 (P4) and P8, as described previously (Kakita and Goldman, 1999). Briefly, mice were anesthetized with isoflurane, after which brains were rapidly removed and stored in ice-cold bicarbonate buffered solution, pH 7.4, gassed with 95% O_2 and 5% CO_2 . Coronal slices (300 μm) were cut on a vibratome. Brain tissue was kept in ice-cold bicarbonate solution during these procedures. The slices were then cultured with Eagle's Basal Medium with Earle's salts (Invitrogen) supplemented with 18.6 mM NaHCO_3 , 1% BSA (fraction 5; Sigma), 5 $\mu\text{g}/\text{ml}$ insulin, 5 $\mu\text{g}/\text{ml}$ transferrin, 5 $\mu\text{g}/\text{ml}$ sodium selenite (Sigma), 20 U/ml penicillin-streptomycin (Invitrogen), 2 mM L-glutamine (Invitrogen), and 27 mM glucose. After that, brain slices were ready for calcium imaging studies.

Immunocytochemistry. At the completion of the calcium-imaging experiment, the cells were stained with antibodies against NG2, O4, O1, PDGF α receptor (PDGF α), and MBP and examined by confocal microscopy. For MBP immunostaining, the cells were rinsed briefly in PBS and fixed in 4% buffered paraformaldehyde for 30 min at room temperature. After rinsing in PBS, the cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature and then processed for immunocytochemistry following the protocol as outlined by Reyes and Campagnoni (2002). Essentially, fixed cells were incubated in a blocking solution (5% goat serum in PBS), followed by an overnight incubation at 4°C with a polyclonal antibody for MBP (1:700). Staining with NG2 (1:50), PDGF α (1:100), O4 (1:20), and anti-galactocerebroside antibody O1 (1:20) was performed on live cells without permeabilization for 1 h at room temperature before fixation. Cells were then incubated with the appropriate secondary antibodies (1:200; Jackson ImmunoResearch) and mounted onto slides with Aquamount (Lerner Laboratories), and fluorescent images were obtained using an Olympus spinning disc confocal microscope.

Cell morphology assessment. To examine differences in morphology, OPCs cultured for 2 d in defined culture media (Agresti et al., 1996), including PDGF-AA (10 ng/ml) and bFGF (10 ng/ml), were stained with

the O4 antibody analyzed as follows: (1) the number of primary processes per OL, i.e., processes that directly bud from the cell; (2) the number of secondary processes per primary process (a process was considered as secondary when it branched directly from a primary process; thin, short processes, which were occasionally found along the entire length of primary process, were not considered as secondary); and (3) the length of primary processes. The percentage of primary processes with lengths corresponding to at least four cell soma diameters was determined. This method has been used previously for monitoring morphological changes in OL as well as other cell types (Yong et al., 1988, 1991; Sisková et al., 2009). The morphological parameters were obtained from 50 cells from at least four independent cultures. The total number of primary and secondary processes and the length of primary processes were measured using the image analysis software SlideBook 4.1 (Intelligent Imaging Innovations).

Time-lapse migration assay. Cultured OPCs were incubated in a stage top chamber with 5% CO_2 at 37°C (live-cell control unit), which was placed over the stage of an Olympus spinning disc confocal inverted microscope equipped with a motorized z-stage. A 20 $\times$ objective was used for acquiring images. Bright-field images were taken every 6 min over a period of 24 h using a CCD camera (Hamamatsu ORCA-ER) and analyzed with image analysis software (SlideBook 4.1; Intelligent Imaging Innovations). Cell migration speed and distances were analyzed offline by tracing individual cells using the motion tracking function of SlideBook software. The brightest part of each cell body was used as the tracking target. Subsequently, migratory values were statistically analyzed under different experimental conditions. Data are presented as mean $\pm$ SEM. Statistical significance was assessed by using the Student's paired *t* test, in which $p < 0.05$ was defined as statistically significant.

Calcium imaging. Methods were similar to those described previously (Colwell, 2000; Michel et al., 2002; Paz Soldán et al., 2003). Briefly, a cooled CCD camera (Hamamatsu ORCA-ER) was added to the Olympus spinning disc confocal microscope to measure fluorescence. To load the dye into cells, primary cultures of oligodendrocytes and brain slices were washed in serum and phenol red-free DMEM and incubated for 45 min at 37°C, 5% CO_2 in the same media containing a final concentration of 4 μM fura-2 AM (Teflabs) plus 0.08% Pluronic F-127 (Invitrogen), then washed four times in DMEM, and stored in DMEM for 0–1 h before being imaged (Paz Soldán et al., 2003). Resting calcium levels were made in serum-free HBSS containing 2 mM Ca^{2+} but no Mg^{2+} . Other measurements were made in HBSS. Calcium influx and resting Ca^{2+} levels were measured on individual cells, and the results were pooled from five separate cell preparations for each condition. The fluorescence of fura-2 was excited alternatively at wavelengths of 340 and 380 nm by means of a high-speed wavelength-switching device (Lambda DG-4; Sutter Instruments). Image analysis software (SlideBook 4.1; Intelligent Imaging Innovations) allowed the selection of several "regions of interest" within the field from which measurements were taken. To minimize bleaching, the intensity of excitation light and sampling frequency was kept as low as possible. In these experiments, measurements were normally made once every 2 s.

Calibration of Ca^{2+} signals. Free $[\text{Ca}^{2+}]$ was estimated from the ratio (*R*) of fluorescence at 340 and 380 nm, using the following equation: $[\text{Ca}^{2+}] = K_d \times \text{slope factor} \times (R - R_{\text{min}})/(R_{\text{max}} - R)$ (Grynkiewicz et al., 1985). The K_d was assumed to be 140 nM, whereas values for R_{min} and R_{max} were all determined using a calibration kit (fura-2 Ca^{2+} imaging calibration; Invitrogen) to estimate values. With this method, glass coverslips were filled with a high- Ca^{2+} solution (fura-2 plus 10 mM Ca^{2+}), a low- Ca^{2+} solution (fura-2 plus 10 mM EGTA), and a control solution without fura-2. Each solution also contains a dilute suspension of 15 μm polystyrene microspheres to ensure uniform coverslip/slide separation and facilitate microscope focusing. The fluorescence (*F*) at 380 nm excitation of the low Ca^{2+} solution was imaged, and the exposure of the camera was adjusted to maximize the signal. These camera settings were then fixed, and measurements were made with 380 and 340 nm excitation of the three solutions: $R_{\text{min}} = F_{340 \text{ nm}} / F_{380 \text{ nm}}$ in low Ca^{2+} ; $R_{\text{max}} = F_{340 \text{ nm}} / F_{380 \text{ nm}}$ in high Ca^{2+} ; $Sf = F_{380 \text{ nm}}$ in low Ca^{2+} ; $Sf = F_{380 \text{ nm}}$ in high Ca^{2+} .

PKC activity assay. A nonradioactive PKC activity assay designed for quantifying the activity of PKC in crude enzyme preparations was used in

accordance with the recommendations of the manufacturer (Assay Designs). This PKC activity assay is based on a solid-phase ELISA that uses a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognizes the phosphorylated form of the substrate. Briefly, cytosolic proteins from untreated and phorbol 12-myristate 13-acetate (PMA)-treated OPCs were added to the appropriate wells, followed by the addition of ATP to initiate the reaction. The kinase reaction was terminated, and a substrate phospho-specific antibody was added to the wells. The phospho-specific antibody was subsequently bound by a peroxidase-conjugated secondary antibody. The assay was developed with tetramethylbenzidine substrate and stopped with acid solution, and finally the intensity of the color was measured in a microplate reader at 450 nm.

Sample preparation. Control and PMA-treated Petri dishes containing $\sim 1.5 \times 10^6$ cells per dish were washed twice with ice-cold PBS. The cells were collected by centrifugation ($600 \times g$ for 10 min at 4°C) and extracted with lysis buffer [20 mM 3-(*N*-morpholino)-propanesulfonic acid, pH 7.4, 50 mM β -glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium vanadate, 5 mM EGTA, 2 mM EDTA, 1% NP-40, 1 mM dithiothreitol, 1 mM benzamide, 1 mM phenylmethylsulfonyl fluoride, and 10 $\mu\text{g/ml}$ leupeptin and aprotinin] for 10 min at 4°C . The insoluble material was cleared by centrifugation (13,000 rpm for 15 min at 4°C), and the resulting protein extracts (cytosolic fraction) were stored at -70°C until use. Protein concentration was determined using the BCA method.

Statistical analysis. Data are presented as mean $\pm$ SEM unless otherwise noted. For fura-2 experiments, the statistical comparisons among different experimental groups were performed by analysis of covariance.

Results

Voltage-operated Ca^{2+} entry in primary oligodendrocyte cultures

Primary cultures of OPCs were first loaded with a membrane-permeable form of the Ca^{2+} indicator dye fura-2, before treatment with 20 mM K^+ to activate voltage-operated Ca^{2+} channels by plasma membrane depolarization. Our data showed that enhanced Ca^{2+} influx in OPCs occurred when high K^+ was introduced into the medium (Fig. 1A; each line represents an analysis of a single cell). High K^+ induced a biphasic increase in OPC $[\text{Ca}^{2+}]_{\text{int}}$. The first phase consisted of a sharp peak characteristic of a transient, large increase in $[\text{Ca}^{2+}]_{\text{int}}$ (Fig. 1A, Peak), which was followed by a second phase of slowly declining internal Ca^{2+} concentrations (Fig. 1A, Plateau phase). Importantly, increases in fura-2 signal in these cells were abolished in the presence of 0 Ca^{2+} and were blocked by Cd^{2+} , verapamil, and nifedipine, confirming that this rise in $[\text{Ca}^{2+}]_{\text{int}}$ resulted from Ca^{2+} influx via L-type VOCCs (Fig. 1B). In support of this, the amplitude of Ca^{2+} uptake was enhanced by Bay K 8644 (1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl)phenyl]-3-pyridinecarboxylic acid, methyl ester), an L-type Ca^{2+} channel agonist that prolongs single-channel open time without affecting the close time (Fig. 1B).

VOCC activity in OPCs is developmentally regulated

Imaging experiments were performed at 1, 2, 3, or 4 d *in vitro* (DIV), allowing analysis of the developmental regulation of voltage-gated Ca^{2+} influx. Immediately after shake-off from mixed glial cultures, OPCs were grown in the presence of mitogens [PDGF (10 ng/ml) and bFGF (10 ng/ml)] for 48 h (1 and 2

Figure 1. *A*, Fura-2 imaging of Ca^{2+} responses to 20 mM K^+ in primary cultures of OPCs. The time of addition of high K^+ -containing external solution is indicated by the horizontal bar. Note that each trace corresponds to a single cell. *B*, K^+ -induced Ca^{2+} uptake was increased in 5 μM Bay K 8644 and abolished in 10 μM Cd^{2+} , 25 μM verapamil, 25 μM nifedipine, and in the absence of external Ca^{2+} ($-\text{Ca}^{2+}$). The graph shows the average amplitude (Peak) calculated from the responding cells, expressed as percentage of change of the emission intensities. Each agonist was applied by a fast and local perfusion system. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 500$ cells for each condition). ** $p < 0.01$, *** $p < 0.001$ versus basal.

DIV) and then induced to exit the cell cycle and differentiate by transferring the cells to a mitogen-free medium (mN2). After shifting to the differentiation medium, there is a sharp decrease in cell division, a decline in early immunocytochemical markers, such as NG2 and A2B5, and an increase in intermediate (e.g., O4) and mature (e.g., O1 and MBP) markers, consistent with differentiation of the OPCs (Paez et al., 2009b). During the first 2 DIV in the presence of growth factors, 75% of the cells were NG2 positive (NG2^+) and 95% of the cells were A2B5 $^+$, 25% of the cells were O4 $^+$ and 8% were O1 $^+$. After switching to a growth factor-free medium (mN2) for 2 d, immunocytochemical staining of the cells demonstrated that $<25\%$ of the cells remained NG2 $^+$, 70% of the cells became O4 $^+$, and 35% of the cells started to express O1. Under these conditions, Ca^{2+} uptake in OPCs after depolarization occurred at 1 and 2 DIV but not at 3 and 4 DIV (Fig. 2H). Similar results were found in OPCs cultured in PDGF alone (Fig. 2I). In the last experiment, the cells were allowed to divide and spontaneously differentiate (i.e., progress through the lineage under the same culture conditions). This experiment demonstrates that high levels of VOCC expression is a property of immature OPCs and does not solely depend on the growth factors used in the culture medium.

Because no culture is perfectly synchronous, we performed Ca^{2+} imaging on individual cells, saved the data, removed the slide, and immunostained for stage-specific markers to determine the phenotype of the cells from which we had just obtained Ca^{2+} entry data. This involved relocating the cells with the computer-driven stage after storing the coordinates of the cells. Such an experiment is shown in Figure 2A–C in which we performed immunocytochemical staining for the NG2 antigen after confocal Ca^{2+} imaging of the field of OPCs that had responded to high K^+ . Examination of the same field after NG2 staining indicated that NG2 $^+$ OPCs responded to depolarization with large increases in intracellular Ca^{2+} (Fig. 2G). In contrast, the more mature O1 $^+$ cell population responded with a significantly smaller increase in the fura-2 signal under the same high K^+ treatment (Fig. 2D–F). Additional experiments measuring $[\text{Ca}^{2+}]_{\text{int}}$ levels after high K^+ depolarization with other differentiation markers (e.g., PDGFR α , O4, and MBP) provided additional evidence for enhanced Ca^{2+} influx in immature cells (PDGFR α^+ , NG2 $^+$, and O4 $^+$), which then declined as the OPCs matured (O1 $^+$ and MBP $^+$)

Figure 2. *A–F*, Immunocytochemical staining for the NG2 (*C*) and O1 antigen (*F*) were used after confocal calcium imaging (*A*, *B* and *D*, *E*) to determine the developmental stage at which OLs responded to high K^+ . Four selected OLs in these microscope fields (*a–d*) responded to high K^+ . Examination of the same field after NG2 (*C*) and O1 (*F*) staining indicated that OLs at NG2⁺ stage responded to high K^+ with large increases in intracellular Ca^{2+} , whereas O1⁺ cells displayed small Ca^{2+} uptake. Intracellular Ca^{2+} in these selected cells are plotted with respect to the time of stimulation in *G*, *H*. Pure OPCs were cultured for 2 d *in vitro* (1 and 2 DIV) in defined culture media plus PDGF (10 ng/ml) and bFGF (10 ng/ml). Then the medium was changed and the cells were cultured in a mitogen-free medium (mN2) for another 2 d (3 and 4 DIV). *I*, Pure OPCs were cultured for 4 DIV in defined culture media plus PDGF (10 ng/ml). The graphs shows the average Ca^{2+} influx amplitude after high K^+ treatment in each experimental condition ($n > 200$ cells for each condition). *J*, Immunocytochemical staining for PDGF α , NG2, O4, O1, and MBP were used after confocal calcium imaging at 2 DIV. The graphs shows the average Ca^{2+} influx amplitude calculated from 50 responding cells for each OL marker, expressed as percentage of change of the emission intensities. Values are expressed as mean $\pm$ SEM of at least four independent experiments. Scale bars: *C*, 20 μ m; *F*, 40 μ m.

(Fig. 2*J*). Together, these data suggest that VOCC influx was greatly reduced with OPC differentiation.

In situ imaging studies reveal voltage-activated Ca^{2+} currents in OPCs

We performed *in situ* experiments in live tissue sections to examine Ca^{2+} influx in GFP-labeled OPCs isolated from transgenic

mice in which the expression of GFP is driven by the proteolipid protein promoter (Mallon et al., 2002). In these mice, GFP expression provides a convenient marker for cells in the oligodendroglial lineage, thus facilitating experiments to identify OPCs and OLs in live tissue slices. We focused our *in situ* measurements of OPC Ca^{2+} influx in slice preparations containing the lateral ventricle subventricular zone (SVZ) and corpus callosum (CC) because these regions have been well studied as sources of OPCs. Our goal in these experiments was to confirm the existence of VOCC activity in OPCs *in situ*. Recordings were made at P4 and P8. OPCs in the SVZ represent precursor and migrating cells, and OPC/OLs in the CC include more mature cells. Slice preparations containing the SVZ and CC were first loaded with the Ca^{2+} indicator dye fura-2, before treating with high K^+ to activate Ca^{2+} entry via VOCCs. Our *in situ* data from the SVZ showed a significant Ca^{2+} influx in OPCs (Fig. 3*A,C*). Importantly, increases in fura-2 signal in these SVZ cells were abolished in the presence of 0 Ca^{2+} and were blocked by verapamil and nifedipine, confirming that this rise in $[Ca^{2+}]_{int}$ results from Ca^{2+} influx via VOCCs (Fig. 3*E*). In agreement with our previous results, Ca^{2+} influx after high K^+ depolarization was significantly greater in immature OPCs from the SVZ versus CC OLs, suggesting that VOCCs may play a role during the early stages of OPC maturation (Fig. 3*B,D,F*).

Influence of PKC/PKA on OPC VOCCs regulation

Because direct phosphorylation by different kinases is one of the most important mechanisms involved in VOCC modulation, we examined the effect of serine-threonine (Ser/Thr) kinases on Ca^{2+} influx mediated by VOCCs in OPCs. Using fura-2 Ca^{2+} imaging and high K^+ depolarization, we tested PKC activation with PMA, a biologically active phorbol ester, and PKC inhibition by chelerythrine. These studies were performed on isolated OPCs in culture. The data indicated that 10 μ M PMA applied 2 min before high K^+ stimulation markedly increased the peak of Ca^{2+} influx evoked by depolarization of OPCs (Fig. 4*B*). In contrast, Ca^{2+} influx induced by high K^+ was markedly reduced when PKC activity was inhibited by 50 μ M chelerythrine (Fig. 4*C*). These results clearly suggest that, in OPCs, PKC activity enhances VOCC function. Because this experiment did not determine the effect of activating PKC after K^+ stimulation, we also examined Ca^{2+} influx by adding PMA 2 min after the K^+ stimulus had begun. As shown in Figure 4*D*, the fura-2 ratio of OPCs responding to 20

mM K^+ was not significantly different compared with control OPCs when PMA was added to the culture medium during K^+ depolarization (Fig. 4*A,E*). These data clearly indicate that PKC modulates VOCC activation without affecting channel inactivation.

The influence of PKA on OPC Ca^{2+} channel function is unknown; several studies in neural cells suggest that PKA acts to depress whole-cell Ca^{2+} currents by affecting a hyperpolarizing shift in the voltage dependence of inactivation for the current. If under basal conditions PKA acts to suppress VOCC function, then an inhibitory influence over PKA would be expected to result in increased VOCC activity. We examined these ideas in the following experiments. First, we examined the effect of H89 (*N*-[2-(*p*-bromo-cinnamylamino)-ethyl]-5-isoquinoline-sulfonamide 2HCl), a specific PKA inhibitor, on Ca^{2+} influx in OPCs. Exposure to high K^+ triggered a significantly larger increase in the $[Ca^{2+}]_{int}$ levels in OPCs pretreated with 25 μ M H89 compared with responses in control cells (Fig. 5*A*). We also examined the effects of PKA activators such as 8-piperidinoadenosine-cAMP (8-PIP-cAMP) and 8-(6-aminohexylamino) adenosine-cAMP (8-AHA-cAMP) on Ca^{2+} responses in OPCs. The results showed that the activation of PKA by either of these compounds significantly decreased the voltage-operated Ca^{2+} entry compared with OPCs that were not exposed to these drugs (Fig. 5*A*). This decrease in Ca^{2+} entry indicates that, under basal conditions, PKA acts to suppress VOCC function in OPCs.

In the experiments described above, Ca^{2+} imaging measurements (high K^+ depolarization) were performed after 2 min of drug exposure. Treating the cells with these PKA modulators before and during depolarization with high K^+ allowed us to analyze the effect of this kinase on both VOCC activation and inactivation. To study the role of PKA in VOCC inactivation, OPCs were treated with 8-PIP-cAMP and 8-AHA-cAMP during the depolarization stimulus. As shown in Figure 5, *A* and *B*, the activation of PKA after K^+ stimulation caused a significant decrease in the $[Ca^{2+}]_{int}$ levels during the peak as well as during the plateau phase, indicating that PKA promotes VOCCs inactivation.

Together, these data suggest that, in primary OPC cultures, Ser/Thr kinases play a significant role in modulating voltage-mediated Ca^{2+} influx in OPCs. The results indicate that PKC and PKA

Figure 3. Brain slices were incubated in a stage top chamber with 5% CO_2 at 37°C. Fura-2 images were obtained for brain slices at 5 s intervals for a total of 15 min. *A, B*, Time-lapse series of P4 GFP-expressing OPCs in the dorsolateral SVZ (*A*) and in the CC (*B*). Each frame represents a single section of a fura-2 time-lapse experiment. An increased fura-2 fluorescence ratio is indicated by warmer colors. Time is denoted in minutes in the top right corner, and the area of the CC and SVZ is indicated in the inset. LV, Lateral ventricle. Scale bars: *A*, 100 μ m; *B*, 50 μ m. *C, D*, VOCC activity was examined in P4 GFP-expressing OPCs from the SVZ area and OLGs from the CC. Note that each trace corresponds to a single cell, and the time of addition of external solution containing high K^+ is indicated by the horizontal bars. *E*, K^+ -induced Ca^{2+} uptake in P4 OPCs from the SVZ was abolished in 25 μ M verapamil, in 25 μ M nifedipine, and in the absence of external Ca^{2+} ($-Ca^{2+}$). The graph shows the average amplitude calculated from the responding cells, expressed as percentage of change of the emission intensities. *F*, VOCC activity was examined in P4 and P8 GFP-expressing OPCs from the SVZ area and OLGs from the CC. The graph shows the average maximal peak values and plateau values during minutes 9–11, calculated from the responding cells, expressed as percentage of change of the emission intensities. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 200$ cells for each condition). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus basal (*E*) and versus SVZ OPCs (*F*).

Figure 4. *A*, Fura-2 imaging of Ca^{2+} response to 20 mM K^{+} in control OPCs (Basal). *B*, *C*, Effect of PMA (10 μM) (*B*) and chelerythrine (50 μM) (*C*) on Ca^{2+} influx induced by high K^{+} in OPCs. As indicated by the horizontal bars, the phorbol ester PMA and the PKC inhibitor chelerythrine were applied 2 min before high K^{+} stimulation. *D*, The PKC activator PMA (10 μM) was applied after K^{+} stimulation. *E*, The graph shows the average maximal peak values and plateau values (minutes 5–7) for each experimental condition, calculated from the responding cells and expressed as percentage of change of the emission intensities. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 200$ cells for each condition). ** $p < 0.01$ versus respective basal levels.

activities influence VOCC-dependent Ca^{2+} influx and that they have opposite effects on the modulation of Ca^{2+} influx evoked by depolarization in OPCs.

The effect of PKC activation on process extension in OPCs is mediated through voltage-operated Ca^{2+} uptake

Several studies in the literature indicate that Ca^{2+} is important in OL and OPC process extension (Pende et al., 1997; Stariha et al., 1997; Yoo et al., 1999). Previous studies in our laboratory indicated that VOCC activation induced OPCs to extend sheets and processes (Paez et al., 2007). This was an important finding not only because it established a link between process extension and intracellular Ca^{2+} levels but also because little attention has been given to the role of VOCCs in OPC or OL function.

Yoo et al. (1999) reported that activation of PKC in OLs caused an increase in process extension as well as an increase in intracellular Ca^{2+} levels. Because increased levels of both PKC activity and intracellular Ca^{2+} lead to process outgrowth in OPCs, we investigated the possibility that PKC was influencing Ca^{2+} levels, leading to process extension by VOCC modulation. OPCs were exposed to varying levels of the PKC activator PMA (0.1, 1, and 5 μM) for 2 d. Process extension was evaluated by determining the percentage of OPCs with processes that had a length equal to or greater than four times the mean cell body diameter of the OPC population. Figure 6*A* shows that there was a positive correlation between the concentration of PMA in the media and the percentage of OPCs with processes longer than four times the cell body diameter. In control media, OPCs with long processes comprised $\sim 30\%$ of the population. As the concentration of PMA was increased, there was a parallel increase in the percentage of these cells. For example, at a PMA concentration of 1 μM , the percentage of OPCs with long processes was $\sim 57\%$ (Fig. 6*A*). Furthermore, the total number of primary and secondary processes was determined in control and PMA-treated OPCs after 48 h. As shown in Figure 6*B*, the total number of primary processes per cell (PP/cell) as well as the number of secondary processes per primary process (SP/PP) was significantly higher compared with control OPCs when PMA was added to the culture medium.

Once the effects of PMA on OPC process formation were determined, we examined the activity of PKC in OPC primary cultures under our experimental conditions. Cytosolic proteins from untreated and PMA-treated OPCs were assayed for PKC activity using a nonradioactive assay (see Materials and Methods). PMA increased PKC activity at least fourfold during the first hour of treatment, whereas the presence of the PKC inhibitor chelerythrine inhibited this activation by $\sim 70\%$ (Fig. 6*C*). The level of inhibition was determined by a comparison of the areas under the activity peaks in Figure 6*C*. These data indicate that PKC activation for 1 h is enough to induce OPC process extension 24–48 h later, suggesting that OPCs do not need continuous PKC activation to sustain process extension.

Calcium-channel blockers were used to test whether voltage-operated Ca^{2+} entry was associated with the morphological transformation of OPCs promoted by PKC activation. OPCs treated with 1 μM PMA were incubated for 48 h in either the presence or absence of the VOCC blockers verapamil and nifedipine, and process elongation was evaluated as described above. In the presence of verapamil and nifedipine, there was strong

inhibition of the elaboration of processes and membrane sheets induced by PMA in OPCs (Fig. 6D). Figure 6E illustrates fluorescent images of OPCs treated with PMA. After 2 d the OPCs, which were generally bipolar in control media, elaborated large processes in the presence of 1 μ M PMA (Fig. 6Eb). This morphological change was significantly reversed in the presence of 25 or 50 μ M verapamil (Fig. 6Ec,Ed). The results of these experiments clearly indicate that VOCCs play a key role in mediating the morphological changes through PKC activation in OPCs.

Modulation of voltage-gated Ca^{2+} influx by tyrosine kinases

To assess the contributions of TK activation on voltage-operated Ca^{2+} uptake, we used genistein, a specific inhibitor of tyrosine protein kinases that acts by binding to the ATP site of the TK, and we also use an inhibitor of tyrosine phosphatases, sodium orthovanadate. Pretreatment of OPC cultures with genistein resulted in a dose-dependent reduction in VOCC Ca^{2+} influx compared with control cells (Fig. 7A,B) (all data not shown). In contrast, OPCs, which were treated with sodium orthovanadate, showed a dose-dependent activation of Ca^{2+} influx across VOCCs (Fig. 7C) (all data not shown). Additionally, orthovanadate increased not only Ca^{2+} influx during high K^{+} stimulation but also caused a sustained increase in $[\text{Ca}^{2+}]_{\text{int}}$ during the plateau phase (Fig. 7C,D). This means that the $[\text{Ca}^{2+}]_{\text{int}}$ remained high after the initial transient influx of Ca^{2+} into the cell. These results clearly suggest that TKs exert an activating influence on VOCC function in OPCs.

PDGF is a potent mitogen that induces early OPCs to proliferate *in vitro* and can prevent premature differentiation *in vivo* (Noble et al., 1988; Raff et al., 1988; Calver et al., 1998). PDGF exerts its effect by interaction with intrinsic TKr and Ca^{2+} signals activated after membrane receptor recruitment. This is one of the most conserved immediate responses triggered in the target cells in which PDGF exerts mitogenic activity. Furthermore, the tyrosine kinase activity of the receptors has been found to be essential for transmission of the mitogenic signal into the cell (Escobedo et al., 1988). Accordingly, we used the PDGF response in OPCs as a model to probe the role of TKr on OPC Ca^{2+} uptake after membrane depolarization. OPCs were incubated with different concentrations of PDGF for 1 h before exposure of the cells to medium containing high K^{+} . Exposure to high K^{+} triggered a significantly larger increase in the fura-2 signal in OPCs pretreated with 20 ng/ml PDGF compared with responses in non-

Figure 5. *A*, Effect of the PKA inhibitor H89 (25 μ M) and the PKA activators 8-PIP and 8-AHA-cAMP (10 μ M) on Ca^{2+} influx induced by high K^{+} in OPCs. The cells were treated with these PKA modulators 2 min before and during depolarization with high K^{+} . The graph shows the average maximal peak values and plateau values during minutes 5–7, calculated from the responding cells, expressed as percentage of change of the emission intensities. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 200$ cells for each condition). * $p < 0.05$, ** $p < 0.01$ versus respective basal levels. *B*, Fura-2 imaging of Ca^{2+} response to 20 mM K^{+} in selected OPCs from control (Basal) and treated cultures with the PKA modulators (8-PIP-cAMP and 8-AHA-cAMP (10 μ M) during high K^{+} depolarization. The time of addition of high K^{+} -containing external solution and the PKA activators is indicated by the horizontal bars.

Figure 6. *A, D*, The percentage of OPCs with processes greater or equal to four times the cell body diameter is indicated as a function of the concentration of PMA (*A*) or verapamil and nifedipine (*D*) used in the incubation media. *B*, Quantitative morphometric analyses of primary (PP) and secondary (SP) process outgrowth in control and PMA-treated OPCs after 2 DIV. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 50$ cells for each condition). * $p < 0.05$, ** $p < 0.01$ versus control cells (Basal). *C*, PKC activity was examined in primary cultures of OPCs. The graph shows the average PKC activity during 24 h under different experimental conditions. PKC activity was detected as described in Materials and Methods, and values are expressed as mean $\pm$ SEM of at least three independent experiments. *E*, Fluorescent images of OPCs immunostained with the O4 antibody after 2 DIV (*a*) and cells treated with PMA (1 μ M) for 2 DIV appeared broader and had elaborated extensive processes (*b*). However, the presence of verapamil (25 or 50 μ M) in the culture media completely inhibited the PMA effect on OPC morphology (*c, d*). Scale bar, 25 μ m.

treated cells (Fig. 8A). As the concentration of PDGF was increased, there was a parallel increase in the Ca^{2+} influx across VOCCs. For example, at a PDGF concentration of 100 ng/ml, the average amplitude of the fura-2 signal was $\sim 45\%$ higher compared with nontreated cells (Fig. 8A). Additionally, in as little as 20 ng/ml PDGF, OPCs incubated with the selective PDGF

Figure 7. *A*, Fura-2 imaging of Ca^{2+} response to 20 mM K^{+} in control OPCs (Basal). *B*, *C*, Effect of genistein (10 μM) (*B*) and the tyrosine phosphatase inhibitor orthovanadate (5 μM) (*C*) on Ca^{2+} influx induced by high K^{+} in OPCs. The time of addition of high K^{+} -containing external solution and the TK modulators is indicated by the horizontal bars. *D*, The graph shows the average maximal peak values and plateau values during minutes 5–7, calculated from the responding cells, expressed as percentage of change of the emission intensities. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 200$ cells for each condition). * $p < 0.05$, ** $p < 0.01$ versus respective basal levels.

TKr inhibitor AG-1296 (6,7-dimethoxy-2-phenylquinoxaline) showed an $\sim 50\%$ decrease in the depolarization-induced Ca^{2+} entry (Fig. 8*B*). The peak $[\text{Ca}^{2+}]_{\text{int}}$ mobilization decreased to 125 ± 5.2 nM in OPCs treated with 1 μM AG-1296 versus 221 ± 3.2 nM in buffer-treated cells ($p \leq 0.01$) (Fig. 8*B,C*). Importantly, differences in Ca^{2+} entry between OPCs treated with 20 and 40 ng/ml PDGF were blocked by genistein and were abolished in the presence of AG-1296 (Fig. 8*C*), confirming that TKs are directly involved in PDGF-mediated modulation of Ca^{2+} influx via VOCCs. These results are consistent with the notion that TKr, such as PDGFr, enhance Ca^{2+} influx induced by depolarization in OPCs.

PDGF promotes OPC migration through TK-modulation of VOCCs

Although the effect of PDGF on OPC migration has been well established (Milner et al., 1997; McKinnon et al., 2005; Frost et al., 2009), little has been known about its mechanism. We recently found increased Ca^{2+} uptake via VOCCs associated with enhanced OPC motility, and this effect was accompanied by increases in the amplitude of spontaneous somatic Ca^{2+} transients (Paez et al., 2009c).

For this reason, we examined the role played by VOCCs on PDGF-induced OPC migration by performing cell migration experiments in the presence of PDGF and pharmacological agents to stimulate or inhibit voltage-gated Ca^{2+} influx. First, we examined the effect of PDGF on OPC migration by means of time-lapse video microscopy performed over a period of 24 h in medium con-

taining different concentrations of PDGF. In this time-lapse cell migration assay, cell movement was assessed by calculating the average cell migration velocity and the total distance traveled by the cell. For this analysis, only OPCs moving $> 50 \mu\text{m}$ in 6 h were scored (Fig. 9*A*). Tracking of cells was performed using the SlideBook 4.1 data analysis program as described previously (Paez et al., 2009c). Under basal experimental conditions (10 ng/ml PDGF), the mean rate of OPC migration was $33 \pm 2.2 \mu\text{m/h}$ (Fig. 9*B*). At higher PDGF concentrations (80 ng/ml), the average OPC velocity was $\sim 70\%$ higher than in basal conditions (Fig. 9*B*). We found a corresponding increase in the total OPC migration distance after 8 h (Fig. 9*C*). Second, we assessed the effect of specific VOCC blockers such as nifedipine and verapamil on PDGF-dependent modulation of OPC migration. These treatments resulted in a significant slowdown of OPC movement (Fig. 10*A*), indicating that VOCCs, known to contribute to homeostatic Ca^{2+} balance in OPCs and other cells, are important components in the mechanism of PDGF on OPC migration. Furthermore, stimulation of Ca^{2+} influx through VOCCs (through high K^{+} treatment) significantly increased the PDGF effect on cell movement (Fig. 10*A*), as did treatment of the cells with Bay K 8644, an L-type VOCC agonist (Fig. 10*A*). These data show that changes in $[\text{Ca}^{2+}]_{\text{int}}$ resulting from the modulation of voltage-gated Ca^{2+} influx provide a powerful means by which OPC migration may be regulated by PDGF. Additionally, they are the first to demonstrate that extracellular Ca^{2+} influx through VOCCs is an important component in the mechanism of PDGF on OPC motility.

Because the PDGFr is directly involved, through its tyrosine kinase activity, in modulating Ca^{2+} influx via VOCCs, we investigated the role of tyrosine protein kinases in PDGF modulation of OPC migration. We tracked OPCs in medium containing 40 ng/ml PDGF and the general TK antagonist genistein. Figure 10*B* shows that the average speed of OPC migration was lower when genistein was present in the media. For example, in 40 ng/ml PDGF, the average migration speed of OPCs was $52 \pm 5.1 \mu\text{m/h}$ ($n = 20$), but as the concentration of genistein was increased, it fell to an average speed of $14 \pm 3.8 \mu\text{m/h}$ ($n = 20$) in the presence of 10 μM genistein (Fig. 10*B*). Furthermore, PDGF modulation of OPC velocity essentially disappeared when the selective PDGF receptor TK inhibitor AG-1296 was added to the external medium (Fig. 10*B*). In contrast, PDGF-induced migration was unaffected by PD173074 (1-tert-butyl-3-[2-[4-(diethylamino)butylamino]-6-(3,5-dimethoxyphenyl)pyrido[2,3-d]pyrimidin-7-yl]urea), a potent and selective inhibitor of bFGF TKr signaling in OPCs (Bansal et al., 2003), and was increased by the tyrosine phosphatase inhibitor sodium orthovanadate (Fig. 10*B*). These results indicate that the tyrosine kinase activity of the PDGFr is a key component of the mechanism of action of PDGF on OPC movement.

Figure 8. *A*, Effect of different concentrations of PDGF on Ca^{2+} influx induced by high K^{+} in OPCs. The graph shows the average maximal peak values and plateau values in the presence of different PDGF concentrations. *B*, Fura-2 imaging of Ca^{2+} response to 20 mM K^{+} in selected OPCs from control and treated cultures with the TKR inhibitor AG-1296 (1 μM) applied 2 min before high K^{+} . *C*, OPCs were treated with AG-1296 (1 μM) or genistein (10 μM) in the presence of 20 or 40 ng/ml PDGF. The graph shows the amplitude of Ca^{2+} uptake (peak) calculated from the responding cells. Values are expressed as mean $\pm$ SEM of at least four independent experiments ($n > 200$ cells for each condition). ** $p < 0.01$, *** $p < 0.001$ versus respective control levels.

Discussion

Oligodendrocytes exhibit increased Ca^{2+} influx after membrane depolarization

Voltage-activated Ca^{2+} currents have been examined in OPC culture preparations from a range of different tissues, providing somewhat differing results. Perinatal progenitor cells taken from rat optic nerve were found to lack Ca^{2+} currents (Barres et al., 1990), although their presence was detected in progenitor cells obtained from adult optic nerve (Borges et al., 1995). Perinatal OPCs from the mouse cortex (Verkhatsky et al., 1990; Blankenfeld et al., 1992) exhibit both low-voltage-activated (LVA) and high-voltage-activated (HVA) Ca^{2+} currents, although the expression of these currents varies from cell to cell (Williamson et al., 1997). Oligodendrocyte Ca^{2+} currents have also been examined *in situ*; recordings from early postnatal slices containing the corpus callosum revealed the presence of both HVA and LVA Ca^{2+} currents in OLs located in this white matter region (Berger et al., 1992). Supporting this finding is the evidence presented here from primary OPC cultures and tissue slices depolarized with high K^{+} . Depolarization consistently leads to plasma membrane Ca^{2+} entry, which is blocked in the presence of VOCC antagonists. Thus, the available evidence indicates the presence of VOCCs in OPCs and immature OLs and that a considerable component of this current is carried by L-type channels.

VOCC influx disappeared with OPC differentiation

Several previous studies have reported that Ca^{2+} responses through VOCCs appear to diminish with maturation of OLs from progenitors to mature cells in culture (Berger et al., 1992;

Figure 9. Cultured OPCs were incubated in a stage top chamber with 5% CO_2 at 37°C, which was placed on the stage of a spinning disc confocal inverted microscope. *A*, Bright-field images were acquired at 6 min intervals for a total of 24 h. Each frame represents a single section of a time-lapse video sequence. Time is denoted in hours in the bottom left corner. Arrows indicate the direction of migration, and therefore the length of the arrow represents the relative velocity of the cell at that moment. Cell migration speed and distances were analyzed offline by tracing individual cells at different times, after which migratory values were statistically analyzed. *B*, OPC average migration speed was calculated from at least 50 cells in each experimental condition. *C*, Total migration distance was followed for 8 h in 150 cells from each experimental condition. Values are expressed as mean $\pm$ SEM of at least four independent experiments. * $p < 0.05$, ** $p < 0.01$ versus control cells (PDGF 10 ng/ml). Scale bar, 30 μm .

Blankenfeld et al., 1992; Takeda et al., 1995). At the same time, other studies have described no difference in the functional expression of VOCCs in immature and mature cultured OLs (Blankenfeld et al., 1992; Paez et al., 2009a). It is possible that voltage-operated Ca^{2+} influx plays a role during the first steps of OL

Figure 10. Average migration speed obtained from cultured OPCs bathed in basal medium (PDGF at 10 ng/ml) or in the presence of 40 ng/ml PDGF. **A**, The effect of VOCC modulators (verapamil, nifedipine, K⁺, and Bay K 8644) on OPC migration is shown. **B**, The role of TKs and TKR on OPC mobility was evaluated using genistein, AG-1296, PD173074, and sodium orthovanadate. OPC average migration speed was calculated from at least 50 cells in each experimental condition. Values are expressed as mean ± SEM of at least four independent experiments. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus control cells (PDGF at 10 ng/ml).

maturation (e.g., migration and proliferation) because expression of VOCCs decreases during development. However, OL cultures are mixtures of cells at different stages of maturation. The previous studies were hampered by an inability to positively identify the phenotypes of individual cultured cells from which the Ca²⁺ influx measurements were made. In the present study, we examined Ca²⁺ influx in individual OPCs. After acquisition of the Ca²⁺ imaging data, the cells were immunocytochemically stained for stage-specific markers. These experiments clearly revealed that developmentally regulated activity of VOCCs, e.g., mature cells expressing MBP and O1, showed a small increase in [Ca²⁺]_{int} after high K⁺ stimulation under culture conditions. *In situ*, Ca²⁺ influx after K⁺ stimulation was significantly greater in immature OPCs from the SVZ compared with more mature OLs found in the CC. Thus, the *in vitro* and *in situ* data indicate that voltage-operated Ca²⁺ influx, present in immature OPCs, disappeared as the cells matured, indicating that VOCCs play a role during the early stages of OPCs maturation.

Regulation of VOCCs by serine–threonine kinases

In this study, the effect of several kinases and phosphatases on depolarization-induced VOCC activity was investigated in OPCs. PKA modulation is a physiologically important mechanism influencing VOCC function in diverse excitable tissues such as heart and muscle. PKA modulates Ca²⁺ influx through phosphorylation of a serine residue on the C terminus distal to the

calmodulin interaction sites of VOCC α1 subunit (Catterall, 2000). The influence of PKA on OPC Ca²⁺ channel function is unknown, but several studies in neuronal cells suggest that PKA acts to depress whole-cell Ca²⁺ currents, causing a hyperpolarizing shift in the voltage dependence of inactivation for the current (Kamp and Hell, 2000; Keef et al., 2001; Johnson et al., 2005). In our experiments, PKA activation promoted two major changes in OPC Ca²⁺ currents: a drastic reduction in the Ca²⁺ current amplitude (peak) and the acceleration of the inactivation kinetics (i.e., significant decrease in the [Ca²⁺]_{int} during the plateau phase). These results clearly indicate that PKA exerts an inhibitory influence on VOCC function in OPCs.

PKC is a multigene family of 10 phospholipid-dependent, serine–threonine kinases central to many signal transduction pathways (Nishizuka, 1992). Several PKCs have been shown to regulate voltage-gated ion channels through the direct phosphorylation of the α1 subunit (Zhu and Ikeda, 1994; Stea et al., 1995). More recently, the formation of a functional PKC–VOCC complex that is critical for rapid and efficient stimulation of Ca²⁺ channel activity by PKC has been described in neurons (Chen et al., 2006). In our studies reported here, we used the PKC activator PMA to demonstrate that PKC has an excitatory influence on VOCC function in OPCs participating in the depolarization-induced activation of the channels. These results were confirmed using the selective PKC inhibitor chelerythrine, which produced a clear decrease in depolarization-induced Ca²⁺ influx in OPCs. Thus, our hypothesis is that PKC and VOCC are part of a signaling cascade implicated in OPC development, of which process remodeling is only one regulated property.

Whereas PKC activation in OPCs leads to a decrease in the number of cells acquiring a mature phenotype (Bhat et al., 1992; Avossa and Pfeiffer, 1993; Radhakrishna and Almazan, 1994; Baron et al., 1998; Heinrich et al., 1999), PKC activation in immature OLs has been reported to enhance elaborate process extensions (Althaus et al., 1991; Yong et al., 1988; 1991). Yoo et al. (1999) have shown that activation of PKC in OL not only causes an increase in process extension but also an increase in intracellular Ca²⁺ levels, suggesting that the PKC pathway for induction of processes is at least partially dependent on increases in [Ca²⁺]_{int} (Yoo et al., 1999).

We showed that the activation of VOCCs in OPC cultures can induce process extension (Paez et al., 2007). The data presented here show that process growth after PKC activation in OPCs is influenced strongly by L-type Ca²⁺ channels because it was significantly reduced by the L-type channel blockers nifedipine and verapamil. These experiments served to define specific channels within the VOCC family that are involved in the morphological remodeling induced by PKC in OPCs. In this regard, astrocyte morphological changes induced by PKC are accompanied by the upregulation and activation of voltage-gated Ca²⁺ channels and are abrogated in the presence of L-type channel blockers (Burgos et al., 2007). Thus, PKC can regulate Ca²⁺ influx in OPCs under depolarizing conditions, and the evidence supports the involvement of VOCCs in OPC process remodeling induced by PKC. This represents a novel regulatory pathway involving VOCC that participates in PKC-dependent oligodendrocyte morphological differentiation.

Role of tyrosine kinases on OPC voltage Ca²⁺ uptake

Although an active and direct participation of TKs in VOCC function in different tissues has been demonstrated (Wang and Lipsius, 1998; Keef et al., 2001; Schröder et al., 2004), nothing is known about VOCC regulation through tyrosine phosphoryla-

tion in OPCs. Using pharmacological approaches, we found that TKs exert an excitatory influence on VOCC function in OPCs. Inhibition of TKs with genistein resulted in a dose-dependent reduction in VOCCs Ca^{2+} influx, whereas inhibition of tyrosine phosphatases (with sodium orthovanadate) showed a dose-dependent activation of Ca^{2+} influx across VOCCs. These data are consistent with those of Cataldi et al. (1996) and Vela et al. (2007) who showed that TK inhibition reduced L-type Ca^{2+} channel activity evoked by high K^{+} in GH3 cells.

The effect of TKr activity on OPC voltage-operated Ca^{2+} influx was examined using PDGF as a model TKr system active in OPCs. Our present findings show that PDGF produces an increase in the activity of VOCC channels in OPCs, suggesting a novel and relevant physiological role of PDGF in the control of the OL lineage progression.

PDGF binds to the PDGF α receptor, which belongs to a family of TKrs that has its own cytoplasmic associated TK activity (Taniguchi, 1995). PDGF promotes OPC proliferation, migration, and survival via PDGFR α , the only PDGF receptor isoform in these cells detected by ligand binding (Pringle et al., 1989) and molecular expression (McKinnon et al., 1990). The TK activity of this receptor has been found to be essential for the transmission of the mitogenic and chemotactic signaling in the cell (Escobedo et al., 1988). Our results are consistent with the notion that TKr, such as PDGFR, enhance Ca^{2+} influx induced by depolarization in OPCs. The PDGF effect on OPC Ca^{2+} entry was abolished in the presence of AG-1296, a selective PDGF receptor TK inhibitor, confirming that the TK activity of PDGFR is essential for VOCC modulation. Furthermore, we have shown that extracellular Ca^{2+} uptake through VOCCs, resulting from PDGFR modulation, is an important component in the mechanism of OPC migration. In this paper, we propose that VOCC modulation by PDGFR is a key component of the migratory mechanism activated by PDGF in OPCs. Our conclusion is supported by experiments in which PDGF promotion of cell migration was efficiently obliterated by specific VOCC inhibitors as well as TKr antagonists.

In summary, the present work demonstrates that PKC and TKr enhance Ca^{2+} influx induced by depolarization in OPCs, whereas PKA has an inhibitory effect. These kinases modulate voltage-operated Ca^{2+} uptake in OPCs and therefore participate in the regulation of essential early OPC developmental functions such as processes extension and migration.

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