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Subcellular Heterogeneity of Voltage-Gated Ca^{2+} Channels in Cells of the Oligodendrocyte Lineage

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KEY WORDS Calcium channels, Oligodendrocytes, Cortex, Development, Confocal microscopy, Fluo-3 fluorescence

ABSTRACT We studied the distribution of voltage-gated Ca^{2+} channels in cells of the oligodendrocyte lineage from retinal and cortical cultures. Influx of Ca^{2+} via voltage-gated channels was activated by membrane depolarization with elevated extracellular K^+ concentration ($[K^+]_e$) and local, subcellular increases in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{in}$) could be monitored with a fluometric system connected to a laser scanning confocal microscope. In glial precursor cells from both retina and cortex, small depolarizations (with 10 or 20 mM K^+) activated Ca^{2+} transients in processes indicating the presence of low-voltage-activated Ca^{2+} channels. Larger depolarizations (with 50 mM K^+) additionally activated high-voltage-activated Ca^{2+} channels in the soma. An uneven distribution of Ca^{2+} channels was also observed in the mature oligodendrocytes; Ca^{2+} transients in processes were considerably larger. Recovery of Ca^{2+} levels after the voltage-induced influx was achieved by the activity of the plasmalemmal Ca^{2+} pump, while mitochondria played a minor role to restore Ca^{2+} levels after an influx through voltage-operated channels. During the development of white matter tracts, cells of the oligodendrocyte lineage contact axons to form myelin. Neuronal activity is accompanied by increases in $[K^+]_e$; this may lead to Ca^{2+} changes in the processes and the Ca^{2+} increase might be a signal for the glial precursor cell to start myelin formation.

INTRODUCTION

Voltage-activated Ca^{2+} channels play a key role for Ca^{2+} signalling in many eucariotic cells. These channels are activated by membrane depolarization, e.g., induced by neurotransmitter receptor activation, leading to a transmembrane Ca^{2+} influx and a resulting increase in cytoplasmic free calcium concentration ($[Ca^{2+}]_{in}$). The increase of $[Ca^{2+}]_{in}$, in turn, plays an unique role in the regulation of various cellular functions, such as cell excitability, secretion, synaptic plasticity, activity of cytoplasmic enzymes and gene expression (Smith and Augustine, 1988; Marty, 1989; McCormack et al., 1990; Szekely et al., 1990; Kasai, 1993). The biophysical and pharmacological properties and the functional importance of Ca^{2+} channels were thoroughly studied in a number of preparations, including muscle, secretory cells, and neurons (Bean, 1989; Kostyuk, 1989; Hess, 1990). In the central nervous system, voltage-gated channels are not restricted to neurons, but are also expressed by the different types of glial cells. Ca^{2+} channels have been described for glial precursor cells, oligodendrocytes and astrocytes (MacVicar, 1984; MacVicar and Tse, 1988; Barres et al., 1988, 1989; Verkhratsky et al., 1990; Blankenfeld et al., 1992). In a cell culture system from the cortex of mice, cells from the oligodendrocyte lineage could be enriched

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at various developmental stages. Combining physiological approaches with immunocytochemical cell identification using stage-specific antibodies, at least four stages of the oligodendrocyte development were distinguished (Sontheimer et al., 1989). Ca^{2+} channels were present in early glial precursor cells, were not detected in late precursors and immature oligodendrocytes, but could be activated in more mature oligodendrocytes. Moreover, in both, early precursors and late oligodendrocytes, two types of voltage-gated Ca^{2+} channels were distinguished, characterized as low- and high-voltage-activated Ca^{2+} channels (Verkhratsky et al., 1990; Blankenfeld et al., 1992). The presence of Ca^{2+} channels was subsequently also demonstrated for glial precursor cells in situ, namely, in a thin slice preparation of the corpus callosum (Berger et al., 1992). Since glial cells do not generate action potentials as an activating event for the voltage-gated Ca^{2+} channels, other depolarizing events were searched for to find a natural stimulus which might activate these channels in brain tissue. Indeed, activation of GABA receptors leads to a depolarizing event sufficient to activate the voltage-gated Ca^{2+} channels and thus triggers an increase in $[Ca^{2+}]_{in}$ (Kirchhoff and Kettenmann, 1992). This Ca^{2+} signal may play a functional role during development, as it has been speculated for immature neurons where a similar response was described (Yuste, R. and Katz, L.C., 1991). GABA mediated Ca^{2+} signalling is operative in glial precursor cells, but not in oligodendrocytes since in the latter cells GABA does not trigger a depolarizing event sufficient to activate the voltage gated Ca^{2+} channels (Gilbert et al., 1989). During the development of white matter tracts, glial precursors of the oligodendrocyte lineage form the first interaction with neuronal elements by contacting primarily large diameter axons with their processes (Sturrock, 1980). We assume that Ca^{2+} might play a role in such cell-to-cell interaction and addressed the question whether Ca^{2+} channels might be concentrated at the process tips or at other specific cellular sites. With the use of microfluorometric methods employing the high spatial resolution of a confocal microscope, we were able to resolve subcellular regions with a high susceptibility for Ca^{2+} entry.

METHODS

Retinal Culture

Cultures were obtained from retinae of 14- to 18-day-old New Zealand white rabbits. Rabbits were killed by an overdose of barbiturate (thiopental), enucleated, and the medullary ray area of the neural retina was isolated as described in Scherer and Schnitzer (1989). This retinal tissue was incubated in a 0.3% trypsine solution for 30 min at 37°C, and after addition of DNase mechanically dissociated with a fire-polished Pasteur pipette. After spinning and resuspending, this cell suspension was plated onto poly-L-lysine coated glass coverslips in 24 well tissue culture plates. Culture medium was a 1:1 mixture of DMEM and Hams F12 according to Gard and Pfeiffer (1989) with minor modifications. 24 h after plating coverslips were flipped over, so that cells were upside down facing the bottom of the culture plate. This resulted in a better cell survival and differentiation. Cultures were kept for up to two weeks, either adding some fresh medium after 1 week or maintaining cells without a change of medium.

Cortical Culture

Cultures were obtained from brains of 14- to 16-day-old embryos of NMRI mice as described by Trotter et al. (1989). After 3–5 days in vitro neurones were eliminated by complement-dependent immunocytolysis using the monoclonal antibody M5, directed against surfaces of central nervous system neurons, and guinea-pig complement (Keilhauer et al., 1985; Trotter et al., 1989). After 1 week an astrocytic cell layer was formed with oligodendrocytes and precursor cells growing on the top. By shaking these cultures of mixed glial cells by hand for a few seconds, oligodendrocytes and precursor cells could be removed from the bed-layer of astrocytes. These cells were replated on coverslips and gave rise to cultures highly enriched in oligodendrocytes and precursor cells. They were plated and maintained in Sato medium (as described in Trotter et al., 1989) containing 10% horse serum for 1 to 14 days. Such cultures contain over 95% oligodendrocytes and putative precursor cells at the initial plating and less than 5% other cell types such as glial fibrillary acidic protein-positive astrocytes.

Antibody Staining

For identifying cell types, cultured cells were stained with O4 and O10 antibodies (Sommer and Schachner, 1981, 1982), prior to recording. Briefly, cells were washed in Hanks balanced salt solution, and incubated for 20 min in either O4, or O10 antibody, respectively, followed by two washes. The binding of monoclonal antibodies was visualized by immunofluorescence using the goat anti mouse-tetramethyl-rhodamine-isothiocyanate (TRITC)-antibody (1:100 Cappel, Durham, NC).

Fluo-3 Measurements

$[Ca^{2+}]_{in}$ transients were measured with the Ca^{2+}-sensitive fluorescent dye fluo-3 (Minta et al., 1989). Cultured oligodendrocytes were loaded with dye by incubation of glass coverslips with adhered cells in normal physiological solution supplemented with 5 μM fluo-3/AM (diluted in DMSO) and 0.02% pluronic-127 detergent for 30 min at 22°C. At the end of this incubation period cells were washed twice in normal solution and then incubated in the same solution for additional 30 min to ensure that the dye was converted into the membrane impermeable form. For measuring the $[Ca^{2+}]_{in}$

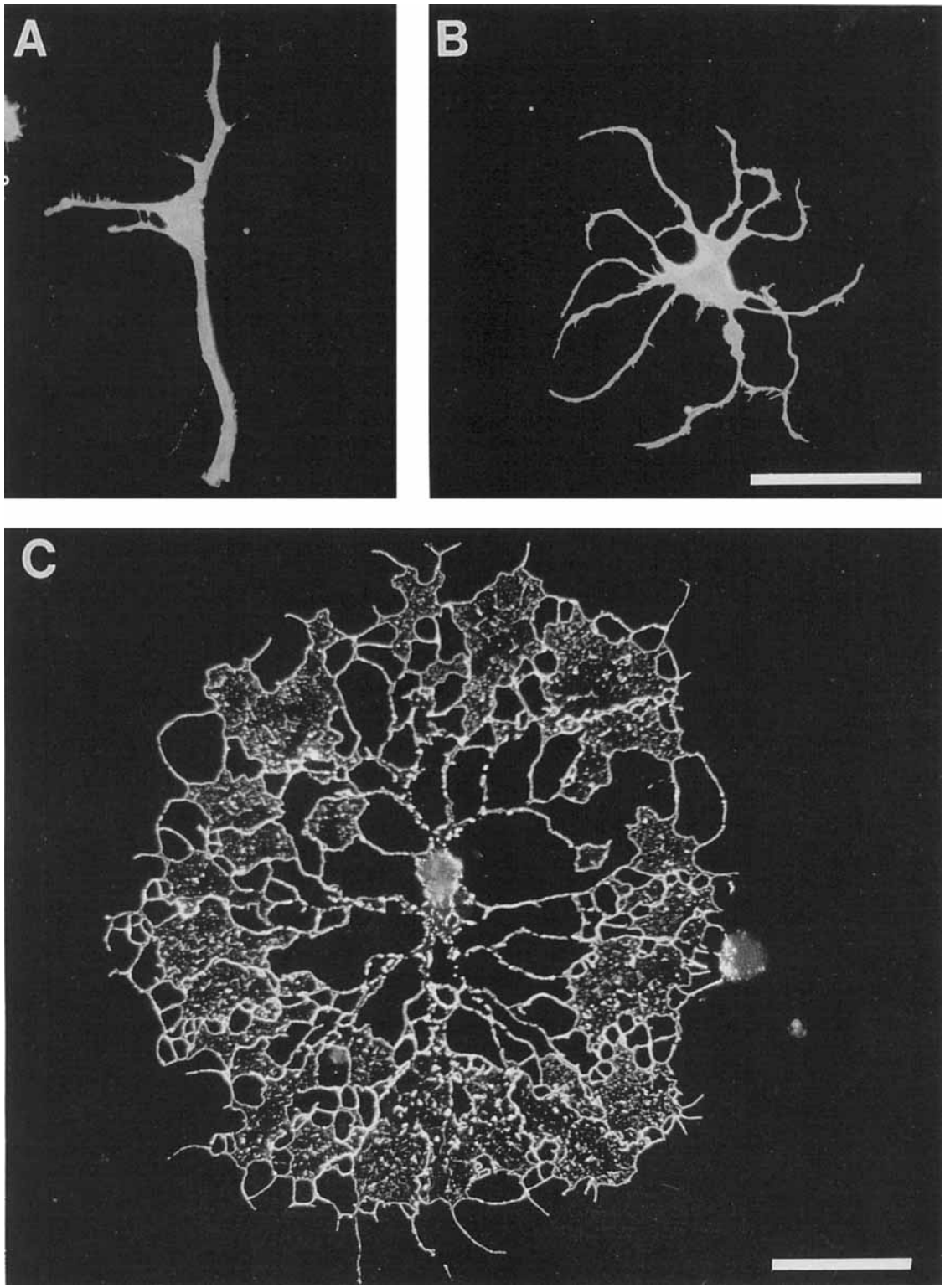


Fig. 1. Immunocytochemical characterization of precursor cells and oligodendrocytes. The photomicrographs show strainings of cells of the oligodendrocyte lineage with antibodies against O4 (A,B) and O10 (C). Cells were cultured for 2 (A), 3 (B), and 7 (C) days. The bar in B corresponds to A and B; bars denote 50 μm. The cell in A has the morphology of a typical simple precursor cell, in B of a more complex precursor cell/immature oligodendrocyte, and in C of a more mature oligodendrocyte.

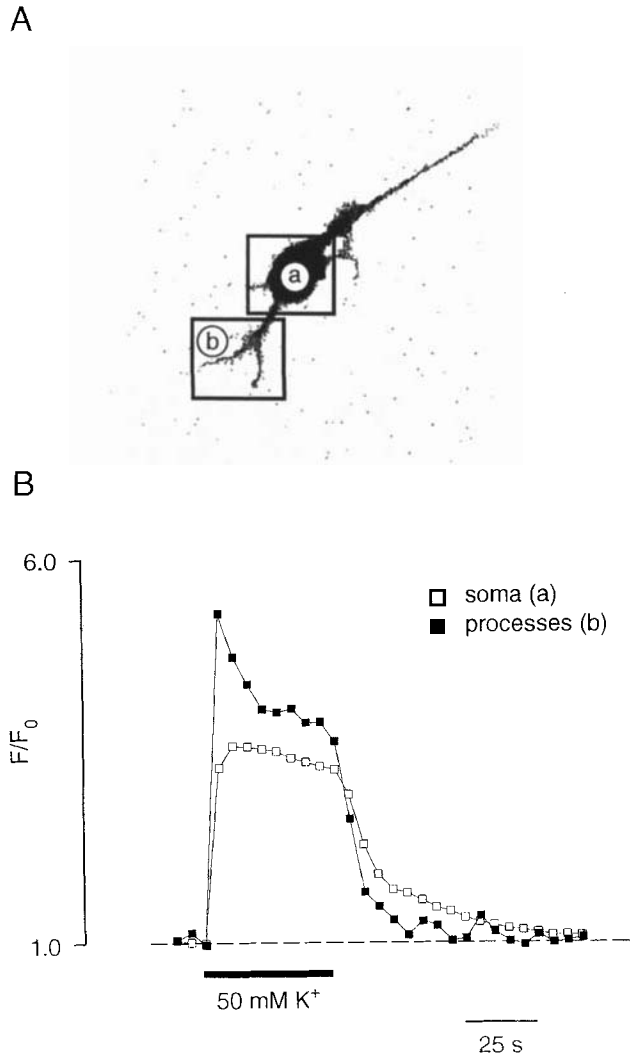


Fig. 2. $[Ca^{2+}]_{in}$ transients elicited by high $[K^+]_e$. An O4-positive cell was selected prior to recording and loaded with fluo-3. In A, the morphological features of the cell are shown in the fluo-3 fluorescence. Two areas were selected demarked as boxes, at the soma (a) and at a process (b). The measurements of a change in the fluo-3 fluorescence signal (F/F_0) in B are taken from the two demarked areas. $[K^+]_e$ was increased from the normal level (5.4 mM) to 50 mM as indicated by bar.

transients we have used the confocal laser scanning microscope Sarastro 2000 (Molecular Dynamics, Sunnyvale, CA). The scanner was mounted on the upright microscope (Axioscope from Zeiss, Germany) equipped with 40× water immersion objective; numerical aperture 0.75; the thickness of the focal plane was estimated to be in a range of 0.5 μm. An argon laser with a wavelength of 488 nm was used for optical excitation. Fluorescence was measured at an emission wavelength of 530 nm selected with an appropriate band pass filter. The power of the laser was set between 9 and 10 mW (the actual energy applied to the cell was less than 1 mW) to minimize fluo-3 bleaching. The images were acquired every 5 s with a resolution of 256 × 256 pixels in the image scan mode of the confocal system. Prior to an experiment for a given cell, the optimal position for the focal plane was determined by evaluating sections at different focal planes. For measuring $[Ca^{2+}]_{in}$ changes with a high time resolution the line scan mode of the confocal system was used. In this configuration a single line was repetitively scanned at a fixed position with a frequency of 100 Hz. Since fluo-3 is not a ratiometric dye, all data are presented as normalized fluorescence intensity and were not calibrated as absolute $[Ca^{2+}]_{in}$ values. To obtain the fluorescence intensity ratio, the resting fluorescence value was determined at the beginning of each experiment. This resting fluorescence was the result of averaging ten images in the image scan mode or 100 lines in the line-scan mode. All subsequently determined images were divided by these resting values.

Dye-loaded cells were mounted on the stage of the microscope, and the recording chamber was continuously superfused with a physiological salt solution at a rate 5 ml/min. Substances were applied by changing the perfusate. All experiments were performed at 20–22°C.

Data Acquisition and Analysis

Data acquisition and image analysis were performed using the software provided by Molecular Dynamics for the Silicon Graphics workstation. To analyze the fluorescent signal, programs for IBM-compatible computer systems were developed (S.K.) and data were transfered from the Silicon Graphics work station. All data are given as mean ± s.e.m.

Solutions and Reagents

All solutions were freshly prepared from refrigerated stock solutions. The bathing salt solution was composed of (in mM): NaCl, 150; KCl, 5.4; $CaCl_2$, 2.5; $MgCl_2$, 1.1; HEPES/NaOH, 10; glucose, 10; pH, 7.4. In the calcium free solution, $CaCl_2$ was omitted, $MgCl_2$ was increased to 2 mM and 0.5 mM EGTA was added, yielding an estimated Ca^{2+} concentration of about 30 nM. The elevated K^+ solution was equivalent to normal bathing solution except that part of Na^+ was replaced by K^+. Fluo-3/AM was obtained from Molecular Probes (Eugene, OR) BAY K 8644 was from RBI (Natick, MA), and all other chemicals were from Sigma Chemical Co. (Taufkirchen, Germany).

RESULTS

Depolarization of Glial Precursors Leads to an Increase in $[Ca^{2+}]_{in}$ With Subcellular Heterogeneity

For the majority of experiments, we studied glial precursor cells in the retinal cultures. By staining the culture with antibodies against O4 prior to the Ca^{2+}

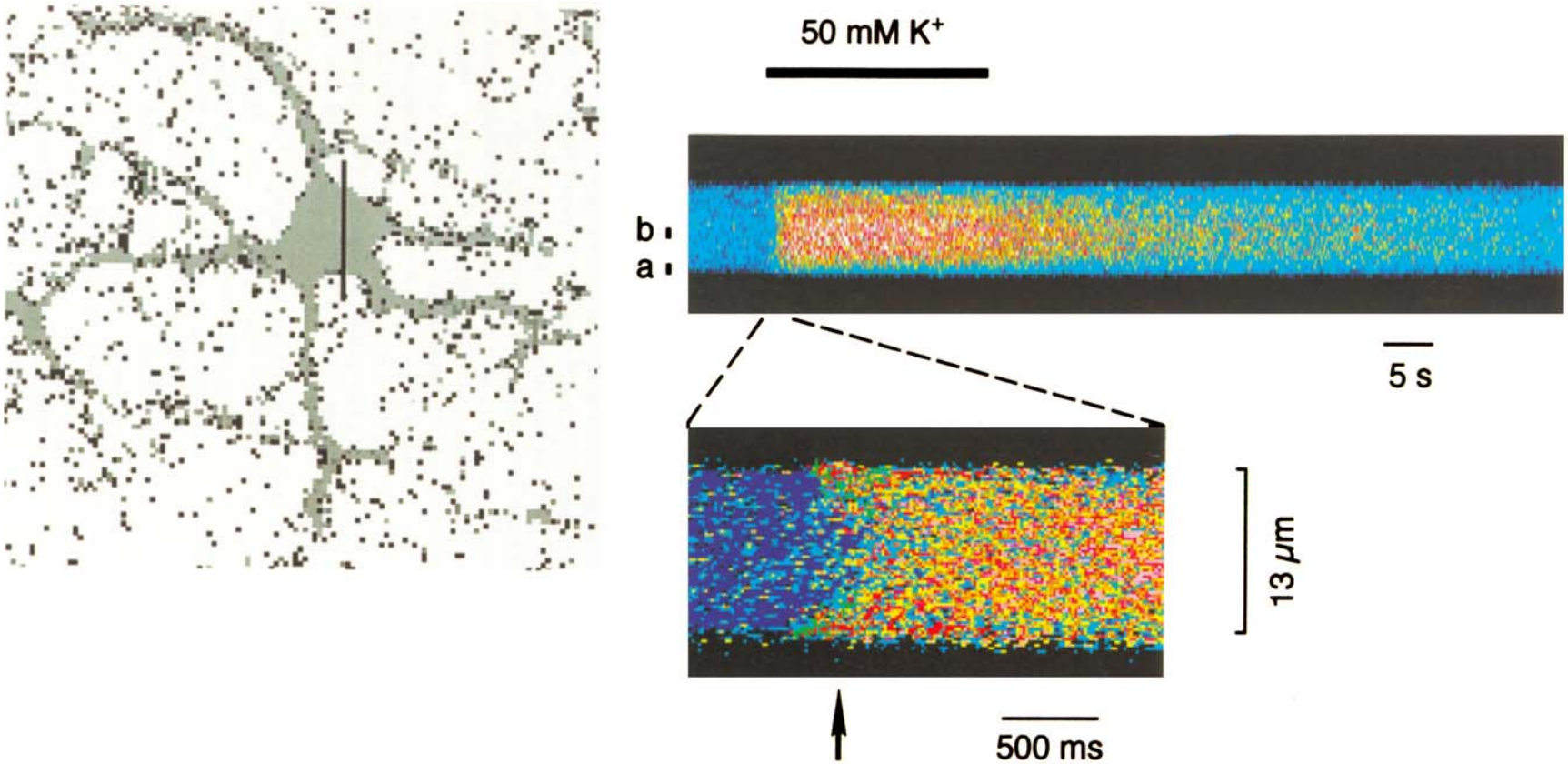


Fig. 3. Fluorescence changes recorded with high time resolution using the line scan mode of the confocal system. An O4-positive cell was selected prior to recording and loaded with fluo-3. On the left, the morphological features of the cell are displayed in the fluo-3 fluorescence. A scanning line was selected along a single focal plane (see vertical line). The colour picture on the top right shows the sequence of scans along the selected line. The two sectors on the line, marked as (a) and (b), correspond to the submembrane region and to the center of the cell soma, respectively. Sampling frequency was 100 Hz. The fluorescence intensity is colour coded; low levels of $[Ca^{2+}]_{in}$ correspond to blue, while the increased levels in $[Ca^{2+}]_{in}$ are coded by red and white. $[K^+]_e$ was increased from the normal level (5.4 mM) to 50 mM as indicated by bar. Below, the onset of the K^+ increase is shown in an expanded time scale. The arrow indicates the line taken for constructing the fluorescence profile shown in Figure 4B.

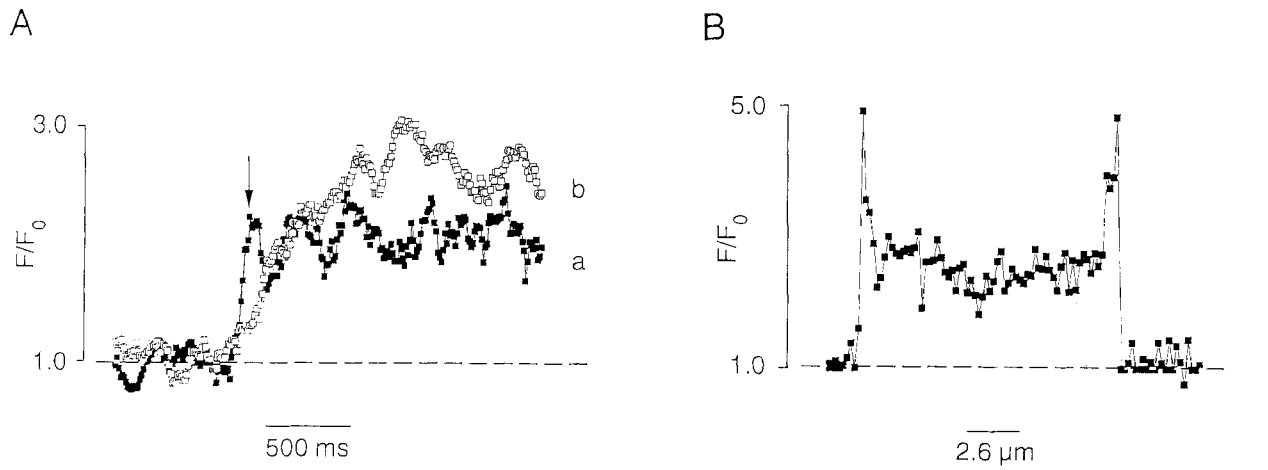


Fig. 4. $[Ca^{2+}]_{in}$ transients recorded with high spatial and temporal resolution The fluorescence ratio (F/F_0) was recorded from the cell shown in Figure 3. A: At the two locations in the cell, marked as (a) and (b) in Figure 3, the fluo-3 fluorescence change was resolved with a high time resolution during the onset of high K^+ application. It is evident that close to the membrane (a) the Ca^{2+} increase occurs much faster than in the center of the cell soma. B: The result of a single line scan is displayed with a high spatial resolution. The line is taken at the time marked by arrows in Figures 3 and 4A. Note the high level in the submembrane region.

measurement, we distinguished the cells of the oligodendrocyte lineage from other glial cells and neurons. To assess the precursor cells, we selected for O4-positive cells with the simplest morphology, namely, cells with 2–3 processes (Fig. 1). A 20 to 30 s increase in K^+ from the resting level of 5.4 to 50 mM evoked a transient increase of the relative fluorescence signal as recorded with the fluo-3 system (N = 73; Fig. 2). The increase of the fluorescence intensity amounted to 100–300% as compared to the resting level. The resting fluo-3 fluorescence appeared to be homogeneous throughout the cells studied, suggesting therefore the spatial uniformity of resting $[Ca^{2+}]_{in}$. In the following we will refer to the fluorescence increase as an increase in $[Ca^{2+}]_{in}$.

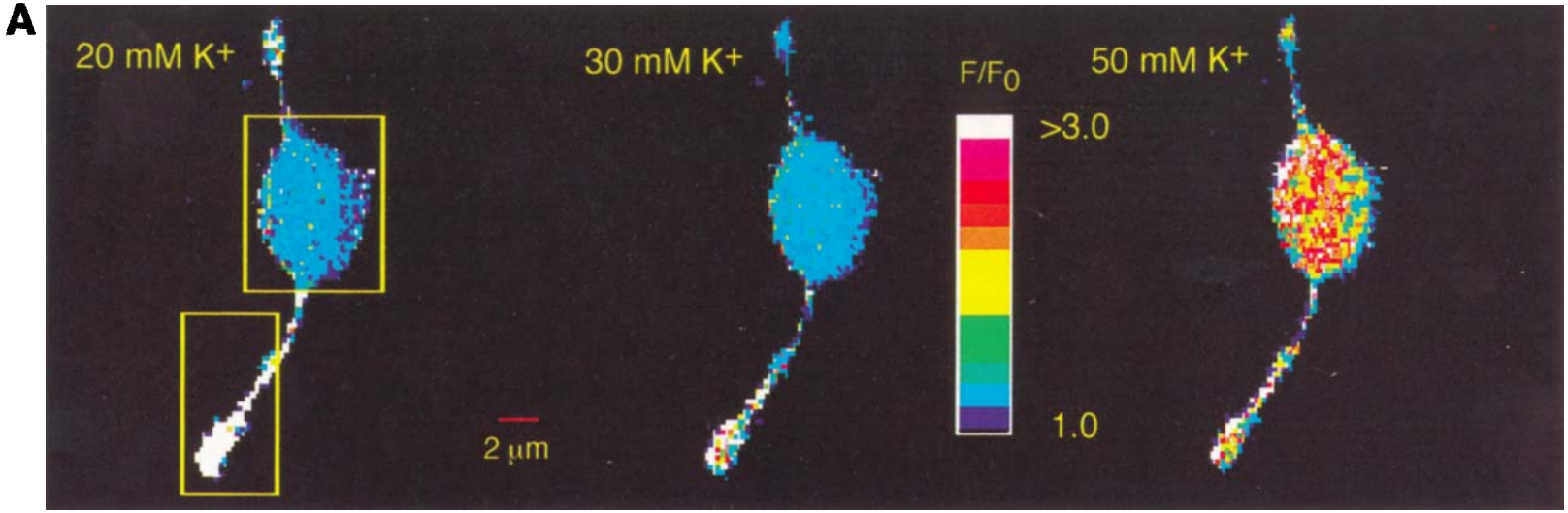
In the majority of experiments, the $[Ca^{2+}]_{in}$ increase induced by the elevation in $[K^+]_e$ was larger in processes as compared to the cell soma (71%, N = 73; Fig. 2). Responses in processes were further distinct in that $[Ca^{2+}]_{in}$ decayed to an intermediate (elevated) level within the 20 s application, while in the soma $[Ca^{2+}]_{in}$ increased to a steady level and remained at that level (Fig. 2). This finding points to a different mechanism of Ca^{2+} entry or removal in processes versus soma.

The Ca^{2+} Influx Is Due to the Activation of Voltage-Gated Ca^{2+} Channels

The $[Ca^{2+}]_{in}$ increase in response to the application of the high-K^+ solution was completely abolished in low Ca^{2+} bathing solution (N = 12) indicating that Ca^{2+} entered the cell via the plasma membrane. The Ca^{2+} increase was furthermore blocked in the presence of the Ca^{2+} channel blockers verapamil (100 μM; N = 4) or Cd^{2+} (100 μM; N = 7). As Cd^{2+} may induce the irreversible quenching of fluo-3 while entering the cell, we specifically tested this possibility. Addition of 100 μM Cd^{2+} to the normal solution did not influence the resting fluo-3 fluorescence. Similarly, we did not observe any changes in fluo-3 fluorescence upon high-K^+ depolarization in the presence of Cd^{2+} in the external solution, indicating the absence of measurable Cd^{2+} entry in our experimental conditions. These observations lead to the conclusion that the depolarization of the glial precursor cells induces $[Ca^{2+}]_{in}$ transients resulting from the activation of voltage-gated Ca^{2+} channels.

To resolve the Ca^{2+} dynamics close to the plasma membrane and further support that Ca^{2+} enters through the plasma membrane, the Ca^{2+} distribution was measured with a line scan. The line was positioned through the center of the cell soma and transient changes in $[Ca^{2+}]$ could be resolved with a time resolution of 10 ms per each line scan. Two regions of the cell were compared, within 1–4 μm from the cell membrane and in the center of the cell soma (Fig. 3A–C). Close to the cell membrane, an increase in $[K^+]_e$ led to a rapid increase in $[Ca^{2+}]$ with a peak after 70–100 ms. Subsequently, $[Ca^{2+}]_{in}$ remained at an intermediate (increased) level. In contrast, in the center of the cell Ca^{2+} increased more slowly, reaching a plateau within 500–600 ms (Fig. 4).

To test for a possible contribution of Ca^{2+}-induced Ca^{2+} release (CICR) from internal stores (Henzi and MacDermott, 1992) to the recorded $[Ca^{2+}]_{in}$ signal, we compared the responses in the presence of ryanodine (50 μM), a blocker of the CICR mechanism in excitable cells (McPherson et al., 1991) with those under control conditions. In all cells studied (N = 9) the amplitude and shape of the depolarization-induced $[Ca^{2+}]_{in}$ transients were not altered in the presence of ryanodine, suggesting no apparent involvement of internal ryanodine-sensitive Ca^{2+} stores in the amplification of the recorded Ca^{2+} signals.



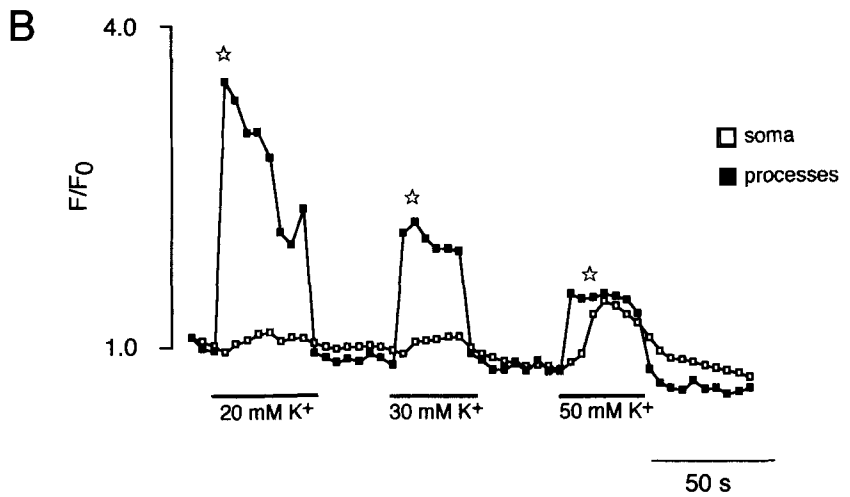


Fig. 5. Differential effect of increasing $[K^+]_e$ on the Ca^{2+} influx. **A:** Pseudocolour images coded as described in Figure 3 were obtained from a precursor cell. The cell was superfused by three different $[K^+]_e$ as indicated. An increase to 20 mM $[K^+]_e$ resulted in a fluorescence change in the processes only, while 50 mM $[K^+]_e$ induced a fluorescence increase within the entire cell. **B:** Two areas were selected (see boxes in A), at the soma and at a process. The fluorescence ratio (F/F_o) was recorded at these locations and is displayed as a continuous record. $[K^+]_e$ was increased as indicated by bars. Stars correspond to the images shown in A.

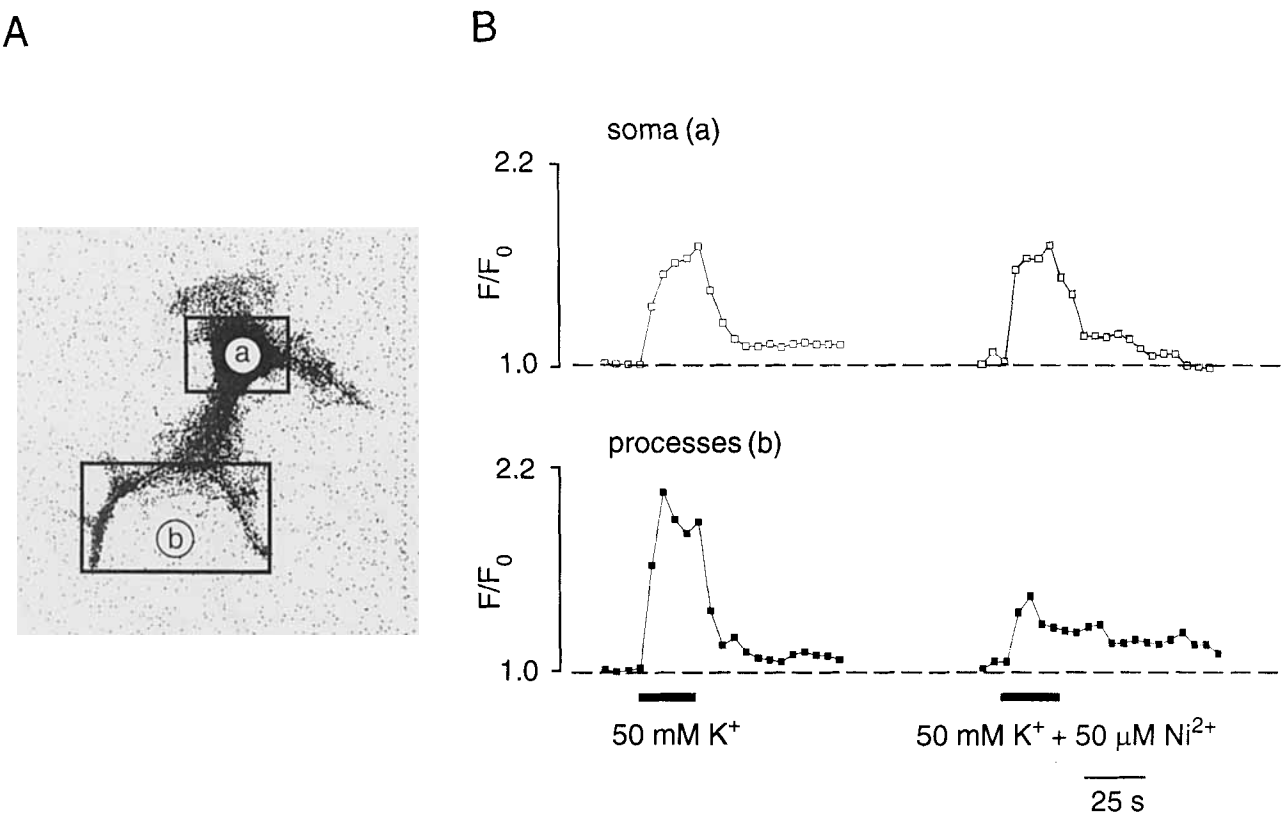


Fig. 6. Distinct pharmacological properties of $[Ca^{2+}]_{in}$ transients recorded in the soma and in processes. **A:** The recordings were obtained from a O4-positive cell with simple morphology. The picture shows the fluo-3 fluorescence after dye loading. **B:** The fluorescence ratio (F/F_0) was recorded at two locations of the cell shown as boxes in A, at the soma (a) and at processes (b). $[K^+]_e$ was increased to 50 mM in the absence (left traces) and presence of Ni^{2+} (right traces). Note that Ni^{2+} markedly depressed the $[Ca^{2+}]_{in}$ increase in processes, while it did not affect the signal recorded from the soma.

The Depolarizing Threshold for the Ca^{2+} Entry Is Different for Processes and Soma

To study the distribution of different types of Ca^{2+} channels, namely, with low and high threshold for activation, precursor cells were depolarized to different levels by applying increasing concentrations of K^+. An increase of K^+ from 5.4 to 20, 30 or 50 mM induced a depolarization by 25, 35, 55 mV from the resting membrane potential (resting potentials of oligodendrocytes and precursors as well as levels of K^+-induced depolarization were measured using conventional patch clamp technique in a current clamp mode; intracellular solution contained, in mM: KCl, 140; $MgCl_2$, 1; EGTA, 1; HEPES/KOH, 10; pH, 7.2). As shown in Figure 5, a moderate depolarization by 20 mM K^+ led to an increase of the $[Ca^{2+}]$ in the processes only, while $[Ca^{2+}]_{in}$ levels in the soma remained unaffected. An increase in $[K^+]_e$ (and thus of the degree of cellular depolarization) resulted in a progressive fall of $[Ca^{2+}]_{in}$ amplitudes in processes, while in the soma $[Ca^{2+}]_{in}$ transients became larger. In average, the amplitude of $[Ca^{2+}]_{in}$ transients in processes was 2.8 ± 0.3 times larger as compared to the soma in response to 20 mM K^+, 1.7 ± 0.2 times in response to 30 mM K^+ and 1.3 ± 0.2 in response to 50 mM K^+ (N = 22).

Moreover, in the presence of Ni^{2+} (50 μM), a specific antagonist of low-voltage-activated Ca^{2+} channels, the depolarization-induced $[Ca^{2+}]_{in}$ transients in the processes were smaller, while the amplitude of $[Ca^{2+}]_{in}$ elevations in the soma was not reduced (N = 5; Fig. 6). In contrast, the agonist of high-voltage-activated Ca^{2+} channels, BAY K 8644 (1 μM) enhanced the amplitude, and markedly prolonged the duration of depolarization-induced $[Ca^{2+}]_{in}$ transients only in the soma (evoked by 50 mM K^+; N = 5), while the amplitude and shape of $[Ca^{2+}]_{in}$ transients in processes were affected to a much lesser extent (not shown).

Ca^{2+} Responses of Cortical Glial Precursor Cells

Similar results were obtained from glial precursor cells from the mouse cortex; increases in $[Ca^{2+}]_{in}$ in processes could be evoked by increasing $[K^+]_e$ to 20 mM or higher while a somatic response was activated by 50 mM $[K^+]_e$ only. The ratio of the fluorescence amplitudes in processes vs. soma were: 2.6 ± 0.4 in re-

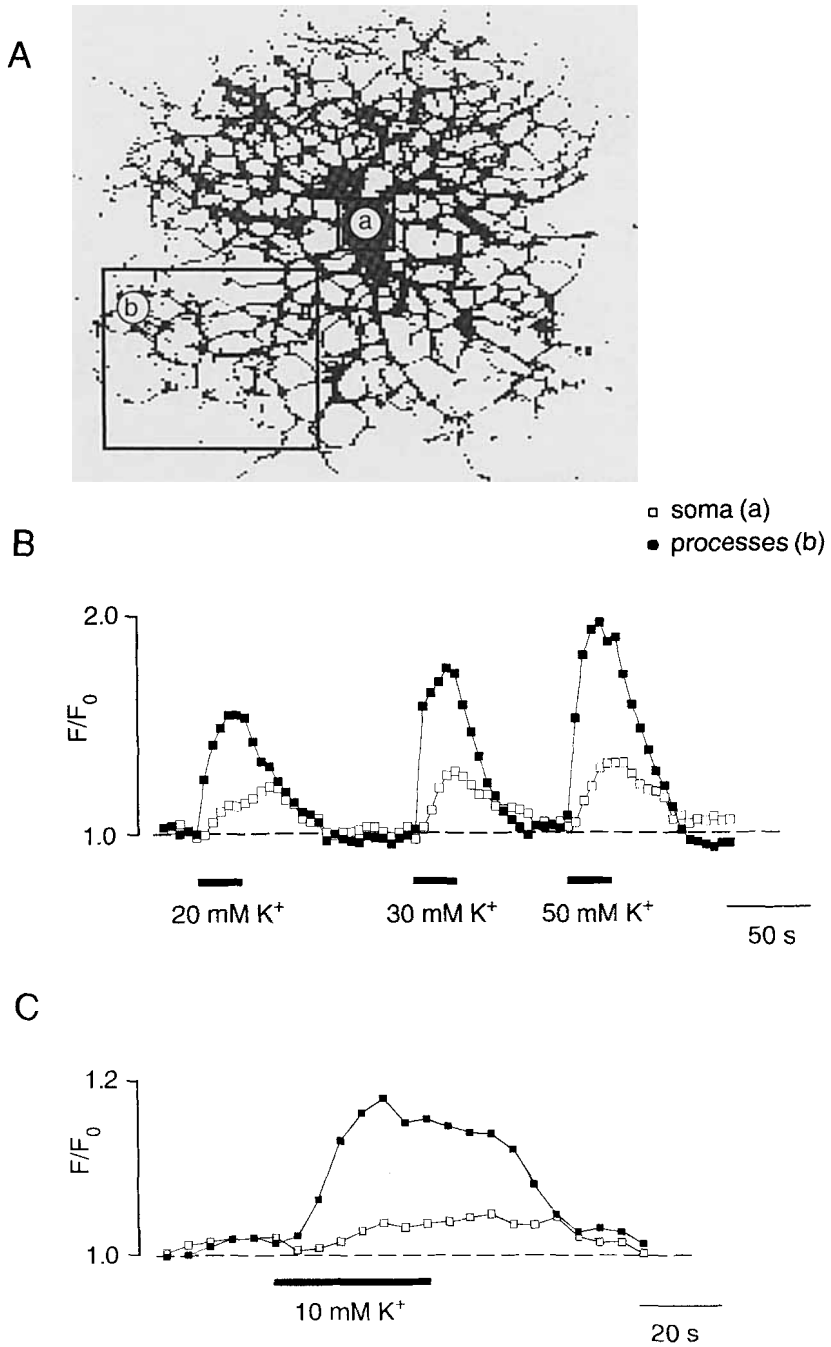


Fig. 7. $[Ca^{2+}]_{in}$ transients in oligodendrocytes. A: An oligodendrocyte was identified by a positive staining against O10. The picture shows the fluo-3 loaded cell. B,C: The fluorescence ratio (F/F_0) was recorded from the two cellular regions specified in A. $[K^+]_e$ was increased as indicated by bars.

sponse to 20 mM K^+, 1.9 ± 0.1 in response to 30 mM K^+, and 1.4 ± 0.2 in response to 50 mM K^+ (N = 17).

Ca^{2+} Responses of Oligodendrocytes Also Show a Heterogeneous Distribution of Ca^{2+} Channels

Oligodendrocytes were identified by a positive staining against O10 prior to the experiment; this marker labels more mature oligodendrocytes characterized by a complex morphology (see Fig. 1). In contrast to precursor cells, responses to increasing depolarization were different in the majority of oligodendrocytes from both retina and cortex. Application of increasing extracellular K^+ concentration (20 mM, 30 mM, and 50 mM) induced a Ca^{2+} signal with increasing amplitude (Fig. 7B). This $[Ca^{2+}]_{in}$ increase was mainly confined to the processes while $[Ca^{2+}]_{in}$ levels in the soma increased

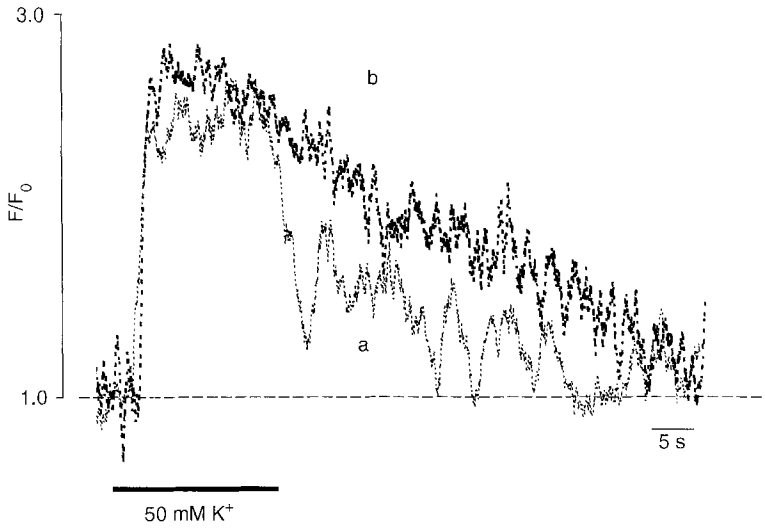


Fig. 8. The recovery of the $[Ca^{2+}]_{in}$ level from a K^+-triggered increase is different in the transmembrane and central region. From the experiment shown in Figure 3, the recovery of the fluorescence ratio (F/F_o) from a K^+-induced increase is illustrated. Note the faster recovery of $[Ca^{2+}]_{in}$ near the membrane (a) versus the center of the soma (b).

only to a much smaller extend (Fig. 7); 50 mM $[K^+]_e$ triggered $[Ca^{2+}]_{in}$ transients in both soma and processes, but the amplitudes of these fluorescence transients were 2.2 ± 0.3 (N = 41) times larger in processes versus soma in retinal and 1.9 ± 0.1 (N = 30) times larger in cortical oligodendrocytes. Thus, a similar uneven distribution of voltage-gated Ca^{2+} channels is also present in oligodendrocytes. To test a more physiological signal, extracellular K^+ was increased to 10 mM. This concentration has been observed in the extracellular space during neuronal activity in white matter (Sykova, 1992). This small increase in $[K^+]_e$ caused a clearly detectable increase in $[Ca^{2+}]_{in}$ (Fig. 7C). From these data we conclude that Ca^{2+} signalling can be activated by physiological stimuli.

The Restoration of the Resting $[Ca^{2+}]_{in}$ Level Is Achieved by the Plasmalemmal Ca^{2+} Pumps

After returning to normal $[K^+]_e$, thus terminating the K^+-induced depolarization, the $[Ca^{2+}]_{in}$ level recovered to the resting value within 15 to 20 s. Measuring the $[Ca^{2+}]_{in}$ with a high time resolution, the decay in the $[Ca^{2+}]_{in}$ occurred considerably faster in the submembrane regions as compared to the center of the soma (Fig. 8). To study the mechanism of the recovery in both immature and mature oligodendrocytes, we used a blocker of the plasmalemmal Ca^{2+} pump, La^{3+} (Kwan et al., 1990, Ganitkevith and Isenberg, 1992) and of the mitochondrial uptake system, carbonyl cyanide m-chlorophenyl-hydrazone (CCCP, Nicholls, 1985). In oligodendrocytes treated by 10 μM CCCP which prevents cytoplasmic Ca^{2+} uptake into mitochondria, the recovery rate of depolarization-mediated $[Ca^{2+}]_{in}$ transients was not significantly changed as compared with the control transients (N = 5, Fig. 9A). Application of CCCP alone caused an elevation of $[Ca^{2+}]_{in}$ due to the liberation of Ca^{2+} ions from the mitochondrial pool. In contrast, blockade of the plasmalemmal Ca^{2+} pumps by La^{3+} effectively prolonged the recovery rate of $[Ca^{2+}]_{in}$ transients evoked by membrane depolarization. In these experiments we used the following protocol: cells were depolarized by an increase of bath $[K^+]_e$ to 50 mM; subsequently the normal $[K^+]_e$ level was restored in the presence of La^{3+} (3 mM). In these experiments we have found that La^{3+} markedly prolonged the duration of the recovery phase of depolarization-induced $[Ca^{2+}]_{in}$ transients (Fig. 9B).

DISCUSSION

Distribution of Ca^{2+} Channels

We demonstrate in this study that Ca^{2+} channels are not evenly distributed in the membrane of glial precursor cells and oligodendrocytes. Such an uneven distribution of ion channels or receptors has been described in many preparations. A classical example is the localization of Na^+ channels at the node of Ranvier in the axonal membrane (Hille, 1992). In Bergmann glial cells, such an uneven distribution has been demonstrated for $GABA_A$ receptors (Müller et al., 1993). In the present study, we found that the two types of Ca^{2+} channels which have been described in the oligodendrocyte cells lineage (Verkhratsky et al., 1990; Blankenfeld et al., 1992) show a differential distribution. In the precursor cells, the low-voltage-activated Ca^{2+} channels are con-

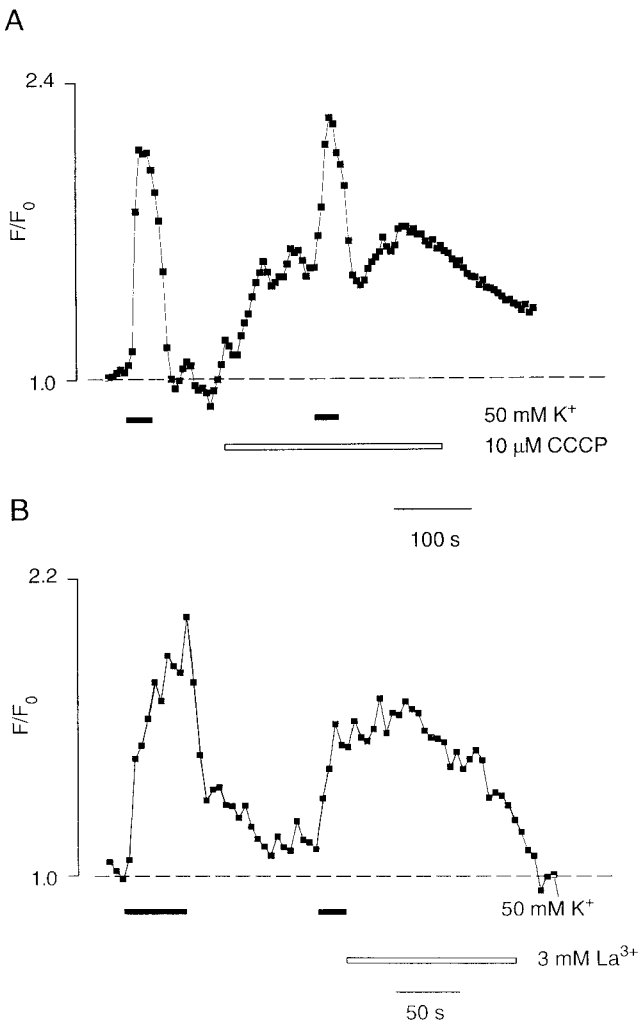


Fig. 9. Effects of Ca^{2+} pump blocker and mitochondrial uncoupler on the recovery of $[Ca^{2+}]_{in}$ from a K^+ induced increase. **A:** The fluorescence ratio (F/F_0) is recorded from an oligodendrocyte identified by a positive staining for O10. $[K^+]_e$ was increased in control conditions and in the presence of CCCP (10 μM). Despite the increased base level, the recovery from the depolarization-induced $[Ca^{2+}]_{in}$ elevation was not significantly altered. **B:** A similar experiment was performed on a different O10-positive oligodendrocyte in the presence of 3 mM La^{3+}. In contrast to CCCP, the recovery of the K^+-induced Ca^{2+} transient was markedly prolonged.

centrated at the processes; the high-voltage-activated are also present in the somatic region. An uneven distribution of voltage-activated Ca^{2+} channels was also found for the O10-positive mature oligodendrocytes. In mature oligodendrocytes presumably both types of Ca^{2+} channels are expressed at higher density in cellular processes. We assume that similar as in neurons, the glial Ca^{2+} channels are linked to the cytoskeleton via linker proteins such as ankyrin or spectrin, similar as other ion channels (Srinivasan et al., 1980).

Mechanisms of $[Ca^{2+}]_{in}$ Recovery

Cytoplasmic calcium concentration is controlled by the interaction of transmembrane Ca^{2+} transport, Ca^{2+} buffering, and Ca^{2+} uptake by internal organelles. All these processes participate in the termination of the Ca^{2+} signal (Pietrobon et al., 1990; Bronner, 1990; Miller, 1991). In particular, there are several mechanisms to remove Ca^{2+} after an influx from the cytoplasm: i) Ca^{2+} binding to cytoplasmic proteins; ii) Ca^{2+} extrusion via plasmalemmal Ca^{2+} pumps and/or Na^+/Ca^{2+} exchanger; and iii) Ca^{2+} accumulation into internal structures, namely, endoplasmic reticulum and mitochondria. We have investigated the contribution of plasmalemmal Ca^{2+} ATP-ases and mitochondrial uptake in the recovery of $[Ca^{2+}]_{in}$ after a depolarization-triggered increase.

Ca^{2+} uptake into mitochondria is mediated via a uniporter driven by the electrical potential difference, the latter is created by the respiratory chain H^+ extrusion mechanism (Bronner, 1990; Meldolesi et al., 1990). However, the mitochondrial system responds only when $[Ca^{2+}]_{in}$ has increased to high levels (Meldolesi et al., 1990). Blockade of the respiratory chain by uncouplers such as CCCP effectively inhibits mitochondrial Ca^{2+} accumulation. Using these tools, we have found that in oligodendrocytes mitochondrial Ca^{2+} uptake did not significantly contribute to the recovery of $[Ca^{2+}]_{in}$ after a K^+-mediated elevation. It is the activity of the La^{3+}-sensitive plasmalemmal Ca^{2+} ATP-ases which is responsible for the Ca^{2+} extrusion after an influx. In addition to ATP-dependent Ca^{2+} extrusion, Ca^{2+} ions could also be expelled via Na^+/Ca^{2+} exchanger; however, our observation that omission of Na^+ ions from the bath did not significantly change the rate of recovery of the depolarization-induced $[Ca^{2+}]_{in}$ transients makes a significant contribution unlikely.

Possible Function of Ca^{2+} Channels in Glial Cells and Their Precursors

Early in development, the extracellular space is much less compacted as compared to the adult nervous system (Ransom et al., 1986). In the white matter, myelin formation coincides with the compaction of the extracellular space indicating that glial precursor cells are surrounded by a large volume fraction (Lehmenkühler et al., 1993). An increase in such a compartment is only possible if large amounts of K^+ are released. Thus, large caliber axons are a more likely source. If we assume that the K^+-triggered increase in $[Ca^{2+}]_{in}$ might be a recognition signal for a glial precursor cell to detect an active axon it would imply that, in particular, the large axons are recognized first. Such a recognition process could be followed by a differentiation step such as myelin formation. Indeed, it has been observed that large caliber axons are myelinated first (Sturrock, 1980). With the beginning of myelination, the extracellular space compacts (Lehmenkühler et al., 1993); as a consequence a small amount of released K^+ will cause a larger change in $[K^+]_e$ concentration in this shrunk space. This would imply that the activity of small axons can now also lead to considerable increases in the $[K^+]_e$. Under these conditions the activity of the small fibers might produce a

K^+ signal large enough to activate Ca^{2+} responses in the precursor cells or even in oligodendrocytes. Indeed, we have shown that increases in $[K^+]_e$ in the physiological range (10 mM) is already sufficient to produce a reasonable $[Ca^{2+}]_{in}$ elevations. As a working hypothesis, we thus propose that neuronal activity leads to a Ca^{2+} influx into the processes of precursor cells and this $[Ca^{2+}]_{in}$ increase is a signal for the glial precursor cell to start myelin formation.

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