Regulation of Store-Operated Calcium Entry by Calcium-Independent Phospholipase A₂ in Rat Cerebellar Astrocytes

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We have studied store-operated Ca²⁺ entry (SOCE) in Bergmann glia and granule cell layer astrocytes in acute brain slices of the rat cerebellum, using the Ca²⁺-sensitive fluorescent dye Fluo-4 and confocal laser scanning microscopy. Astrocytes were identified by their morphology, location, and their Ca²⁺ response in K⁺-free solution. Depletion of Ca²⁺ stores by cyclopiazonic acid (CPA) (20 μM) induced SOCE in both types of astrocyte. A similar Ca²⁺ influx was elicited by the calmodulin antagonist calmidazolium (CMZ) (1 μM). The SOCE channel blocker 2-aminoethoxy-diphenylborate (2-APB) (100 μM) and the Ca²⁺ release-activated channel blocker 3,5-bis(trifluoromethyl) pyrazole derivative (BTP2) (20 μM) suppressed the CPA- and the CMZ-induced Ca²⁺ influx. Pretreatment of acute slices with the specific Ca²⁺-independent phospholipase A₂ (iPLA₂) inhibitor bromoenol lactone (BEL) (25 μM) blocked the CPA- and the CMZ-induced Ca²⁺ influx. The lysophospholipid products of iPLA₂, lysophosphatidylcholine (250 nM) and lysophosphatidylinositol (250 nM), but not lysophosphatidic acid (250 nM), induced a BTP2- and 2-APB-sensitive, but BEL-insensitive, Ca²⁺ influx. CPA or CMZ enhanced the BEL-sensitive enzymatic activity of iPLA₂ in cerebellar astrocyte culture. Inhibition of iPLA₂ expression by specific antisense oligodeoxynucleotide of iPLA₂ reduced the SOCE and the Ca²⁺ store refilling in cultured astrocytes. Spontaneous Ca²⁺ oscillations in astrocytes *in situ* were reduced after inhibiting SOCE channels or iPLA₂ activity. The results suggest that the depletion of Ca²⁺ stores activates iPLA₂ to open Ca²⁺ channels in the plasma membrane by the formation of lysophospholipids in astrocytes, presumably to refill the stores and allow normal Ca²⁺ signaling.

Key words: Fluo-4; cyclopiazonic acid; calmidazolium; lysophospholipids; calcium stores; Bergmann glia

Introduction

Depletion of intracellular Ca²⁺ stores can induce influx of Ca²⁺ from the extracellular space through channels in the plasma membrane, a mechanism known as store-operated Ca²⁺ entry (SOCE) or capacitative Ca²⁺ entry (Parekh and Putney, 2005). SOCE appears to be the predominant pathway of regulated Ca²⁺ influx in non-excitable cells but may also play a major role in some types of excitable cells, including neurons (Fagan et al., 2000; Emptage et al., 2001; Akbari et al., 2004; Verkhratsky, 2005). SOCE plays a major role not only in replenishing Ca²⁺ stores but also in various physiological processes ranging from short-term responses, such as exocytosis, contraction, and Ca²⁺ oscillations, to long-term control of gene transcription, cell cycle, and apoptosis (Berridge et al., 2000; Parekh and Putney, 2005).

A model proposed for SOCE channel activation implies the existence of a diffusible messenger termed Ca²⁺ influx factor (CIF), which can be released from the stores into the cytosol during depletion, thereby transmitting the signal to the plasmale-

mmal SOCE channels to trigger Ca²⁺ entry (Randriamampita and Tsien, 1993). Smani et al. (2004) have recently demonstrated that CIF activates SOCE channels in the plasma membrane through its interaction with Ca²⁺-independent phospholipase A₂ (iPLA₂). In this paradigm, CIF can displace inhibitory calmodulin (CaM) from iPLA₂, resulting in activation of iPLA₂ and formation of lysophospholipids, which in turn activates SOCE. During refilling of Ca²⁺ stores and termination of CIF production, calmodulin inhibits iPLA₂ activity by binding to the enzyme, thereby suppressing SOCE. The EF hand protein stromal-interacting molecule (STIM1) was recently identified as a possible intracellular messenger, which migrates from the endoplasmic reticulum (ER) to the plasma membrane during depletion of Ca²⁺ stores, suggesting that STIM1 functions as the missing link between Ca²⁺ store depletion and SOCE activation (Liou et al., 2005; Roos et al., 2005; Zhang et al., 2005).

In glial cells, SOCE has been studied in some detail only in cell cultures. SOCE can be induced, e.g., in cultured rat cerebellar astrocytes (Jung et al., 2000; Lo et al., 2002) and C6 glioma cells (Wu et al., 1997), in which it is enhanced by either cAMP (Wu et al., 1999) or nitric oxide (Li et al., 2003), and reduced by arachidonic acid (AA) (Sergeeva et al., 2003; Yang et al., 2005). In glial cells in acute brain slices, however, mechanisms of regulating SOCE have not been investigated. The present study aims to elucidate the signaling pathway that activates SOCE in rat cerebellar astrocytes *in situ*. We investigated the SOCE in the two major types of astrocyte in the cerebellar cortex, the Bergmann glia and

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astrocytes in the granule cell layer (GCL), by imaging cytosolic Ca²⁺ with confocal microscopy. We found that SOCE exists in both types of astrocyte and is mediated by specific lysophospholipid products of iPLA₂. Furthermore, we show that spontaneous Ca²⁺ oscillations of astrocytes in the cerebellum are reduced by inhibiting SOCE and by the iPLA₂ inhibitor bromoenol lactone (BEL).

Materials and Methods

Slice preparation. Cerebellar brain slices were prepared following the method of Edwards et al. (1989). In brief, a juvenile rat [postnatal day 8 (P8) to P15] was decapitated, and the cerebellum was isolated rapidly in an ice-cold, bicarbonate-buffered (5% CO₂/95% O₂, pH 7.4), Ca²⁺-reduced solution (0.5 mM; MgCl₂ increased to 2.5 mM). Sagittal slices of the vermis (250 μm thick) were obtained using a vibratome (VT 1000; Leica, Darmstadt, Germany) and stored for 1 h in gassed Ca²⁺-reduced saline at 30°C before dye loading and later at room temperature (21–24°C). Cells of interest were Bergmann glial cells of the Purkinje cell layer and granule cell layer astrocytes of the cerebellar cortex. The cell types were identified as explained in Results.

Cell culture. Astrocyte primary cultures were prepared from the cerebellar hemispheres of newborn rats (P1–P3) by a method similar to that described by Fischer (1984). Briefly, cerebella were rapidly excised and maintained in an ice-cold Ca²⁺-free isotonic salt solution containing the following (in mM): 120 NaCl, 5.4 KCl, 0.8 MgCl₂, 25 Tris-HCl, and 15 glucose, pH 7.2. After removing the meninges, cerebella were digested with 0.1% trypsin for 5 min at room temperature, before trypsin was replaced with 10% fetal calf serum (FCS) in Eagle's Basal Medium (BME) (Sigma, St. Louis, MO). The tissue was triturated using successively decreasing bore size fire-polished Pasteur pipettes in the same medium. Cells were centrifuged at 600 rpm for 5 min and resuspended in DNase-containing solution. Centrifugation was repeated and the pellet was resuspended in DMEM (Sigma) (supplemented with 2.0 g of NaHCO₃, 2.0 g of glucose, 1000 U/L penicillin, 1 g/L streptomycin, and 5% FCS). Cells were plated in poly-D-lysine (PDL)-coated (0.001%) bottles with DMEM and incubated at 37°C in a humidified atmosphere with 10% CO₂. After the cells had reached confluence (~10 d), the cells were dissociated by 0.1% of trypsin, and this step was stopped by fetal calf serum (10% in BME). Then the cells were incubated with DNase, centrifuged, resuspended in DMEM, plated on glass coverslips coated with PDL (0.001%), and incubated as described previously. The experiments were performed on secondary astrocyte cultures between 2 and 10 d after plating at room temperature (21–24°C). Cultures showed >95% staining for the astrocyte-specific marker glial fibrillary acidic protein (GFAP).

Transfection with oligodeoxynucleotides. A 20-base-long antisense (AS) oligodeoxynucleotide (ODN) corresponding to the conserved nucleotides 59–78 in the murine group IV iPLA₂ sequence was used (AS-ODN, 5'-fluorescein-ACTCCTTCACCCGGAATGGGT-3'; MWG-Biotech, Ebersberg, Germany). The corresponding sense (S) oligodeoxynucleotide complement (S-ODN, 5'-fluorescein-CCCATTCCGGGTGAAGAG-3'; MWG-Biotech) was used as a control. Both S-ODN and AS-ODN contained nuclease-resistant phosphorothioate linkages to limit their degradation and were labeled with fluorescein to detect transfected cells (see below, Calcium imaging). Secondary astrocytes at 60% confluence were transfected using Lipofectamine 2000 (LF) (Invitrogen, Karlsruhe, Germany) in the dark following the protocol of the manufacturer. The experiments were performed 36–48 h after transfection. Transfection efficiency was determined with fluorescein-labeled ODNs and amounted to 71% in the astrocyte cultures used.

Dye loading. Cerebellar slices were loaded with a solution containing 3 μM Fluo-4 AM (Invitrogen, Carlsbad, CA), made from a 6 mM stock solution in dimethylsulfoxide (DMSO) with 20% pluronic acid, in a Petri dish inside a small oxygenated closed box for 60 min at 20–24°C. After dye loading, the slices were kept on a mesh of nylon in artificial CSF (ACSF) (see below). All salines for acute brain slices were gassed with 5% CO₂/95% O₂ to maintain pH and oxygen levels at physiological levels. Cerebellar astrocyte cultures were incubated in 2 μM Fluo-4 AM-

containing solutions for 45 min in the dark at room temperature. ODN-transfected and nontransfected cultures were loaded in a 1 μM fura-2 AM-containing saline, made from a 4 mM stock solution in DMSO, for 30 min at room temperature.

Calcium imaging. Experiments with acute brain slices and cultures were performed with a confocal laser scanning microscope (LSM 510; Zeiss, Oberkochen, Germany). The Ca²⁺-sensitive dye Fluo-4 was excited by the 488 nm line of an argon laser, and images were taken with a frequency of 0.3–1.0 Hz. Excitation and emission signals were separated by a dichroic mirror at 500 nm. The emission signal was truncated by a 505 nm optical long-pass filter. The sequence of fluorescence images was sampled in one focal plane for Ca²⁺ measurements at room temperature (21–24°C). Regions of interest (ROIs) were defined in the first image, and the relative fluorescence changes ΔF (percentage) were measured throughout the series, related to the basic, normalized fluorescence intensity at the beginning of the experiment (defined as 100%). Hence, Ca²⁺ changes were referred to as this percentage change of the Fluo-4 fluorescence. All settings of the laser, optical filters, and the microscope as well as the data acquisition were controlled by personal computer software (Zeiss). Additional details have been described previously (Beck et al., 2004).

Experiments on ODNs-transfected and nontransfected cell cultures were performed with an Olympus Optical (Tokyo, Japan) upright microscope (BX50W1) using a Ca²⁺ imaging system (T.I.L.L. Photonics, Munich-Martinsried, Germany). Monochromator setting and data acquisition were controlled by software for a personal computer system. The transfected cells were identified by imaging the fluorescein (excitation at 488 nm, emission at 510 nm). Fura-2-loaded cells were excited by monochromatic wavelengths of 340 and 380 nm. The fluorescence emissions of several ROIs (each covering one single cell body) were simultaneously recorded with the CCD camera IMAGO (T.I.L.L. Photonics), using a 440 nm long-pass filter. The signals were sampled at 0.2–0.5 Hz, computed into relative ratio units F_{340}/F_{380} . The decay of intracellular Ca²⁺ transients was fitted by an equation for monoexponential decay using Origin software (Microcal Software, Northampton, MA), resulting in a time constant τ .

Antibody staining. Cerebellar slices were fixed in 4% paraformaldehyde in PBS, pH 7.4, overnight at 4°C. Slices were rinsed with PBS and permeabilized in 0.5% Triton X-100 in PBS/10% normal goat serum (NGS), and the primary antibody (rabbit anti-glial fibrillary acidic protein, 1:1000; Z0334; DakoCytomation, Carpinteria, CA) in PBS containing 0.05% Triton X-100 was incubated overnight at 4°C. The slices were then rinsed with PBS and incubated in secondary antibody (Alexa 488 goat anti-rabbit IgG, 1:200; Invitrogen, Carlsbad, CA) and propidium iodide (5 μM) for 3 h at room temperature. After rinsing, slices were mounted on a coverslip and imaged using confocal microscopy. GFAP staining was visualized at 488 nm excitation and 510 nm emission, whereas the nuclei (labeled by propidium iodide) were visualized at 544 nm excitation and 620 nm emission.

ODNs-transfected cultures were washed with PBS and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. After washing, cells were permeabilized in 0.1% Triton X-100 in PBS/10% NGS for 1 h. Incubation with primary antibody [rabbit iPLA₂ (type IV) polyclonal antiserum, 1:500 (Cayman Chemical, Ann Arbor, MI)] was performed in PBS containing 0.05% Triton X-100 overnight at 4°C. Cells were then incubated with secondary antibody (Alexa 546 goat anti-rabbit IgG, 1:200; Invitrogen, Carlsbad, CA) for 1 h, washed, and mounted. Control staining was done in the same way but without the primary antibody. Cell cultures of all treatments, i.e., S-ODN, AS-ODN, and LF alone, were processed simultaneously with the same solutions and incubation times. The expression of iPLA₂ in random fields was assessed by the iPLA₂ staining using confocal microscopy. The transfected cells were identified based on the labeling of fluorescein, and iPLA₂ staining was visualized at 543 nm excitation and with a 560 nm long-pass emission filter, whereas fluorescein was detected as described (see above, Calcium imaging). S-ODN-transfected cells, AS-ODN-transfected cells, and LF-treated cells were imaged with the same laser and microscope settings. Anti-iPLA₂-dependent fluorescence was quantified to compare the amount of iPLA₂ in the cells.

Figure 1. Identification of rat cerebellar astrocytes *in situ*. **A**, Labeling of astrocytes with anti-GFAP antibody (green) and cell nuclei with propidium iodide (red) in different cell layers of a cerebellar slice. ML, Molecular layer; PCL, Purkinje cell layer. **B**, At higher magnification, Bergmann glial cell processes in the molecular layer (arrowheads). Purkinje cell somata appear as black holes in the Purkinje cell layer (asterisk). **C**, Astrocytes (arrowheads) in the granule cell layer. **D**, **F**, Fluorescence images of Fluo-4-loaded Bergmann glia (**D**) and astrocytes in the GCL (**F**) during ATP application (20 μM for 30 s), when they respond with Ca^{2+} signals. Somata of Bergmann glial cells and GCL astrocytes are indicated by circles (regions of interest, in which cytosolic Ca^{2+} was recorded). **E**, **G**, The fluorescence recording, given as the relative change in fluorescence related to the value at the beginning of the experiments (ΔF), show typical Ca^{2+} responses of a Bergmann glial cell (**D**) and a GCL astrocyte (**G**) to ATP and 0 mM K^+ and to CPA (20 μM) in the absence of extracellular Ca^{2+} , followed by readdition of Ca^{2+} . Scale bars: **A**, 100 μm ; **B**, 50 μm ; **C**, 30 μm ; **D**, **F**, 20 μm .

Therefore, a stack of images was recorded, and the brightest image was chosen, in which the mean fluorescence intensities of the astrocyte somata were measured.

Solutions. The standard saline for acute brain slices (ACSF) contained the following (in mM): 125 NaCl, 2.5 KCl, 2 CaCl_2 , 1 MgCl_2 , 25 D-glucose, 26 NaHCO_3 , 1.25 NaH_2PO_4 , and 0.5 L-lactate (gassed with 5% CO_2 /95% O_2 to adjust the pH to 7.4). In Ca^{2+} -reduced (0.5 mM) saline, 1.5 mM Ca^{2+} was replaced by 1.5 mM MgCl_2 and L-lactate was omitted. In salines with varying K^+ concentrations, KCl was exchanged by or for NaCl. Ca^{2+} -free saline was prepared by replacing CaCl_2 by equimolar amounts of MgCl_2 and by adding 1 mM EGTA. The standard saline for cultured astrocytes contained the following (in mM): 145 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 D-glucose, and 10 HEPES, pH set to 7.4. Ca^{2+} -free saline was prepared by replacing CaCl_2 by equimolar amounts of MgCl_2 and by adding 0.5 mM EGTA. The experimental chamber containing either an acute slice or cell cultures was continuously perfused with the saline at room temperature. Cyclopiazonic acid (CPA) (Alexis Biochemicals, San Diego, CA), calmidazolium chloride (CMZ) (Sigma), BEL (Sigma), 1,1,1-

trifluoro-2-heptadecanone (PACOCF₃) (Tocris Cookson, Ballwin, MO), propranolol hydrochloride (Sigma), 2-aminoethoxydiphenylborate (2-APB) (Tocris Cookson), AA (Tocris Cookson), and N5-[4-[3,5-bis(trifluoromethyl)-1H-pyrazole-1-yl]phenyl]-4-methyl-1,2,3-thiadiazole-5-carboxamide] (BTP2) (Calbiochem, La Jolla, CA) were dissolved in DMSO as stock solutions. Lysophosphatidylcholine (LPC) (Sigma), lysophosphatidylinositol (LPI) (Sigma), ADP (Sigma), ATP (Sigma), and 9-(tetrahydro-2-furanyl)-9H-purin-6-amine (SQ 22536) (Alexis Biochemicals) were dissolved in deionized water and lysophosphatidic acid (LPA) (Sigma) was dissolved in an equal volume of ethanol/water to make a stock solution and used at concentrations as mentioned in Results. All drugs were added to the experimental saline immediately before use. The final concentration of DMSO never exceeded 0.1%.

iPLA₂ activity assay. *iPLA₂* activity was measured in cerebellar astrocyte culture using cPLA₂ assay kit (Cayman Chemical) as described previously (Smani et al., 2003, 2004). In brief, cells were grown in six-well dishes until 80% confluency. The required wells of the cells were exposed to different treatments, as described in Results, and washed with PBS (in mM: 171 NaCl, 10 Na_2HPO_4 , 3.4 KCl, and 1.8 KH_2PO_4 , pH 7.4) before and after each treatment. All steps were performed on ice. The cells were collected with a scraper and homogenized using mechanical homogenizer in 50 μl (per well) of ice-cold buffer (in mM: 300 sucrose and 10 Tris-HCl, pH 7.0) with protease inhibitor cocktail (Roche, Indianapolis, IN). Cell homogenates were centrifuged at 20,000 $\times g$ for 20 min at 4°C, and the supernatant was collected. Protein content was measured using Bio-Rad (Hercules, CA) reagent, and the *iPLA₂* assay was done on the same day following the instructions of the manufacturer. Supernatants from control cultures and treated cultures, containing the same amount of protein (2–3 mg/ml) in a final volume of 10 μl , were added to microtiter plate wells consisting of 5 μl of a modified assay buffer (300 mM NaCl, 0.5% Triton X-100, 60% glycerol, 4 mM EGTA, 10 mM HEPES at pH 7.4, and 2 mg/ml BSA). The reaction was initiated by the addition of 200 μl of arachidonoyl thiophosphatidylcholine and incubated at 20°C for 60 min. The reaction was terminated by the addition of 10 μl of 5,5'-dithio-bis-2-nitrobenzoic acid for 5 min, and the absorbance was measured at 405 nm in a 96-well microplate reader (Multiscan EX; Thermo Labsystems, Dreieich, Germany). To determine the BEL-sensitive *iPLA₂* activity, the optical density obtained in the presence of BEL was subtracted from all readings, and the resulting optical density was expressed in percentage of respective positive control (see Results).

Statistics. Measurements are expressed as mean values $\pm$ SEM; *n* indicates the number of cells (first value given) and the number of brain slices or culture plates (second value given), from which cells were analyzed (each experiment in acute brain slices was repeated in different animal preparations). All statistical tests were done with either SigmaPlot (Systat Software, Point Richmond, CA) or Origin 7.0 (Microcal Software). Statistical differences were tested using Student's *t* test in Origin 7.0. Means were defined as statistically different at an error probability of **p* < 0.05, ***p* < 0.01, or ****p* < 0.005.

Results

Identification of cell types in acute rat brain slices

We used acute slices of the rat cerebellum to monitor cytosolic Ca²⁺ in somata of Bergmann glia and of astrocytes in the GCL *in situ*. Bergmann glial cells are a special kind of cerebellar astrocytes with their cell bodies grouped around a Purkinje neuron and extend radial or Bergmann fibers toward the pial layer (Fig. 1*A,B*). In mammals, most of the radial glial cells disappear during development; only a vestige persists in some specialized areas, such as Bergmann glia in cerebellar cortex, in which they adapt to perform the local functional requirements (Rakic, 2003). Although Bergmann glial cells are classified as belonging to the astrocyte lineage (Campbell and Götz, 2002), we treated these cells as a distinct glial cell category because of their functional importance in the cerebellum. Bergmann glial cells were readily identified by their location, typical radial morphology, and their ability to generate Ca²⁺ transients during application of ATP or ADP (Kirischuk et al., 1995; Hammer, 2006). After dye loading, the cell body and the radial dendrites of Bergmann glia were clearly visible during their response to ATP (20 μ M for 30 s) (Fig. 1*B,D,E*), whereas Purkinje neurons were hardly stained by the dye. Ca²⁺ responses to ATP indicated the functional status of the intracellular Ca²⁺ stores. Bergmann glial cells, however, often lacked the Ca²⁺ rise in response to K⁺-free saline (0 mM K⁺) typical for astrocytes, which was used to identify astrocytes in the GCL (Fig. 1*C,F,G*) (Dallwig and Deitmer, 2002; Beck et al., 2004).

Application of CPA (20 μ M), an inhibitor of the sarcoendoplasmic Ca²⁺-ATPase, in the absence of external Ca²⁺ evoked a transient cytosolic Ca²⁺ rise (Fig. 1*E,G*), indicative of Ca²⁺ leak from the intracellular Ca²⁺ stores. Readdition of Ca²⁺ in the presence of CPA resulted in another cytosolic Ca²⁺ rise (Fig. 1*E,G*), indicative of SOCE after the depletion of the intracellular Ca²⁺ stores. These Ca²⁺ responses were recorded in both Bergmann glial cells and astrocytes in the GCL.

Store-operated Ca²⁺ entry after Ca²⁺ store depletion by cyclopiazonic acid

First, we studied SOCE as induced by CPA in Bergmann glia and in astrocytes of the GCL with respect to its sensitivity to different drugs (Fig. 2). In Bergmann glia, CPA-induced depletion of Ca²⁺ stores resulted in Ca²⁺ transients during readdition of Ca²⁺ (SOCE), with a mean amplitude of $48 \pm 8\%$ ΔF ($n = 92$ cells/8 slices). This Ca²⁺ transient was significantly reduced to $21 \pm 2\%$ ΔF ($p < 0.05$; $n = 42/3$) by 2-APB (100 μ M), an inhibitor of SOCE channels (Ma et al., 2000; Bakowski et al., 2001; Prakriya and Lewis, 2001; Voets et al., 2001). 2-APB was applied 5 min

Figure 2. SOCE in Bergmann glia and GCL astrocytes. *A, C*, Original recordings of the cytosolic Ca²⁺ concentration in somata of a single Bergmann glial cell (*A*) and in a single GCL astrocyte (*C*). Acute brain slices were exposed to 20 μ M CPA (indicated by arrowhead) for 7 min in Ca²⁺-free condition before 2 mM Ca²⁺ was readded, and the resulting cytosolic Ca²⁺ rise was indicative of SOCE. Traces are for the cells treated with CPA alone, with CPA and drugs added, or preincubated: 100 μ M 2-APB added (arrow), 20 μ M BTP2 incubated for 15 min, 25 μ M BEL preincubated for 30–40 min at 37°C, and 10 μ M PACOCF₃ added 15 min before the readdition of Ca²⁺. Cells only kept in Ca²⁺-free solution for 8–10 min served as control (without CPA, bottom trace). *B, D*, Bar plots showing the average, relative amplitude of the fluorescence signal (ΔF), indicative of cytosolic Ca²⁺ rise after readdition of Ca²⁺. The Ca²⁺ signals (SOCE) were reduced by the SOCE blockers 2-APB and BTP2 and by the iPLA₂ inhibitors BEL and PACOCF₃ in Bergmann glia (*B*) and GCL astrocytes (*D*). The insets in *B* and *D* show Ca²⁺ responses to 20 μ M ATP in a Bergmann glial cell (*B*) and a GCL astrocyte (*D*) after preincubation of slices in BEL to demonstrate functional status of the Ca²⁺ release capacity of the cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ versus CPA-induced SOCE; n indicates the number of cells tested/number of slices.

before the readdition of Ca²⁺ in the presence of CPA (Fig. 2*A,B*). Comparable results were obtained with BTP2 (20 μ M) (Ishikawa et al., 2003; Zitt et al., 2004), a structurally distinct blocker of Ca²⁺ channels that are activated by store depletion. BTP2, preincubated for 15–20 min, significantly reduced SOCE to $12 \pm 1.3\%$ ΔF ($p < 0.01$; $n = 46/3$) (Fig. 2*A,B*). To test whether iPLA₂ plays a role in SOCE in Bergmann glia, the specific, irreversible inhibitor of iPLA₂, BEL (25 μ M) (Ackermann et al., 1995; Winstead et al., 2000), was used. BEL requires the basal activity of iPLA₂ and thus inhibits the enzyme more effectively at physiological temperature (37–40°) than at room temperature (20–22°), when the enzyme activity is low. Moreover, it permeates only slowly into intact cells and hence requires relatively long incubation times (Balsinde et al., 1995). Because the efficacy of BEL to block iPLA₂ is temperature and time dependent, acute brain slices were preincubated in BEL for at least 30 min at 37°C. BEL was removed by washing for 15–20 min before starting the pro-

Figure 3. Calmodulin inhibitor calmidazolium evokes a Ca²⁺ influx in Bergmann glia and GCL astrocytes. **A, C**, Original recordings of the cytosolic Ca²⁺ concentration in somata of a single Bergmann glial cell (**A**) and a single GCL astrocyte (**C**). Acute slices were exposed to 1 μM CMZ (arrowhead) for 7 min in Ca²⁺-free saline before Ca²⁺ was readded (2 mM; filled bar). Traces are for cells treated with CPA alone, with CPA and 100 μM 2-APB added (arrow), with CPA and pretreated with 20 μM BTP2 for 15 min, and with CPA and pretreated with 25 μM BEL for 30–40 min at 37°C. Cells only kept in Ca²⁺-free solution for 8–10 min served as control (without CPA, bottom trace). **B, D**, Bar plots showing the average, relative amplitude of the fluorescence signal (ΔF), indicative of cytosolic Ca²⁺ rise after readdition of Ca²⁺. The Ca²⁺ signals (SOCE) were reduced by the SOCE blockers 2-APB and BTP2 and by the iPLA₂ inhibitor BEL in Bergmann glia (**B**) and in GCL astrocytes (**D**). ***p* < 0.01, ****p* < 0.005 versus CMZ-induced Ca²⁺ influx; *n* indicates the number of cells tested/number of slices.

tol of measuring cytosolic Ca²⁺ changes. In BEL-treated Bergmann glial cells, the mean amplitude of the SOCE was reduced to $4.5 \pm 0.2\%$ ΔF (*n* = 62/6; *p* < 0.01) (Fig. 2*A, B*). In addition to iPLA₂ inhibition, BEL has been shown to inhibit another cellular phospholipase, the Mg²⁺-dependent phosphatidate phosphohydrolase (PAP-1) (Balsinde and Dennis, 1996; Fuentes et al., 2003), which is known to be involved in the synthesis of diacylglycerol. To check the possible role of PAP-1 in BEL-induced inhibition of SOCE, we used propranolol (50–100 μM) to inhibit PAP-1 (Fuentes et al., 2003). The acute slices were pretreated with propranolol for 30 min at room temperature. Propranolol did not inhibit the CPA-induced Ca²⁺ influx in either Bergmann glial cells or astrocytes of the GCL (data not shown), which indicates that PAP-1 is not involved in SOCE regulation in cerebellar astrocytes.

BEL has also been reported to induce cell death by inhibiting endoplasmic iPLA₂ (Cummings et al., 2002) or PAP-1 (Fuentes et

al., 2003). After BEL treatment, Bergmann glial cells still generated Ca²⁺ transients in response to ATP (20 μM, 30 s), which evokes IP₃-mediated Ca²⁺ release from ER stores (Shao and McCarthy, 1995) (Fig. 2*B*, inset). This indicates the functional status of the Ca²⁺ stores, ruling out the possibility that BEL, at the concentration used here, caused cell death in Bergmann glia. We also used a second iPLA₂ blocker, PACOCF₃ (10 μM), which belongs to a different chemical family than BEL and is known to inhibit iPLA₂ with an IC₅₀ value of 3.8 μM (Ackermann et al., 1995), to further confirm the involvement of iPLA₂ in regulating SOCE. The Ca²⁺ transient during Ca²⁺ readdition in the presence of CPA was significantly reduced to $5.0 \pm 0.8\%$ ΔF (*n* = 73/6; *p* < 0.01) (Fig. 2*A, B*) by PACOCF₃ (Fig. 2*A, B*). In the presence of BEL or PACOCF₃, the Ca²⁺ transient during Ca²⁺ readdition was even smaller than in control cells (the mean amplitude being $10.2 \pm 1\%$ ΔF; *p* < 0.05; *n* = 52/5), which were not exposed to CPA but left for the same time period in Ca²⁺-free solution. This suggests that some iPLA₂ activity is present even when Ca²⁺ stores are not depleted by CPA (Fig. 2*B*).

Like in Bergmann glial cells, readdition of Ca²⁺ induced an influx of Ca²⁺ in GCL astrocytes in the presence of CPA (Fig. 2*C, D*). During readdition of Ca²⁺ to the extracellular solution, cytosolic Ca²⁺ increased by $70 \pm 8\%$ ΔF (*n* = 62/8). This SOCE was significantly reduced by 2-APB (100 μM) to $32 \pm 2.5\%$ ΔF (*p* < 0.01; *n* = 31/4) and by BTP2 (20 μM) to $13 \pm 1\%$ ΔF (*p* < 0.005; *n* = 22/3). Preincubation with BEL or PACOCF₃ strongly inhibited SOCE to $5.0 \pm 0.5\%$ ΔF (*p* < 0.005; *n* = 57/6) and to $8 \pm 1\%$ ΔF (*p* < 0.005; *n* = 53/6), respectively, indicating that iPLA₂ is essential for the activation of SOCE (Fig. 2*C, D*). The PAP-1 inhibitor propranolol (50–100 μM) did not affect SOCE induced

in the presence of CPA (data not shown). GCL astrocytes responded to ATP (20 μM, 30 s) with a Ca²⁺ transient after BEL treatment, which indicated the functional Ca²⁺ stores in the cells (Fig. 2*D*, inset). In control cells, readdition of external Ca²⁺ induced a Ca²⁺ transient of $22 \pm 1.3\%$ ΔF (*p* < 0.005; *n* = 59/5), which was still larger than in cells treated with BEL or PACOCF₃ (*p* < 0.01) (Fig. 2*C, D*). These results suggest that iPLA₂ activation by depletion of Ca²⁺ stores in both Bergmann glia and GCL astrocytes is required for the SOCE in these cells. Thus, similar results were obtained in experiments using either BEL or PACOCF₃ to inhibit iPLA₂ activity, and, for the following experiments, BEL was chosen as potent iPLA₂ inhibitor.

Ca²⁺ influx induced by calmidazolium

After obtaining evidence for a role of iPLA₂ in CPA-induced Ca²⁺ influx, we wanted to know whether the activation of iPLA₂ itself, without previous Ca²⁺ store depletion, induces influx of

Ca²⁺ in Bergmann glial cells and GCL astrocytes. The activity of iPLA₂ is regulated by CaM, which binds to the C terminal of the enzyme and thereby inhibits its activity (Wolf and Gross, 1996a,b; Wolf et al., 1997). We used the CaM antagonist CMZ at a concentration of 1 μ M to dissociate CaM from iPLA₂. At this concentration, CMZ does not cause release of Ca²⁺ from stores itself (Smani et al., 2004), which was confirmed in the present study by comparing the cytosolic Ca²⁺ response induced by ADP (20 μ M) in untreated cells and cells treated with 1 μ M CMZ. No significant difference in the amplitude of the Ca²⁺ transients evoked by ADP was found in untreated ($n = 24/3$) and CMZ-treated ($n = 29/3$) cells (data not shown), indicating that CMZ does not affect the filling state of the Ca²⁺ stores.

CMZ was applied in the absence of external Ca²⁺, and a Ca²⁺ transient was recorded in Bergmann glial cells, when Ca²⁺ was readmitted to the extracellular solution (Fig. 3A). This Ca²⁺ transient was similar to that induced by the depletion of Ca²⁺ stores by CPA and had an amplitude of $39 \pm 4\%$ ΔF ($n = 61/7$) (Fig. 3B). To determine the mechanism by which CMZ activates Ca²⁺ influx in these cells, we examined the effect of SOCE channel inhibitors 2-APB (100 μ M) and BTP2 (20 μ M) on the Ca²⁺ rise in the presence of CMZ. In Bergmann glia, this Ca²⁺ rise was reduced to $13 \pm 1.3\%$ ΔF in the presence of 2-APB ($p < 0.005$; $n = 40/3$), and pretreatment of the slices with BTP2 for 15 min decreased this Ca²⁺ rise to $11 \pm 1.2\%$ ΔF ($p < 0.005$; $n = 25/3$). The iPLA₂ inhibitor BEL reduced the Ca²⁺ rise in CMZ even to $1.0 \pm 0.1\%$ ΔF ($p < 0.005$; $n = 39/3$) compared with the untreated control slices, in which the Ca²⁺ rise was $10 \pm 1\%$ ΔF ($p < 0.005$; $n = 52/5$) (Fig. 3A,B).

CMZ has been shown to inhibit the activity of adenylyl cyclase (AC), albeit at greater concentrations than those used in the present study (Haunso et al., 2003). We tested whether the CMZ-induced augmentation of the Ca²⁺ transient during Ca²⁺ readmission can be mimicked by the inhibition of AC activity. We used the AC inhibitor SQ 22536 (200 μ M) in the absence of CMZ for 15 min before reading Ca²⁺. Ca²⁺ readmission resulted in a Ca²⁺ influx with a mean amplitude of $13 \pm 0.9\%$ ΔF ($n = 42/5$; data not shown), which was not significantly different from control slices (same treatment except without SQ 22536). Hence, inhibition of AC does not increase the Ca²⁺ rise after readmission of external Ca²⁺, as CMZ does.

In GCL astrocytes, the Ca²⁺ rise in the presence of CMZ decreased from $78 \pm 8\%$ ΔF ($n = 61/5$) to $35 \pm 6.5\%$ ΔF in the presence of 2-APB ($p < 0.01$; $n = 36/4$) and to $25 \pm 2\%$ ΔF in the presence of BTP2 ($p < 0.005$; $n = 25/3$). Preincubation of BEL almost completely blocked the Ca²⁺ rise in CMZ to a mean

Figure 4. Lysophospholipids evoke Ca²⁺ influx in Bergmann glia. **A**, Original recordings of cytosolic Ca²⁺ concentration in soma of a single Bergmann glial cell. LPI at 250 nM was added in Ca²⁺-free saline (arrowhead) 3 min before Ca²⁺ (2 mM) was readmitted. Traces are for the cells exposed to LPI alone, to LPI and pretreated with 25 μ M BEL for 30–40 min at 37°C, to LPI and 100 μ M 2-APB added before (arrow), and to LPI and pretreated with 20 μ M BTP2 for 15 min. Cells only kept in Ca²⁺-free solution for 8–10 min served as control (without LPI, bottom trace). **B**, Bar plot showing the average, relative amplitude of the Ca²⁺ rise after readdition of Ca²⁺ in Bergmann glial cells. The LPI-induced Ca²⁺ influx was not affected by the iPLA₂ inhibitor BEL but suppressed by SOCE blockers 2-APB and BTP2. **C**, Bar plot showing the average, relative amplitude of the Ca²⁺ rise in the presence of 250 nM LPC after readdition of Ca²⁺; LPC-induced Ca²⁺ influx was not affected by the iPLA₂ inhibitor BEL but reduced by the SOCE blockers 2-APB and BTP2. **D**, Comparison of the Ca²⁺ rise after readdition of Ca²⁺ in the presence of 250 nM LPI, LPC, or LPA and without lysophospholipids as control. LPI and LPC, but not LPA, induced a significant Ca²⁺ influx. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ versus LPI/LPC-induced Ca²⁺ influx; n indicates the number of cells tested/number of slices.

amplitude of $2.5 \pm 0.4\%$ ΔF ($p < 0.005$; $n = 27/3$). This was significantly smaller than in control cells ($p < 0.005$), in which the readdition of Ca²⁺ induced a Ca²⁺ rise of $22 \pm 1.3\%$ ΔF ($p < 0.005$; $n = 59/5$) (Fig. 3C,D). When the adenylyl cyclase inhibitor SQ 22536 was present, Ca²⁺ readmission evoked a Ca²⁺ influx with a mean amplitude of $26 \pm 1\%$ ΔF ($n = 44/5$; data not shown), which was not significantly different from control cells. Our experiments show that inhibiting calmodulin with CMZ induces a Ca²⁺ influx that is dependent on the activation of iPLA₂ and SOCE channels. Furthermore, these results indicate that CMZ activates a Ca²⁺ influx even when intracellular Ca²⁺ stores are not depleted.

Figure 5. Lysophospholipids evoke Ca²⁺ influx in GCL astrocytes. **A**, Original recordings of cytosolic Ca²⁺ concentration in soma of a single GCL astrocyte. LPI at 250 nM was added in Ca²⁺-free saline (arrowhead) 3 min before Ca²⁺ (2 mM) was readded. Traces are for the cells exposed to LPI alone, to LPI and pretreated with 25 μM BEL for 30–40 min at 37°C, to LPI and 100 μM 2-APB added before (arrow), and to LPI and pretreated with 20 μM BTP2 for 15 min. Cells only kept in Ca²⁺-free solution for 8–10 min served as control (without LPI, bottom trace). **B**, Bar plot showing the average, relative amplitude of the Ca²⁺ rise after readdition of Ca²⁺ in GCL astrocytes. The LPI-induced Ca²⁺ influx was not affected by the iPLA₂ inhibitor BEL but was suppressed by SOCE blockers 2-APB and BTP2. **C**, Bar plot showing the average, relative amplitude of the Ca²⁺ rise in the presence of 250 nM LPI after readdition of Ca²⁺; LPC-induced Ca²⁺ influx was not affected by iPLA₂ inhibitor BEL but was reduced by SOCE blockers 2-APB and BTP2. **D**, Comparison of the Ca²⁺ rise after readdition of Ca²⁺ in the presence of 250 nM LPI, LPC, or LPA and without lysophospholipids as control. LPI and LPC, but not LPA, induced a significant Ca²⁺ influx. **p* < 0.05, ***p* < 0.01, ****p* < 0.005 versus LPI/LPC-induced Ca²⁺ influx; *n* indicates the number of cells tested/number of slices.

Ca²⁺ influx induced by lysophospholipids

Because the activation of iPLA₂ releases lysophospholipids and a free fatty acid (AA), we tested whether these products can lead to Ca²⁺ influx into astrocytes. We used three different lysophospholipids: LPI (250 nM), LPC (250 nM), and LPA (250 nM). In Ca²⁺-free saline, all three lysophospholipids were added 3 min before readdition of Ca²⁺ to the extracellular solution. LPC and LPI induced a larger Ca²⁺ rise during Ca²⁺ readdition in Bergmann glia (Fig. 4) and GCL astrocytes (Fig. 5), whereas Ca²⁺ rises in the presence of LPA were not significantly changed compared with those in control experiments in the absence of lysophospholipids (Figs. 4D, 5D). Notably, both LPI and LPC still induced augmented Ca²⁺ rises after incubation of the slices with BEL (Figs. 4A–C, 5A–C). Because LPI and LPC are products of iPLA₂ activity and appear downstream of iPLA₂ activity, this indicates that BEL does not suppress Ca²⁺ influx per se. Moreover, this

suggests that iPLA₂, by producing lysophospholipids, promotes Ca²⁺ influx.

In Bergmann glia, the Ca²⁺ rise during Ca²⁺ readdition had an amplitude of 42 ± 6% Δ*F* (*n* = 54/5) in the presence of added LPI. This Ca²⁺ rise was reduced to 16 ± 2.7% Δ*F* (*n* = 51/4) and 13 ± 2.3% Δ*F* (*n* = 28/3) in the presence of 100 μM 2-APB and after preincubation of 20 μM BTP2, respectively (*p* < 0.05) (Fig. 4A,B). The mean amplitude of the Ca²⁺ rise in the presence of added LPC was 36 ± 3.7% Δ*F* (*n* = 89/7), which was reduced to 11 ± 1.3% Δ*F* (*n* = 72/4) and 12 ± 1.3% Δ*F* (*n* = 32/3) by 2-APB and BTP2, respectively (*p* < 0.01) (Fig. 4C). The Ca²⁺ rise in the presence of LPA was 15 ± 1.6% Δ*F* (*n* = 67/5) and was not significantly different from control cells without any added lysophospholipids, under which condition the Ca²⁺ rose by 9 ± 1% Δ*F* (*n* = 32/5) (Fig. 4D).

In GCL astrocytes, the Ca²⁺ rise during Ca²⁺ readdition in the presence of LPI had an amplitude of 58 ± 6.2% Δ*F* (*n* = 61/5). This Ca²⁺ rise was inhibited to 27 ± 4.4% Δ*F* by 2-APB (*p* < 0.05; *n* = 48/4) and 25 ± 2.1% Δ*F* after preincubation with BTP2 (*p* < 0.05; *n* = 31/3) (Fig. 5A,B). The mean amplitude of this Ca²⁺ rise in the presence of LPC was 53 ± 7% Δ*F* (*n* = 89/7) and was reduced to 26 ± 4 and 22 ± 2.5% Δ*F* by 2-APB (*n* = 67/4) and BTP2 (*n* = 35/3), respectively (*p* < 0.05) (Fig. 5C). In the presence of LPA, the Ca²⁺ rise had an amplitude of 24 ± 1.7% Δ*F* (*n* = 67/6), which was not significantly different from that in control cells, which was 23 ± 2.5% Δ*F* (*n* = 43/5) (Fig. 5D). These results show that LPI and LPC, but not LPA, induce a Ca²⁺ influx mediated by 2-APB- and BTP2-sensitive ion channels.

We then asked whether the Ca²⁺ influx after Ca²⁺ readdition uses the same pathway after depleting the stores with CPA or with added LPI and LPC. Therefore, we tested whether LPI and LPC led to an additional Ca²⁺ influx after inducing SOCE in CPA.

After depletion of Ca²⁺ stores by CPA, readdition of external Ca²⁺ evoked a Ca²⁺ increase (Fig. 6A–D), as described above (Fig. 2). In the continued presence of CPA and external Ca²⁺, LPI and LPC, both at 250 nM, were added (Fig. 6A,C, arrows). We observed no additional Ca²⁺ rise by either lysophospholipid in Bergmann glia (Fig. 6A,B) or in GCL astrocytes (Fig. 6C,D), suggesting that CPA- and lysophospholipid-induced Ca²⁺ influx are not additive.

Ca²⁺ influx induced by arachidonic acid

Because iPLA₂-mediated hydrolysis of membrane phospholipids also generates AA, we also examined the effect of AA, which has been demonstrated to activate Ca²⁺ influx through arachidonate-regulated channels in a variety of different cell types, which is clearly distinct from SOCE (Shuttleworth and

Thompson, 1999). Acute slices were exposed to AA (10 μ M) in Ca²⁺-free solution 3 min before external Ca²⁺ was readdd (Fig. 7A). In GCL astrocytes, AA induced a significant increase of the Ca²⁺ rise after readdition of Ca²⁺, which was often oscillatory and had a mean maximal amplitude of $48 \pm 5\%$ ΔF ($p < 0.05$; $n = 53/6$). In comparison, the Ca²⁺ influx in control cells without added AA had an amplitude of $23.5 \pm 2\%$ ΔF ($n = 38/5$). The larger Ca²⁺ rise in the presence of AA was not significantly changed in the presence of 2-APB ($n = 47/6$) (Fig. 7A,B), indicating that AA activated Ca²⁺ channels, which were insensitive to 2-APB in contrast to those activated in the presence of CPA, CMZ, and lysophospholipids. The Ca²⁺ rise in Bergmann glia in the presence of AA was not significantly larger compared with control experiments without AA (Fig. 7C), although their mean amplitude was $17 \pm 3.3\%$ ΔF ($n = 36/4$) compared with $8 \pm 1.2\%$ ΔF ($n = 23/3$) in control cells. 2-APB had no effect on the Ca²⁺ rise in the presence of AA.

BEL-sensitive SOCE and iPLA₂ activity in cultured astrocytes

To confirm the involvement of iPLA₂ in SOCE in cerebellar astrocytes, we wanted to measure the activity of iPLA₂ under store-depleted condition and after inhibition of CaM, in astrocyte culture. First, we established that the Ca²⁺ rises during readdition of external Ca²⁺ in the presence of CPA and CMZ were sensitive to the iPLA₂ inhibitor BEL (Fig. 8A–C). Addition of CPA (10 μ M) in Ca²⁺-free saline evoked a Ca²⁺ transient in all cells. During readdition of external Ca²⁺ after store depletion by CPA, a Ca²⁺ rise, indicative of SOCE, was measured with a mean amplitude of $75 \pm 4\%$ ΔF ($n = 150/5$). This Ca²⁺ rise was much larger than in control cells ($12 \pm 0.5\%$ ΔF ; $p < 0.005$; $n = 163/5$), in which the cells were not exposed to CPA. In astrocyte cultures pretreated with 25 μ M BEL, the SOCE induced in the presence of CPA was reduced to $4 \pm 0.9\%$ ΔF ($p < 0.005$; $n = 141/5$) (Fig. 8A,C). In the presence of 1 μ M CMZ, the cytosolic Ca²⁺ rose to $87 \pm 7\%$ ΔF ($n = 132/5$), which was reduced to $4 \pm 0.8\%$ ΔF ($p < 0.005$; $n = 146/5$) after preincubation with BEL (Fig. 8B,C). Again, the Ca²⁺ rise induced by CMZ was significantly smaller after BEL treatment than the Ca²⁺ rise in control cells without CMZ ($p < 0.005$).

We determined BEL-sensitive iPLA₂ activity in cerebellar astrocyte cultures by absorbance measurements using an iPLA₂ activity assay (see Materials and Methods). Maximal iPLA₂ activity (100%) was measured in the presence of 10 mM EGTA to suppress all Ca²⁺/calmodulin-dependent iPLA₂ inhibition. Basal iPLA₂ activity was $4 \pm 1.4\%$ of the maximal iPLA₂ activity as measured in cell homogenates from untreated cerebellar astro-

A Bergmann glia

C Astrocytes in GCL

B

D

Figure 6. Ca²⁺ influx induced by lysophospholipids (LPL) is not additive to SOCE in Bergmann glia and GCL astrocytes. **A, C**, Original recordings of the cytosolic Ca²⁺ in the soma of a single Bergmann glial cell (**A**) and in a single GCL astrocyte (**C**) as induced after depleting the intracellular Ca²⁺ stores with CPA (20 μ M) and addition of lysophospholipids (LPL, arrow) after readdition of Ca²⁺ (2 mM). Traces are for the cells exposed to CPA and then LPI, to CPA and then LPC, and to CPA alone (bottom trace, control). **B, D**, Bar plots showing the average, relative amplitude of the Ca²⁺ rise just after lysophospholipids addition in 2 mM Ca²⁺ in Bergmann glia (**B**) and GCL astrocytes (**D**). The lysophospholipids did not evoke a Ca²⁺ influx additional to the SOCE in CPA. The number of cells tested/number of slices is indicated by n in the bars.

cytes (Fig. 8D). In astrocytes treated with 10 μ M CPA (10 min at 37°C) or 1 μ M CMZ (10 min at 37°C), the iPLA₂ activity increased to 62 ± 1.2 and $55 \pm 3.0\%$ of the maximal iPLA₂ activity, respectively. These results suggest that iPLA₂ activity is increased under conditions when SOCE is activated in cerebellar astrocytes.

Antisense inhibition of iPLA₂

In another approach to examine the role of iPLA₂ in SOCE, we used antisense technology to knock down the expression of iPLA₂. Cerebellar astrocyte cultures were transfected with S-ODN and AS-ODN of iPLA₂-A isoforms using a Lipofectamine-assisted transfection protocol (Balsinde et al., 1997; Smani et al., 2003). Control cells were exposed to the transfection protocol without ODNs to check whether LF alone had any nonspecific effects. Cultured astrocytes were loaded with

Figure 7. AA evokes a 2-APB-insensitive Ca²⁺ influx in GCL astrocytes and Bergmann glia. **A**, Original recording of the cytosolic Ca²⁺ concentration in GCL astrocytes in response to AA. Acute slices were exposed to 10 μM AA (arrowhead) for 3 min in Ca²⁺-free solution before 2 mM Ca²⁺ was readded. Traces are for the cells exposed to AA alone with different Ca²⁺ response patterns (top 2 traces) and with AA together with 100 μM 2-APB added before (arrow). Cells only kept in Ca²⁺-free solution for 8–10 min served as control (without AA, bottom trace). **B**, **C**, Bar plots of the average, relative amplitude of the Ca²⁺ rise after Ca²⁺ readdition in the presence of AA alone, with 2-APB added and in control cells in GCL astrocytes (**B**) and in Bergmann glia (**C**). AA-induced Ca²⁺ influx was not blocked by 2-APB, suggesting that AA activated a SOCE-independent pathway for Ca²⁺ influx. **p* < 0.05 versus AA-induced Ca²⁺ rise versus control; *n* indicates the number of cells tested/number of slices.

fura-2, and the fluorescence ratio at 340 and 380 nm excitation was measured, indicative of the Ca²⁺ concentration in the cells. First, we used ATP (20 μM, 20 s) to check the functional status of the Ca²⁺ stores. ATP induced a similar Ca²⁺ rise in LF-treated and S-ODN-transfected cells, with mean amplitudes of 0.55 ± 0.13 ratio units (*n* = 176/6) and 0.59 ± 0.12 ratio units (*n* = 142/8) (Fig. 9A,C), respectively. In AS-ODN-transfected cells, the Ca²⁺ transient induced by ATP was strongly reduced to 0.17 ± 0.08 ratio units (*p* < 0.001; *n* = 153/8) (Fig. 9B,C), possibly reflecting the impaired filling status of the intracellular Ca²⁺ stores, when iPLA₂ expression was inhibited. CPA (10 μM) was then applied in the absence of extracellular Ca²⁺ for 7 min, which led to a slow transient Ca²⁺ rise in all cells. The absolute mean amplitude of Ca²⁺ transient after the addition of CPA in LF-treated cells was 0.16 ± 0.03 ratio units (*n* = 176/6), which was similar to that of S-ODN-treated cells, being 0.15 ± 0.03 ratio units (*n* = 142/8). The AS-ODN-transfected cells showed a reduced Ca²⁺ transient during CPA addition of 0.08 ± 0.04 ratio units (*p* < 0.001; *n* = 153/8) (Fig. 9B,C), suggesting reduced Ca²⁺ release from the stores, possibly attributable to an incomplete filling status of the Ca²⁺ stores after interference with iPLA₂ expression.

Because the Ca²⁺ store content appeared to be reduced by AS-ODN, we wanted to check whether this reduction might be attributable to dysregulated ER-Ca²⁺ homeostasis. The decay time constant τ of the Ca²⁺ transients evoked by added ATP was analyzed in the S-ODN- and AS-ODN-treated cells. In S-ODN-treated astrocytes, τ was 10 ± 3.8 s (*n* = 62), which was not

significantly different from that in AS-ODN-treated cells, with a τ of 11 ± 5.7 s (*n* = 51; data not shown); this finding is in line with an unimpaired ER homeostasis in AS-ODN-treated cells. We also measured the basal Ca²⁺-dependent fura-2 ratio level in S-ODN- and AS-ODN-transfected cells to get an indication of a change in resting cytosolic Ca²⁺ level after inhibiting iPLA₂. The basal ratio was 0.48 ± 0.03 (*n* = 142/8) in S-ODN-transfected cells, which was indeed significantly different from the AS-ODN-transfected cells, with a ratio of 0.41 ± 0.03 (*p* < 0.001; *n* = 153/8; data not shown), suggesting that the reduced cytosolic Ca²⁺ level in the AS-ODN-transfected cells might be attributable to the impaired iPLA₂ activity.

During readdition of Ca²⁺, the Ca²⁺ rise was similar in LF-treated cells and cells transfected with the S-ODN, with a mean amplitude of 0.30 ± 0.06 ratio units (*n* = 176/6) and 0.33 ± 0.09 ratio units (*n* = 142/8), respectively. There was a significant reduction, however, in the Ca²⁺ transient evoked by Ca²⁺ readdition in AS-ODN-transfected cells to 0.16 ± 0.07 ratio units (*p* < 0.001; *n* = 153/8) (Fig. 9B,C). Hence, only transfection with iPLA₂ antisense-ODN, but not sense-ODN or LF alone, affected SOCE in cultured astrocytes, indicating a crucial role of iPLA₂ for generating SOCE.

To ascertain the effective knockdown of iPLA₂ expression in AS-ODN-transfected cells, we labeled the cells with anti-iPLA₂ antibody and measured the relative fluorescence. Antibody staining of all transfected cells (LF, S-ODN, and AS-ODN) was performed 48 h after transfection. All cells were processed in parallel with the same protocol, and the anti-iPLA₂ immunoreactivity was assessed under the same settings of the confocal microscope. The relative fluorescence intensity of iPLA₂ expression was measured only in cells labeled with fluorescein, indicating successful transfection, and is given in arbitrary units (AU). In LF-treated and S-ODN-transfected cells, the mean fluorescence was 110 ± 1.5 AU (*n* = 324) (Fig. 9D,G) and 130 ± 1.6 AU (*n* = 330) (Fig. 9E,G), respectively. In AS-ODN-treated cells, the fluorescence intensity was significantly reduced to 69 ± 1.5 AU (*p* < 0.001; *n* = 509) (Fig. 9F,G), consistent with a reduction of iPLA₂ expression in AS-ODN treated cells. The background fluorescence was measured to be 17 ± 0.8 AU (*p* < 0.001; *n* = 290) (Fig. 9G) in control cells, which were stained only with the secondary antibody.

Spontaneous Ca²⁺ oscillations in rat cerebellum

If SOCE was indeed instrumental for the refilling of intracellular Ca²⁺ stores, suppression of SOCE would be expected to affect spontaneous Ca²⁺ oscillations (Nett et al., 2002; Zur Nieden and Deitmer, 2006). We therefore examined the effects of two substances, known to inhibit SOCE, on spontaneous Ca²⁺ oscillations in astrocytes *in situ*. Bergmann glia and GCL astrocytes showed spontaneous activity in cerebellar brain slices (Fig. 10A,B), although Bergmann glial cells generated Ca²⁺ oscilla-

tions less frequently than GCL astrocytes. We analyzed the relative number of cells with spontaneous Ca²⁺ oscillations and the frequency of the Ca²⁺ oscillations. Over 20 min, spontaneous Ca²⁺ signals were recorded in the standard solution as control. The experimental protocol continued for another 20 min after the SOCE channel blocker 2-APB was applied. Ca²⁺ signals were analyzed for at least 15 min, leaving the first 5 min to allow the drugs to work. Spontaneous Ca²⁺ responses could be recorded in 28% ($n = 87$ of 313/31) of the cells identified as Bergmann glia and in 47% ($n = 107$ of 227/27) of the GCL astrocytes. The overall frequency of spontaneous Ca²⁺ transients averaged 0.17 ± 0.06 transients/min ($n = 87/31$) in Bergmann glial cells and 0.40 ± 0.07 transients/min ($n = 107/27$) in GCL astrocytes.

Inhibiting iPLA₂ by preincubation with BEL for 30–40 min, as described above, decreased the frequency of the Ca²⁺ signals in GCL astrocytes (Fig. 10A) on average to 40% of the control, whereas the number of spontaneously active cells decreased to 46% (Fig. 10B,C). In Bergmann glial cells, the number of active cells decreased to 28%, and the frequency of Ca²⁺ signals decreased to 39% of the control.

In the presence of 2-APB, Ca²⁺ signals were observed in only 58% of the Bergmann glial cells and 62% of the GCL astrocytes that exhibited spontaneous Ca²⁺ oscillations under control conditions (Fig. 10B). The frequency of the Ca²⁺ oscillations was reduced to 49% ($n = 17/11$) in Bergmann glia and to 55% ($n = 24/11$) in GCL astrocytes in the presence of 2-APB (Fig. 10C). These results suggest that the functional activity of iPLA₂ and 2-APB-sensitive SOCE channels are required for maintaining spontaneous Ca²⁺ oscillations in Bergmann glia and GCL astrocytes at their normal level.

Discussion

The present study provides evidence that SOCE in rat cerebellar astrocytes *in situ* is mediated by the activation of iPLA₂. We showed here the following: (1) depletion of intracellular Ca²⁺ stores by CPA activates Ca²⁺ influx (SOCE), which can be suppressed by inhibiting iPLA₂ activity by BEL and PACOCF₃; (2) inhibiting calmodulin by CMZ to relieve the calmodulin-dependent block of iPLA₂ induces a Ca²⁺ influx, which shares the pharmacological profile of SOCE as obtained in CPA; (3) lysophospholipid products of iPLA₂, LPI and LPC, but not LPA, evoke a Ca²⁺ influx, which could be suppressed by SOCE channel blockers; (4) depletion of Ca²⁺ stores by CPA and inhibition of calmodulin by CMZ increased BEL-sensitive iPLA₂ enzyme activity in cultured cerebellar astrocytes; and (5) the specific antisense inhibition of iPLA₂ reduced SOCE. Finally, the SOCE channel blocker 2-APB and the iPLA₂ inhibitor BEL reduced spontaneous Ca²⁺ oscillations in astrocytes *in situ*, indicating functional significance of iPLA₂-mediated SOCE for normal

Figure 8. CPA- and CMZ-related Ca²⁺ influx and iPLA₂ in cerebellar astrocyte culture. **A, B**, Original recordings of the cytosolic Ca²⁺ concentration in single, cultured astrocytes in the presence of CPA or CMZ. Astrocytes were exposed to either 10 μM CPA (**A**) or 1 μM CMZ (**B**) (indicated by arrowhead) for 7 min in Ca²⁺-free condition before 2 mM Ca²⁺ was readmitted. Traces are for the cells exposed to CPA or CMZ alone and to CPA or CMZ in cells pretreated with 25 μM BEL for 30 min at 37°C. Cells only kept in Ca²⁺-free solution for 8–10 min served as control (bottom trace). **C**, Bar plot of the average, relative amplitude of Ca²⁺ rise after readdition of Ca²⁺ as obtained from experiments as shown in **A** and **B**, showing that BEL suppressed the influx of Ca²⁺ in the presence of either CPA or CMZ. **D**, Bar plot showing the percentage of BEL-sensitive iPLA₂ enzyme activity as induced by either CPA or CMZ relative to the value in cells treated with 10 mM EGTA (100%), as obtained in the *in vitro* enzyme assay. * $p < 0.05$ versus 10 mM EGTA; *** $p < 0.005$ versus CPA/CMZ-related Ca²⁺ influx; n indicates the number of astrocytes/number of culture plates.

Ca²⁺ signaling in astrocytes. This is the first study demonstrating the involvement of iPLA₂ activity for SOCE in acute tissue slices and in glial cells *in situ*.

CPA-induced SOCE requires the functional activity of iPLA₂ in astrocytes

The possible link of iPLA₂ activation to store depletion was first reported in rat smooth muscle cells (Wolf et al., 1997) and pancreatic islets (Nowatzke et al., 1998), in which the depletion of intracellular Ca²⁺ stores resulted in an increased iPLA₂-mediated phospholipid hydrolysis and accumulation of free fatty acids. According to the proposed molecular mechanism for cultured smooth muscle cells, the generated lysophospholipids activate SOCE channels and capacitative Ca²⁺ influx, leading to the refilling of Ca²⁺ stores (Smani et al., 2004).

In rat brain, >70% of the PLA₂ activity could be attributed to iPLA₂ (Yang et al., 1999). iPLA₂ was detected in the cerebellar cortex in all cell layers by means of *in situ* hybridization and antibody staining (Shirai and Ito, 2004). In our study, the activation of SOCE by store depletion appeared to be attributable to stimulation of iPLA₂, because iPLA₂ impairment by the iPLA₂ inhibitors BEL and PACOCF₃ completely prevented Ca²⁺ influx in response to CPA in acute brain slices and in culture (BEL), as

Figure 9. Antisense inhibition of iPLA₂ reduced the CPA-related Ca²⁺ influx. **A, B**, Original recordings of the cytosolic Ca²⁺ concentration in single, cultured, transfected astrocytes in the presence of CPA. Astrocytes were briefly exposed to 20 μ M ATP (20 s) to check functional status of Ca²⁺ stores, before 10 μ M CPA was added for 7 min in Ca²⁺-free condition, followed by readdition of Ca²⁺ (2 mM). Traces in **A** are for the cells transfected with S-ODN of iPLA₂ and LF alone. Traces in **B** show cells transfected with AS-ODN with two different response patterns. **C**, Bar plot of the average amplitude of Ca²⁺ transients evoked by ATP and CPA and of the Ca²⁺ rise after the readdition of Ca²⁺ as obtained from experiments as shown in **A** and **B**. **D–G**, Labeling of transfected astrocytes with anti-iPLA₂ antibody, showing similar iPLA₂ expression in LF-treated cells (**D**) and in cells treated with S-ODN (**E**). AS-ODN-treated cells showed reduced iPLA₂ expression (**F**). **G**, Bar plot of the relative fluorescence in AU in transfected cells as shown in **D–F**. Control represents the background fluorescence from astrocytes labeled only with secondary antibody. *** $p < 0.005$; n indicates the number of transfected astrocytes investigated. Scale bars, 25 μ m.

outlined in the hypothetical diagram in supplemental Figure 1 (available at www.jneurosci.org as supplemental material). Moreover, iPLA₂ activity increased when ER stores had been depleted in astrocyte culture. Importantly, the specific iPLA₂ AS-ODN impaired SOCE, presumably by a reduction of iPLA₂ expression in the cells. Notably, the CPA-mediated Ca²⁺ influx in the presence of BEL or PACOCF₃ was even smaller than the Ca²⁺ influx in control cells, in which Ca²⁺ stores were not depleted by CPA. This suggests some constitutive activity of iPLA₂, which might be independent of the filling state of intracellular Ca²⁺ stores. The Ca²⁺ influx evoked by lysophospholipids in BEL-treated brain slices indicated that BEL did not suppress Ca²⁺ influx per se and presumably did not affect SOCE directly. This drug has also been reported to inhibit PAP-1, but the CPA-mediated Ca²⁺ entry was not prevented by the PAP-1 inhibitor propranolol in our study, indicating that the BEL-induced inhibition of SOCE was not attributable to suppressing PAP-1 activity. Furthermore, ATP-induced Ca²⁺ transients in BEL-pretreated astrocytes demonstrated intact Ca²⁺ stores, suggesting that BEL did not induce conditions with impaired ER integrity or apoptosis, under our experimental conditions.

In cells not challenged with thapsigargin or CPA, CaM binds to iPLA₂ and thereby suppresses the activity of this enzyme. After the dissociation of CaM, iPLA₂ becomes functionally active (Wolf and Gross, 1996a,b). In cultured smooth muscle cells, the CaM inhibitor CMZ induces Ca²⁺ release as well as 2-APB-insensitive Ca²⁺ influx at concentrations larger than 1 μ M, whereas lower doses of CMZ (≤ 1 μ M) only evoke 2-APB-

sensitive Ca²⁺ entry (Smani et al., 2004). In HeLa cells, CMZ at concentrations larger than 1 μ M induced Ca²⁺ entry that involves a non-SOCE pathway and was sensitive to AA (Peppiatt et al., 2004). In the present study, we used 1 μ M CMZ to evoke Ca²⁺ influx in acute brain slices and found that CMZ induced a Ca²⁺ influx without depleting the Ca²⁺ stores. This Ca²⁺ influx is sensitive to 2-APB and BTP2 and is suppressed after inhibiting iPLA₂. Because the Ca²⁺ signals induced by CPA-mediated store depletion and by CMZ-mediated CaM inhibition exhibit the same pharmacological profile, we suggest a similar signaling pathway in glial cells as reported for smooth muscle cells (Smani et al., 2004) (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Hence, store depletion as well as CMZ would release the inhibitory interaction of CaM on iPLA₂ and then allow SOCE. This is supported by the enhanced enzyme activity of iPLA₂ in cerebellar astrocyte culture after store depletion by CPA and after inhibiting CaM with CMZ.

Activation of SOCE channel appears to be mediated by the products of iPLA₂ because both lysophospholipids LPC and LPI activated a SOCE-like Ca²⁺ influx. LPA did not generate a Ca²⁺ influx, which may be attributable to the lack of a large head group in LPA, in contrast to LPI and LPC. Suppression of iPLA₂ activity by BEL

did not affect the LPI- and LPC-induced Ca²⁺ influx, in line with the hypothesis that the lysophospholipid-mediated activation of Ca²⁺ entry is downstream to iPLA₂ activation (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Moreover, our results suggest that LPI- and LPC-induced Ca²⁺ influx passes through 2-APB- and BTP2-sensitive channels. Lysophospholipid-activated Ca²⁺ entry in Bergmann glial cells and GCL astrocytes apparently uses a pathway similar, if not identical, to that triggered by store depletion. It is yet unknown, however, how the generation of lysophospholipids activates SOCE. Lysophospholipids may open the SOCE channels either indirectly by affecting the lipid environment of the channels or directly by interacting with the membrane channel protein. Notably, a direct interaction of lysophosphatidylcholine with the cation channel TRPC5 was recently suggested in transfected HEK293 cells and arterial smooth muscle cells (Flemming et al., 2006).

Ca²⁺ oscillations rely on SOCE in cerebellar astrocytes

Spontaneous Ca²⁺ oscillations have been reported in astrocytes in culture (Fatatis and Russell, 1992; Harris-White et al., 1998) and *in situ* (Parri et al., 2001; Aguado et al., 2002; Nett et al., 2002; Zur Nieden and Deitmer, 2006). Ca²⁺ signaling in the form of repetitive oscillations are associated with regulating a variety of physiological processes, including gene expression (Dolmetsch et al., 1998), K⁺ conductance (Chen et al., 1997), and secretion (Thomas et al., 1996). The number of spontaneously active astrocytes and the number of Ca²⁺ transients exhibited by each astro-

Figure 10. Spontaneous Ca²⁺ oscillations in acute rat cerebellar slices were reduced by suppressing SOCE and by inhibiting iPLA₂ activity. **A**, Continuous, original recordings of spontaneous Ca²⁺ transients for 20 min in GCL astrocytes. Traces are for untreated slices (control, top trace) and for slices pretreated with BEL (25 μM for 30–40 min at 37°C; bottom trace). **B**, **C**, Bar plots showing the percentage of oscillating cells (**B**) and the relative frequency of Ca²⁺ transients (**C**) relative to control conditions (untreated slices, 100%) and for slices after treatment with 2-APB and with BEL in Bergmann glia and in GCL astrocytes.

cyte increased by increasing the external Ca²⁺ (Parri and Crunelli, 2003) and was reduced by inhibiting metabotropic glutamate receptors of groups I and II (Zur Nieden and Deitmer, 2006). SOCE and Ca²⁺ store refilling are required to maintain both spontaneous Ca²⁺ oscillations (Nett et al., 2002; Zur Nieden and Deitmer, 2006) and Ca²⁺ oscillations evoked by stimulation of metabotropic glutamate receptors in type I cortical astrocytes (Pizzo et al., 2001). It has been shown in rat hippocampal astrocytes that Ca²⁺ oscillations were attributable to phospholipase C-mediated Ca²⁺ release from intracellular stores (Zur Nieden and Deitmer, 2006). 2-APB effectively inhibits SOCE channels in many cell types (Bakowski et al., 2001; Braun et al., 2001; Broad et al., 2001; Gregory et al., 2001; Kukkonen et al., 2001; Prakriya and Lewis, 2001) but can also be a membrane-permeable inhibitor of IP₃ receptors (Wu et al., 2000). We observed that the number of spontaneously active astrocytes as well as the frequency of oscillations were reduced by blocking SOCE channels and by inhibiting iPLA₂ activity. Our results provide evidence that iPLA₂-mediated Ca²⁺ influx through SOCE channels is needed for the maintenance of Ca²⁺ signaling in astrocytes *in situ*, which requires continuous refilling of Ca²⁺ stores.

In summary, our results suggest that iPLA₂ is a major regula-

tor of SOCE in cerebellar astrocytes; during depletion of Ca²⁺ stores, iPLA₂ could be activated to open the channels in plasma membrane by the formation of lysophospholipids (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). A CIF would transfer the signal from depleted stores to relieve the block of iPLA₂ by CaM in the plasma membrane, consistent with studies of iPLA₂-mediated SOCE in other cell types evoked by emptying stores by either inhibiting SERCA pumps (Smani et al., 2004; Van den Abeele et al., 2004) or activating translocon pores (Flourakis et al., 2006). CIF has been suggested to act through the activation of iPLA₂ (Smani et al., 2004; Van den Abeele et al., 2004); however, it remains unresolved how the formation of CIF is triggered in response to depletion of Ca²⁺ stores. The STIM1 is likely the sensor (CIF) of depleted Ca²⁺ inside the ER Ca²⁺ stores, which translocates in response to store depletion from the ER to the plasma membrane (Roos et al., 2005; Zhang et al., 2005) and also controls the operation of the SOCE channels within the plasma membrane (Spassova et al., 2006). Recently, the plasma membrane four-transmembrane-spanning protein Orail or CRACM1 (Ca²⁺ release-activated channel blocker) has been reported to be necessary for SOCE and CRAC channel activity (Feske et al., 2006; Vig et al., 2006). Orail and STIM1 are likely to be functionally coupled and sufficient to mediate SOCE (Peinelt et al., 2006; Soboloff et al., 2006). Whether STIM1 is the first step in the molecular cascade to activate iPLA₂- and LPL-induced Ca²⁺ entry to refill intracellular Ca²⁺ stores in astrocytes still needs experimental evidence.

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