Ceramide Inhibits Inwardly Rectifying K^+ Currents via a Ras- and Raf-1-Dependent Pathway in Cultured Oligodendrocytes

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Ceramide is a lipid mediator implicated in apoptosis induced by proinflammatory cytokines in many cell types, including oligodendrocytes (OLGs). To determine whether ceramide modulates transmembrane signaling events in OLGs, we studied its effect on intracellular Ca^{2+} (Ca_i), resting membrane potential and inwardly rectifying K^+ currents (I_{Kir}) in cultured neonatal rat OLGs. We report here that (1) exposure to C2-ceramide (cer) rarely increases OLG Ca_i , whereas sphingosine elicits sustained increase in Ca_i ; (2) cer causes OLG depolarization, an effect mimicked by sphingosine-1-phosphate but not by sphin-

gosine; and (3) cer, but not its inactive analog dihydroceramide, inhibits OLG I_{Kir} . The cer effect is attenuated by Ras antibody Y13-259, by protein kinase C inhibitory peptide (19–36), and by suppression of c-Raf-1 expression with antisense raf-1 oligonucleotides. We conclude that cer-induced OLG depolarization is mediated via inhibition of I_{Kir} by a Ras- and raf-1-dependent pathway, which results in the phosphorylation of the inward rectifier K^+ channel protein.

Key words: K^+ channels; sphingolipids; protein kinases; phosphorylation; glial cells; depolarization

Oligodendrocytes (OLGs) are responsible for myelination in the CNS and are known to be susceptible to immune-mediated injury. One of the cytokines involved in immune-mediated injury of OLGs is tumor necrosis factor- α (TNF- α) (Robbins et al., 1987; Selmaj and Raine, 1988; Soliven et al., 1991; Louis et al., 1993; D'Souza et al., 1996). Many second messenger pathways have been implicated in TNF-mediated signaling, including activation of phospholipase A_2 , which causes the release of arachidonic acid (AA), and activation of sphingomyelinases (SMases), which cleave sphingomyelin to form ceramide (cer), sphingosine (sph), and sphingosine-1-phosphate (SPP) (Kim et al., 1991; Jayadev et al., 1994; Wiegmann et al., 1994). Chakravorty et al. (1997) reported that SMase activity in myelin is activated by TNF- α . Binding of nerve growth factor to p75 receptor, a member of the TNF receptor superfamily, increases cer levels and causes apoptosis in cultured neonatal rat OLGs (Casaccia-Bonnet et al., 1996b). Exactly how cer works is not fully understood, but there is evidence to suggest that activation of cJun N-terminal kinase is involved in a signal transduction pathway leading to cell death (Casaccia-Bonnet et al., 1996b; Verheij et al., 1996).

Our previous studies indicate that modulation of ionic fluxes, membrane depolarization and K^+ channels constitutes one of the important signaling mechanisms used by cytokines and growth factors to influence cellular functions in OLGs and progenitors. Modulation of the delayed rectifier (I_K) is associated with changes in proliferation of OLG progenitors (Gallo et al., 1996; Attali et al., 1997), whereas inhibition of the inward rectifier (I_{Kir})

leads to depolarization and decrease in phosphorylation of myelin proteins (Soliven et al., 1994; Takeda et al., 1997). Treatment of OLGs with TNF- α causes process inhibition, membrane depolarization, and decreased K^+ current amplitudes but does not alter intracellular Ca^{2+} (Ca_i) (Soliven et al., 1991). AA exerts a direct inhibitory action on OLG K^+ currents (Soliven and Wang, 1995), but unlike TNF- α , it elicits a sustained increase in OLG Ca_i , which results in OLG lysis (Soliven et al., 1993). Thus AA appears to exert effects on OLGs that are distinct from those mediated by TNF- α . It is possible that the effect of TNF- α on OLGs may be attributable to other mediators such as sphingolipid products.

The goal of this study was to investigate the effect of sphingolipid products, cer in particular, on the electrophysiological properties of OLGs. Cer, sph, and SPP are interconvertible, hence the effect of cer was compared to those with sph and SPP. We report here that (1) cer, sph, and SPP differentially modulate the Ca_i and resting membrane potential (RMP) of OLGs; and (2) cer causes OLG depolarization by inhibition of I_{Kir} via a ras- and raf1-dependent pathway. To our knowledge, this is the first report of Ras- and raf1-dependent modulation of glial K^+ channels.

MATERIALS AND METHODS

Cell culture. Primary glial cultures were established from 3- to 5-d-old Holtzman rat pups (Harlan Sprague Dawley, Madison, WI) as previously described (McCarthy and de Vellis, 1980), and were maintained in DMEM (1.0 gm/l deoxyglucose) supplemented with 10% FCS plus 1% penicillin and streptomycin (Life Technologies, Grand Island, NY). Bipolar progenitor cells were detached from mixed glial cultures after 10–14 d by overnight shaking (200 rpm) at 37°C, collected and plated on poly-L-lysine-coated 35 mm Petri dishes. After 24 hr, the culture medium was changed to DMEM plus 0.5% FCS and 5 μ g/ml insulin, 5 μ g/ml transferrin, and 5 ng/ml selenium (ITS) (Sigma, St. Louis, MO). The medium was changed every other day up to days 4–6 when experiments were initiated.

Fluorescence imaging studies. Ca_i imaging experiments were performed in OLGs loaded with fura-2 AM (5 μ M) as described previously (Soliven et al., 1993). For membrane potential studies, OLGs were loaded with a voltage-sensitive dye, bis(1,3-dibutylbarbituric acid)trime-thine oxonol (DiBAC $_4$) (2.0 μ M); Molecular Probes, Eugene, OR), for 30

Received April 14, 1998; revised Aug. 18, 1998; accepted Aug. 20, 1998.

This work was supported by National Multiple Sclerosis Society Grant RG2195-C4, by grants from the Spinal Cord Research Foundation and the Brain Research Foundation, and by a gift from Mr. M. P. Miller (all to B.S.). We thank Dr. D. Nelson for the privilege of using her imaging setup.

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min at room temperature, and measurements were performed in the presence of extracellular DiBAC₄. Drugs to be tested were added to solutions containing 2.0 μ M DiBAC₄. Cells were illuminated using a 75 W xenon light source at an excitation wavelength of 490 nm. In imaging experiments, a 20 $\times$ Nikon Fluor (numerical aperture, 0.75) objective was used to gather fluorescent data from a number of cells in the field of view. Emitted fluorescence was collected through a 515 nm long-pass filter with a Hamamatsu c2400 SIT video camera connected to a PTI image processor. A variable number of frame averages (depending on the fluorescence intensity) were collected at a predetermined interval and stored on a hard disk for off-line analysis. Bis-barbituric oxonols, a term that includes DiSBAC2 and DiBAC₄, in which alkyl is diethyl and dibutyl, respectively, have been used to monitor membrane potential changes in rat basophilic leukemia cells (Mohr and Fewtrell, 1987) and many other cell types (Laskey et al., 1992; Plásek and Sigler, 1996). The distribution of these negatively charged oxonol dyes across the membrane is potential-dependent. They enter depolarized cells, resulting in an increase in fluorescence. Hyperpolarization results in extrusion of the dye and thus a decrease in fluorescence.

Electrophysiology. Current recordings were obtained using the whole-cell configuration of the patch-clamp technique as previously described (Soliven et al., 1991). Pipette resistance ranged from 2 to 5 M Ω . Cells were studied at room temperature. The pipette (intracellular) solution consisted of either of the following (in mM): (1) 140 KCl, 2 CaCl₂, 2 MgCl₂, 11 EGTA, and 10 HEPES, pH 7.35; or (2) 140 CsCl, 2 CaCl₂, 2 MgCl₂, 11 EGTA, and 10 HEPES, pH 7.35. The bathing solution consisted of the following (in mM): 145 NaCl, 5.4 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, and 10 sucrose, pH 7.4 (normal bath solution). For high-K⁺-containing solutions, 35 mM KCl was used to replace equimolar concentrations of NaCl. Current records were filtered at 2 kHz using an eight-pole Bessel filter and sampled at 5 kHz.

Ras assay. Cultured OLGs were washed twice with phosphate-free DMEM, ITS, and 0.5% dialyzed FBS. Cells were labeled with 400 μ Ci/ml [³²P]orthophosphate (Amersham, Arlington Heights, IL) for 5 h. After washing three times with ice-cold PBS, cells were lysed with 600 μ l of ras lysis buffer (500 mM NaCl, 0.05% SDS, 1 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 0.5% sodium deoxycholate). Cell lysates were centrifuged at 12,000 $\times$ g for 5 min at 4°C, followed by immunoprecipitation with 5 μ l of agarose-conjugated anti-H-Ras IgG1 (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hr. After repeated washing 8 $\times$ in ras washing buffer (in mM: 25 Tris-HCl, pH 7.5, 150 NaCl, and 20 MgCl₂), bound nucleotides were eluted with a solution containing (in mM) 20 EDTA, 1 GDP, and 1 GTP at 68°C. The eluate was analyzed by thin-layer chromatography. Four-microliter aliquots were spotted onto PEI-cellulose plates (Alltech, Deerfield, IL) and run in the developing solvent (1 M KH₂PO₄, pH 3.4). Dried polyethyleneimine (PEI) cellulose plates were exposed to autoradiographic films. Results were calculated from the following equation based on densitometric analysis (BioImage): $GTP/[GTP + (1.5 \times GDP)] \times 100$. The factor of 1.5 corrects for the incorporation of three [³²P]orthophosphate molecules into GTP and two into GDP.

Transfection experiments. Purified phosphorothioate antisense (5'-TCCCCTGTATGTGCTCCAT-3'), sense (5'-ATGGAGCACATACAG-GGA-3'), and nonsense (5'-TTTTTGCACAGCTTGCC-3') oligodeoxynucleotides (ODNs) of c-raf-1 were obtained from GENSET (Muszynski et al., 1995). OLGs (4 d *in vitro*) were treated with 5 μ M ODNs for 4 h in serum-free DMEM followed by changing back to DMEM containing ITS and 0.5% FBS. Because of the slow turnover rate of c-raf-1, the treatment was repeated the next day. After another 24 hr, Western blot analysis of raf-1 expression or electrophysiological experiments were performed.

Western blot analysis of raf-1. Cultured OLGs were lysed in a buffer containing 300 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 25 mM HEPES, pH 7.7, 20 mM β -glycerophosphate, 0.1% Triton X-100, 0.1% SDS, 0.1 mM Na₃VO₄, 100 μ g/ml PMSF, 2 μ g/ml leupeptin, and 0.5% sodium deoxycholate. Samples were resolved using 12% SDS-PAGE and electroblotted to polyvinylidene difluoride membranes. Blots were blocked with 5% nonfat milk in 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20 for 1 hr at room temperature. The expression of raf-1 was detected using a monoclonal antibody to c-raf-1 (Transduction Laboratories, Lexington, KY), followed by the ECL detection method (Amersham).

Data analysis. Results for both electrophysiological and biochemical experiments are expressed as mean $\pm$ SEM with the number of experiments in parentheses. Data were analyzed with ANOVA, followed by

Scheffé *F* test for multiple group experiments and Student's *t* test for unpaired samples.

Materials. Drugs or agents used in this study were obtained from the following sources: C2-cer, staurosporine, quinacrine, phorbol 12-myristate 13-acetate (PMA), and okadaic acid (Sigma); dihydro-cer (dh-cer), H8, and wortmannin (Calbiochem, San Diego, CA); protein kinase C (PKC) inhibitor (19–36) (Life Technologies); and PKI (5–25) and neutralizing antibody to ras (Y13–259) (Santa Cruz Biotechnology). Cer, dh-cer, wortmannin, and okadaic acid were dissolved in ethanol (10 mM stock), whereas sphingosine, PMA, and staurosporine were dissolved in DMSO (10 mM stock). Aliquots of stock solutions were stored at –20°C and diluted to the final concentrations on the day of experiment. The final concentrations of ethanol or DMSO did not exceed 0.3%. For SPP, 1 mM stock in 100% methanol was prepared, dried down, and stored at –20°C. Before experiments, SPP was redissolved in PBS as a complex with BSA (4 mg/ml) to a final concentration of 1 mM. BSA by itself had no effect on Ca_i, but interfered with DiBAC₄ experiments. For the latter experiments, SPP was redissolved in PBS only.

RESULTS

Effect of sphingolipid products on Ca_i and RMP of cultured neonatal OLGs

Fluorescence imaging techniques were used to screen for modulatory actions of cer on transmembrane signaling events in OLGs. C2-cer, a cell-permeable analog of ceramide was used in these studies. The effect of cer was compared with other sphingolipid products, sph and SPP. Cer (10–20 μ M) and SPP (5–10 μ M) evoked a Ca_i response (measured as 340:380 fluorescence intensity ratio) in only 10 of 77 (13%) and in 7 of 50 (14%) of OLGs, respectively. In contrast, Ca_i increases were frequently observed in response to 10–30 μ M sph (65 of 79 OLGs or 82%) but not to lower concentrations (100 nM, 1 μ M). Exposure to 0.1% ethanol, 0.1–0.3% DMSO, or 0.1% BSA had no effect on OLG Ca_i (n = 22–39 OLGs for each condition). Figure 1*A* shows an example of heterogeneous Ca_i responses to 10 μ M cer but not to 1 μ M cer. Ca_i responses induced by sph were characterized by a sustained increase to a new plateau after a variable delay and were not reversible by washout with normal bath solutions (NB) (Fig. 1*B*). Perfusion of Ca²⁺-free solutions (0 mM Ca²⁺ and 1 mM EGTA) after sph-induced Ca_i response decreased the 340:380 ratio to baseline levels, indicating that Ca_i increases were derived primarily from Ca²⁺ influx. Note that subsequent perfusion with normal bath solutions containing 2 mM Ca²⁺ resulted in the restoration of the Ca_i increase. Perfusion of sph in Ca²⁺-free solutions did not elicit Ca_i increases (n = 14) (data not shown). In contrast to sph, Ca_i responses to SPP were transient but still evident under Ca²⁺-free conditions, indicating a mobilizing action of SPP on intracellular Ca²⁺ stores (Fig. 1*C,D*).

Next, we examined whether cer modulates the RMP in OLGs, and if so, whether the effect could be mimicked by sph or SPP. Changes in RMP were measured in OLGs loaded with DiBAC₄ (2 μ M). An increase in fluorescence intensity indicates membrane depolarization. Neither 0.1% ethanol nor 0.1–0.3% DMSO had any effect on DiBAC₄ fluorescence (n = 34 OLGs for each condition). Brief exposure to cer (10 μ M) and SPP (5 μ M) resulted in membrane depolarization in 33 of 44 (75%) and 28 of 28 (100%) of OLGs, respectively. In contrast, exposure to sph elicited hyperpolarization in 73 of 75 (97%) of OLGs, as depicted in Figure 2, *A* and *B*. The effect of sph was reversible by washout with normal bath solutions or with its vehicle DMSO. A depolarizing response to solutions containing 70 mM K⁺ (high K) was used as positive control. Given that sph is a known inhibitor of PKC, we examined whether pre-exposure to PMA would attenuate the hyperpolarizing response to sph. Perfusion with PMA (32–320 nM) alone attenuated the hyperpolarizing response to 10

Figure 1. Representative traces illustrating the effect of cer, sph, and SPP on 340:380 fluorescence intensity ratio in fura-2-loaded neonatal OLGs. An increase in 340:380 ratio indicates an increase in Ca_i . *A*, cer (1–10 μ M). *B*, sph (30 μ M), followed by washout with NB and Ca^{2+} -free solutions. *C*, SPP (5 μ M). Note that 0.1% BSA and 0.1% DMSO had no effect on OLG Ca_i . *D*, cer, SPP, and sph in Ca^{2+} -free solution (1 EGTA, 0 Ca^{2+}).

Figure 2. Representative traces illustrating the effect of cer (10 μ M), sph (10 μ M), and SPP (5 μ M) on DiBAC₄ fluorescence in cultured rat OLGs. An increase in fluorescence intensity indicates depolarization. *A*, Cer-induced depolarization and sph-induced hyperpolarization. Note that 0.1% ethanol (EtOH) and 0.1% DMSO had no effect on OLG RMP. *B*, SPP-induced depolarization and sph-induced hyperpolarization compared with depolarization induced by high K^+ (70 mM). *C*, Effect of sph in the presence of PMA (320 nM). *D*, Effect of sph and cer in Ca^{2+} -free conditions.

μ M sph. The average decrease in oxonol fluorescence intensity induced by sph was $46.5 \pm 1.5\%$ ($n = 21$) in the presence of PMA versus $65.2 \pm 2.2\%$ ($n = 37$) in normal bath solutions ($p < 0.00001$ for sph vs sph plus PMA). To determine whether sph-induced hyperpolarization was related to Ca_i increases, we repeated experiments in Ca^{2+} -free solutions. The average decrease in oxonol fluorescence intensity induced by sph was $38.6 \pm 2.8\%$ ($n = 25$) in Ca^{2+} -free solutions ($p < 0.00001$ for sph versus sph

plus EGTA). Figure 2, *C* and *D*, shows examples of sph-induced hyperpolarization in the presence of PMA or external EGTA. These results indicate that sph-induced hyperpolarization is partially PKC- and Ca^{2+} -dependent. Note that cer-induced OLG depolarization was still evident in Ca^{2+} -free solutions. These results demonstrate that (1) cer, sph, and SPP differentially modulate Ca_i and RMP of OLGs; and (2) cer induces OLG depolarization that is not caused by Ca^{2+} influx. Subsequent studies

Figure 3. Cer inhibits $I_{K_{ir}}$ in cultured rat OLGs. *A*, Cer ($10\ \mu\text{M}$) induced a decrease in $I_{K_{ir}}$ amplitudes at 5 min after perfusion. Step pulses ranging from -120 to 80 mV in 20 mV increments were applied for 360 msec from holding potential of -40 mV. The pipette solution contained 140 mM Cs^+ instead of K^+ . Bath solutions contained 5.4 mM K^+ . *B*, The corresponding I - V plots.

focused on the mechanism's underlying cer-induced OLG depolarization.

Cer inhibits $I_{K_{ir}}$ in cultured oligodendrocytes

Whole-cell patch-clamp technique was used to study the effect of cer on $I_{K_{ir}}$, the predominant ionic current in OLGs. Step pulses of 360 msec were applied from a holding potential of -80 mV. Inward currents activated at hyperpolarized potentials were sensitive to external Ba^{2+} and external K^+ concentration, as expected for $I_{K_{ir}}$. At a strongly hyperpolarized potential (-100 to -120 mV), $I_{K_{ir}}$ frequently exhibited current inactivation. Steady-state current amplitudes measured at -120 mV were analyzed and normalized to the initial current amplitude (amp). In the presence of normal bath and pipette solutions, OLG RMP estimated from zero current potential was -74.2 ± 0.4 mV ($n = 20$). Perfusion with cer ($10\ \mu\text{M}$) resulted in a decrease in $I_{K_{ir}}$ amp to $65.2 \pm 7.3\%$ ($n = 10$) of the initial current amp and a depolarizing shift in the RMP of 7.0 ± 1.9 mV ($n = 10$) at 5 min after perfusion. In comparison, $I_{K_{ir}}$ amp remained at $98.2 \pm 2.9\%$ of the initial amp with a minimal depolarizing shift in RMP [1.3 ± 0.8 mV ($n = 10$)] under control conditions over the same period ($p < 0.01$ for cer vs ctrl with regard to shift in RMP, and $p < 0.0001$ for cer vs ctrl with regard to percentage of initial current amp).

To isolate $I_{K_{ir}}$, step pulses from a holding potential of -40 mV were applied in the presence of Cs pipette (intracellular) solution, which blocks outward K^+ currents. Bath solutions contained either 5.4 mM K^+ (normal bath solution) or 35 mM K^+ (high- K^+ solution). Steady-state current amplitudes at -80 mV were analyzed. Because results obtained in normal K^+ and high- K^+ conditions were similar, the normalized data were pooled. As shown in Figure 3, perfusion of OLGs with cer ($10\ \mu\text{M}$) resulted

Figure 4. Cer-induced $I_{K_{ir}}$ inhibition involves Ras-dependent pathways. *A*, Representative traces before (*pre*) and after (*post*) perfusion with $10\ \mu\text{M}$ cer. Only currents at -80 mV are shown for simplicity in this and subsequent figures. *IgG* (+), Inclusion of $0.2\ \mu\text{g}/\text{ml}$ control IgG in the intracellular (pipette) solution. *RasAb* (+), Inclusion of $0.2\ \mu\text{g}/\text{ml}$ neutralizing antibody against Ras (Y13-259) in the pipette solution. *B*, Summarized data showing $I_{K_{ir}}$ modulation by cer ($10\ \mu\text{M}$) but not by inactive cer analog dh-cer ($10\ \mu\text{M}$) (* $p < 0.0001$ for overall ANOVA; $p < 0.0001$ for ctrl vs cer; $p < 0.001$ for cer vs dh-cer). Y13-259 prevented the effect of cer on $I_{K_{ir}}$ (** $p < 0.02$ for Y13-259 vs ctrl IgG).

in $I_{K_{ir}}$ inhibition. $I_{K_{ir}}$ amplitudes were decreased to $75.6 \pm 2.6\%$ of the initial current amp at 5 min after perfusion ($n = 23$; $p < 0.0001$ for cer vs ctrl), and to $68.0 \pm 2.8\%$ at 10 min ($n = 14$). Cer was effective at concentrations $\geq 1\ \mu\text{M}$ (data not shown). This effect was specific in that the inactive cer analog dh-cer ($10\ \mu\text{M}$) did not inhibit $I_{K_{ir}}$ (% initial $I_{K_{ir}}$ amp, $99.2 \pm 5.2\%$; $p < 0.001$ for cer vs dh-cer). Perfusion with SPP ($10\ \mu\text{M}$) also did not inhibit OLG $I_{K_{ir}}$ (% initial $I_{K_{ir}}$ amp, 96.8 ± 3.3 , $n = 7$). For all subsequent experiments, only data at 5 min after perfusion were analyzed.

Cer-induced inhibition of $I_{K_{ir}}$ involves Ras activation and kinase-dependent channel phosphorylation

Given that apoptosis induced by TNF- α or Fas ligand has been reported to involve Ras activation in other cell types (Gulbins et al., 1995; Trent et al., 1996), we examined whether Ras was involved in cer-induced $I_{K_{ir}}$ inhibition by inclusion of a neutralizing antibody (Y13-259) in the intracellular solution (Hattori et al., 1987). As shown in Figure 4, the effect of cer on $I_{K_{ir}}$ was significantly attenuated by Y13-259 ($0.2\ \mu\text{g}/\text{ml}$) but not by the same concentration of control IgG (anti-GFAP antibody) [% initial $I_{K_{ir}}$ amp for Ras antibody (Ab), $96.5 \pm 3.4\%$; $n = 11$; control IgG, $73.8 \pm 3.5\%$; $n = 5$; $p < 0.02$ for Ras Ab vs control IgG]. Next, we examined whether cer caused Ras activation in cultured OLGs. As shown in Figure 5, exposure of OLGs to cer ($10\ \mu\text{M}$) led to increased Ras activation within 5 min, as evidenced by an increase of radiolabeled GTP bound to Ras in

Figure 5. Examples of autoradiograms illustrating Ras activation by cer in OLGs. Radiolabeled GDP and GTP that bind Ras are detected with thin layer chromatography. Treatment of cer ($10 \mu\text{M}$) for 5 min increased radiolabeled GTP compared with controls.

treated OLGs compared with untreated OLGs. The percent GTP-bound ras was $34.7 \pm 7.1\%$ ($n = 6$) under control conditions and $62.2 \pm 8.8\%$ ($n = 6$) in OLGs treated with cer for 5 min ($p < 0.03$ for control vs cer).

To determine whether cer-induced I_{Kir} inhibition was mediated by kinase-dependent ion channel phosphorylation, OLGs were pretreated with kinase inhibitors such as staurosporine, a PKC inhibitor, and H8, a nonspecific kinase inhibitor, for 1 hr before perfusion with cer. The effect of cer on I_{Kir} was attenuated by both staurosporine (50 nM) and H8 ($30 \mu\text{M}$) [% initial I_{Kir} amp, $97.7 \pm 2.1\%$ ($n = 7$) for staurosporine-treated OLGs; $98.3 \pm 4.3\%$ ($n = 7$) for H8-treated cells]. In contrast, cer-induced I_{Kir} inhibition was not affected by pretreatment with the phospholipase A_2 inhibitor quinacrine ($10 \mu\text{M}$) (% initial current amp, $79.9 \pm 7.5\%$; $n = 7$). The effect of cer was mimicked by okadaic acid (50 nM), an inhibitor of protein phosphatase IIA (% initial I_{Kir} amp, $78.1 \pm 4.3\%$; $n = 6$). These results suggest that cer inhibits I_{Kir} via kinase-dependent phosphorylation of K^+ channels.

To further delineate the role of kinases in cer-induced I_{Kir} phosphorylation, we investigated whether the effect of cer could be blocked by the inclusion of a PKC pseudosubstrate (PKCI, $10 \mu\text{M}$) or a protein kinase inhibitory peptide (PKI, $10 \mu\text{M}$) in the intracellular solution. As shown in Figure 6, cer-induced I_{Kir} inhibition was blocked by PKCI but not by PKI [% initial I_{Kir} amp, $95.1 \pm 6.3\%$ ($n = 6$) for PKCI; $68.7 \pm 8.6\%$ ($n = 6$) for PKI]. Next, we examined which PKC isoforms were involved in cer action. One of the targets of Ras is PKC- ζ , a phorbol ester-insensitive PKC isoform that can be activated via the phosphatidylinositol-3-kinase (PI-3 kinase) pathway (Diaz-Meco et al., 1994). OLGs were treated for 1–2 hr with wortmannin ($5 \mu\text{M}$), a potent inhibitor of PI-3 kinase, or for 24 hr with PMA ($1 \mu\text{M}$) to downregulate PMA-sensitive PKC isoenzymes. Cer was still able to inhibit I_{Kir} in wortmannin-treated OLGs, but not in OLGs in which conventional and novel PKC isoforms were downregulated [% initial I_{Kir} amp, $74.9 \pm 8.8\%$ ($n = 6$) for wortmannin; $90.5 \pm 6.0\%$ ($n = 7$) for PMA 24 hr]. These results were statistically significant ($p < 0.05$ for cer vs PKCI and for cer vs PMA 24 hr) (Fig. 6B).

Cer-induced I_{Kir} inhibition involves a raf-1-dependent pathway

Figure 7 shows a schematic diagram illustrating possible signaling pathways in which both Ras and PKC are involved in cer-induced

Figure 6. The role of protein kinases in cer-induced I_{Kir} inhibition. *A*, Representative traces illustrating the effect of PKC and PKA inhibitors on cer action. *Left*, Inclusion of PKC (19–36) pseudosubstrate ($10 \mu\text{M}$) in the pipette solution. *Right*, Inclusion of PKA inhibitor peptide PKI (5–24) ($10 \mu\text{M}$) in the pipette solution. *B*, Summarized data showing that pretreatment of OLGs with wortmannin ($5 \mu\text{M}$), a PI-3-kinase inhibitor, or inclusion of PKI in the pipette solution did not block the effect of cer. In contrast, inhibition of PKC activity by PKCI or by PKC downregulation with 24 hr PMA treatment significantly attenuated cer-induced I_{Kir} inhibition (* $p < 0.05$ for cer vs PKCI and for cer vs PMA 24 h).

Figure 7. Schematic diagram of possible signaling pathways involved in cer-induced I_{Kir} inhibition.

I_{Kir} inhibition. One possible convergent target of Ras and PKC is raf-1 (Kolch et al., 1993), which could also be activated by cer-activated protein kinase (CAPK) (Yao et al., 1995). An antisense approach was used to investigate whether raf-1 was involved in cer-induced I_{Kir} inhibition. As shown in Figure 8A, treatment of OLGs with antisense ODN ($5 \mu\text{M}$) but not sense and nonsense raf-1 ODNs inhibited the expression of raf-1 without

Figure 8. Cer-induced I_{Kir} inhibition involves raf-1-dependent pathways. **A**, Western blot analysis of raf-1 protein expression in cultures treated with 5 μ M sense, antisense, or nonsense raf-1 ODNs. Raf-1 protein was detected using a monoclonal antibody against raf-1. Data shown are representative of three experiments. **B**, Summarized data showing that cer-induced I_{Kir} inhibition was prevented by antisense raf-1 ODN but not by sense or nonsense raf-1 ODNs ($p < 0.03$ for antisense ODNs vs sense and nonsense ODNs). See Materials and Methods for details.

inducing cytotoxicity in OLGs (see Materials and Methods). I_{Kir} after cer was $78.2 \pm 4.3\%$ ($n = 7$) of the initial current amp in OLGs treated with sense and nonsense ODNs and $91.5 \pm 3.3\%$ ($n = 9$) in OLGs treated with antisense ODNs ($p < 0.03$ for antisense ODNs vs sense and nonsense ODNs) (Fig. 8B). These results indicate that cer-induced I_{Kir} inhibition involves a raf-1-dependent pathway.

We have previously reported that PMA inhibits OLG I_{Kir} (Hertz et al., 1990). Figure 9A shows examples of I_{Kir} inhibition by PMA (1 μ M) in the presence of Ras Ab (Y13-259) in the pipette solution (Ras Ab, $67.6 \pm 5.4\%$; $n = 5$). In addition, the inhibitory effect of PMA on I_{Kir} was still observed in OLGs treated with raf-1 antisense ODNs (antisense, $67.7 \pm 6.4\%$; $n = 6$; nonsense, $62.9 \pm 7.3\%$; $n = 6$; $p > 0.05$ between antisense vs sense and nonsense ODNs for PMA effect). (Fig. 9B). Hence, Ras Ab and raf-1 antisense ODNs attenuate the inhibitory action of cer on I_{Kir} but not that of direct PKC-mediated Kir channel phosphorylation.

DISCUSSION

This study demonstrates that cer, sph, and SPP differentially modulate transmembrane signaling events in cultured OLGs, even though these sphingolipid products are interconvertible. Ca_i increases in OLGs were induced by sph but only infrequently by cer or SPP. Our results confirm that sphingolipid products contribute to the regulation of Ca_i homeostasis or Ca_i signaling in OLGs but differ from findings of Fatatis and Miller (1996) in some aspects. We did not observe oscillatory Ca_i responses, and the frequency of Ca_i responses was less than that observed by Fatatis and Miller (1996). Possible explanations include (1) use of primary OLG cultures in our study versus the CG4 cell line and

Figure 9. PMA-induced I_{Kir} inhibition in OLGs. **A**, Representative traces illustrating the effect of PMA in the presence of Ras Ab or in OLGs treated with antisense raf-1 ODN. **B**, Summarized data illustrating that PMA-induced I_{Kir} inhibition was not prevented by Ras Ab or by antisense Raf-1 ODN ($p = NS$ for PMA vs Ras Ab + PMA and for PMA vs antisense + PMA).

transformed OLGs in their study; (2) differences in the culture medium used between the two studies; and (3) use of sphingosine kinase inhibitor by Fatatis and Miller (1996) but not by us. In addition to their effects on Ca_i homeostasis, sphingolipid products regulate OLG RMP. Cer and SPP caused OLG depolarization, whereas sph caused OLG hyperpolarization; the latter appeared to be partially Ca^{2+} - and PKC-dependent. Further studies are required to characterize the conductances underlying sph-induced hyperpolarization. We found that cer-induced but not SPP-induced depolarization is mediated by inhibition of OLG I_{Kir} . It is possible that SPP acts via other mechanisms such as inhibition of $Na^+-K^+-ATPase$. Hence, sphingolipid products may play a critical role in the regulation of membrane excitability and ion channels, in addition to their putative role in cell growth and programmed cell death (Spiegel and Merrill, 1996).

Cer has been shown to induce cell death in cultured rat OLGs but not in astrocytes (Casaccia-Bonnet et al., 1996a; Larocca et al., 1997). There is evidence to suggest that cer acts via Ras-dependent signaling pathways in Jurkat cells and fibroblasts (Gulbins et al., 1995; Trent et al., 1996). Our results from Ras activation assay and neutralizing antibody (Y13-259) experiments indicate that Ras is involved in cer-induced I_{Kir} inhibition as well. Y13-259 binds to switch II region of Ras, blocking conformational changes, and influences the binding of effectors (Sigal et al., 1986; Hattori et al., 1987). Although ras-p21 has been reported to induce expression of a calcium-activated K^+ channel in murine fibroblast cell lines (Huang and Rane, 1994) and to inhibit coupling of muscarinic receptors to atrial K^+ channels (Yatani et al.,

1990), it is unclear whether ras-p21 can directly interact with ion channels, as is the case for heterotrimeric G proteins (Schreibmayer et al., 1996). Our finding that protein kinase inhibitors attenuated the inhibitory action of cer on OLG I_{Kir} indicates that the latter involves ion channel phosphorylation and not a direct inhibition of Kir channel by Ras.

The target proteins of Ras include PI-3K, PKC- ζ , and raf-1 (Avruch et al., 1994; Marshall, 1995). Therefore, we tested whether the above proteins are involved in the inhibitory action of cer on I_{Kir} (see Fig. 7). PKC- ζ is activated by phosphatidylinositol-(3,4,5)-triphosphate via a PI-3K pathway, although direct regulation of PKC- ζ by Ras has also been reported (Diaz-Meco et al., 1994). Cer has been shown to induce phosphorylation and activation of PKC- ζ (Muller et al., 1995; Hannun, 1996). Our data indicate that cer-induced I_{Kir} inhibition does not involve PKC- ζ , but involves phorbol ester-sensitive PKC isoforms (conventional and novel PKCs). One might find it difficult to conceptualize the involvement of both Ras and PKC in cer-induced I_{Kir} inhibition. It is recognized that PKC can function downstream of Ras, that is, at the level of raf-1 activation (Kolch et al., 1993; Cai et al., 1997). Whether PKC signals can occur upstream of Ras remains controversial, but El-Shermerly and coworkers (1997) have recently demonstrated that PMA acts upstream of Ras and SOS in NIH3T3 cells. It should also be noted that cer has been reported to cause inactivation rather than activation of phorbol ester-sensitive PKC isoforms such as PKC- α , PKC- δ , PKC- ϵ in other cell types (Jones and Murray, 1995; Lee et al., 1996; Sawai et al., 1997). Nonetheless, data from PKC downregulation and PKCI experiments indicate that basal PKC activity is required for the action of cer in our study.

We postulated that Ras and PMA-sensitive PKC isoforms converge on a common downstream target, which then phosphorylates I_{Kir} or activates another effector to phosphorylate I_{Kir} . One such common target is raf-1, a 74 kDa serine/threonine kinase that is activated by PKC (Kolch et al., 1993; Cai et al., 1997), by CAPK (Yao et al., 1995), and by Ras. The mechanism of raf-1 activation is complex and tightly regulated by multiple phosphorylation events. It is possible that basal PKC activity is required for optimal activation of raf-1 by ras or CAPK. Raf-1 has been shown to be activated by TNF- α via neutral sphingomyelinase (Belka et al., 1995). Huang and Rane (1994) reported that raf-1 induces a Ca^{2+} -activated K^+ channel in transformed fibroblasts. Results from our antisense experiments reveal that cer-induced I_{Kir} inhibition is mediated by a raf-1-dependent pathway, but the data do not permit us to distinguish between direct phosphorylation of Kir channels by raf-1 versus K^+ channel phosphorylation by raf-1-dependent effectors. The effect of raf-1 is specific in that antisense raf-1 ODN did not block PKC-mediated phosphorylation of Kir channels. These findings are in agreement with studies demonstrating a direct PKC-mediated modulation of cloned inward rectifiers expressed in *Xenopus* oocytes (Fakler et al., 1994; Henry et al., 1996). Kir subunits identical to ROMK1 and IRK1 have been identified in OLGs (Karschin and Wischmeyer, 1995).

Although cer also inhibits outward K^+ currents in OLGs (Hida and Soliven 1996), the role of raf-1 versus other kinases in this action of cer remains to be clarified. Interestingly, a brief application of cer results in inhibition of the N-type K^+ channel in lymphocytes, an effect that is mediated via activation of a Src-like tyrosine kinase p56lck (Gulbins et al., 1997). The N-type K^+ channel is also inhibited by activation of Fas receptor in Jurkat T-lymphocytes, suggesting that it plays a role in the regulation of Fas-induced apoptosis (Szabo et al., 1996). In contrast, neuronal

apoptosis induced by staurosporine or serum deprivation is associated with the enhancement of the delayed rectifier and loss of intracellular K^+ (Yu et al., 1997). Aside from differences in apoptotic stimuli and cell type in the above studies, the latter study also differs in the experimental design in that currents are recorded from a population of neurons after a prolonged exposure (6–11 hr) to the apoptotic stimuli. In OLGs, prolonged incubation with TNF- α results in the inhibition of both outward K^+ currents and I_{Kir} (Soliven et al., 1991). Thus the role of different K^+ channel subtypes in the regulation of apoptotic mechanisms or cell cycle may be complex and may depend on the cell type (proliferating vs nonproliferating) and/or the apoptotic stimulus.

We conclude that (1) cer exerts modulatory actions on transmembrane signaling events in OLGs that are distinct from those exerted by sph or SPP; (2) although both cer and SPP cause OLG depolarization, only cer inhibits I_{Kir} ; and (3) cer-induced I_{Kir} inhibition is mediated by a ras- and raf-1-dependent pathway. Wilk-Blaszczak and coworkers (1998) have recently demonstrated that a mitogen-activated protein kinase (MAPK) p38 is involved in the regulation of the N-type Ca^{2+} channel by G-protein-coupled receptors in a neuroblastoma $\times$ glioma hybrid cell line. Others have reported that MAPKs Erk-1 and Erk-2 are required for the activation of volume-activated Cl^- current in cultured astrocytes (Crpel et al., 1998). Results from our study provide further support to the concept that MAPK cascades play an important role in the regulation of ion channels, membrane excitability, and synaptic transmission.

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