Granule neurons in cerebellum express distinct splice variants of the inositol trisphosphate receptor that are modulated by calcium

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Granule neurons in cerebellum express distinct splice variants of the inositol trisphosphate receptor that are modulated by calcium. Am J Physiol Cell Physiol 287: C971–C980, 2004. First published June 9, 2004; 10.1152/ajpcell.00571.2003.—Primary cultures of granule cells (GC) from rat cerebellar cortex were used to determine whether bioelectric activity, via a Ca\(^{2+}\)/calmodulin-dependent kinase (CaMK) signaling cascade, modulates expression and exon selection in the inositol trisphosphate receptor type 1 (IP\(_3\)R1). IP\(_3\)R1 contains or lacks three exons (S1, S2, and S3) that are regulated in a regionally and temporally specific manner. The neuronal, or long, form of IP\(_3\)R1 is distinguished from peripheral tissues by inclusion of the S2 exon. Although previous studies indicated that IP\(_3\)R1 are undetectable in the cerebellar granular layer in vivo, receptor protein and mRNA are induced in cultured GC grown in medium supplemented with 25 mM KCl or NMDA, two trophic agents that promote long-term survival, compared with GC grown in 5 mM KCl. IP\(_3\)R1 induction in response to 25 mM KCl or NMDA is attenuated by coaddition of voltage-sensitive calcium channel or NMDA receptor antagonists, respectively. Actinomycin D, CaMK, and calcineurin antagonists likewise inhibit IP\(_3\)R1 expression in GC. Although previous studies indicated that IP\(_3\)R1 are undetectable in the cerebellar granular layer in vivo, receptor protein and mRNA are induced in cultured GC grown in medium supplemented with 25 mM KCl or NMDA, two trophic agents that promote long-term survival, compared with GC grown in 5 mM KCl. IP\(_3\)R1 induction in response to 25 mM KCl or NMDA is attenuated by coaddition of voltage-sensitive calcium channel or NMDA receptor antagonists, respectively. Actinomycin D, CaMK, and calcineurin antagonists likewise suppress induction. Unlike the major variants of IP\(_3\)R1 in Purkinje neurons, which lack S1 and S3, GC grown with trophic agents express mRNA containing these exons. Both neuronal types contain S2. Evidence obtained using mutant mice with Purkinje cell lesions, laser-microdissected GC neurons from slices, and explant cultures indicates that GC predominantly express the S1-containing variant of IP\(_3\)R1 in vivo.

Inositol trisphosphate receptors (IP\(_3\)R) are encoded by three genes (IP\(_3\)R1, IP\(_3\)R2, and IP\(_3\)R3) that differ in their affinities for IP\(_3\) (33) and mediate the release of Ca\(^{2+}\) from intracellular stores in response to ligands that stimulate the hydrolysis of membrane phospholipids (for review, see Ref. 43). IP\(_3\)R1 predominates in neurons and can include sequences encoded by three alternatively spliced exons (S1, S2, and S3) that are regulated in a regionally and temporally specific manner (30), although the unique functions of the splice variants are still under investigation. The receptor is most enriched in Purkinje neurons of the cerebellar cortex, but significant amounts of mRNA and protein are also present in hippocampus, cerebral cortex, nucleus accumbens, caudate putamen, and deep cerebellar nuclei (9, 32). Although previously available methods of assay rendered IP\(_3\)R1 undetectable in the granular layer of cerebellar cortex (24, 30, 32, 40), work done at our laboratory (35) previously demonstrated that primary cultures of granule cells (GC) grown in medium supplemented with elevated KCl (to promote long-term survival; see Ref. 11) express significant amounts of immunoreactive protein compared with those grown in medium containing 5 mM KCl. Genazzani et al. (12) verified this observation and further showed an increase in mRNA encoding the long, i.e., S2-containing, form and attenuation of depolarization-dependent IP\(_3\)R1 induction by FK506 (also called tacrolimus) and cyclosporin A (CsA), agents that inhibit calcineurin (also called protein phosphatase 2B). Studies in hippocampal neurons also point to regulatory roles for depolarization and calcineurin in the induction of IP\(_3\)R1 mRNA and suggest that the transcription factor nuclear factor of activated T cells (NF-ATc) mediates this effect (13). Taken together, these data suggest that depolarization-dependent activation of calcineurin leads to hypophosphorylation and nuclear translocation of NF-ATc and subsequent induction of IP\(_3\)R1 in GC and other neurons. However, the IP3R gene is regulated by multiple transcription factors, including those that synergize with NF-ATc as well as those that are NF-ATc independent (7, 16). In particular, the effects of Ca\(^{2+}\)/calmodulin-dependent kinase (CaMK)-dependent signaling cascades on IP3R1 expression and splicing have not been examined. Perhaps more important, the significance of IP3R1 expression in cultured GC, given its apparent paucity in vivo, has not been considered. These questions prompted the present investigation.

Materials and Methods

Materials. Sprague-Dawley neonatal rats were purchased from Taconic Farms (Germantown, NY). Purkinje cell degeneration (pcd) mice and normal littermates were purchased from Jackson Laboratories (Bar Harbor, ME). All procedures involving the use of animals were approved by the institutional review committee in accordance with government guidelines. KN-62, CsA, and (5R,10S)-(+-)5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-iminomaleate (MK-801) were purchased from Calbiochem (La Jolla, CA). D,L-2-Amino-5-phosphonopentanoic acid (AP5) was purchased from Sigma (St. Louis, MO). FK506 was purchased from A. G. Scientific (San Diego, CA). Texas red-conjugated secondary antibody of multiple-labeling grade was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Antibodies specific for type I IP3 R, raised in rabbit and affinity purified (49), were generously provided by Dr. R. J. H. Wojcikiewicz (SUNY Upstate Medical University). The RNeasy Mini Kit was purchased from Qiagen (Valencia, CA). Reagents for RT, including Moloney murine leukemia virus (MMLV), were purchased from Promega (Madison, WI). Thermus aquaticus polymerase (AmpliTaq Gold or AmpliTaq), 10× PCR buffer, and MgCl\(_2\) were purchased from Applied Biosystems (Foster City, CA). [\(^{32}\)P]dCTP was obtained from NEN (Wilmington, DE). Oligonucleotide primer sequences are listed in Table 1.
otide primers were purchased from Genset (La Jolla, CA). PCR products were imaged with a Molecular Dynamics PhosphorImager obtained from Amersham Pharmacia Biotechnology (Piscataway, NJ) or on a Fluor-S from Bio-Rad (Hercules, CA). All other reagents were tissue culture or molecular biology grade, as required, and were obtained from commercial sources.

**Cerebellar GC cultures.** Cerebellar cortices from postnatal day 8 (P8) Sprague-Dawley rats were minced, trypsinized, and triturated to dissociate cells as described previously by investigators at our laboratory (23). Cells were plated on polylysine-coated (10 μg/ml) 35-mm dishes at a density of 2.5 × 10^5 cells/2 ml of medium (for RNA and protein extraction studies) or 6–6.5 × 10^5 cells/ml of medium in 24-well Corning dishes containing glass coverslips (for immunocytochemical studies) and then incubated at 37°C in a humidified atmosphere of 5% CO_2-air. Cultures were supplemented with 10 μM cytosine β-d-arabinofuranoside 24 h after plating to inhibit nonneuronal cell, i.e., glial, proliferation. The growth medium consisted of basal Eagle’s medium with Earle’s salts, supplemented with final concentration (f.c.) heat-inactivated fetal calf serum (10%), gentamicin sulfate (100 μg/ml), and L-glutamine (2 mM). Where indicated, standard growth medium (containing 5 mM KCl) was augmented with 20 mM KCl or 140 mM N-methyl-D-aspartate (NMDA) plus 5 mM KCl [Note: 10 mM KCl alone does not enhance Neuron survival.](#)

**Neuron survival.** GC viability was examined using an assay in which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is converted to a blue formazan product by the mitochondria of viable cells as described previously (29). MTT was added directly to the culture medium at a final concentration of 0.5 mg/ml, and cultures were incubated at 37°C for 10 min. The solution was then aspirated and replaced with an equal volume of dimethyl sulfoxide. The absorbance of samples was quantified using a Hitachi U-2000 spectrophotometer at a wavelength of 540 nm. Our laboratory previously showed that the color yield was proportional to the number of viable cells, assessed by phase-contrast microscopy in conjunction with fluorescein diacetate-propidium iodide (FDA/PI; live/dead assay) (45).

**Immunofluorescence/Western immunoblotting.** GC or explants attached to coverslips were washed with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min. Afterward, cultures were washed three times for 5 min each with PBS, permeabilized with 0.3% Triton-X in 2% donkey serum, 1% bovine serum albumin (BSA) in PBS for 20 min at RT, rinsed three times for 5 min each with PBS, blocked with 2% donkey serum, 1% BSA in PBS for 30 min, and then incubated overnight with antibody specific for IP_3_R1 protein (1:10). Subsequently, the coverslips were washed three times for 5 min each in PBS and incubated with Tras再现-red labeled donkey anti-rabbit IgG (1:100 dilution, 1 h at RT), washed three times for 5 min each in PBS, and then mounted with Vectashield antifade on glass slides. Photomicrographs were produced using a Zeiss microscope.

For Western immunoblotting analysis, GC were rinsed with PBS, and then protein was collected in 100 μl of homogenization buffer (63 mM Tris·HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 5 mM β-mercaptoethanol, 100 μM PMSF). For IP•R1 protein detection, 15 μg/lane of whole cell homogenate was resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5% gels. After electrophoresis, proteins were transferred to nitrocellulose and incubated with 1:100 dilution of polyclonal antibody specific for IP•R1 protein (49) and then peroxidase-conjugated secondary antibody. Immunoreactivity was detected using enhanced chemiluminescence.

**RT-PCR.** Whole cell RNA from GC, whole cerebellum, and hippocampus were isolated using the guanidinium isothiocyanate method (4). After the final ethanol precipitation, the RNA pellet was resuspended in sterile water. The concentration and purity of RNA were assessed with a spectrophotometer using nucleotide absorption at 260 nm and ratio at 260/280 nm (nucleotide/protein), respectively. Where indicated, RNA was isolated using a Qiagen RNeasy Mini kit. Harvested RNA was used in a RT reaction to produce cDNA for use as a template in PCR. RNA (0.1–0.2 μg) in 4 μl of sterile water was heated to 94°C for 1 min and then cooled on ice. Six microliters of a mixture containing (f.c.) MMLV RT (100 U), RT buffer (in mM: 50 Tris·HCl, pH 8.3, 75 KCl, 3 MgCl_2, 10 dithiothreitol), the four dNTPs (0.5 mM each), RNasin ribonuclease inhibitor (20 U), and hexamer random primers (5 μM) were added to each RNA sample (final volume 10 μl). The RT reaction was initiated by incubation at 37°C for 60 min to promote synthesis of cDNA and terminated by heating to 95°C for 5 min and then placing the tubes on ice and diluting to 1:5–5.0 μg/ml using sterile H_2O. After RT, PCR was performed in a final volume of 100 μl containing Taq buffer (10 mM Tris·HCl, pH 8.8, 50 mM KCl), 2 mM MgCl_2, 0.17 μg/ml BSA, 2.5 units of Taq polymerase (AmpliTaq Gold), 2 μM [α-32P]dCTP, and 0.05 mM of each dNTP, as well as 25 pmol of each oligonucleotide primer as follows: S1 upstream (5’-GTTCAACCTCCGTCA- ACTGTAAC-3’; sense) and S1 downstream (5’-ACGAGGAGCACTTAT-3’; antisense); S2 upstream (5’-GTTT CACTGCAACCTAATAAC-3’; sense) and S2 downstream (5’-AATGTTTCTTTGATGACTTCGTC-3’; antisense); S3 upstream (5’-TGACACTACCCCTCCCATTAG-3’; sense) and S3 downstream (5’-TGATATGATGATGCTACGTTG-3’; antisense). Before PCR, samples were incubated at 94°C (S1 and S2) or at 96°C (S3) for 7 min. PCR was performed for 35 cycles in a PerkinElmer Tempcycler model 480 as follows: S1, 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s; S2, 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min; and S3, 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min. After PCR, the samples were heated to 72°C for 5 min. The amplified products were resolved on 8% (S1 and S2) or 20% (S3) polyacrylamide gels, and radioactivity was exposed on a PhosphorImager. Where indicated, variants containing or lacking the S1 exon were distinguished by restriction analysis with BpmI.

**PCR of the region flankling the mouse IP•R1 S1 splice site was amplified in a final volume of 100 μl containing Taq buffer (10 mM Tris·HCl, pH 8.8, 50 mM KCl), 2 mM MgCl_2, 0.17 mg/ml BSA, 2.5

(Notes and references are omitted for brevity.)
units of Taq polymerase (AmpliTaq Gold), and 0.05 mM of each dNTP, as well as 25 pmol of each oligonucleotide primer: upstream (5'-GGAAAGAAGCCAAGAATTGAC-3'; sense) and downstream (5'-CAGGGGACACGGAATCTTGG-3'; antisense). Before PCR, the samples were incubated at 94°C for 9 min. PCR was performed for 40 cycles in a PerkinElmer model 480 Tempycler as follows: 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s. After PCR, the samples were heated to 72°C for 5 min. The amplified products were resolved on 1.5% agarose gels and photographed under UV illumination.

PCR for actin was performed using the primers specified by Kudo et al. (22). PCR for CaMKIV was performed in a final volume of 100 μl containing Taq buffer (10 mM Tris·HCl, pH 8.8, 50 mM KCl), 2 mM MgCl₂, 0.17 mg/ml BSA, 2.5 units of Taq polymerase (AmpliTaq Gold), and 0.05 mM of each dNTP, as well as 25 pmol of each oligonucleotide primer: upstream (5'-TGCAAGGTAGAAAGG-GACTCG-3'; sense) and downstream (5'-GTACTGGAGGTGAC-GACTCG-3'; antisense). Before PCR, the samples were incubated at 94°C for 7 min. PCR was performed for 40 cycles in a PerkinElmer model 480 Tempycler as follows: 94°C for 30 s, 56°C for 30 s, and 72°C for 45 s. After PCR, the samples were heated to 72°C for 5 min. The amplified products were resolved on 8% polyacrylamide gels and photographed under UV illumination.

**Statistical analysis.** Values are means ± SE and were analyzed for significance using Student’s t-test or ANOVA followed by Tukey’s test as appropriate. *P < 0.05 was considered significant.

**RESULTS**

**Survival-promoting agents that increase intracellular Ca²⁺ upregulate IP₃ R1.** In vivo, IP₃ R1 expression is particularly robust in Purkinje neurons of the cerebellar cortex but is undetectable in the granular layer. Similarly, when GC are cultured in vitro in medium containing 5 mM KCl, a growth condition that does not sustain long-term survival of the majority of neurons (e.g., Fig. 1A), immunoreactive IP₃ R1 is low or undetectable (Fig. 1B). However, IP₃ R1 is readily detected in GC neurons when the growth medium is supplemented with 20 mM KCl (i.e., 25 mM) or 140 μM NMDA plus 5 mM KCl (Fig. 1B), two agents that promote long-term survival by activating Ca²⁺ influx through voltage-sensitive calcium channels (VSCC; inhibited by nimodipine or nifedipine) or NR (inhibited by MK-801 or AP5), respectively (e.g., Fig. 1A). Consistent with this, upregulation of IP₃ R1 by elevated KCl is attenuated when the VSCC antagonist nifedipine (5 μM) is added (Fig. 1C; similar results are observed using NR antagonists in GC grown in medium supplemented with NMDA; data not shown). Note that brief, overnight addition of VSCC or NR antagonists to the growth medium does not compromise GC viability, unlike longer term exposure. Note also that the observed increase in IP₃ R1 (per mg of homogenate protein) in GC is well below the amount observed in lysates of whole cerebellar cortex, which include Purkinje neurons (Fig. 1B).

Ca²⁺ influx through VSCCs and NR induces mRNA containing S1, S2, and S3 exons. In contrast to cerebellum of adult rodents, cerebella from neonatal rodents express substantial amounts of a variant of IP₃ R1 mRNA that includes the S1 exon, i.e., IP₃ R1 S1+ (30), which is assumed to be localized in Purkinje neurons. Interestingly, the dominant expression of IP₃ R1 S1+ corresponds to the period of active GC migration and synaptogenesis. To explore the possibility that IP₃ R1 S1+ is expressed in developing GC, we examined whether exposure to 25 mM KCl or NMDA induces corresponding increases in IP₃ R1 mRNA containing S1+ and, if so, whether these require activation of VSCC and NR, respectively. The effects of these agents on selection of the S2 exon, as well as S3, which is not expressed in cerebellum in vivo (34), were also examined. GC were grown for 5 days in vitro (DIV) in media containing 140 μM NMDA plus 5 mM KCl, 5 or 25 mM KCl, in the presence

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**Fig. 1.** Survival-promoting agents that increase intracellular Ca²⁺ upregulate inositol trisphosphate receptor type 1 (IP₃ R1). A: viability of granule cells (GC) grown in medium containing 5 mM KCl, 25 mM KCl ± 5 μM nimodipine (Nimo), or 140 μM N'-methyl-aspartate (NMDA) ± 5 mM KCl ± 10 μM (5,10S)-5-methyl-10,11-dihydro-5H-benzo[a,d]cyclohepten-5,10-iminemaleate (MK-801). Drugs were added at 2 days in vitro (2 DIV) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was done at 7–9 DIV (n = 4). *Significantly different vs. 25 mM KCl. **Significantly different vs. NMDA. B: top: cultures were grown for 8 DIV in medium supplemented with 20 mM KCl (final concentration, 25 mM), whole cell homogenates were harvested, and 10–40 μg of homogenate protein were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose blotting membrane, and probed with an antibody against IP₃ R1. Bottom: adult cerebellum (AdCb), and GC cultured in medium containing 5 mM KCl, 25 mM KCl, or NMDA were grown for 5 DIV, and then whole cell homogenates were harvested for Western immunoblotting. Each well was loaded with 15 μg of protein and probed with an antibody against IP₃ R1. C: immunofluorescence photomicrograph comparing GC grown in 25 mM KCl ± overnight treatment with nifedipine (5 μM), added at 7 DIV. At 8 DIV, cultures were fixed and incubated with antibody against IP₃ R1. Similar results were observed in 3 separate cell preparations. Magnification, ×63. Exposure time, 10 s.
or absence of the VSCC antagonist nimodipine (5 µM) or the NR antagonist MK-801 (10 µM), added at 4 DIV. Samples were then individually processed for RT-PCR using oligonucleotide primers (Fig. 2A) flanking the three exons and specifying amplicons of 691 bp (S1⁺), 517 bp (S2⁻) or 400 bp (S3⁻), and 102 bp (S3⁺) or 75 bp (S2⁺). As shown, amplicons were barely visible in GC grown in 5 mM KCl but were readily detectable after sustained growth in medium containing 25 mM KCl or NMDA (Fig. 2B). In all cases, amplicons were also detectable in GC grown overnight in 25 mM KCl, although to a lesser extent than chronic treatment. The KCl-mediated increases in IP₃R1 mRNA were attenuated by overnight addition of nimodipine, and the NMDA-mediated increases were attenuated by overnight addition of MK-801. Volumetric analysis of S3 amplicons (sum total) was used to obtain estimates of the relative increases in IP₃R1 mRNA, which were statistically robust (Fig. 2C). Taken together, these data indicate that 25 mM KCl and NMDA induce expression of IP₃R1 mRNA by activating VSCC and NR, respectively.

Interestingly, only amplicons corresponding to S1⁺ mRNA (691 bp) were detected in GC grown in elevated KCl or NMDA. In 12 of 12 cell preparations tested, GC exclusively expressed this variant, regardless of the developmental stage or culture condition used. This is in contrast to adult cerebellar cortex, in which the IP₃R1 S1⁻ variant predominates (646 bp) and is localized in Purkinje neurons (30). Low amounts of transcripts encoding both variants of S2 were detected in GC grown in 5 mM KCl, with proportionately more S2⁻. Moreover, statistically significant increases in those containing the S2 exon were observed after sustained growth in medium containing 25 mM KCl or NMDA, compared with 5 mM KCl (% inclusion of S2: 5 mM, 41.7 ± 3.7; 25 mM, 81.9 ± 2.8; NMDA, 73.3 ± 5.7; n = 4). Likewise, statistically significant increases in transcripts containing the S3 exon were observed after growth in medium containing 25 mM KCl or NMDA, compared with 5 mM KCl (% inclusion of S3: 5 mM, 49.6 ± 2.8; 25 mM, 67.5 ± 1.6; NMDA, 61.8 ± 1.4; n = 5). Note that no effort was made to distinguish between the different subtypes of S2, i.e., S2ABC⁺ (520 bp) vs. S2B⁻ (517 bp, the major form in neurons) (30).

CaMK inhibitors attenuate Ca²⁺-dependent induction of IP₃R1. To discern the possible role of CaMK in GC IP₃R1 expression, cultures were grown in medium containing 5 or 25 mM KCl, or 140 mM NMDA, with or without 20 mM KCl added at 4 DIV to 5 mM KCl cultures (5 Δ 25, 24 h); and nimodipine (5 µM) or MK-801 (10 µM) were added at 4 DIV to 25 mM KCl or NMDA cultures, respectively. (representative experiment; n = 5). Amplicons were resolved on 8% polyacrylamide gels and exposed on a PhosphorImager. C: volumetric analysis of radiolabeled samples from 3 cell preparations was performed to estimate differences in IP₃R1 amplicon (sum of S3 variants). *Significant differences vs. 5 mM KCl-containing samples, set at 100%. **Significant differences vs. respective treatment (e.g., 5 Δ 25 or NMDA alone).

A

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B

C

Fig. 2. IP₃R1 S1, S2, and S3 mRNA variants are upregulated in a Ca²⁺-dependent manner. A: sequences of the primers used for PCR and the predicted sizes of the amplified fragments. B: RT-PCR (40 cycles; 5 ng of RNA) was performed on GC cultured for 5 DIV in medium containing 5 mM KCl, 25 mM KCl, or 140 µM NMDA + 5 mM KCl. As indicated, 20 mM KCl was added at 4 DIV to 5 mM cultures (5 Δ 25, 24 h); and nimodipine (5 µM) or MK-801 (10 µM) were added at 4 DIV to 25 mM KCl or NMDA cultures, respectively. (representative experiment; n = 5). Amplicons were resolved on 8% polyacrylamide gels and exposed on a PhosphorImager. C: volumetric analysis of radiolabeled samples from 3 cell preparations was performed to estimate differences in IP₃R1 amplicon (sum of S3 variants). *Significant differences vs. 5 mM KCl-containing samples, set at 100%. **Significant differences vs. respective treatment (e.g., 5 Δ 25 or NMDA alone).
mM KCl, and, where indicated, KN-62 (5 μM; IC₅₀ in vitro 900 nM) was added at 4 DIV. The selection of agent for these studies was based on a rigorous analysis of the specificity of numerous kinase inhibitors in which the fidelity of KN-62 as a selective and potent inhibitor of CaMKII and CaMKIV was supported (8). Note that brief, overnight addition of KN-62 to the growth medium does not compromise GC viability, unlike longer-term exposure. GC were harvested at 5 DIV and processed for RT-PCR using the S1, S2, and S3 oligonucleotide primers (Fig. 2A). Consistent with the data in Fig. 2B, cultures grown in 25 mM KCl-containing media expressed amplicons corresponding to S1+ , S2+, and S3+ IP₃R1 mRNA. Importantly, overnight addition of KN-62 attenuated the KCl-mediated upregulation of all three variants (Fig. 3A) as well as attenuation of immunoreactive IP₃R1 (Fig. 3B). Volumetric analysis of the S3 variants (sum total), readily measurable in the 5 mM samples, was performed to obtain estimates of the depolarization-induced increases in IP₃R1 mRNA. These increases were statistically significant relative to 5 mM KCl or 25 mM KCl cultures receiving KN-62 (% of 5 mM KCl, set at 100; 25 mM + KN-62 = 273.3 ± 27.3; 25 mM KCl = 716.7 ± 112.0; n = 3). Note that attenuation of IP₃R1 mRNA induction by 25 mM KCl was also observed after overnight treatment with KN-93, a less selective CaMK inhibitor that triggers some toxicity (not shown). These data indicate that activation of a target of KN-62, likely CaMKII or CaMKIV, is required for Ca²⁺-dependent induction of IP₃R1 in GC.

**Actinomycin D and calcineurin inhibitors attenuate Ca²⁺-dependent induction of IP₃R1.** Uprogation of the IP₃R1 mRNA by agents that increase Ca²⁺ influx could be due to increased stability and/or enhanced gene transcription. To distinguish between these possibilities, actinomycin D, a pleiotropic transcription inhibitor, was used. GC were grown in medium containing 5 mM KCl, and, as indicated, some cultures were supplemented with 20 mM KCl alone or with 20 mM KCl plus actinomycin D (1 μg/ml), added at 4 DIV. At 5 DIV, RNA was harvested and S1-specific amplicons were compared. Overnight addition of 20 mM KCl alone induced IP₃R1 S1+ mRNA, whereas simultaneous addition of actinomycin D (1 μg/ml) prevented this effect (Fig. 4A). This result indicates that depolarization-dependent upregulation of IP₃R1 is due to enhanced gene transcription.

As discussed, FK506 or CsA, two calcineurin inhibitors, attenuates depolarization-dependent induction of IP₃R1 in GC (12) and hippocampal neurons (13). To verify this effect in our cultures, GC were grown for 4 DIV and then vehicle, FK506 (0.2 μg/ml), or CsA (1 μg/ml) was added. After 24 h, whole cell RNA was harvested for RT-PCR analysis using oligonucleotide primers specifying the S1 variants of IP₃R1. Figure 4B shows that these agents reduced the depolarization-dependent induction of IP₃R1 S1+ mRNA in GC. Similar results were observed in 4 of 4 different cell preparations treated with FK506 and 2 of 2 preparations treated with CsA. Collectively, these data indicate that depolarization-dependent transcription of IP₃R1 in GC requires both calcineurin and CaMK.

**IP₃R1 S1⁺ is the predominant variant in cerebella of mutant mice lacking Purkinje neurons.** Investigators have largely ignored functional studies of IP₃R1 in GC neurons in vivo because of the lack of detectable expression of mRNA and protein. As indicated, IP₃R1 S1⁺ is prominent in the neonatal, but not adult, cerebellar cortex (30). After migration is complete (P21 in rat), there is a switch from prominent expression of IP₃R1 S1⁺ to the adult form, S1⁻. An intriguing possibility is that GC in vivo express IP₃R1 S1⁻, and it contributes to the Ca²⁺⁻dependent process of migration and/or synaptic refinement. To explore this, oligonucleotide primers encoding the S1⁺ variant were used to compare the relative proportions of S1⁺ and S1⁻ variants in the cerebella of mutant mice with specific Purkinje cell lesions (designated as pcd) vs. wild-type controls.

The two major neuronal types in cerebellar cortex are Purkinje and GC. In pcd mice, there is a rapid degeneration of nearly all Purkinje neurons beginning at approximately P15, without significant loss of GC (17). Thus, if GC in vivo predominately express IP₃R1 S1⁺ mRNA (as observed in cultures under all conditions of assay), then an increase in the relative proportion of the S1⁺ variant should be observed in the cerebellar cortex of pcd⁻/⁻ mice, compared with wild-type (+/+ ) littermates. In other words, by eliminating the major source of IP₃R1 in cerebellum, namely, IP₃R1 S1⁻ in Purkinje neurons, a detectable amount of IP₃R1 S1⁺ mRNA may be revealed in the remaining GC. Initially, a RT-PCR assay specifying S1⁺ and S1⁻ variants in mice was tested. Despite a 99.6% similarity in the amino acid sequences between rat and mouse, different oligonucleotide primers were designed because of potential mismatches between the rat primers and the murine sequences flanking the S1 site. The predicted sizes of the amplicons are 158 and 113 bp (Fig. 5A). On the basis of in

![Fig. 3. CaMKs mediate IP₃R1 variant expression. A: RT-PCR (S1, 40 cycles; S2, 35 cycles; S3, 35 cycles; actin, 26 cycles; 5 ng of RNA) was performed on GC cultured for 5 DIV in medium containing 5 or 25 mM KCl. As indicated, KN-62 (5 μM) was added overnight. Amplicons were resolved on 8% (S1, S2, and actin) or 20% (S3) polyacrylamide gels and exposed on a PhosphorImager. B: immunoaffluorescence photomicrograph comparing GC grown in 25 mM KCl ± overnight treatment with KN-62 (5 μM) added at 7 DIV. At 8 DIV, cultures were fixed and incubated with antibody against IP₃R1. Similar results were observed in 2 separate cell preparations. Magnification, ×63. Exposure time, 10 s.](image-url)
mal mice expressed primarily the S1/H11002 variant in 30-day-old mice. The (H9262 cyclosporin A (CsA, 1 medium containing 5 or 25 mM KCl. As indicated, FK506 (FK, 0.2 actin, 26 cycles; 5 ng of RNA) was performed on GC cultured for 5 DIV in acrylamide gels and exposed on a PhosphorImager. RNA was harvested at 5 DIV. RT-PCR analysis with oligonucleotide primers (H11001 specifying mRNA corresponding to the S1/H11001 variant predominated. Also, overall size of the cerebella of pcd mice (%S1/H11002 in wild-type vs. null mouse cerebellum: 82.5 ± 0.85 vs. 44.7 ± 2.02%; n = 4). In addition, the overall amount of IP3R1 amplicon was lower than that of wild type. These data verify that S1/H11002 is the major variant in Purkinje cells. Importantly, they suggest that GC predominately express IP3R1 S1/H11002 in vivo.

**Fig. 5. IP3 R1 S1 mRNA expression in Purkinje cell degeneration (pcd) vs. wild-type mice. A:** schematic diagram of oligonucleotide primers flanking the S1 splice site. B: *left:* RT-PCR analysis of mRNA in adult murine hippocampus (Hip) and cerebellum (Cb). Hippocampus from normal mice expressed primarily the S1+/variant (158 bp), whereas the cerebella from normal mice expressed primarily the S1− variant (113 bp). *Right:* RT-PCR analysis of mRNA harvested from wild-type (++) and matched mutant/pcd−/− cerebella of 30-day-old mice. The (+/−) mice expressed primarily the S1− variant (113 bp). In the pcd−/− mouse, the S1− variant predominated. Also, overall size of the cerebella and expression of the S1 mRNA were reduced compared with wild type. Amplicons were resolved on ethidium bromide-stained gels and photographed under UV illumination.

**Fig. 4.** Actinomycin D and calcineurin inhibitors attenuate Ca2+/dependent induction of IP3R1. A: RT-PCR (S1, 40 cycles; actin, 26 cycles; 5 ng of RNA) was performed on GC cultured for 5 DIV in medium containing 5 mM KCl. As indicated, 20 mM KCl + actinomycin D (A, 1.0 μg/ml) or cyclosporin A (CsA, 1 μg/ml) was added overnight to 5 mM KCl cultures. RNA was harvested at 5 DIV. RT-PCR analysis with oligonucleotide primers that specify mRNA corresponding to the S1+/variant or actin was performed. Amplicons were resolved on 8% polyacrylamide gels and exposed on a PhosphorImager. B: RT-PCR (S1, 40 cycles; actin, 26 cycles; 5 ng of RNA) was performed on GC cultured for 5 DIV in medium containing 5 or 25 mM KCl. As indicated, FKS06 (FK, 0.2 μg/ml) or cyclosporin A (CsA, 1 μg/ml) was added overnight to 25 mM KCl cultures (and also to 5 mM cultures, although no effect was observed; data not shown). RNA was harvested at 5 DIV. RT-PCR analysis with oligonucleotide primers that specify mRNA corresponding to the S1+/variant or actin was performed. Amplicons were resolved on ethidium bromide-stained gels and photographed under UV illumination.

**Fig. 6.** GC neurons in the granular laminae express IP3R1 S1+/+. The innovative technique of laser microdissection provides a means of obtaining highly enriched tissue from distinct laminae of the cerebellar cortex. If GC neurons express IP3R1 S1+/+ in vivo, then it should be enriched in the granular layer of slices from rat cerebellar cortex compared with the cell-sparse medullary layer (1). To test this hypothesis, slides were prepared from cerebellar cortices of P13–P18, medullary and granular layers were microdissected (Fig. 7), and samples were subjected to RT-PCR analysis using oligonucleotide primers specifying mRNA corresponding to IP3R1 S1+/−. CaMKIV, expressed in hippocampus, and the major variant in cerebellum was S1−. The identity of this S1− variant was verified by restriction site analysis (with BpmI; data not shown). Next, RNA from the cerebella of 30-day-old wild-type and pcd−/− mice was compared (Fig. 5B). Again, the predominant variant in wild-type mice was S1−. In contrast, a statistically significant increase in the relative proportion of amplicon corresponding to S1− mRNA was observed in pcd mice (%S1− in wild-type vs. null mouse cerebellum: 82.5 ± 0.85 vs. 44.7 ± 2.02%; n = 4). In addition, the overall amount of IP3R1 amplicon was lower than that of wild type. These data verify that S1− is the major variant in Purkinje cells. Importantly, they suggest that GC predominately express IP3R1 S1− in vivo.

**GC in cerebellar explant cultures express IP3R1.** If IP3R1 has a role in GC maturation, it may be possible to detect IP3R1 expression in GC grown in standard culture medium (e.g., 5 mM KCl) at an early stage of development. Connor et al. (5) reported that GC in explant cultures of rodent cerebellar cortex migrate outward along glial fibers that radiate from the explant core. As the GC migrate outward, they develop the biochemical and electrophysiological properties of maturing GC in vivo (5). We cultured explants derived from the cerebellar cortices at P3 and verified by phase-contrast microscopy the temporal migration of GC away from the core along radial glial fibers. To assess the expression of immunoreactive IP3R1 in GC cultures were grown for 3 DIV and then fixed and processed for immunocytochemistry. As shown in Fig. 6, GC throughout the culture were positively stained with IP3R1 antibodies.
This result is consistent with the preponderance of IP$_3$ R1 S1 and NR, thereby activating downstream signaling pathways, in particular CaMK (e.g., CaMKIV) and phosphatases (e.g., calcineurin). These enzymes in turn decode and relay this highly specific information to the nucleus via coordinate activation of various transcription factors (for review, see Refs. 7, 18, and 27).

In the present study, evidence for activation of CaMK in IP$_3$R1 induction in GC is based on the use of highly selective pharmacological agents. Accordingly, supplementation of the growth medium with elevated KCl or NMDA supported GC survival (11) and also induced IP$_3$R1 protein and mRNA. Overnight addition of the l-type VSCC antagonists, nifedipine or nimodipine, effectively reversed 25 mM KCl-mediated induction of IP$_3$R1 protein and mRNA (Figs. 1C and 2B). Likewise, the competitive and noncompetitive NR antagonists, APS and MK-801, respectively, effectively reversed NMDA-mediated induction of IP$_3$R protein and mRNA (Fig. 2B). Thus, although the route of Ca$^{2+}$ entry into neurons can differentially affect gene transcription, i.e., hippocampal neurons (28), both routes of Ca$^{2+}$ entry induced IP$_3$R1 in GC. The selective and potent cell-permeable CaMK inhibitor KN-62, which competes with calmodulin for binding to the regulatory domains of CaMKIV and CaMKII (6, 8, 44), also attenuates IP$_3$R1 induction (Fig. 3). Similar effects were observed with the less selective agent, KN-93. On the basis of existing reports that these compounds attenuate transcriptional events mediated by CaMKIV in diverse cell types (e.g., Refs. 15, 38, 50), we suggest that nuclear CaMKIV is a component of this signaling cascade in GC. However, a role for CaMKII cannot formally be excluded.

**DISCUSSION**

It is well established that activity-dependent alterations in intracellular Ca$^{2+}$ concentration and downstream Ca$^{2+}$-sensitive enzymes translate changes in bioelectrical activity into distinct patterns of gene expression, ultimately affecting complex biological processes such as survival, differentiation, and plasticity (for review, see Refs. 2, 39, 41, and 48). In many central neurons, the excitatory transmitter glutamate initiates these processes by regulating the influx of Ca$^{2+}$ through VSCC and NR, thereby activating downstream signaling pathways, in particular CaMK (e.g., CaMKIV) and phosphatases (e.g., calcineurin). These enzymes in turn decode and relay this highly specific information to the nucleus via coordinate activity.
Besides the present study and an earlier report (35), only one
group of investigators has examined depolarization-de-
pendent induction of IP3-R1 in GC (12). Those authors dem-
strated that pharmacological blockade of calcineurin with
FK506 or CsA attenuated depolarization-dependent expression
of IP3-R1 (independently verified herein, Fig. 4B). Depolariza-
tion- and calcineurin-dependent induction of IP3-R1 was sub-
sequently reported in hippocampal neurons, and a NF-ATc
pathway was shown to be critical (13; see also Ref. 16). Taken
together, these studies support roles for both calcineurin, acting
through NF-ATc, and CaMK, probably CaMKIV, as regulators
of IP3-R1 expression in GC.

IP3-R1 promoter activity appears to be regulated through
several cis elements present in a short region within the
5′-flanking region, which contains a TATA box, and binding
sites for the CAMP-responsive element, PU box motif,
CCAAT-binding transcription factor/NF-1, AP2, basic helix-
loop-helix factor, TPA-responsive element, AP3, B sequence,
CCAGG-containing sequence, and POU/homeobox transcription
factor binding sequence. These cis elements confer wide-
spread and differential expression of the IP3-R1 gene in brain
and peripheral tissues (10). An interesting possibility in GC is
that the basic helix-loop-helix promoter site in the IP3-R1 gene
is synergistically activated by calcineurin and CaMKIV. Along
these lines, the myocyte enhancing factor-2 (MEF-2) can
dimerize with basic helix-loop-helix proteins, and synergism
between CaMKIV and calcineurin in transactivation through
MEF-2 has been described in T cells, ventricular myocytes,
and skeletal muscle (for review, see Refs. 18 and 39). Indeed,
MEF-2 is expressed in newly generated GC and is activated by
depolarization and Ca2+,
where it contributes to survival (25).
Moreover, MEF-2 activity in GC appears to be enhanced by
calcineurin under depolarizing conditions because this process is
sensitive to inhibition by CsA and FK506 (26). More
recently, Groth and Mermelstein (16) reported that inhibition of
NF-AT signaling only partially reduced expression of IP3-R1
in response to brain-derived neurotropic factor (BDNF), indi-
cating that multiple transcription factors can control the IP3-R1
gene, some independently of NF-AT. In contrast, inhibition of
NF-AT signaling completely abolished BDNF expression, indi-
cating that although multiple transcription factors may regu-
late BDNF, NF-AT is required. Further studies are needed to
determine whether a synergistic link between CaMKIV and
calcineurin regulates Ca2+,
dependent IP3-R1 induction in GC.

Ca2+ and CaM kinases induce selection of a distinct IP3-R1
variant in GC. In the present study, depolarizing agents that
increase Ca2+ influx in GC produced splice variants that
differed from those that predominate in Purkinje neurons or in
GC grown in 5 mM KCl. GC grown in NMDA or in 25 mM
KCl-containing medium exhibited significantly higher levels
of amplicons that included all three exons. In contrast, Purkinje
neurons predominantly express variants containing S2 but
lacking the S1 and S3 exons (30, 34), while GC grown in 5 mM
KCl predominantly express variants containing S1 with pro-
portionately less S2+ and similar amounts of S3+- (Figs. 2
and 3). These data suggest that Ca2+,
calcineurin, and/or
CaMK influence exon choice in IP3-R1. Indeed, a handful of
reports have indicated that Ca2+,
dependent signaling cascades influence exon selection in excitable cells. For example, depo-
larization-dependent Ca2+ influx through VSCC or NR alters
exon selection in pre-mRNA encoding Ca2+ -ATPases (51),
NR (46, 47), and the slo K+ channel (50). Using transfected
cell lines, Xie and Black (50) specifically demonstrated that
overexpression of CaMKIV regulates splicing of the STREX
exon in slo K+ channel and two exons (5 and 21) in NR1, hinting that
CaMKIV may have a broad role in the regulation of alternative
splicing. To identify specific features of the STREX exon
needed for CaMKIV repression, deletions in the STREX ex-
onic sequences that were similar to either purine-rich enhancer
or pyrimidine-rich repressor elements, previously identified in
other pre-mRNA, were mutated. The pyrimidine-rich repressor
deletion increased exon inclusion with almost no repression by
CaMKIV, leading to the conclusion that the pyrimidine-rich
region is required for CaMKIV-mediated repression of the
STREX exon (50). An additional repressor element in the
upstream 3′ splice site, termed the CaMKIV-responsive RNA
element (CaRRE), that confers sensitivity to CaMKIV was also
identified. However, a thorough sequence analysis of potential
purine- and pyrimidine-rich sequences in and near the S2 and
S3 exons in IP3-R1 was unrevealing. Furthermore, we were
unable to identify a CaRRE sequence in the 3′ region of the S2
and S3 exons. In both cases, structurally distinct but function-
ally homologous sequences may underlie Ca2+,
dependent exon selection, and GC should prove useful in studying such
questions in the future.

Is IP3-R1 physiologically relevant in cerebellar GC neurons in vivo? As indicated, previously available methods of assay
did not reveal IP3-R1 protein or mRNA in the cerebellar
granular layer in vivo (24, 30, 32, 40). The inability to detect a
low amount of immunoreactive IP3-R1 may be further com-
pounded by the fact that the adjacent Purkinje cell layer is by
far the richest source known. Nevertheless, IP3-R1-specific
mRNA can be detected in GC grown in standard medium (5
mM KCl) when a highly sensitive RT-PCR assay is used (e.g.,
Figs. 2–4). Moreover, immunoreactive IP3-R1 is detectable in
migrating GC in explant cultures grown in culture medium
containing standard concentrations of KCl (5 mM) (Fig. 6). In
all conditions tested, we observed only the S1+ variant of
IP3-R1, the form that predominates in neonatal, but not adult,
cerebellar cortex (30). These data suggest that IP3-R1 S1+
may have a role in developing, but not mature, GC. Along these
lines, Kirischuk et al. (19) compared agonist-induced Ca2+-
mobilization in GC from acutely prepared slices from murine
cerebella at two ages. IP3-mediated Ca2+ mobilization was
observed in GC in slices from neonates (P6) but not adults
(P30).

Herein we provide the first evidence that IP3-R1 S1+
is expressed in GC in vivo. First, cerebella from pcd mice express
proportionally more amplicon corresponding to the S1+ variant
than do their wild-type littermates (Fig. 5). This result is
consistent with the selective loss of the S1+ variant due to
degeneration of Purkinje cells relative to the S1+-expressing
GC, which are spared. Second, microdissected tissue from the
granular layer of cerebellar slices from neonatal rats specifi-
cally express amplicons corresponding to IP3-R1 S1+ (Fig. 7).
Although previous investigators failed to detect IP₃R₁ mRNA or immunoreactive protein in GC, there is evidence for IP₃R₁ promoter activity. Thus moderate expression of a transgene containing a strong IP₃R₁ promoter was detected in cerebellar GC neurons (10), and Ohkawa et al. (36) reported weak but significant AP₂-dependent IP₃R₁ promoter activity in GC. Possibly, IP₃R₁ contributes to migration or synaptic refinement in developing GC, two Ca²⁺-dependent processes that depend critically on the concerted action of CaM kinases and phosphatases (for review, see Refs. 7, 20, 21). In this regard, emerging evidence suggests an important link between IP₃R₁ activity and neuronal morphogenesis (3, 9, 14, 16, 42). Notably, functional inactivation of IP₃R₁ in growth cones in dorsal root ganglia results in growth arrest and neurite retraction (42), and axonal outgrowth is compromised in NF-ATc null mice (14). Thus neurons may use IP₃R₁ in conjunction with other pathways known to integrate Ca²⁺ signals to strengthen maturing synapses. The data presented herein provide a strong rationale for pursuing functional studies of IP₃R₁ in developing GC.

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REFERENCES

33. Newton CL, Mignery GA, and Sudhof TC. Co-expression in vertebrate tissues and cell lines of multiple inositol 1,4,5-trisphosphate (InsP₃)}