Selective Blockade of P/Q-Type Calcium Channels by the Metabotropic Glutamate Receptor Type 7 Involves a Phospholipase C Pathway in Neurons

Julie Perroy,[1] Laurent Prezeau,[1] Michel De Waard,[2] Ryuichi Shigemoto,[3] Joel Bockaert,[1] and Laurent Fagni[1]

[1]*Centre National de la Recherche Scientifique, Unité Propre de Recherche 9023, Centre CNRS-INSERM de Pharmacologie et d'Endocrinologie, 34000 Montpellier, France,* [2]*Institut National de la Santé et de la Recherche Médicale, U-464, Faculté de Médecine Nord, 13916 Marseilles, France, and* [3]*Laboratory of Cerebral Structure, National Institute for Physiological Sciences, CREST Japan Science and Technology Corporation, Myodaiji, Okazaki 444–8585, Japan*

Although presynaptic localization of mGluR7 is well established, the mechanism by which the receptor may control Ca^{2+} channels in neurons is still unknown. We show here that cultured cerebellar granule cells express native metabotropic glutamate receptor type 7 (mGluR7) in neuritic processes, whereas transfected mGluR7 was also expressed in cell bodies. This allowed us to study the effect of the transfected receptor on somatic Ca^{2+} channels. In transfected neurons, mGuR7 selectively inhibited P/Q-type Ca^{2+} channels. The effect was mimicked by GTPγS and blocked by pertussis toxin (PTX) or a selective antibody raised against the G-protein αo subunit, indicating the involvement of a G_o-like protein. The mGuR7 effect did not display the characteristics of a direct interaction between G-protein $\beta\gamma$ subunits and the α1A Ca^{2+} channel subunit, but was abolished by quenching $\beta\gamma$ subunits with specific intracellular peptides. Intracellular dialysis of G-protein $\beta\gamma$ subunits did not mimic the action of mGluR7, suggesting that both G-protein $\beta\gamma$ and αo subunits were required to mediate the effect. Inhibition of phospholipase C (PLC) blocked the inhibitory action of mGluR7, suggesting that a coincident activation of PLC by the G-protein $\beta\gamma$ with αo subunits was required. The Ca^{2+} chelator BAPTA, as well as inhibition of either the inositol trisphosphate (IP_3) receptor or protein kinase C (PKC) abolished the mGluR7 effect. Moreover, activation of native mGluR7 induced a PTX-dependent IP_3 formation. These results indicated that IP_3-mediated intracellular Ca^{2+} release was required for PKC-dependent inhibition of the Ca^{2+} channels. Possible control of synaptic transmission by the present mechanisms is discussed.

Key words: mGluR7; Ca^{2+} channels; G-protein; PLC; cerebellar granule cells; transfection

The physiological actions of the neurotransmitter glutamate are mediated by ionotropic and metabotropic receptors (Nakanishi, 1992). Eight genes encoding mGluRs have been identified and classified into three groups. mGluR1 and mGluR5 belong to group I and activate phospholipase C (PLC) through stimulation of a G_q protein, in heterologous and homologous systems (Conn and Pin, 1997). The group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6, mGluR7, and mGluR8) mGluRs are coupled to $G_{i/o}$ protein in neuron (Prezeau et al., 1994) and heterologous expressing cells (Conn and Pin, 1997). These receptors are widely distributed throughout the mammalian brain (Kinzie et al., 1995; Ohishi et al., 1995; Bradley et al., 1996; Kinoshita et al., 1998), but the mGluR7 subtype displays peculiar properties in that it is almost exclusively localized at presynaptic sites (Shigemoto et al., 1996, 1997; Kinzie et al., 1997). Because of a lack of specific pharmacology, functional discrimination between mGluR7 and the other group III mGluR subtypes can only be achieved according to their different affinity for L-2-amino-4-phosphonobutyrate (L-AP-4), a selective group III mGluR agonist. Indeed the affinity of mGluR7 for L-AP-4 is clearly lower (EC_{50} = 160–500 μM; Okamoto et al., 1994; Saugstad et al., 1994) than that of mGluR4, 6, and 8 (EC_{50} = 0.2–1.2, 0.9, and 0.06–0.60 μM, respectively; Pin et al., 1999).

In behavioral studies, young mGluR7 knock-out mice display deficits in the fear response and conditioned taste aversion, whereas the adult mutants develop lethal spontaneous epileptic seizures (Masugi et al., 1999). *In vitro* studies showed that mGluR7 stimulation mediates neuroprotective effects in cultured cerebellar granule cells by decreasing glutamate release (Lafon-Cazal et al., 1999a) and promotes excitotoxicity in cultured striatal neurons by inhibiting GABA release (Lafon-Cazal et al., 1999b). Group III mGluRs, presumably mGluR7, have been shown to inhibit glutamate autaptic currents in hippocampal neurons (O'Connor et al., 1999). These studies, together with those showing the presynaptic localization of the receptor in the murine adult brain, suggest that mGluR7 plays an important role in modulation and plasticity of synaptic transmission.

The mechanism by which mGluR7 may control neurotransmitter release is still unknown. Indeed, previous studies have shown that L-AP-4 inhibits high-threshold voltage-gated Ca^{2+} channels in various neuronal preparations (Trombley and Westbrook, 1992; Rothe et al., 1994; Choi and Lovinger, 1996; Takahashi et al., 1996; Shen and Slaughter, 1998). Nevertheless, in these studies, the maximal inhibitions were obtained for relatively low concentrations of L-AP-4 (<100 μM) that should have selectively activated group III mGluRs, but with the exception of mGluR7. Moreover, inhibition of adenylyl cyclase by mGluR7 has only been shown in heterologous expression systems (Okamoto et al., 1994; Saugstad et al., 1994), and to our knowledge there is no clear study precluding that a different mechanism may function in neurons. Therefore, in the present study we investigated whether mGluR7 could modulate specific Ca^{2+} channel subtypes in cultured cerebellar granule cells and which coupling mechanism could be involved in this effect. We found that the receptor selectively inhibited P/Q-type Ca^{2+} channels by activating a G_o-like protein and, unexpectedly, through a PLC-dependent pathway.

Received April 3, 2000; revised July 20, 2000; accepted Aug. 16, 2000.

This work was supported by Centre National de la Recherche Scientifique and grants from Association Française contre les Myopathies, Fondation pour la Recherche Médicale, Bayer (France), and Hoechst-Marrion-Roussel (FRHMR1/9702). We thank J. P. Pin and F. Ango for constructive discussion of this work. We also thank Dr. J. Saugstad (Atlanta, GA) for the rat mGluR7a cDNA, J. M. Sabatier (Marseille, France) for the synthesis of the 68 AA peptide, V. Homburger (Montpellier, France) for the anti-Gαo antibody, and B. Mouillac (Montpellier, France) for the anti-cMyc monoclonal antibody.

Correspondence should be addressed to Dr. L. Fagni, Centre National de la Recherche Scientifique, Unité Propre Recherche 9023, CNRS-INSERM de Pharmacologie et d'Endocrinologie, 141 Rue de la Cardonille, 34000 Montpellier, France. E-mail: fagni@bacchus.montp.inserm.fr.

 0270-6474/00/207896-09$15.00/0

MATERIALS AND METHODS

Cell culture. Primary cultures of cerebellar cells were prepared as previously described (Van Vliet et al., 1989). Briefly, 1-week-old newborn mice were decapitated and cerebellum-dissected. The tissue was then gently triturated using fire-polished Pasteur pipettes, and the homogenate was centrifuged at 500 rpm. The pellet was resuspended and plated in tissue culture dishes previously coated with poly-L-ornithine. Cells were maintained in a 1:1 mixture of DMEM and F-12 nutrient (Life Technologies, Gaithersburg, MD), supplemented with glucose (30 mM), glutamine (2 mM), sodium bicarbonate (3 mM) and HEPES buffer (5 mM), decomplemented fetal calf serum (10%), and 25 mM KCl to improve neuronal survival. One-week-old cultures contained 125×10^3 cells/cm^2.

Plasmids and transfection. The N-terminal epitope-tagged mGluR7a receptor was constructed as follows. The Myc epitope was inserted in the extracellular domain, immediately downstream from the signal peptide. We used a mGluR5a-containing plasmid (pRKG5a-N-Myc) as the starting vector, in which the signal peptide was followed by the Myc-coding sequence at the N terminus of the protein, and then by a *Mlu*I site (Ango et al., 1999). The mGluR7a-coding sequence (except the signal peptide) was introduced into this vector using the *Mlu*I site and *Xba*I (3′ of the coding sequence), by following two steps: first we used a PCR and the oligonucleotide S, creating an *Mlu* I site in frame with the coding sequence of the vector (5′-gccAcgcgtatgtacgccccgcac-3′), and the oligonucleotide AS containing the *Xho*I site present in mGluR7 (5′-ttttctagaggaaggaatcaggcgg gacca-3′); second the fragment *Xho*I–*Xba*I was inserted by classical subcloning. The sequence was verified by sequencing. The resulting plasmid (pRKG7a-N-Myc) was referred as Myc-mGluR7. Functional coupling of Myc-mGluR7 was verified in human embryonic kidney 293 cells according to the protocol described elsewhere (Parmentier et al., 1998).

Immediately before plating, or 24 hr after plating, cerebellar cultures were transfected with the Myc-mGluR7 expression plasmid for immunocytochemical experiments or cotransfected with the transfection marker, green fluorescent protein (GFP)-containing plasmid, pEGFP-N1 (Clontech, Palo, Alto, CA), and non epitope-tagged mGluR7, for electrophysiological recordings, by using Transfast (Promega, Madison, WI), as described elsewhere (Ango et al., 1999).

Immunocytochemistry. Cultured cerebellar granule cells were fixed in a 4% paraformaldehyde and 0.1 M glucose-containing PBS solution. The culture was permeabilized with 0.05% Triton X-100, and fluorescent immunolabeling of native mGluR7 was performed by using a previously characterized anti-mGluR7a/b primary antibody (Shigemoto et al., 1996, 1997). The presence of Myc-mGluR7 protein at the cell surface of cultured neurons was examined in nonpermeabilized cerebellar cultures exposed to a monoclonal mouse anti-Myc primary antibody (a gift from B. Mouillac) diluted at 1:300 in a PBS–gelatin (0.2%) solution. After overnight incubation in the presence of either one of these primary antibodies at room temperature, cells were then rinsed and exposed to a goat Texas Red-conjugated anti-rabbit IgG secondary antibody or to a goat Texas Red-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, PA; 1:1000 dilution), for 2 hr at room temperature. Then cells were rinsed again with PBS and mounted on glass coverslips for observation on an Axiophot 2 Zeiss microscope.

Electrophysiology. Whole-cell patch-clamp Ba^{2+} currents were recorded at room temperature from GFP and mGluR7 cotransfected cerebellar granule cells, after 9 ± 1 days *in vitro* as previously described (Ango et al., 1999). The bathing medium contained (in mM): $BaCl_2$ 20, HEPES 10, tetraethylammonium acetate 10, TTX 3×10^{-4}, glucose 10, sodium acetate 120, and MK801 1×10^{-3}, adjusted to pH 7.4 with NaOH and 330 mOsm with sodium acetate. Drug solutions were prepared in this bathing medium, and the pH was adjusted to 7.4. The NMDA receptor-channel blocker MK-801 (1 μM) was added to all the solutions to avoid activation of this receptor by the D isoform of D,L-AP-4 (our unpublished observation). Patch pipettes were made from borosilicate glass, coated with Sylgard, and the tip was fire-polished. Pipettes had resistances of 3–5 MΩ when filled with the following internal solution (in mM): Cs-acetate 100, $MgCl_2$ 2, HEPES 10, glucose 15, CsCl 20, EGTA 20, Na_2ATP 2, and cAMP 1, adjusted to pH 7.2 with CsOH and 300 mOsm with CsOH. In some experiments, intracellular EGTA was replaced by BAPTA.

Ba^{2+} currents were evoked by voltage-clamp pulses of 500 msec duration, from a holding potential of −80 mV to a test potential of 0 mV. Voltage pulses were applied at a rate of 0.1 Hz. Current signals were recorded with an Axopatch 200 amplifier, filtered at 1 kHz with an 8-pole Bessel filter, and sampled at 3 kHz on a Pentium II personal computer. Linear leak and capacitive currents were digitally subtracted from records before analysis by using the P/N procedure of the pClamp6 software of Axon Instruments (Foster City, CA). Analyses were performed by using the Clampfit subprogram of pClamp6. Ba^{2+} currents were measured at their peak amplitude and expressed as mean ± SEM of the indicated number (n) of experiments. In experiments in which neurons were dialyzed with compounds, current measurements were started at least 5 min after breaking the patch.

The intracellular I-II loop of the α1A Ca^{2+} channel subunit, which contained the binding site of G-protein βγ subunits and P/Q-type Ca^{2+} channel β subunit, was generated in the laboratory, according to the following procedure. The 68-mer peptide, corresponding to the sequence from 360 to 427 of the α1A (BI-2) subunit, was synthesized by the solid-phase method (Merrifield, 1986) by means of an automated peptide synthesizer (model 433A; Applied Biosystems, Foster City, CA). The peptide chain was assembled by a double-coupling strategy using Fmoc amino acid hydroxybenzotrioazol active esters. The crude peptide was purified to homogeneity by C18 reversed-phase HPLC and characterized by amino acid analysis after acidolysis, Edman sequencing, and mass spectrometry. The experimental values obtained were all in agreement with the theoretically deduced values.

Measurement of inositol phosphate accumulation. The procedure we used to measure inositol triphosphate (IP_3) accumulation in neurons was adapted from one previously described (Blahos et al., 1998). One-week-old cerebellar granule cell cultures were incubated for 14 hr in culture medium containing 2 μCi/ml *myo*-(^{3}H)inositol (23.4 Ci/mol) (NEN, Paris, France). Cells were then washed three times and incubated for 1 hr at 37°C, in 1 ml of HEPES saline buffer (in mM: NaCl 146, KCl 4.2, $MgCl_2$ 0.5, and HEPES 20, glucose 0.1%, pH 7.4) supplemented with 1 U/ml glutamate pyruvate transaminase (Boehringer Mannheim, Meylan, France) and 2 mM pyruvate (Sigma, Lisle d'Abeau, France). Cells were then washed again with the same buffer, and LiCl was added to a final concentration of 10 mM. The agonist was applied 15 min later and left for 5 min. The reaction was stopped by replacing the incubation medium with 0.5 ml of perchloric acid (5%) on ice. Supernatants were recovered, and IPs were purified on Dowex columns (Berridge et al., 1983). Total radioactivity remaining in the membrane fraction was counted after treatment with 10% Triton X-100 and 0.1 N NaOH for 30 min and used as a standard. Results were expressed as the ratio of [^{3}H]IP production over radioactivity present in the membranes. Experiments were performed in triplicates for statistical analyses.

Materials. L-AP-4, Dihydroxy-phenyl-glycine (DHPG), and MK-801 were purchased from Tocris Cockson. PTX, Nimodipine, GF109203X, U73122, and U73343 were purchased from Research Biochemicals (Natick, MA). ω-Agatoxin-IVA and ω-Conotoxin-GVIA were from Alomone Labs (Jerusalem, Israel). The G-protein βγ subunits purified from bovine brain were from Calbiochem. The inhibitor peptide of the catalytic subunit and the competitive inhibitor of the regulatory subunit of protein kinase A, protein kinase A inhibitor peptide (PKI), and Rp-cAMPS respectively, were also from Calbiochem. GTPγS was from Sigma, and PDBu and PMA were from Fluka. An antibody raised against the G_o-protein was a generous gift from V. Homburger. This antibody has been previously shown to specifically recognize the G-protein αo but not αi subunit (Lledo et al., 1992). The pcDNA3-CD8-βARK plasmid, which was composed of the CD8 antigen membrane receptor and a domain containing the G-protein βγ subunit-binding site of βARK, was a generous gift from Dr. J. Lang. The pEGFP-N1 expression plasmid was purchased from Clontech.

RESULTS

Absence of native mGluR7 expression in the soma of cultured cerebellar granule cells

Immunolabeling of native mGluR7 in permeabilized cerebellar granule cells revealed a somatic exclusion and a neuritic punctate pattern of distribution of the receptor (Fig. 1*A*). D,L-AP-4, up to 1 mM concentration, did not significantly alter the whole-cell Ba^{2+} current of neurons transfected (Fig. 2*A*) or not (data not shown) with GFP alone (control). Together these results indicated that the native mGluR7 was absent at the surface of the soma of cultured cerebellar granule cells. Therefore these neurons were potentially a good model to study the effect of transfected mGluR7 on somatic Ca^{2+} channels in these neurons, providing that the transfected receptor would be expressed at the cell body membrane.

Selective inhibition of P/Q-type Ba^{2+} current by transfected mGluR7 at the soma of cultured cerebellar granule cells

Eight to ten days after transfection of the Myc-mGluR7 expression plasmid in cultured cerebellar granule cells, Myc immunostaining revealed the presence of both somatic and neuritic cell surface clusters of the recombinant receptors (Fig. 1*B*). In these transfected neurons, D,L-AP-4 (500 μM) decreased the amplitude of the total Ba^{2+} current, without significantly affecting its activation and inactivation kinetics (Fig. 2*B*). The effect started after a delay of ~20 sec, developed slowly, and reached a plateau over a 1 min 30 sec application of the agonist. This inhibition was accompanied by a slight but not significant modification of voltage-dependent activation (Fig. 2*C*) and no alteration of steady-state inactivation (Fig. 2*D*) properties of the current. The D,L-AP-4 effect was dose-dependent, the threshold effect being obtained for 100 μM, and the maximal effect (38% inhibition) for 500 μM concentrations (Fig. 2*A,B*). The current inhibition lasted for at least 10 min after washout of the agonist (Fig. 2*E*). This long-lasting D,L-AP-4-

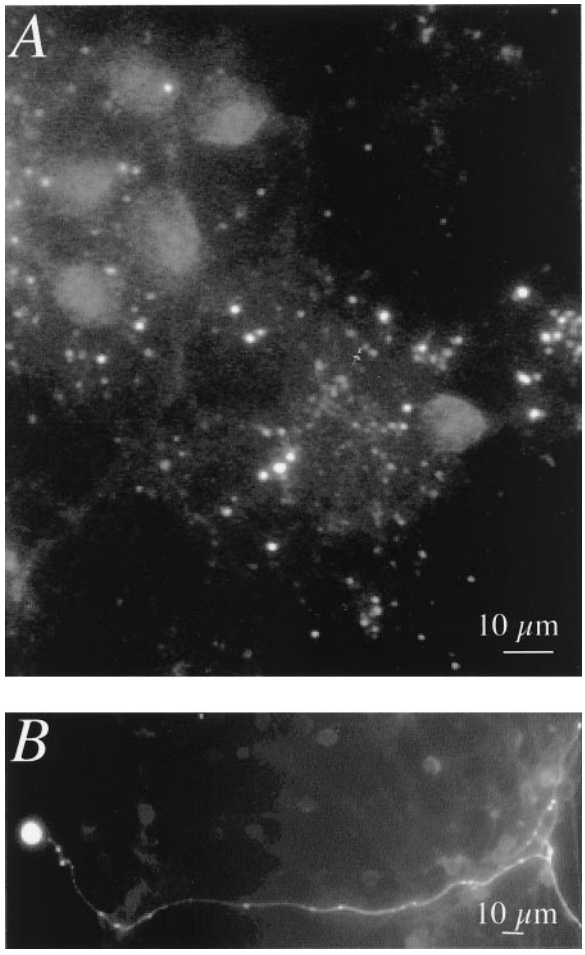

Figure 1. Localization of native and transfected mGluR7 in cultured cerebellar granule cells. *A,* Native mGluR7 immunolabeling in permeabilized cultured cerebellar granule cells. *B,* Nonpermeabilized cultured cerebellar granule cell transfected with the Myc-mGluR7 expression plasmid and labeled with an anti-Myc antibody. Note the presence of neuritic clusters in *A* and *B* and presence of somatic immunolabeling only in *B*.

mediated inhibition of Ba^{2+} currents did not result from an agonist-independent run-down of the current, because the amount of inhibition was stable for several minutes during wash-out of the agonist (Fig. 2*E*). Moreover, no significant decrease of the current was observed over a period of 45 min, in the absence of D,L-AP-4 (data not shown).

In cultured cerebellar granule cells transfected with GFP alone (control), P/Q-type (ω-Agatoxin-IVA; 250 nM), N-type (ω-Conotoxin-GVIA; 1 μM), and L-type (nimodipine; 1 μM) Ca^{2+} channel blockers inhibited the whole-cell Ba^{2+} current by 41, 10, and 22%, respectively. The remaining 27% of total Ba^{2+} current were of the R-type. Similar results were obtained in cultured cerebellar granule cells cotransfected with GFP and mGluR7 or nontransfected cultured cerebellar granule cells (Fig. 2*F*). Therefore, our transfection procedure did not alter functional expression of native Ca^{2+} channels in the studied cells.

To determine which types of Ca^{2+} channels were inhibited by transfected mGluR7, D,L-AP-4 (500 μM) was applied first, followed by perfusion of different selective Ca^{2+} channel blockers, on neurons cotransfected with mGluR7 and GFP. After application of D,L-AP-4, the remaining Ba^{2+} current was not significantly affected by application of ω-Agatoxin-IVA (250 nM), but was further depressed by ω-Conotoxin-GVIA (1 μM) or nimodipine (1 μM), and in similar proportions as those obtained in control cells (transfected with GFP alone; Fig. 2*G*). It is worth noting that the fractional inhibition induced by D,L-AP-4 in cotransfected neurons (38%; Fig. 2*A*) was not significantly different from the fraction of ω-Agatoxin-IVA-sensitive current obtained in control neurons (41%; Fig. 2*G*).

In a second series of experiments, an initial application of a given channel blocker was immediately followed by application of D,L-AP-4 (500 μM). When ω-Agatoxin-IVA was applied first, the ω-Agatoxin-IVA-resistant Ba^{2+} current was not affected by subsequent perfusion of D,L-AP-4 (500 μM; 3 ± 1% inhibition, $n = 7$, Fig. 3*A*). On the other hand, when ω-Conotoxin-GVIA or nimodipine were applied first, the drug/toxin-insensitive Ba^{2+} current was further depressed by subsequent application of D,L-AP-4 (37 ± 2% inhibition, $n = 5$, after ω-Conotoxin-GVIA, Fig. 3*B*; 36 ± 4% inhibition, $n = 5$, after nimodipine, Fig. 3*C*; 36 ± 2% inhibition, $n = 5$, after ω-Conotoxin-GVIA and nimodipine, Fig. 3*D*). Finally, after coapplication of all three Ca^{2+} channel blockers, D,L-AP-4 did not further inhibit the remaining Ba^{2+} current (3 ± 2% inhibition, $n = 4$). After application of D,L-AP-4, ω-Conotoxin-GVIA (Fig. 3*A,C*) and nimodipine (Fig. 3*A,B*) further inhibited the Ba^{2+} current (by 10 and 20%, respectively), whereas ω-Agatoxin-IVA did not (Fig. 3*B,D*). Altogether these results demonstrated that mGluR7 selectively blocked the P/Q-type Ca^{2+} channels, without significantly affecting N-, L-, and R-types.

mGluR7-mediated activation of a G_o-like protein

To examine if a G-protein was involved in the mGluR7-mediated inhibition of P/Q-type Ba^{2+} currents, we intracellularly applied the nonselective G-protein activator, GTPγS (100 μM). Under these conditions, the D,L-AP-4-mediated inhibition of Ba^{2+} current was highly reduced (Fig. 4*A*). Indeed, ω-Agatoxin-IVA inhibited only 12 ± 2% ($n = 5$) of the current, indicating that the P/Q-type Ca^{2+} channels were already significantly inhibited by GTPγS. Overnight incubation of the culture in the presence of PTX (200 ng/ml) abolished the inhibitory effect of D,L-AP-4 (Fig. 4*A*). Together these observations showed that a $G_{i/o}$-like protein was involved in the D,L-AP-4-mediated inhibition of P/Q-type Ca^{2+} channels.

A specific antibody raised against the G-protein αo subunit, which did not recognize the G-protein αi subunit (see Materials and Methods), was used to determine which type of G-protein was involved in the L-AP-4 effect. This antibody significantly inhibited the effect of D,L-AP-4 on the Ba^{2+} current (Fig. 4*A*) without significantly altering the current density in the absence of agonist (89 ± 7 pA/pF, $n = 10$ without antibody; 79 ± 8 pA/pF, $n = 6$, with antibody). The boiled antibody was without effect (36 ± 4%, D,L-AP-4-induced inhibtion of Ba^{2+} current, $n = 5$). These results showed that a G_o-, rather than a G_i-like protein, was involved in the D,L-AP-4-mediated effect.

We then investigated the involvement of the G-protein αo and βγ subunits. In heterologous expression systems, it has been shown that the P/Q-type Ca^{2+} channels can be blocked by a direct binding of G-protein βγ subunits on the I-II loop of the α1A Ca^{2+} channel subunit (De Waard et al., 1997; Bourinet et al., 1999). To test the implication of the native G-protein βγ subunits in the mGluR7-mediated inhibition of Ba^{2+} current in transfected cerebellar granule cells, these subunits were quenched in two ways. First, a peptide derived from the I-II loop of the α1A Ca^{2+} channel subunit (10 μM) was applied into the cell via the recording electrode. Second, the cDNA coding for a chimera composed of the CD8 membrane receptor antigen and of a domain containing the G-protein βγ subunit binding site of βARK, was cotransfected with mGluR7 in the cerebellar neurons. Under both conditions, the inhibitory effect of D,L-AP-4 was strongly reduced (Fig. 4*A*). We verified that after dialysis of the I-II loop of the α1A or transfection of the CD8-βARK chimera, ω-Agatoxin IVA still inhibited the whole-cell Ba^{2+} current (35 ± 6% inhibition, $n = 5$, in dialyzed neurons; 37 ± 2% in transfected neurons), indicating that the P/Q-type Ca^{2+} channels were not significantly affected by the tested peptides. Also, perfusion of a peptide-free solution or transfection of the CD8-βARK chimera deleted of its binding site for G-protein βγ subunits did not affect the action of D,L-AP-4 on Ba^{2+} currents (33 ± 3% inhibition, $n = 5$ in dialyzed neurons; 39 ± 3%, $n = 5$ in transfected neurons). These results indicated that mGluR7-mediated Ca^{2+} channel inhibition involved G-protein βγ subunits.

However, dialysis of G-protein βγ subunits (both at 50 μg/ml)

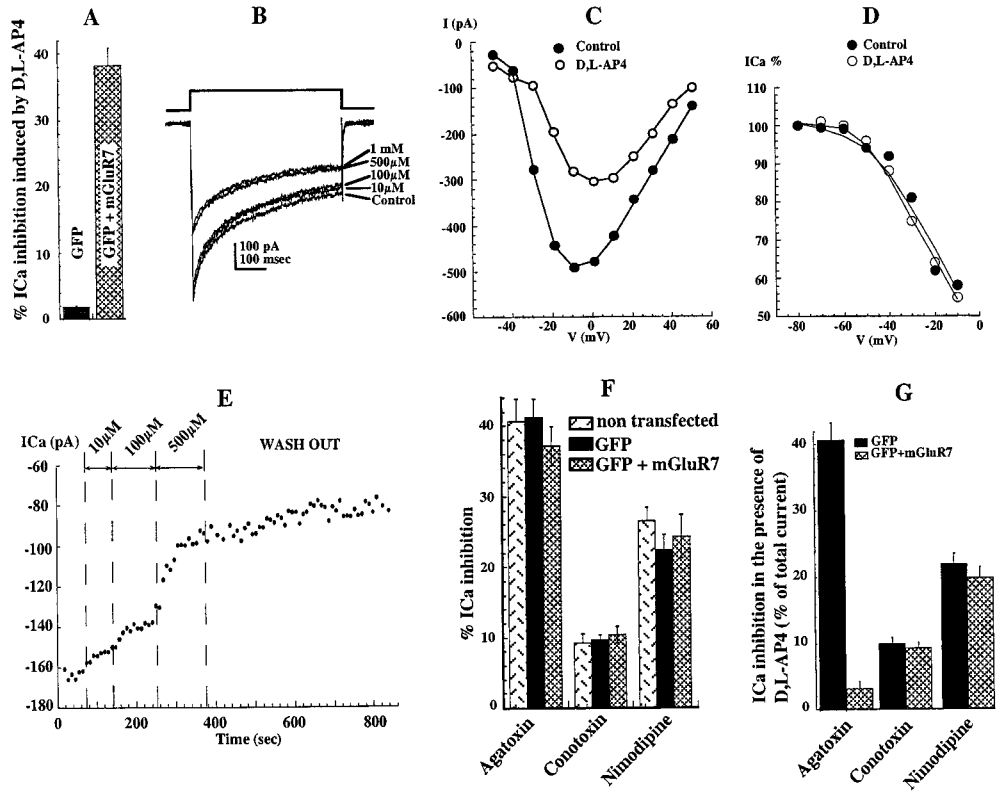

Figure 2. Inhibitory effect of D,L-AP-4 on Ba^{2+} currents in mGluR7-transfected cerebellar granule cells. *A,* Each bar of the histogram represents the mean (± SEM; $n = 10$ to 18) of fractional reduction of whole-cell Ba^{2+} current induced by D,L-AP-4 (500 μM) applied alone, in cultured cerebellar granule cells transfected with GFP alone, or cotransfected with GFP + mGluR7. Note that D,L-AP-4 alone inhibited Ba^{2+} currents only in cotransfected cells. *B,* Ba^{2+} currents recorded in a mGluR7-transfected cell, in the absence and presence of 10 μM, 100 μM, 500 μM, or 1 mM D,L-AP-4. Please note the absence of change in activation kinetics in the presence of the agonist. *C, D,* Activation (*C*) and inactivation (*D*) curves of whole-cell Ba^{2+} currents obtained from two different granule cells, in the absence (control) and presence of D,L-AP-4 (500 μM). Similar results were obtained from five other cells. *E,* Time course and concentration-dependent effect of D,L-AP-4 on Ba^{2+} currents in a mGluR7-transfected cerebellar granule cell. *F,* Inhibitory effects of ω-Agatoxin-IVA (250 nM), ω-Conotoxin-GVIA (1 μM), and nimodipine (1 μM) on Ba^{2+} currents obtained in nontransfected cultured cerebellar granule cells or transfected with GFP alone or cotransfected with GFP + mGluR7. Each bar of the histogram represents the mean (± SEM) of at least seven experiments. *G,* Inhibitory effects of ω-Agatoxin-IVA (250 nM), ω-Conotoxin-GVIA (1 μM), and nimodipine (1 μM) on Ba^{2+} currents obtained in the presence of D,L-AP-4 (500 μM), in cultured cerebellar granule cells transfected with GFP alone or cotransfected with GFP + mGluR7. Each bar of histogram represents the mean (± SEM) of at least 10 experiments. Note that the percentage of Ba^{2+} current inhibited by each toxin was similar in control and cotransfected cells, except for ω-Agatoxin-IVA, which was ineffective only in cotransfected cells.

did not significantly modify activation and inactivation kinetics of the whole-cell Ba^{2+} current (data not shown), and neither significantly altered the fraction of Ba^{2+} current that was inhibited by D,L-AP-4 (Fig. 4*A*), in mGluR7/GFP-cotransfected cerebellar granule cells. The tested G-protein βγ subunits inhibited and slowed activation kinetics of P/Q-type Ca^{2+} channels expressed in *Xenopus* oocytes, indicating that the absence of effect of the tested G-protein βγ subunits in cultured cerebellar granule cells did not result from a lack of activity of these molecules (data not shown). Together, these observations indicated that G-protein βγ subunits were required, but not sufficient to mediate the mGluR7-induced inhibition of Ba^{2+} current.

We further examined whether the mGluR7-mediated Ba^{2+} current inhibition could result from the well characterized direct interaction of G_o-protein βγ subunits with the I-II loop of α1A Ca^{2+} channel subunit (De Waard et al., 1997; Bourinet et al., 1999). A positive prepulse relieves this interaction and inhibition (voltage-dependent facilitation) of the P/Q-type Ba^{2+} current. In mGluR7-transfected cerebellar granule cells, no such voltage-dependent facilitation was observed in the absence or presence of D,L-AP-4 (Fig. 4*B*). Thus the ratio, *R*, between amplitudes of Ba^{2+} currents evoked with and without depolarizing prepulse was measured in the absence and presence of D,L-AP-4. We obtained values of *R* that were not significantly different from 1 ($R = 1.10 \pm 0.07\%$, $n = 9$, in the absence of agonist; $R = 1.04 \pm 0.06\%$, $n = 9$, in the presence of D,L-AP-4). This result indicated the absence of both tonic and mGluR7-mediated inhibition of Ca^{2+} channels through direct interaction of G_o-protein βγ subunits in mGluR7-transfected cerebellar granule cells.

mGluR7-mediated PKC-dependent blockade of Ba^{2+} current

Because mGluR7 appeared to inhibit P/Q-type Ca^{2+} channels via an indirect action of a G_o-like protein, we searched for any involvement of additional intracellular factors. The protein kinase A inhibitors, Rp-cAMPS (10 μM) and PKI (1 μM), added to the recording pipette solution, failed to inhibit Ba^{2+} currents in the absence or presence of D,L-AP-4, in nontransfected as well as mGluR7-transfected cerebellar granule cells (data not shown). On the other hand, the PKC activator PDBu (1 μM) inhibited the whole-cell Ba^{2+} current of cultured cerebellar granule cells by $27 \pm 5\%$ ($n = 7$). Similar results were obtained with the other PKC agonist, PMA (200 nM; $21 \pm 7\%$ inhibition, $n = 4$). These effects were not additive to the inhibitory effect of ω-Agatoxin IVA ($5 \pm 3\%$ inhibition after PDBu application, $n = 7$; Fig. 5*A*), indicating that P/Q-type Ca^{2+} channels in these neurons were PKC-sensitive. The hypothesis that mGluR7 activated a PKC-dependent pathway was therefore tested. In mGluR7-transfected granule neurons, a 30 min pretreatment with the selective PKC inhibitor GF109203X (10 μM) almost abolished the inhibitory effect of D,L-AP-4 (Fig. 5*B*). We then studied whether PLC was also involved. The D,L-AP-4-mediated inhibitory effect was altered by the PLC inhibitor U73122 (2 μM), whereas the inactive analog U73343 (same concentration) was without effect (Fig. 5*B*). These results indicated that mGluR7

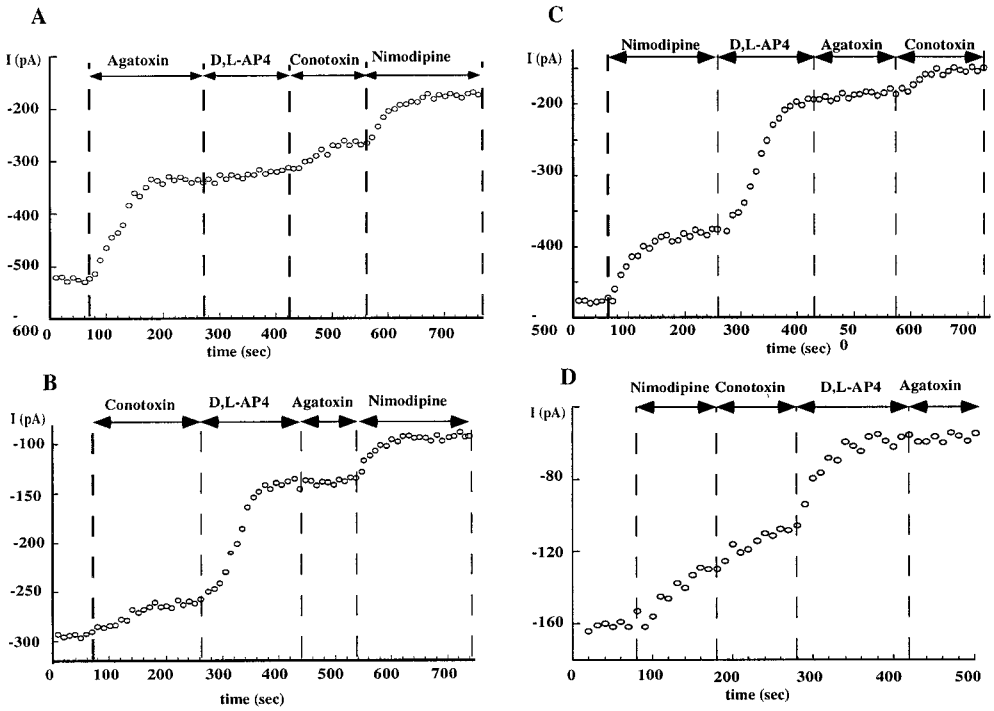

Figure 3. Selective blockade of P/Q-type Ba^{2+} currents by D,L-AP-4. Absence of effect of D,L-AP-4 (500 μM) on ω-Agatoxin-IVA- (250 nM, *A*) and inhibitory effect of the agonist on ω-Conotoxin GVIA- (1 μM, *B, D*) and nimodipine- (1 μM, *C, D*) insensitive Ba^{2+} currents. Graphs *A–D* were obtained from four different mGluR7-transfected cerebellar granule cells. Similar results were obtained from at least five other cells, for each graph.

blocked P/Q-type Ca^{2+} channels via a PLC/PKC-dependent pathway.

Because inhibition of Ba^{2+} currents by transfected mGluR7 was PLC-dependent, we tested whether the native receptor was able to induce IP_3 formation in the studied neurons. In nontransfected cerebellar cultures, D,L-AP-4 at concentrations that stimulated mGluR7 (500 μM or 1 mM), increased IP_3 formation by more than twofold, whereas lower concentrations of D,L-AP-4 (100 μM and 200 μM) had no significant effect on basal IP_3 formation (Fig. 6*A*). The D,L-AP-4-induced formation of IP_3 was abolished by an overnight pretreatment of the cultures with PTX (200 ng/ml; Fig. 6*A*). Therefore, native mGluR7 in cerebellar granule cells induced formation of IP_3 via a G_o-protein-dependent pathway. This result was in agreement with our electrophysiological data. Similar increases of IP_3 formation were obtained in mGluR7 transfected cerebellar cultures (Fig. 6*A*). This apparent absence of effect of transfected mGluR7 can be explained by the low rate of transfection (2–5%) obtained with our method (Ango et al., 1999).

The observed mGluR7-induced IP_3 formation should lead to diacylglycerol synthesis and intracellular Ca^{2+} release from IP_3-sensitive stores. The released Ca^{2+}, together with diacylglycerol, should then activate PKC, which in turn blocks P/Q-type Ca^{2+} channels. In agreement with this hypothesis, the inhibitory effect of D,L-AP-4 on Ba^{2+} currents was antagonized by the IP_3 receptor blocker heparin (400 μg/ml; Fig. 6*B*). Although resistant to 20 mM EGTA, the L-AP-4 induced P/Q type Ca^{2+} channel inhibition was abolished by the faster Ca^{2+} chelator BAPTA (20 mM), added to the intracellular recording medium (Fig. 6*B*).

DISCUSSION

The present results indicated that activation of mGluR7 selectively inhibited P/Q-type Ca^{2+} channels in cultured cerebellar granule cells. This blockade involved a G_o-protein and unexpectedly for a group III mGluR, PLC, intracellular Ca^{2+}, and PKC activation. Consistent with these results, we found that mGluR7 stimulated neuronal IP_3 formation in a PTX-dependent manner. We therefore propose a model in which mGluR7 activates a G_o-protein, the $\beta\gamma$ subunits of which directly stimulated PLC, likely in combination with the αo subunit, and induced IP_3 and diacylglycerol formation. This in turn results in intracellular Ca^{2+} release from IP_3-sensitive Ca^{2+} stores, PKC activation, and P/Q-type Ca^{2+} channel inhibition (Fig. 7).

Transfected granule cells as a model to study the mGluR7 signaling

The cultured cerebellar granule cell preparation provided a more physiological environment than the classical heterologous expression system to study the transduction signaling of transfected neuronal receptors. At the concentrations presently used (0.5 and 1 mM), D,L-AP-4 could not distinguish between the different group III mGluRs. Because the agonist did not affect Ba^{2+} currents in the absence of transfected mGluR7, native functional group III mGluRs were likely absent at the somatic plasma membrane of cultured cerebellar granule cells. This was confirmed, at least for mGluR7, by our immunolabeling experiments. Indeed, the native receptor was strictly localized in neuritic processes, whereas the transfected receptor was also detected at the somatic membrane. Together, these observations indicated that the D,L-AP-4 effects that we observed on Ba^{2+} currents in mGluR7-transfected cells certainly resulted from selective activation of this receptor, with the exclusion of any other group III mGluRs.

It could be argued that the unexpected coupling of the transfected mGluR7 with PLC pathway resulted from overexpression of the receptor in the cell body. Although not definitively proved, the following results argued against this hypothesis. First, the native neuritic mGluR7 also activated PLC, as indicated by the D,L-AP-4-mediated IP_3 formation observed in nontransfected cerebellar cultures. Second, cotransfection and overexpression of mGluR7 with mGluR2 did not change the coupling characteristics of the latter receptor (our unpublished observations), i.e., selective inhibition of N- and L-type Ca^{2+} channels (Chavis et al., 1995, 1998).

Indirect G_o-protein-mediated inhibition of P/Q-type Ca^{2+} channels

The mechanisms by which mGluRs block Ca^{2+} channels in neurons remain controversial. On one hand, a membrane delimited action mediated by direct interaction between G_o-protein $\beta\gamma$ subunits and Ca^{2+} channels has been proposed (Trombley and Westbrook, 1992; Choi and Lovinger, 1996; Ikeda, 1996). On the other hand, a slow inhibition of Ca^{2+} channels recorded in cell-attached patches has also been found and was consistent with the involvement of a soluble intracellular messenger (Chavis et al., 1994). The nature of this messenger remains however to be determined. In the present study, inhibition of P/Q-type Ca^{2+} channels involved a G_o-like protein, the $\beta\gamma$ subunits of which did not seem to directly

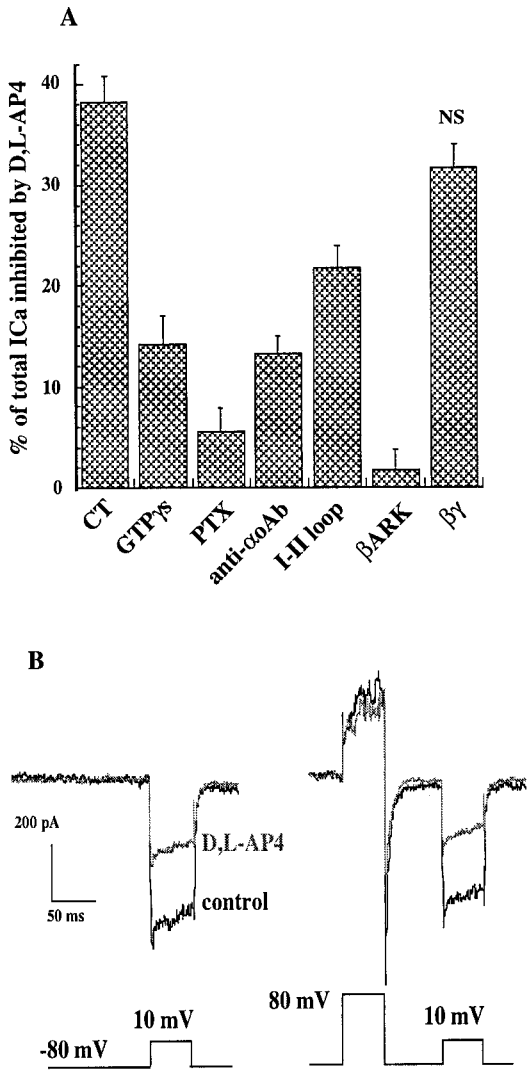

Figure 4. D,L-AP-4 inhibited Ba^{2+} current through an indirect action of G_o-protein. *A,* Mean (± SEM; $n = 6–10$) fractional reduction of the whole-cell Ba^{2+} current induced by D,L-AP-4 (500 μM) in mGluR7-transfected cerebellar granule cells, under different conditions (from *left* to *right*): control condition (*CT*), in the presence of intracellular GTPγS (100 μM), after an overnight PTX treatment (200 ng/ml), after intracellular dialysis of an antibody raised against the G-protein αo subunit (1:100 dilution; *anti-αoAb*), after intracellular dialysis of the α1A I-II loop peptide (10 μM; *I-II loop*), in cells cotransfected with CD8-βARK chimera (*βARK*), after intracellular dialysis of purified G-protein βγ subunits (50 μg/ml; *βγ*). *NS,* Not significantly different from control. *B,* Whole-cell Ba^{2+} currents evoked by depolarizing steps to +10 mV, from a holding potential of −80 mV, preceded (*right*) or not (*left*) by a prepulse to +80 mV. Note that D,L-AP-4 (500 μM) induced similar Ba^{2+} current inhibition in the presence or absence of prepulse depolarization. Similar results were observed in eight other mGluR7-transfected neurons.

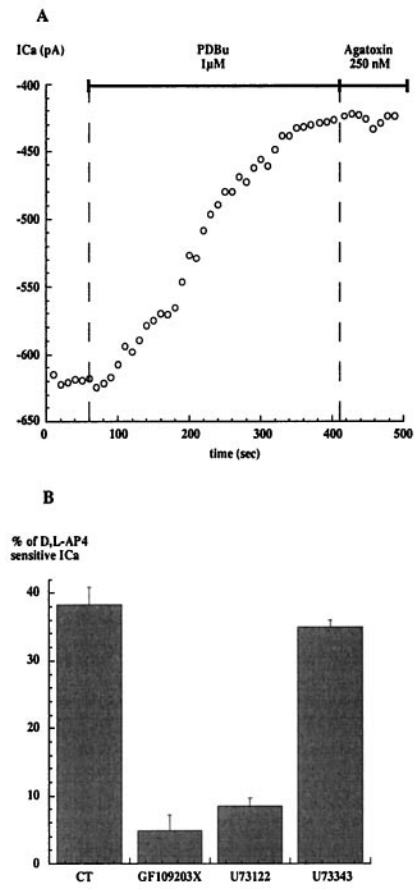

Figure 5. D,L-AP-4-mediated inhibition of Ba^{2+} currents involved a PLC/PKC pathway in mGluR7-transfected cerebellar granule cells. *A,* Time course of the inhibitory effect of the PKC activator PDBu (1 μM, 30 min) on whole-cell Ba^{2+} currents and absence of effect of ω-Agatoxin-IVA (250 nM) after the PDBu-mediated inhibition. *B,* Mean (± SEM; $n = 5–10$) fractional reduction of the whole-cell Ba^{2+} current recorded in mGluR7-transfected cerebellar granule cells induced by D,L-AP-4 (500 μM), under the following conditions (from *left* to *right*): in control cells (*CT*), in cells pretreated for 30 min with the PKC antagonist GF109203X (10 μM), and after 5 min dialysis of the PLC antagonist U73122 (2 μM) or the inactive analog U73343 (same concentration).

interact with the Ca^{2+} channel, because this inhibition was neither accompanied by slow activation kinetics of the Ba^{2+} current, nor removed by a depolarizing prepulses. A direct voltage-insensitive action of G-protein αo subunit on Ca^{2+} channels has been reported in sympathetic neurons (Delmas et al., 1998). However, such a mechanism did not seem to be involved in cerebellar granule cells because inhibition of PLC or PKC completely abolished the effect of D,L-AP-4 on I_{Ca} (Fig. 5*B*).

Now, cloned P/Q-types Ca^{2+} channels are generally inhibited by direct interaction of G-protein βγ subunits with the I-II loop of the α1A Ca^{2+} channel subunit (De Waard et al., 1997). However, it has been recently reported that alternative splicing of the α1A gene generates α1A-a and α1A-b subunits with distinct properties. Thus, the presence of a Val_{421} residue in the I-II linker domain of the α1A-b (absent in the α1A-a) splice variant subunit confers to the Ca^{2+} channel the faculty of being directly inhibited by G-protein βγ subunits (Bourinet et al., 1999). The authors' data also predict that most of α1A transcript in cerebellar neurons should be of the α1A-a subtype. Together these data are consistent with the hypothesis that cultured cerebellar granule cells may predominantly express the α1A-a splice variant subunit, which could explain the absence of direct effect of G_o-protein βγ subunits on P/Q-type Ca^{2+} currents in these neurons on application of mGluR7 agonist.

Activation of G_o-protein generally leads to inhibition of N-type Ca^{2+} channels (Hille, 1994) through a direct action of the G-protein βγ subunits on the channels (Ikeda, 1996), whereas in the present study N-type Ca^{2+} channels were spared. It is worth noting that in cultured cerebellar granule cells, inhibition of N-type Ca^{2+} channels was mediated by the G_o-protein-coupled mGluR2/3, and this effect did not display the fast kinetics and membrane-delimited voltage-dependent characteristics of a direct action of G_o-protein βγ subunits on these channels (Chavis et al., 1994, 1995). This suggests that other mechanisms are involved in our preparation and mediate a selective inhibition of P/Q-type versus N-type Ca^{2+} channels by mGluR7 and mGluR2/3, respectively. Two tentative hypotheses can be proposed to explain such a selectivity. mGluRs may be colocalized with specific Ca^{2+} channels in functional microdomains, probably through interaction with

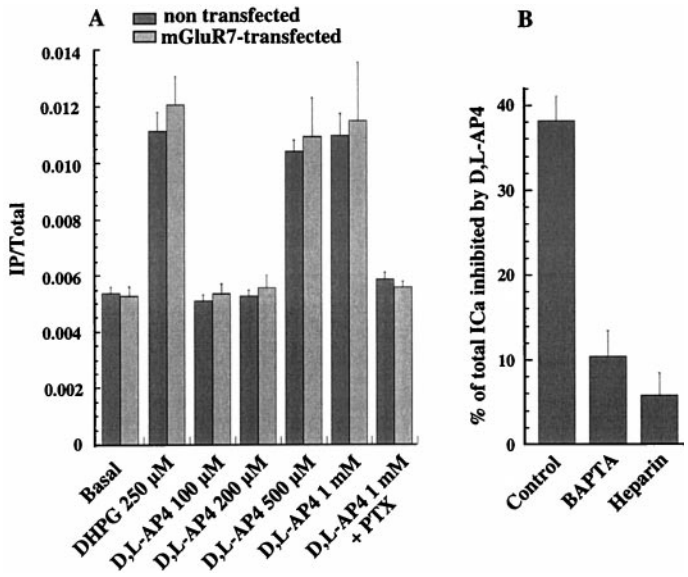

Figure 6. D,L-AP-4-induced IP_3 formation in nontransfected or mGluR7-transfected cultured cerebellar granule cells. *A,* IP formation was determined in nontransfected or mGluR7-transfected cerebellar cultures (from *left* to *right*) in the absence (*Basal*) and presence of the mGluR1 agonist DHPG (positive control), or different concentrations of D,L-AP-4. The last bar of the histogram on the *right* was obtained in PTX-treated cells. Each bar of the histogram represents the mean ± SEM of four independent experiments performed in triplicate. *B,* Mean (± SEM; $n = 5$–10) fractional reduction of the whole-cell Ba^{2+} current recorded in mGluR7-transfected cerebellar granule cells induced by D,L-AP-4 (500 µM), under the following conditions (from *left* to *right*): in control cells, in cells recorded with an intracellular medium containing 20 mM BAPTA, and after 5 min dialysis of the IP_3 receptor antagonist heparin (400 µg/µl).

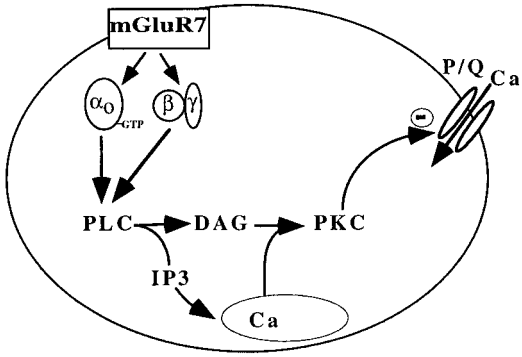

Figure 7. Model for mGluR7-induced inhibition of the P/Q-type Ca^{2+} channels in cerebellar granule cells. mGluR7 activates a G_o protein, the αo and βγ subunits of which stimulates a PLC. This results in IP_3 and diacylglycerol (*DAG*) formation. IP_3-induced Ca^{2+} release and DAG stimulate PKC, which in turn blocks the P/Q-type Ca^{2+} channel. Whether PKC directly phosphorylates the channel or acts on an intermediate protein is not determined.

scaffold proteins, the nature of which remains however to be identified. Alternatively, particular G-protein subunit combinations may activate specific signaling pathways. For instance, it has been reported that distinct αo, β, and γ subunit combinations display different efficacy in modulating βARK (Muller et al., 1993) or voltage-gated Ca^{2+} channel activity (Kleuss et al., 1993; Kalkbrenner et al., 1995).

Cellular determinants of the GluR7-mediated activation of PLC

Unexpectedly, we found that the mGluR7-mediated inhibition of the Ca^{2+} channels was PLC-dependent. The effect was abolished by treatments with PTX or a specific antibody raised against the G-protein αo subunit, indicating that a G_o protein activated PLC in cultured cerebellar granule cells. This finding was reminiscent of the G_o-mediated activation of PKC in enteric neurons (Pan et al., 1997). The effect of mGluR7 was mimicked by the nonselective G-protein activator GTPγS and blocked by quenching the G-protein βγ subunits with intracellular specific peptides. A likely hypothesis is that G-protein βγ subunits directly acted on a PLCβ in our preparation, as it is the case in various heterologous expression systems (Blank et al., 1992; Boyer et al., 1992, 1994; Camps et al., 1992; Blitzer et al., 1993). However, it is worth noting that intracellular dialysis of purified G-protein βγ subunits did not mimic the inhibitory effect of mGluR7 on Ba^{2+} currents. Together these observations indicated that G-protein βγ subunits were required, but not sufficient to activate PLC in neurons, and that G-protein αo and βγ subunits were both involved. Although G-protein αo subunit reconstituted in phospholipid vesicles was not required to activate PLC, this subunit shifted to the left the concentration-effect curve for βγ-mediated activation of PLCβ and increased the maximal activity of PLCβ (Boyer et al., 1992). It is therefore possible that under our experimental conditions, such a synergistic effect of G-protein αo subunits on activation of a PLCβ by the βγ subunits was required to reach a level of PLC activity sufficient to activate PKC and inhibit P/Q-type Ca^{2+} channels.

It has been shown that PKC phosphorylates a site located in the domain I-II linker of the cloned α1A Ca^{2+} channel subunit, which results in upregulation of the P/Q-type Ca^{2+} current (Bourinet et al., 1999). Because phorbol esters or mGluR7 inhibited native P/Q-type Ca^{2+} channels in cultured cerebellar granule cells, a different PKC phosphorylation site was involved in this effect. An alternative hypothesis is that PKC phosphorylated an intermediate protein that in turn downregulated the Ca^{2+} channel.

A reason why native P/Q-type Ca^{2+} channels in cultured cerebellar granule cells were not sensitive to the facilitatory effect of PKC could be that these neurons express the α1A-a Ca^{2+} channel subunit isoform, as suggested above. Indeed, in addition to be little sensitive to G-protein βγ subunits interaction, this subunit isoform harbors low sensitivity to upregulation mediated by PKC (Bourinet et al., 1999).

Possible physiological consequences of the mGluR7-mediated inhibition of P/Q-type Ca^{2+} channels

Activation of PLC by mGluR7 was sufficient to inhibit Ca^{2+} channels in cultured cerebellar granule cells. This observation was consistent with the absence of effect of PKA inhibitors on Ba^{2+} currents, in the absence or presence of D,L-AP-4, in mGluR7-transfected cerebellar granule cells. Moreover, it has been reported that L-AP-4 activates cAMP-independent and PLC-dependent pathways in mitral olfactory bulb (Schoppa and Westbrook, 1997) and retinal ganglion cells (Shen and Slaughter, 1998) respectively. Also, the efficiency of the coupling between mGluR7 and adenylyl cyclase in BHK cells is relatively low (Saugstad et al., 1994). Altogether these results suggest that the classical inhibition of adenylyl cyclase by group III mGuRs, which has been described in heterologous expression systems (Okamoto et al., 1994; Saugstad et al., 1994; Wu et al., 1998), would not be the primary transduction pathway by which mGluR7 triggers its physiological effects in neurons. We therefore propose that in natural systems this receptor, like group I mGluRs, acts through a PLC-dependent cascade.

To further understand the role of mGluR7 in synaptic transmission, one needs to transpose our results to neuronal synaptic terminals. Like mGluR7 (Shigemoto et al., 1996, 1997; Kinzie et al., 1997), IP_3-sensitive Ca^{2+} stores, PKC activity (Rodriguez-Moreno et al., 1998), and P/Q-type Ca^{2+} channels (Turner et al., 1992; Takahashi and Momiyama, 1993; Regehr and Mintz, 1994; Dunlap et al., 1995) have been found at presynaptic sites and control neurotransmitter release. Therefore, our results anticipate that mGluR7 would downregulate synaptic transmission through activation of PKC. This hypothesis is in apparent discrepancy with the classical phorbol ester-mediated facilitation of transmitter release, but it has been shown that these compounds act indepen-

dently of PKCγ, the major brain isoform of PKC (Goda et al., 1996).

This does not exclude the possibility that other presynaptic receptors can mediate direct effects of G_o-protein on N-type Ca^{2+} channels. Indeed, it has been shown that although N-type Ca^{2+} channels can be inhibited by direct and indirect effects of G-proteins in sympathetic neuron somata, only the direct pathway seems to mediate inhibition of transmitter release (Koh and Hille, 1997). The relative importance of a direct versus indirect inhibition of presynaptic Ca^{2+} channels may depend on the colocalization of Ca^{2+} channels with G_o-protein-coupled receptors. Thus, although the model we propose here (Fig. 7) provides a possible physiological mechanism by which mGluR7 could dampen synaptic transmission, it may not be predominant under low-frequency synaptic activity. Indeed, whereas neurotransmitter glutamate normally lasts shortly in the synaptic cleft, the mGluR7-induced inhibition of Ba^{2+} current needed a long time to develop and lasted long after washout. Because of these kinetic properties, the mGluR7-activated pathway may be more important under sustained synaptic activity such as during induction of synaptic plasticity or subthreshold epileptic neuronal discharges. In agreement with this hypothesis, inhibition of excitatory synaptic transmission by group III mGluRs, during high- but not low-frequency synaptic activity, has been observed in locus coeruleus (Dube and Marshall, 2000). This hypothesis provides the mGluR7 pathway as neuroprotective and is consistent with physiological studies showing that adult mGluR7 knock-out mice died from epileptic seizures (Masugi et al., 1999).

REFERENCES

Ango F, Albani-Torregrossa S, Joly C, Robbe D, Michel JM, Pin JP, Bockaert J, Fagni L (1999) A simple method to transfer plasmid DNA into neuronal primary cultures: functional expression of the mGlu5 receptor in cerebellar granule cells. Neuropharmacology 38:793–803.

Berridge MJ, Dawson RM, Downes CP, Heslop JP, Irvine RF (1983) Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. Biochem J 212:473–482.

Blahos II J, Mary S, Perroy J, de Colle C, Brabet I, Bockaert J, Pin JP (1998) Extreme C terminus of G protein alpha-subunits contains a site that discriminates between Gi-coupled metabotropic glutamate receptors. J Biol Chem 273:25765–25769.

Blank JL, Brattain KA, Exton JH (1992) Activation of cytosolic phosphoinositide phospholipase C by G-protein beta gamma subunits. J Biol Chem 267:23069–23075.

Blitzer RD, Omri G, De Vivo M, Carty DJ, Premont RT, Codina J, Birnbaumer L, Cotecchia S, Caron MG, Lefkowitz RJ, Landau EM, Iyengar R (1993) Coupling of the expressed alpha 1B-adrenergic receptor to the phospholipase C pathway in *Xenopus* oocytes. The role of Go. J Biol Chem 268:7532–7537.

Bourinet E, Soong TW, Sutton K, Slaymaker S, Mathews E, Monteil A, Zamponi GW, Nargeot J, Snutch TP (1999) Splicing of alpha 1A subunit gene generates phenotypic variants of P- and Q-type calcium channels. Nat Neurosci 2:407–415.

Boyer JL, Waldo GL, Harden TK (1992) Beta gamma-subunit activation of G-protein-regulated phospholipase C. J Biol Chem 267:25451–25456.

Boyer JL, Graber SG, Waldo GL, Harden TK, Garrison JC (1994) Selective activation of phospholipase C by recombinant G-protein alpha- and beta gamma-subunits. J Biol Chem 269:2814–2819.

Bradley SR, Levey AI, Hersch SM, Conn PJ (1996) Immunocytochemical localization of group III metabotropic glutamate receptors in the hippocampus with subtype-specific antibodies. J Neurosci 16:2044–2056.

Camps M, Carozzi A, Schnabel P, Scheer A, Parker PJ, Gierschik P (1992) Isozyme-selective stimulation of phospholipase C-beta 2 by G protein beta gamma-subunits. Nature 360:684–686.

Chavis P, Shinozaki H, Bockaert J, Fagni L (1994) The metabotropic glutamate receptor types 2/3 inhibit L-type Ca channels via a Pertussis toxin-sensitive G-protein in cultured cerebellar granule cells. J Neurosci 14:7067–7076.

Chavis P, Fagni L, Bockaert J, Lansman JB (1995) Modulation of calcium channels by metabotropic glutamate receptors in cerebellar granule cells. Neuropharmacology 34:929–937.

Chavis P, Ango F, Michel JM, Bockaert J, Fagni L (1998) Modulation of big K^+ channel activity by ryanodine receptors and L-type Ca^{2+} channels in neurons. Eur J Neurosci 10:2322–2327.

Choi S, Lovinger DM (1996) Metabotropic glutamate receptor modulation of voltage-gated Ca^{2+} channels involves multiple receptor subtypes in cortical neurons. J Neurosci 16:36–45.

Conn PJ, Pin JP (1997) Pharmacology and functions of metabotropic glutamate receptors. Annu Rev Pharmacol Toxicol 37:205–237.

De Waard M, Liu H, Walker D, Scott VES, Gurnett CA, Campbell KP (1997) Direct binding of G-protein βγ complex to voltage-dependent calcium channels. Nature 385:446–450.

Delmas P, Abogadie FC, Dayrell M, Haley JE, Milligan G, Caulfield MP, Braown DA, Buckley NJ (1998) G-proteins and G-protein subunits mediating cholinergic inhibition of N-type calcium currents in sympathetic neurons. Eur J Neurosci 10:1654–1666.

Dube GR, Marshall KC (2000) Activity-dependent activation of presynaptic metabotropic glutamate receptors in locus coeruleus. J Neurophysiol 83:1141–1149.

Dunlap K, Luebke JI, Turner TJ (1995) Exocytotic Ca^{2+} channels in mammalian central neurons. Trends Neurosci 18:89–98.

Goda Y, Stevens CF, Tonegawa S (1996) Phorbol ester effects at hippocampal synapses act independently of the gamma isoform of PKC. Learn Mem 3:182–187.

Hille B (1994) Modulation of ion-channels function by G-protein-coupled receptors. Trends Neurosci 17:531–536.

Ikeda SR (1996) Voltage-dependent modulation of N-type calcium channels by G-protein βγ subunits. Nature 380:255–258.

Kalkbrenner F, Degtiar VE, Schenker M, Brendel S, Zobel A, Heschler J, Wittig B, Schultz G (1995) Subunit composition of Go proteins functionally coupling galanin receptors to voltage-gated calcium channels. EMBO J 14:4728–4737.

Kinoshita A, Shigemoto R, Ohishi H, van der Putten H, Mizuno N (1998) Immunohistochemical localization of metabotropic glutamate receptors, mGluR7a and mGluR7b, in the central nervous system of the adult rat and mouse: a light and electron microscopic study. J Comp Neurol 393:332–352.

Kinzie JM, Saugstad JA, Westbrook GL, Segerson TP (1995) Distribution of metabotropic glutamate receptor 7 messenger RNA in the developing and adult rat brain. Neuroscience 69:167–176.

Kinzie JM, Shinohara MM, van den Pol AN, Westbrook GL, Segerson TP (1997) Immunolocalization of metabotropic glutamate receptor 7 in the rat olfactory bulb. J Comp Neurol 385:372–384.

Kleuss C, Scherubl H, Heschler J, Schultz G, Wittig B (1993) Selectivity in signal transduction determined by β subunits of heterotrimeric G proteins. Science 258:832–834.

Koh DS, Hille B (1997) Modulation by neurotransmitters of catecholamine secretion from sympathetic ganglion neurons detected by amperometry. Proc Natl Acad Sci USA 94:1506–1511.

Lafon-Cazal M, Fagni L, Guiraud MJ, Mary S, Lerner-Natoli M, Pin JP, Shigemoto R, Bockaert J (1999a) mGluR7-like metabotropic glutamate receptors inhibit NMDA-mediated excitotoxicity in cultured mouse cerebellar granule neurons. Eur J Neurosci 11:663–672.

Lafon-Cazal M, Viennois G, Kuhn R, Malitschek B, Pin JP, Shigemoto R, Bockaert J (1999b) mGluR7-like receptor and GABA(B) receptor activation enhance neurotoxic effects of *N*-methyl-D-aspartate in cultured mouse striatal GABAergic neurones. Neuropharmacology 38:1631–1640.

Lledo PM, Homburger V, Bockaert J, Vincent JD (1992) Differential G protein-mediated coupling of D2 dopamine receptors to K^+ and Ca^{2+} currents in rat anterior pituitary cells. Neuron 8:455–463.

Masugi M, Yokoi M, Shigemoto R, Muguruma K, Watanabe Y, Sansig G, van der Putten H, Nakanishi S (1999) Metabotropic glutamate receptor subtype 7 ablation causes deficit in fear response and conditioned taste aversion. J Neurosci 19:955–963.

Merrifield B (1986) Solid phase synthesis. Science 232:341–347.

Muller S, Hekman M, Lhose MJ (1993) Specific enhancement of β-adrenergic receptor kinase activity by defined G-protein β and γ subunits. Proc Natl Acad Sci USA 90:10439–10443.

Nakanishi S (1992) Molecular diversity of glutamate receptors and implications for brain function. Science 258:597–603.

O'Connor V, El Far O, Bofill-Cardona E, Nanoff C, Freissmuth M, Karschin A, Airas JM, Betz H, Boehm S (1999) Calmodulin dependence of presynaptic metabotropic glutamate receptor signaling. Science 286:1180–1184.

Ohishi H, Akazawa C, Shigemoto R, Nakanishi S, Mizuno N (1995) Distributions of the mRNAs for L-2-amino-4-phosphonobutyrate-sensitive metabotropic glutamate receptors, mGluR4 and mGluR7, in the rat brain. J Comp Neurol 360:555–570.

Okamoto N, Hori S, Akazawa C, Hayashi Y, Shigemoto R, Mizuno N, Nakanishi S (1994) Molecular characterization of a new metabotropic glutamate receptor mGluR7 coupled to inhibitory cyclic AMP signal transduction. J Biol Chem 269:1231–1236.

Pan H, Wang HY, Friedman E, Gershon MD (1997) Mediation by protein kinases C and A of Go-linked slow responses of enteric neurons to 5-HT. J Neurosci 17:1011–1024.

Parmentier ML, Joly C, Restituito S, Bockaert J, Grau Y, Pin JP (1998) The G protein-coupling profile of metabotropic glutamate receptors, as determined with exogenous G proteins, is independent of their ligand recognition domain. Mol Pharmacol 53:778–786.

Pin JP, De Colle C, Bessis AS, Acher F (1999) New perspectives for the development of selective metabotropic glutamate receptor ligands. Eur J Pharmacol 375:277–294.

Prezeau L, Carrette J, Helpap B, Curry K, Pin JP, Bockaert J (1994) Pharmacological characterization of metabotropic glutamate receptors in several types of brain cells in primary cultures. Mol Pharmacol 45:570–577.

Regehr WG, Mintz IM (1994) Participation of multiple calcium channel

types in transmission at single climbing fiber to Purkinje cell synapses. Neuron 12:605–613.
Rodriguez-Moreno A, Sistiaga A, Lerma J, Sanchez-Prieto J (1998) Switch from facilitation to inhibition of excitatory synaptic transmission by group I mGluR desensitization. Neuron 21:1477–1486.
Rothe T, Bigl V, Grantyn R (1994) Potentiating and depressant effects of metabotropic glutamate receptor agonists on high-voltage-activated calcium currents in cultured retinal ganglion neurons from postnatal mice. Pflügers Arch 426:161–170.
Saugstad JA, Kinzie JM, Mulvihill ER, Segerson TP, Westbrook GL (1994) Cloning and expression of a new member of the D-2-amino-4-phosphonobutyric acid-sensitive class of metabotropic glutamate receptors. Mol Pharmacol 45:367–372.
Schoppa NE, Westbrook GL (1997) Modulation of mEPSCs in olfactory bulb mitral cells by metabotropic glutamate receptors. J Neurophysiol 78:1468–75.
Shen W, Slaughter MM (1998) Metabotropic and ionotropic glutamate receptors regulate calcium channel currents in salamander retinal ganglion cells. J Physiol (Lond) 510:815–828.
Shigemoto R, Kulik A, Roberts JD, Ohishi H, Nusser Z, Kaneko T, Somogyi P (1996) Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature 381:523–525.
Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, Flor PJ, Neki A, Abe T, Nakanishi S, Mizuno N (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 17:7503–7522.
Takahashi T, Momiyama A (1993) Different types of calcium channels mediate central synaptic transmission. Nature 366:156–158.
Takahashi T, Forsythe ID, Tsujimoto T, Barnes-Davies M, Onodera K (1996) Presynaptic calcium current modulation by a metabotropic glutamate receptor. Science 274:594–597.
Trombley PQ, Westbrook GL (1992) L-AP-4 inhibits calcium currents and synaptic transmission via a G- protein-coupled glutamate receptor. J Neurosci 12:2043–50.
Turner TJ, Adams ME, Dunlap K (1992) Calcium channels coupled to glutamate release identified by omega-Aga- IVA. Science 258:310–313.
Van Vliet BJ, Sebben M, Dumuis A, Gabrion J, Bockaert J, Pin JP (1989) Endogenous amino acid release from cultured cerebellar neuronal cells: effect of tetanus toxin on glutamate release. J Neurochem 52:1229–1239.
Wu S, Wright RA, Rockey PK, Burgett SG, Arnold JS, Rosteck Jr PR, Johnson BG, Schoepp DD, Belagaje RM (1998) Group III human metabotropic glutamate receptors 4, 7 and 8: molecular cloning, functional expression, and comparison of pharmacological properties in RGT cells. Brain Res Mol Brain Res 53:88–97.